The Dissertation Committee for Claudia A. Soto certifies that this is the approved version of the following dissertation:

Protein-Protein Interactions with Serotonin G Protein Coupled Receptors: Novel Targets for Neurotherapeutics Drug Discovery

Committee:

Kathryn A. Cunningham, Ph.D., Mentor

Noelle C. Anastasio, Ph.D.

Lisa A. Elferink, Ph.D.

Scott R. Gilbertson, Ph.D.

F. Gerard Moeller, M.D.

Dean, Graduate School

Protein-Protein Interactions with Serotonin G Protein Coupled Receptors: Novel Targets for Neurotherapeutics Drug Discovery

by

Claudia A. Soto, B.S.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas Medical Branch

August, 2017

Dedicación

Para mi mama, por siempre recordarme lo importante que es que la mujer tenga una carrera profesional y por su apoyo incondicional.

Acknowledgements

I would like to express my sincerest gratitude to my mentor Dr. Kathryn A. Cunningham for her many edits, insightful conversations and above all her unwavering support throughout this process. I am also thankful to Dr. Noelle C. Anastasio for teaching me how to develop sound experimental designs and continuously challenging me to think deeper. I thank Robert G. Fox for his excellent technical assistance, expert troubleshooting and extensive intellectual contributions to this work. Additionally, I am grateful for the assistance from Dr. Scott R. Gilbertson and his group, for without their compounds this work would not have been possible. I also thank the rest of my dissertation committee, Dr. F. Gerald Moeller and Dr. Lisa A. Elferink, for their support and critical feedback throughout the development of these studies. Finally, I would like to thank past and present members of the Cunningham and Anastasio laboratories for all of their support and the many memories we shared along the way. This work was supported by the National Institute on Drug Abuse grants DA0333935, DA020087, DA07287, DA030977, DA033374, DA07287 the Center for Addiction Research and UTMB. A portion of this work was supported by the NIH Intramural Research Programs of the National Institute on Drug Abuse and the National Institute of Alcohol Abuse and Alcoholism.

Protein-Protein Interactions with Serotonin G Protein Coupled Receptors: Novel Targets for Neurotherapeutics Drug Discovery

Publication No._____

Claudia A. Soto, Ph.D.

The University of Texas Medical Branch, 2017

Supervisor: Kathryn A. Cunningham

G protein coupled receptors (GPCRs) are involved in many physiological processes and are important therapeutic targets for a variety of human health disorders. In fact, approximately 30% of current FDA-approved medications target GPCRs and that number is likely to continue to rise with current GPCR-targeted drug discovery efforts. The main approach to pharmacologically manipulate GPCRs is to develop synthetic agonists, inverse agonists and antagonists that target the orthosteric site. While this approach has yielded several important medications, the development of selective orthosteric ligands is challenging and has led to the loss of many promising therapeutic candidates from the drug development pipeline due to adverse events attributed to off-target effects. Therefore, alternative strategies to develop GPCR drugs with enhanced selectivity are necessary. One alternative approach is to target GPCR protein-protein interactions (PPIs) that are selective for the GPCR of interest and involved in the regulation of the receptor function. This dissertation provides proof-of-concept data of two PPIs within the serotonin (5-HT) 5-HT_2 receptor (i.e., 5-HT_{2C}R and 5-HT_{2A}R) system which may be promising targets for the treatment of neuropsychiatric disorders. The 5-HT_{2C}R has been implicated in anxiety,

binge eating disorder, depression, impulsivity, movement disorders, obesity, schizophrenia and substance use disorders and selective 5-HT_{2C}R agonists display therapeutic potential. We show that disruption of the 5-HT_{2C}R interaction with the protein phosphatase and tensin (PTEN) via peptide-based PPI disrupters enhance 5-HT_{2C}R-mediating signaling *in vitro* and potentiates selective 5-HT_{2C}R agonists in behavioral rodent models. On the other hand, antagonism of the 5-HT_{2A}R is postulated to be a critical component in the actions of atypical antipsychotics (e.g., clozapine) and has been shown to improve symptomatology in preclinical models of psychostimulant addiction, anxiety, depression and sleep disorders. Here, we propose that targeting 5-HT_{2A}R:5-HT_{2A}R receptor-receptor interactions with bivalent ligands that have two pharmacophores that bind the orthosteric site tethered via a chemical linker provide potential therapeutically beneficial compounds. This dissertation provides initial characterization of 5-HT_{2A}R:5-HT_{2A}R homobivalent ligands that retain antagonism properties both *in vitro* and *in vivo*. Based on the cases explored here and other promising examples in the field, PPIs provide a much needed alternative approach for the selective regulation of GPCRs and should be considered, studied and exploited for their potential clinical implications.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Schemes

List of Abbreviations

3L4F	3^{rd} Loop 4^{th} Fragment
5-HT	Serotonin
5-HT_{2A}R	Serotonin 5-HT_{2A} Receptor
5-HT_{2B}R	Serotonin 5-HT_{2B} Receptor
5-HT_{2C}R	Serotonin 5-HT_{2C} Receptor
B_2-AR	β_2 Adrenergic Receptor
Ca_i^{2+}	Intracellular Calcium
CNS	Central Nervous System
DAG	Diacylglycerol
$ERK_{1/2}$	Extracellular Signal Regulated Kinases 1/2
FDA	Food and Drug Administration
FRET	Fluorescence Resonance Energy Transfer
G protein	Guanin Nucleotide Binding Protein
GABA	γ-Aminobutyric Acid
GPCR	G Protein Coupled Receptor
GRK	G Protein Couple Receptor Kinase
h5-HT_{2A}R-CHO	Chinese Hamster Ovary Cell Expressing the human 5-HT_{2C}R
HBSS	Hank's Balanced Salt Solution
IP_3	Inositol triphosphate
HEK	Human Embryonic Kidney Cell
mGluR	Metabotropic Glutamate Receptor

OR	Opioid Receptor
$pERK_{1/2}$	Phosphorylated Extracellular Signal Regulated Kinases ½
PKC	Protein Kinase C
PLC_{β}	Phospholipase C_{β}
PPI	Protein-Protein Interaction
PTEN	Phosphatase and Tensin Homolog
$PI(3,4,5)P_3$	Phosphatidylinositol (3,4,5) Triphosphate
$PI(4,5)P_2$	Phosphatidylinositol (4,5) Diphosphate

Chapter 1: Introduction

G Protein Coupled Receptors are Important Drug Targets

G-protein coupled receptors (GPCRs) are important therapeutic targets for a variety of human health disorders. In fact, approximately 30% of current FDA-approved medications target GPCRs and that number is likely to continue to rise with current drug discovery efforts (Bunnage, 2011; Jacoby et al., 2006). Although vastly diverse, this superfamily of proteins retain key structural components that classify them as GPCRs. These structural components include seven transmembrane α-helical domains, linked by alternating extracellular and intracellular loops as well as an extracellular amino (N-) terminus and an intracellular carboxyl (C-) terminus. The family of GPCRs responds to a variety of ligands such as small molecule neurotransmitters, photons, peptides, proteins, amino acids and ions which induce coupling of heterotrimeric G (guanine nucleotide-binding) proteins to transmit extracellular signals into intracellular processes (for review, (Rajagopal et al., 2010)). GPCRs are organized in three main classes, Class A, Class B and Class C, based on receptor structure. Class A, known as rhodopsin-like receptors, are characterized by several highly conserved residues within the transmembrane domains and intracellular loops, such as the D-R-Y motif that is present in the second intracellular loop of all class A GPCRs (Gether, 2000). This class is the best studied and largest class of GPCRs, containing approximately 85% of all GPCRs. Class A GPCRs are subdivided into six sub-categories to include the biogenic amine receptors for serotonin, dopamine, norepinephrine and muscarinic receptors as well as opioid, chemokine and olfactory receptors, to name a few (Gether, 2000; Jacoby et al., 2006). Clinically approved

medications that target class A GPCRs include Allegra/Telfast® (the histamine H_1 antagonist fexofenadine) for the treatment of allergies, Belviq® (the serotonin 5-HT_{2C} agonist lorcaserin) for weight loss and Diovan® (the angiotensin AT_1 antagonist valsartan) for hypertension. The GPCR class B is composed of peptide, hormone and neuropeptide receptors including secretin, calcitonin and parathyroid hormone receptors and are characterized by a long N-terminus tail (Bortolato et al., 2014). Clinically approved medications that target class B GPCRs include Forteo® (the parathyroid hormone PTH-1 peptide agonist teriparatide) for osteoporosis and Tanzeum® (the glucagon-like peptide GLP-1 peptide agonist albiglutide) for type 2 diabetes (for review) (Bortolato et al., 2014). The Class C GPCRs are characterized by very large N- and C-terminus tails and includes metabotropic glutamate (mGlu) and γ-aminobutyric acid type B ($GABA_B$) receptors (Jacoby et al., 2006). Clinically approved medications that target class C GPCRs include Neurontin® (the $GABA_B$ agonist GABApentin) for neurological pain.

APPROACHES TO TARGET GPCRS

The main approach to pharmacologically manipulate GPCRs that is currently the predominant focus of drug discovery efforts is to develop synthetic agonists, inverse agonists and antagonists that target the orthosteric site (Conn et al., 2009). While this approach has yielded several important medications, the development of selective orthosteric ligands is challenging and has led to the loss of many promising therapeutic candidates from the drug development pipeline due to adverse events attributed to off-target effects (Bunnage, 2011). Selective targeting of the orthosteric site is challenging due to the high degree of homology this site possesses between GPCRs that accommodate the same endogenous ligand. A prominent example of this translational barrier is observed

within the serotonin (5-HT) family, which consists of at least 14 receptors and will be discussed in depth later in this chapter. Overall, the current rate of FDA-approved and launched medications is dramatically slow when the number of promising targets that come out of preclinical work is considered. Therefore, alternative approaches to modulate these important druggable targets will have vast clinical implications.

The allosteric modulation of GPCRs offers an alternative approach to manipulate the receptors of interest. Allosteric modulation is defined as modulation of receptor signaling by targeting sites that are topographically distinct from the orthosteric site (Christopoulos and Kenakin, 2002; Conn et al., 2009; May et al., 2007; Wild et al., 2014). This type of modulation can be accomplished via targeting allosteric binding sites on the GPCR or stabilizing/disrupting allosteric protein-protein interactions (Christopoulos and Kenakin, 2002), both of which are discussed here.

Modulation via allosteric receptor binding sites is a promising avenue of investigation for a variety of receptor targets, including metabotropic glutamate receptors (Hopkins et al., 2009; Menniti et al., 2013), the muscarinic M_4 receptor (Chan et al., 2008) and the serotonin (5-HT) 5-HT_{2C} receptor (Ding et al., 2012; Im et al., 2003). This approach is based on the postulation that GPCRs include binding sites that are distinct from the orthosteric site. These distinct sites are not under evolutionary pressure to accommodate the endogenous ligand and, thus, can theoretically be more variable between receptors that accommodate the same endogenous ligand (Melancon et al., 2012). The proposed heterogeneity of this site could provide an enhanced opportunity to selectively target the desired receptor over the other highly homologous receptors within its family. Small molecules that target an allosteric site on the receptor may stabilize receptor conformations

that, on their own, do not induce signal transduction, but can fine-tune receptor function in the presence of the orthosteric ligand (Wild et al., 2014). The lack of agonist activity of allosteric modulators is postulated to alleviate issues that arise from chronically stimulating the orthosteric site, such as receptor desensitization, tolerance and up/down regulation at the membrane (Kenakin, 2009; Wild et al., 2014). Allosteric modulators have been reported for receptors within all three GPCR classes (Conn et al., 2009), some of which have been FDA-approved, such as Sensipar® (the calcium-sensing receptor positive allosteric modulator Cinacalcet; Amgen, FDA-approved 2004) for the treatment of hyperparathyroidism (Gustafsson et al., 2010) and Selzentry® (the chemokine receptor 5 negative allosteric modulator maraviroc; Pfizer, FDA-approved 2007) for the treatment of HIV/AIDS (Dorr et al., 2005). Targeting receptor allosteric binding sites represents an avenue which will yield new clinically approved therapeutics and solidify the concept of allosteric modulators for key GPCRs.

Allosteric regulation of GPCR signaling can also be achieved via manipulations of GPCR interactions with other proteins (Milligan and White, 2001). In recent years, various intracellular interacting proteins have been shown to modulate GPCR signal transduction (Brady and Limbird, 2002). Canonically, the family of interacting proteins that have been most widely studied are the G proteins which are the key proteins involved in GPCR signal transduction. Following G proteins, the role of β-arrestins and GPCR kinases (GRKs) is most delineated in their relation to both internalization/desensitization as well as G protein-independent signaling (for review) (Reiter and Lefkowitz, 2006) However, there are many more GPCR interacting proteins, some of which are selective for particular GPCRs that

can also modulate receptor signaling and, thus, present attractive new targets for drug discovery (Milligan and White, 2001).

These GPCR protein-protein interactions (PPIs) can form between receptor-accessory proteins, as well as receptor-receptor proteins (Brady and Limbird, 2002; Milligan and White, 2001). Accessory proteins include scaffolding proteins, kinases, phosphatases and chaperones that interact with a GPCR under given circumstances. Some of these interactions are ubiquitous for all GPCRs such as the interaction with G proteins, GRKs or β-arrestins. Since these accessory proteins interact with many GPCRs, selective modulation of a specific receptor would not be possible by targeting these PPIs since ligands that regulate these interactions would likely alter signal transduction of a large number of GPCRs. However, there are several examples in which PPIs are specific for one GPCR over highly homologous receptors within the same family. Georgoussi and colleagues thoroughly reviewed accessory protein interactions within the opioid receptor (OR) family which is composed of the δ-OR, κ-OR and μ-OR. In this paper, the authors clustered accessory protein PPIs as they relate to interference of G protein signaling, internalization/desensitization, lysosomal targeting and trafficking, linkage to the cytoskeleton, sorting into large sense-core vesicles, chaperones, transcription factors, and regulation of neurotransmitter release (Georgoussi et al., 2012). Thus, the example of ORs illustrates the breath of receptor regulation afforded by accessory proteins. Some of these interactions are shared among all three ORs and some are not, such as the interaction that occurs between the κ-OR and GEC1 (also named $GABA_A$ receptor-associated protein like 1 and Apg8L), which facilitates trafficking of κ-OR from the endoplasmic reticulum to the plasma membrane (Chen et al., 2006). This interaction does not occur with either δ-OR or

μ-OR and could thus be a target to specifically modulate κ-OR localization and ultimately function.

Another set of important PPIs that regulate GPCR function are receptor-receptor interactions. Receptor-receptor interactions can occur between two or more of the same GPCR or between different GPCRs, often, but not necessarily, within the same GPCR family. These receptor-receptor PPIs have been shown to alter both receptor localization and signaling and, thus, add a previously unappreciated complexity to the GPCR field (Hiller et al., 2013). These interactions further add important drug targets for the development of ligands that may have higher selectivity and reduced side effect profiles.

The functional importance of GPCR receptor-receptor interactions is exemplified by the interaction between the Class C GPCRs $GABA_B$ receptor 1 ($GABA_BR1$) and $GABA_B$ receptor 2 ($GABA_BR2$) (for review) (Brady and Limbird, 2002). Heterologous cells expressing either $GABA_BR1$ or $GABA_BR2$ alone do not result in fully functional receptors. In the case of $GABA_BR1$ expressing cells, receptors are retained in intracellular compartments and do not traffic to the plasma membrane. In the case of $GABA_BR2$ expressing cells, receptors are trafficked to the membrane, but cannot bind GABA or promote intracellular signaling. However, when cells express both receptors simultaneously, they are fully functional, located at the plasma membrane and responsive to GABA stimulation (Ng et al., 1999). It is proposed that this phenomenon occurs due to an endoplasmic reticulum retention sequence in the C-terminus of $GABA_BR1$ that is occluded by interaction with $GABA_BR2$ which allows the heterodimer to traffic to the plasma membrane. As illustrated by this example, it is important to delineate the functional significance of receptor-receptor interactions as they pertain to receptor signaling,

localization and function. Furthermore, since receptor-receptor interactions, be it hetero- or homo-oligomerization, can alter receptor function, targeting these PPIs is interesting and provides a new approach for GPCR drug discovery.

The development of selective modulators to manipulate, normalize and/or even promote PPIs with therapeutic benefit is still in its infancy. This type of molecules are most developed for non-GPCR PPIs (Arkin and Wells, 2004; Zinzalla and Thurston, 2009). PPI modulators can be classified as allosteric disrupters, allosteric stabilizers, direct disrupters and direct stabilizers. "Allosteric" in this context means that the modulator binds one of the protein partners to stabilize a conformation that either promotes or prevents interaction with the other partner in the case of PPI stabilizers or disrupters, respectively (Thiel et al., 2012). Direct modulators induce their effects by targeting the interface of the PPI. For example, direct PPI disrupters may disrupt the interaction by mimicking one of the partners and competing for interaction with the second partner. Conversely, direct PPI stabilizers may bind in the interface of an existing interaction and increase the binding affinity of the proteins involved (Thiel et al., 2012).

One strategy to modulate receptor-receptor interactions is bivalent ligands which consist of two pharmacophores linked via a tether of optimal length (Hiller et al., 2013). The pharmacophores can either be the same entity resulting in a homobivalent ligand or different chemical entities resulting in a heterobivalent ligand (Brogi et al., 2014). These two pharmacophores each interact with two separate receptors and can thus serve as a tool to probe homo/heterodimers (Brogi et al., 2014; George et al., 2002). Due to the need to interact at two binding sites rather than one, these tethered ligands may provide increased target selectivity (Hiller et al., 2013). One disadvantage of bivalent ligands for central

nervous system targets is their high molecular weight and, thus, projected poor penetration through the blood brain barrier. Despite this problem, several bivalent compounds have shown efficacy in preclinical models, although none are currently FDA approved (Brogi et al., 2014). In an interesting example of the therapeutic implications of bivalent ligands, Portoghese and colleagues demonstrate that morphine-induced tolerance and dependence in mice is modulated by linker length between a μ-OR agonist and a δ–OR antagonist bivalent ligand (MDAN) (Daniels et al., 2005). Two major side effects of analgesics like morphine that limit their clinical utility are the development of tolerance and physical dependence. Portoghese and colleagues demonstrate that MDAN with short linker lengths display both tolerance and physical dependence that is comparable to treatment with morphine or the tethered μ-OR agonist lacking the δ–OR antagonist pharmacophore. Excitingly, MDAN with longer linker lengths did not result in either tolerance or physical dependence but retained analgesic properties, while the μ-OR agonist with a tether of the same length lacking the δ–OR antagonist pharmacophore displayed both side effects. Furthermore, MDAN with the optimal linker length displayed comparable bioavailability and blood-brain barrier penetration in comparison to morphine (Daniels et al., 2005). Together these results suggest that a bivalent ligand with a pharmacophore targeting the μ-OR and one targeting the δ–OR could have extensive therapeutic implications as well as facilitate the study of this proposed heterodimer and lends support to the investigation of bivalent ligands with other receptor-receptor pharmacophores.

There are several approaches to improve the drug-like properties of peptide-based PPI modulators, which are mostly aimed to increase the peptide resistance to proteases. These approaches include: (i) protecting N and C terminal regions, (ii) replacing L amino

acids with D- enantiomers which are not found endogenously, (iii) cyclization to increase steric hindrance and rigidity, and (iv) replacement of some of the chemical groups in the peptide bonds with non-peptide chemical linkers which makes the peptide derivatives not recognizable by proteases (Adessi and Soto, 2002; Di, 2015).

One important approach for PPI-based drug discovery is the identification of the key region for the interaction termed as the PPI "hotspot" (Thangudu et al., 2012). The PPI hotspot can identified by mutating individual amino acids in the interaction interface with an alanine; an approach termed alanine scanning. The alanine replacement residues that do not allow for the interaction to occur, or that decrease the PPI binding affinity, form part of the PPI hotspot (Arkin and Wells, 2004). If identified, a small molecule can be designed that will fit into the hotspot and either stabilize or disrupt the PPI. This type of small molecules might be very useful to target specific PPIs, resulting in selective modulation of the receptor activity.

Overall, GPCR PPIs provide an alternative approach for the regulation of GPCR signaling and are important targets to consider for the development of therapeutics with enhanced selectivity.

DEVELOPMENT OF PPI-BASED DRUGS FOR THE SEROTONIN RECEPTOR FAMILY

One receptor family which nicely portrays the importance of receptor selectivity is the serotonin (5-HT) receptor family. Thus, as discussed below, targeting PPIs may offer a great opportunity to develop selective drugs for this receptor family. Before discussing this approach, I will briefly introduce the 5-HT receptor family and highlight the reasons that support the need for selective drugs.

Serotonin neurotransmission is implicated in many important physiological functions, and disruption of 5-HT neurotransmission is involved in a variety of disorders including anxiety, obesity, schizophrenia, and depression (Hoyer et al., 2002). Therefore, GPCRs within the 5-HT family are important therapeutic targets of major translational relevance. Serotonin signals through at least 14 receptors, 13 of which are GPCRs and many of them are localized in the central nervous system (Hoyer et al., 2002). Serotonin receptors are divided into seven families (i.e., 5-HT_{1-7}) (for review) (Barnes and Sharp, 1999). Some of these receptors exert inhibitory control over neuronal firing while others have excitatory control after agonist stimulation (Pytliak et al., 2011). Thus, overall 5-HT neurotransmission not only depends on the level of 5-HT itself, but also on the receptor composition and distribution within the brain and other tissues. Serotonin receptors, which are classified into seven families based on pharmacological properties, all accommodate 5-HT within the orthosteric binding site, and thus, selectively targeting individual 5-HT receptors is challenging.

The serotonin 5-HT_2 receptor (5-HT_2R) subfamily is composed of the 5-HT_{2A} receptor (5-HT_{2A}R), 5-HT_{2B}R and 5-HT_{2C}R, all three of which preferentially couple to $G_{q/11}$ proteins to result in phospholipase C_β-mediated downstream signaling, including intracellular calcium (Ca_i^{2+}) release and phosphorylation of extracellular signal regulated kinases 1 and 2 ($ERK_{1/2}$) (Hoyer et al., 2002). The 5-HT_{2A}R and 5-HT_{2C}R can also activate phospholipase A_2 (PLA_2) and generate arachidonic acid (AA) through an (unidentified) pertussis toxin-sensitive G protein (Felder et al., 1990) as well as phospholipase D (PLD) via $G\alpha_{12/13}$ (McGrew et al., 2002; Moya et al., 2011).These three receptors share a high degree of sequence homology, regulate a vast number of physiological properties, and are

implicated in various pathological processes, thus making them important drug targets, as discussed below.

SELECTIVE ACTIVATION OF 5-HT$_{2C}$R HAS IMPORTANT THERAPEUTIC POTENTIAL

The 5-HT$_{2C}$R has been implicated in anxiety, binge eating disorder, depression, impulsivity, movement disorders, obesity, schizophrenia and substance use disorders (for review) (Di Giovanni and De Deurwaerdere, 2016a). In particular, genetic, biochemical and pharmacological analyses have implicated 5-HT$_{2C}$R hypofunction as a regulator of behaviors related to a variety of neuropsychiatric disorders (for reviews) (Cunningham and Anastasio, 2014; Di Giovanni and De Deurwaerdere, 2016a, b; Howell and Cunningham, 2015). For example, selective 5-HT$_{2C}$R agonists have shown efficacy and potency to reduce food consumption (Fletcher et al., 2009; Gustafson et al., 2013), impulsivity (Anastasio et al., 2013; Fletcher et al., 2007; Navarra et al., 2008), and the reinforcing (Cunningham et al., 2011; Fletcher et al., 2004; Higgins et al., 2012; Kasper et al., 2013; Neelakantan et al., 2017; Neisewander and Acosta, 2007; Swinford-Jackson et al., 2016) and subjective effects (Callahan and Cunningham, 1995; Higgins et al., 2012) of drugs of abuse (i.e., cocaine, ethanol, methamphetamine, nicotine, oxycodone), among other behaviors. Importantly, we have shown that the 5-HT$_{2C}$R is an important mediator of the behavioral effects of cocaine. For example, a selective 5-HT$_{2C}$R agonist suppresses intake and sensitivity to cocaine-associated cues in rat self-administration models (Anastasio et al., 2014; Cunningham et al., 2011; Swinford-Jackson et al., 2016) while engineered 5-HT$_{2C}$R hypofunction triggers enhanced cocaine-seeking (Anastasio et al., 2014). These data suggest that strategies to maximize 5-HT$_{2C}$R signaling may provide therapeutic

efficacy in several important disorders and as such development of selective 5-HT_{2C}R ligands is an important avenue of investigation.

One major barrier to selective 5-HT_{2C}R agonist development has historically been the potential for adverse effects that arise with activation of the 5-HT_{2A}R and 5-HT_{2B}R. Activation of 5-HT_{2A}R can result in hallucinogenic effects (Nichols, 2004), which leads to difficulty in obtaining FDA approval for 5-HT_{2A}R therapeutics. For example, the "first-in-class" 5-HT_{2C}R agonist lorcaserin (Belviq®) was FDA approved in 2012 as a weight loss drug in obese individuals (ww.us.eisai.com/)(Pharmaceuticals, 2007; Smith et al., 2008). However, lorcaserin is classified as schedule IV (Shram et al., 2011) because of the *potential* for dysphoregenic properties due to its affinity for and partial agonism at the 5-HT_{2A}R (Smith et al., 2008), which illustrates one translational barrier in targeting the 5-HT_{2C}R. Furthermore, selective 5-HT_{2C}R agonists that have affinity for the 5-HT_{2B}R also face approval barriers and safety concerns due to their potential to cause valvulopathies and cardiac pathology (Fitzgerald et al., 2000; Rothman et al., 2000). This is illustrated by the 5-HT releaser fenfluramine which was approved as a weight loss medication. Fenfluramine was prescribed as a combination therapy along with phentermine (Fen-Phen) with the idea that lower doses of each would synergize and produce weight loss with lower incidence of adverse events (Weintraub et al., 1992). The Fen-Phen combination, which produced rapid and sustained weight loss, was thought to be a magic pill to treat obesity and prescriptions exceeded 18 million in 1996 (Capriotti, 1998; Connolly et al., 1997). However, in the late 1990s, reports emerged demonstrating an alarming rate of cardiac events in patients on Fen-Phen (Connolly et al., 1997) which eventually led to the removal of Fen-Phen from the market. Years later, fenfluramine and some of its metabolites were

shown to exhibit 5-HT_{2B}R agonist actions which were responsible for these adverse events (Fitzgerald et al., 2000; Rothman et al., 2000). Since that time, compounds with agonist actions at the 5-HT_{2B}R have faced approval issues for safety concerns and as such, 5-HT_{2C}R agonists potentially useful for therapeutic medications must lack the ability to stimulate the 5-HT_{2B}R.

5-HT_{2C}R:PTEN COMPLEX AS A PPI TARGET – DIRECT PPI INHIBITION

One approach to selectively target the 5-HT_{2C}R is through PPIs that are selective for 5-HT_{2C}R over 5-HT_{2A}R and 5-HT_{2B}R and which can regulate 5-HT_{2C}R signaling. In that regard, one PPI of interest is the interaction between the 5-HT_{2C}R and accessory protein phosphatase and tensin (PTEN) which occurs in the third intracellular loop of the 5-HT_{2C}R (Anastasio et al., 2013; Ji et al., 2006). Disruption of 5-HT_{2C}R:PTEN complex potentiates 5-HT_{2C}R agonist effects in a variety of assays and thus this PPI can be classified as an allosteric modulator of the 5-HT_{2C}R. PTEN does not interact with the 5-HT_{2A}R (Anastasio et al., 2013), suggesting that PPI disrupters of the 5-HT_{2C}R:PTEN complex can selectively enhance 5-HT_{2C}R signaling without effects at 5-HT_{2A}R. Therefore, this approach may represent a promising strategy to attenuate the current limitations of orthosteric 5-HT_{2C}R agonists.

The PPI between 5-HT_{2C}R and PTEN was first demonstrated by co-immunoprecipitation assays in PC12 cells and rat ventral tegmental area (Ji et al., 2006) and subsequently extended to medial prefrontal cortex (Anastasio et al., 2013). Amino acids Pro283-Arg297 of the rat 5-HT_{2C}R (analogous to Pro280-Arg295 in the human 5-HT_{2C}R) are within the third loop of the receptor, and are critical for the 5-HT_{2C}R:PTEN

interaction. A small peptide, termed 3L4F (3rd loop, 4th fragment), homologous to this sequence disrupts the 5-HT_{2C}R:PTEN complex both in 5-HT_{2C}R cellular models and rodent ventral tegmental area (Anastasio et al., 2013; Ji et al., 2006). Treatment with 3L4F in cells stably expressing the human 5-HT_{2C}R potentiates intracellular calcium (Ca_i^{2+}) release evoked by 5-HT or the selective 5-HT_{2C}R agonist WAY163909 (Anastasio et al., 2013). These data suggest that disruption of the 5-HT_{2C}R:PTEN complex enhances agonist-induced 5-HT_{2C}R signaling. Treatment with 3L4F alone does not induce Ca_i^{2+} release suggesting that, similar to allosteric modulators discussed above, disruption of the 5-HT_{2C}R:PTEN complex may "tune up" 5-HT_{2C}R agonist signal transduction rather than induce signaling on its own. Treatment with 3L4F in 5-HT_{2A}R expressing cells did not induce Ca_i^{2+} release either on its own or in the presence of 5-HT, consistent with the lack of 5-HT_{2A}R and PTEN co-immunoprecipitation in cells (Anastasio et al., 2013).

The initial *in vivo* profile of 5-HT_{2C}R:PTEN complex disruption was assessed using the rat 3L4F analog (r3L4F) conjugated to a short cell penetrant peptide TAT (YGRKKRR) (Vives et al., 1997) at the N terminus to promote blood brain barrier permeability (TAT-r3L4F) (Anastasio et al., 2013). Selective 5-HT_{2C}R agonists are known to suppress spontaneous locomotor activity (Cunningham et al., 2013; Cunningham et al., 2011; Fletcher et al., 2002; Grottick et al., 2000; Halford et al., 1997). Treatment with TAT-r3L4F alone dose-dependently (0.1 and 1 µmol/kg) suppressed horizontal, but not vertical activity, consistent with elevation of 5-HT_{2C}R signaling (Anastasio et al., 2013). The low dose of TAT-r3L4F (0.1 µmol/kg), which did not suppress motor activity on its own, enhanced the efficacy of a sub-effective dose of the selective 5-HT_{2C}R agonist WAY163909 to suppress motor activity (Anastasio et al., 2013). Selective 5-HT_{2C}R

agonists have also been shown to suppress, while 5-HT_{2C}R antagonists increase, indices of impulsive action as measured by choice serial reaction time tasks (Cunningham et al., 2011; Fletcher et al., 2007; Navarra et al., 2008; Winstanley et al., 2004). Treatment with TAT-r3L4F synergized with a sub-effective dose of WAY163909 to suppress measures of impulsive action (Anastasio et al., 2013). Lastly, TAT-r3L4F blocked Δ^9-tetrahydrocannabinol-induced conditioned place preference, an effect that was mimicked by the 5-HT_{2C}R agonist Ro-600175 and reversed by 5-HT_{2C}R antagonist SB242084 (Ji et al., 2006). The suppression of these behaviors cannot be explained by suppression of spatial learning or spatial memory retrieval, both of which are unaffected by TAT-r3L4F treatment (Maillet et al., 2008). Together, these results suggest that disruption of the 5-HT_{2C}R:PTEN complex by TAT-r3L4F enhances 5-HT_{2C}R-mediated effects *in vivo*.

Disruption of the 5-HT_{2C}R:PTEN complex resulted in potentiation of effects of a 5-HT_{2C}R agonist in a variety of assays which suggests that this PPI may be a viable therapeutic target. As discussed above, major disadvantages of peptide-based PPI disrupters include low bioavailability and reduced pharmacokinetic properties, which are concerns for the therapeutic use of 3L4F. Approaches to overcome this barrier include shortening of the peptide fragment as well as chemical modifications that enhance the pharmacokinetic properties of the peptide (Di, 2015). The ideal endpoint is to determine the PPI hotspot and generate a small molecule that can target that region directly thereby harnessing the advantages of targeting PPIs along with the enhanced drug-like potential of small molecules. In an effort to move towards that goal, our laboratory identified that the first eight amino acids within the 3L4F sequence (Pro280-Arg287) are sufficient for retaining 3L4F activity *in vitro* (Anastasio et al., 2013). This dissertation provides further

evidence that disruption of 5-HT_{2C}R:PTEN complex has the potential for therapeutic benefits for disorders marked by 5-HT_{2C}R hypofunction as well as moves forward in generating new direct PPI disrupters that may have enhanced pharmacokinetic properties (Chapter 2).

SELECTIVE 5-HT_{2A}R ANTAGONISTS HAVE IMPORTANT THERAPEUTIC POTENTIAL

Most atypical antipsychotics are classified as possessing 5-HT_{2A}R inverse agonist/antagonist properties (Meltzer et al., 2012a; Meltzer et al., 2012b) which is postulated to be a critical component in the actions of atypical antipsychotics (e.g., clozapine) that underlies the enhanced therapeutic benefits over typical antipsychotics (e.g., haloperidol) (Meltzer et al., 2012b; Meltzer et al., 1989). As such, 5-HT_{2A}R blockade is an important avenue of investigation. Specifically, the main clinical barrier for typical antipsychotics, like haloperidol, are extrapyramidal side effects that include dystonia, tardive dyskinesia, Parkinsonism, akinesia, akathisia and neuroleptic malignant syndrome (Blair and Dauner, 1992). Extrapyramidal effects, which often lead to discontinuation of medication treatment, are a result of dopamine D_2 receptor blockade. On the contrary, most atypical antipsychotics have higher affinity for 5-HT_{2A}R over D_2 receptor (Meltzer et al., 1989) and are hypothesized to distally modulate serotonergic and dopaminergic neurotransmission through 5-HT_{2A}R blockade in the medial prefrontal cortex (Meltzer et al., 2012b). Atypical antipsychotics do not result in extrapyramidal effects and are thus more tolerated by patients and have become the first line of treatment.

The FDA has recently approved the 5-HT_{2A}R inverse agonist/antagonist pimavanserin (tradename NUPLAZID®) (ACP-103; ACADIA Pharmaceuticals) for Parkinson's disease psychosis (Meltzer et al., 2010). Pimavanserin, which does not block

D_2 receptors (Vanover et al., 2006), has provided the first in-class 5-HT_{2A}R stand-alone antipsychotic and supports the idea that 5-HT_{2A}R inverse agonist/antagonism may be useful as a monotherapy for psychosis. Selective 5-HT_{2A}R inverse agonists/antagonists also show promise to improve symptomatology in preclinical models of psychostimulant addiction (Anastasio et al., 2011; Burmeister et al., 2004; Fink et al., 2015; Fletcher et al., 2012; Nic Dhonnchadha and Cunningham, 2008; Nic Dhonnchadha et al., 2009), anxiety (Carr and Lucki, 2011; Pillay and Stein, 2007; Weisstaub et al., 2006), depression (Berg et al., 2008; Celada et al., 2004; Zaniewska et al., 2010) and sleep disorders (Holshoe, 2009; Landolt and Wehrle, 2009; Popa et al., 2005; Teegarden et al., 2008).

Thus, selective inhibition of 5-HT_{2A}R has important therapeutic implications. However, the potential for inverse agonism/antagonism at the 5-HT_{2C}R, and other GPCRs (*i.e.*, histamine H_1, adrenergic α_{1A} and 5-HT_6 receptors), may present translational barriers for 5-HT_{2A}R inverse agonist/antagonist development, as illustrated by atypical antipsychotics (e.g., clozapine) (Kroeze et al., 2003). Although atypical antipsychotics do not induce extrapyramidal side effects like typical antipsychotics, the main adverse effect of this medication class is substantial weight gain which affects 40-80% of patients on these medications (Masand, 1999; Umbricht et al., 1994). The weight gain, which often exceeds 20% of ideal body weight (Masand, 1999; Umbricht et al., 1994), can result in increased incidence of metabolic-associated diseases including type 2 diabetes, hyperglycemia, and hypertension, as well as lower medication compliance (Green et al., 2000; Meltzer and Roth, 2013). In an interesting epidemiological study, Fountaine and colleagues estimated that clozapine treatment, which is the current gold-standard, resulted in avoidance of 492 suicide deaths per 100,000 schizophrenic patients in 10 years.

However, the authors also estimated that clozapine resulted in an additional 416 deaths related to antipsychotic-induced weight gain. Therefore, the clinical benefit induced by clozapine is outweighed by the adverse effect of weight gain (Fontaine et al., 2001). Interestingly, weight gain is an adverse effect associated predominantly with atypical, over typical, antipsychotics (Green et al., 2000), and could be attributed to enhanced serotonergic involvement in this class of medications. Inverse agonism/antagonism of 5-HT_{2C}R, which is present in most atypical antipsychotics, is thought to contribute to the metabolic side effects of atypical antipsychotics (Kroeze et al., 2003). This adverse effect of 5-HT_{2C}R antagonism is not unexpected due to the fact that activation of the 5-HT_{2C}R has been repeatedly shown to result in weight loss. Therefore, development of ligands that selectively antagonize the 5-HT_{2A}R with little to no activity as a 5-HT_{2C}R antagonist could have the potential of harnessing the current therapeutic benefits of atypical antipsychotics while avoiding weight gain and could thus greatly benefit this patient population.

5-HT_{2A}R HOMODIMERS AS PPI TARGET – PPI STABILIZATION

One approach to develop 5-HT_{2A}R antagonists with enhanced therapeutic potential may be to target 5-HT_{2A}R:5-HT_{2A}R homodimer interactions. Traditionally, GPCRs were conceptualized as existing as monomers, however emerging evidence suggest that GPCRs can form receptor-receptor complexes which can alter localization and signaling. Brea and colleagues provided evidence supporting the existence of homodimeric 5-HT_{2A}R:5-HT_{2A}R interactions through the use of co-immunoprecipitation and fluorescence resonance energy transfer (FRET). They expressed N-terminus FLAG-tagged 5-HT_{2A}R as well as c-myc-tagged 5-HT_{2A}R in human embryonic kidney (HEK) cells and showed that anti-FLAG antibody immunoprecipitation followed by c-myc immunoblotting results in a clear 55 kDa

immunoreactive band which is consistent with 5-HT_{2A}R molecular weight. Similarly they showed that HEK cells expressing yellow fluorescent protein-tagged 5-HT_{2A}R and cyan fluorescent protein-tagged 5-HT_{2A}R produced a robust and saturable FRET signal. These data suggest that at least two distinct 5-HT_{2A}R are in complex together which provides support for this receptor existing, at least in part, as a homodimer (Brea et al., 2009). Furthermore, 5-HT_{2A}R:5-HT_{2A}R homodimers have been proposed to be the minimum functioning unit of the PLCβ and PLA_2 mediated signaling pathways induced by 5-HT_{2A}R agonists *in vitro* (Iglesias et al., 2016). Additionally, molecular dynamics modeling studies suggest that the putative 5-HT_{2A}R ligand binding sites displace differently in simulations of monomers vs. homodimers, which may suggest that receptor-receptor interactions may prefer different 5-HT_{2A}R ligands (Bruno et al., 2011). Therefore, development of tools to explore 5-HT_{2A}R homodimer relevance in signal transduction are necessary.

Here, we propose that targeting 5-HT_{2A}R:5-HT_{2A}R homodimers with bivalent ligands that have two pharmacophores that bind the orthosteric site tethered via a chemical linker will provide therapeutically beneficial compounds. This dissertation provides initial characterization of 5-HT_{2A}R:5-HT_{2A}R homobivalent ligands that retain antagonism properties both *in vitro* and *in vivo* (Chapter 3). Although our homobivalent ligands do not display enhanced selectivity for the 5-HT_{2A}R over the 5-HT_{2C}R, these ligands may be used as tools for the exploration of homodimer impact in behaviors where 5-HT_{2A}R inverse agonists/antagonists show efficacy. Since GPCR dimers are known to alter ligand binding sites, signaling properties and trafficking (Hiller et al., 2013), it is possible that 5-HT_{2A}R:5-HT_{2A}R homodimers may differentially regulate behaviors implicated in these important disorders, which has been difficult to study, especially *in vivo*.

Along with enhance therapeutic potential, 5-HT_2R ligands with enhanced selectivity are necessary to advance the scientific understanding of the distinct roles of 5-HT_2Rs in physiological and pathological processes. At the moment, it is difficult to disentangle the exact roles for these receptors due to a lack of highly specific ligands (Di Giovanni and De Deurwaerdere, 2016a). Better delineation of *in vivo* receptor function and thus biological contribution will further expand and inform drug discovery efforts as well as the understanding of the development of important psychiatric disorders. Therefore, approaches that allow for the selective manipulation of GPCRs within the 5-HT_2R subfamily are necessary. Overall, this dissertation explores the hypothesis that direct PPI stabilizers and disrupters can allosterically regulate 5-HT_2R signaling and are therefore promising targets for drug development.

Chapter 2: *In Vivo* and *In Vitro* Analyses of Novel Peptidomimetic Disruptors for the Serotonin 5-HT_{2C} Receptor Interaction with Phosphatase and Tensin Homolog (PTEN) (Soto et al.)

INTRODUCTION

The serotonin (5-HT) 5-HT_{2C} receptor (5-HT_{2C}R) is engaged in normal physiology (e.g., appetite) (Heisler et al., 2003) while 5-HT_{2C}R dysfunction is implicated in multiple pathological disorders (e.g., anxiety, depression, obesity, substance use disorders) (Bubar and Cunningham, 2008; Howell and Cunningham, 2015; Miller, 2005; Tecott et al., 1995). In particular, genetic, biochemical and pharmacological analyses have implicated 5-HT_{2C}R hypofunction as a regulator of behaviors related to a variety of neuropsychiatric disorders (for review) (Cunningham and Anastasio, 2014; Di Giovanni and De Deurwaerdere, 2016b; Howell and Cunningham, 2015)). For example, selective 5-HT_{2C}R agonists have shown efficacy and potency to reduce food consumption (Fletcher et al., 2009; Gustafson et al., 2013), impulsivity (Anastasio et al., 2013; Fletcher et al., 2007; Navarra et al., 2008), and the reinforcing (Cunningham et al., 2011; Fletcher et al., 2004; Higgins et al., 2012; Kasper et al., 2013; Neelakantan et al., 2017; Neisewander and Acosta, 2007; Swinford-Jackson et al., 2016) and subjective effects (Callahan and Cunningham, 1995; Higgins et al., 2012) of drugs of abuse (i.e., cocaine, ethanol, methamphetamine, nicotine, oxycodone), among other behaviors. As such, therapeutic molecules that enhance 5-HT_{2C}R activity have important clinical implications.

Agonist binding to the 5-HT_{2C}R results in dynamic changes in receptor conformation and induction of a variety of intracellular signaling pathways (Berg et al.,

1994b; Labasque et al., 2008; Werry et al., 2005). Best characterized is coupling of the 5-HT_{2C}R to $G\alpha_{q/11}$ proteins to activate phospholipase C_β (PLC_β) resulting in increased intracellular calcium (Ca_i^{2+}) release (Hannon and Hoyer, 2008; Millan et al., 2008), among various other signaling outcomes (Berg et al., 1994b; McGrew et al., 2002). The sequence of ligand binding to a GPCR and subsequent activation of downstream signaling can be positively or negatively modulated by binding of ligands at allosteric sites which are topographically distinct from the orthosteric ligand binding site (Christopoulos and Kenakin, 2002; Conn et al., 2009; May et al., 2007; Wild et al., 2014). There are theoretical reasons (i.e., increased selectivity, upper ceiling effects, separate control of affinity and efficacy) as to why allosteric ligands may be preferred therapeutic chemical targets (Kenakin and Miller, 2010). Therefore, 5-HT_{2C}R allosteric modulators present a novel drug design strategy to selectively tune up or down signaling in response to endogenous 5-HT or synthetic 5-HT_{2C}R agonists in disorders marked with 5-HT_{2C}R hypofunction.

Allosteric modulation can be accomplished via targeting allosteric binding sites on the GPCR or stabilizing/disrupting allosteric protein-protein interactions involved in signal transduction (Christopoulos and Kenakin, 2002). One protein-protein interaction of interest occurs between 5-HT_{2C}R and protein phosphatase and tensin (PTEN) in the third intracellular loop of the 5-HT_{2C}R (Anastasio et al., 2013; Ji et al., 2006). PTEN is a dual phosphatase that contains distinct lipid and protein phosphatase activities and is involved in the suppression of cell proliferation pathways through the lipid phosphatase activity (Lee et al., 1999; Maehama and Dixon, 1998; Ning et al., 2004; Stambolic et al., 1998). Disruption of the 5-HT_{2C}R:PTEN complex enhances selective 5-HT_{2C}R agonist-induced effects in both cellular and rodent models (Anastasio et al., 2013; Ji et al., 2006). As such,

disruption of the 5-HT_{2C}R:PTEN complex is a potential target to selectively potentiate agonist activity at the 5-HT_{2C}R and may hold therapeutic promise.

Previous studies have employed a 16 amino acid peptide homologous to a fragment of the third intracellular loop of the human 5-HT_{2C}R (**h3L4F** (**1**); **Figure 2.1**; Ac-PNQDQNARRRKKKERR-NH_2; Pro280-Arg295) and its shorter version (peptide **2**; **Figure 2.1;** Ac-PNQDQNAR-NH_2; Pro280-Arg287) to disrupt the 5-HT_{2C}R:PTEN complex (Anastasio et al., 2013; Ji et al., 2006). Peptides **h3L4F** (**1**) and **2** enhance 5-HT_{2C}R agonist-mediated Ca_i^{2+} release in Chinese hamster ovary cells stably expressing the human 5-HT_{2C}R (h5-HT_{2C}R-CHO) (Anastasio et al., 2013). Additionally, treatment with peptides **h3L4F** (**1**) and **2** alone do not induce Ca_i^{2+} release in h5-HT_{2C}R-CHO cells, suggesting that these peptides do not possess agonist activity but rather fine tune agonist-induced 5-HT_{2C}R signaling. The initial *in vivo* profile of 5-HT_{2C}R:PTEN complex disruption was assessed using a peptide homologous to an analogous fragment of the rat 5-HT_{2C}R third intracellular loop (**r3L4F**; Ac-PNPDQKPRRKKKEKR-NH_2) conjugated to a short cell penetrant peptide TAT (YGRKKRR) (Vives et al., 1997) at the N terminus to promote blood brain barrier permeability (**TAT-r3L4F** (**3**); **Figure 2.1**) (Anastasio et al., 2013). Selective 5-HT_{2C}R agonists are known to suppress spontaneous locomotor activity (Cunningham et al., 2013; Cunningham et al., 2011; Fletcher et al., 2002; Grottick et al., 2000; Halford et al., 1997). Treatment with **TAT-r3L4F** alone dose-dependently (0.1 and 1 µmol/kg) suppressed horizontal, but not vertical activity, consistent with elevation of 5-HT_{2C}R signaling (Anastasio et al., 2013). The low dose of **TAT-r3L4F** (0.1 µmol/kg), which did not suppress motor activity on its own, enhanced the efficacy of a sub-effective dose of selective 5-HT_{2C}R agonist WAY163909 to suppress motor activity (Anastasio et

al., 2013). Selective 5-HT_{2C}R agonists have also been shown to suppress, while 5-HT_{2C}R antagonists increase, indices of impulsive action as measured by choice serial reaction time tasks (Cunningham et al., 2011; Fletcher et al., 2007; Navarra et al., 2008; Winstanley et al., 2004). Treatment with **TAT-r3L4F** synergized with a sub-effective dose of WAY163909 was found to suppress measures of impulsive action (Anastasio et al., 2013). Lastly, **TAT-r3L4F** blocked Δ^9-tetrahydrocannabinol-induced conditioned place preference, an effect that was mimicked by the 5-HT_{2C}R agonist Ro-600175 and reversed by 5-HT_{2C}R antagonist SB242084 (Ji et al., 2006). Together, these results suggest that disruption of the 5-HT_{2C}R:PTEN complex by **TAT-r3L4F** enhances 5-HT_{2C}R-mediated effects *in vivo*.

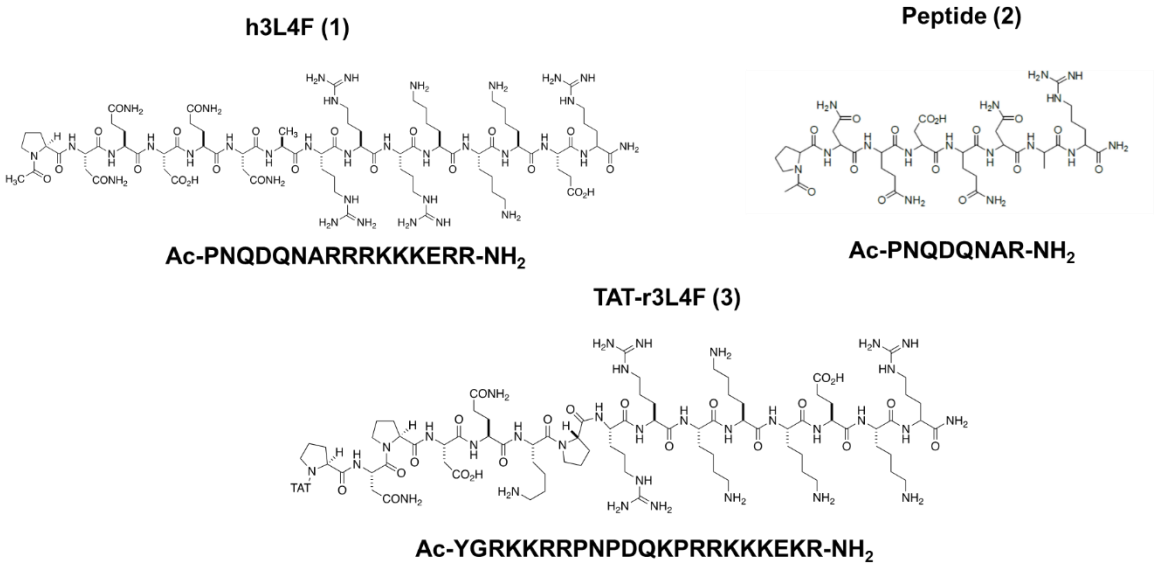


Figure 2.1: Parent peptides h3L4F (1), peptide (2) and TAT-r3L4F (3).

In the present study, we further investigate the effects of **TAT-r3L4F** *in vivo* as well as explored the signal transduction pathways recruited in **TAT-r3L4F**-mediated effects *in vivo* through the use of a drug discrimination paradigm. Drug discrimination is a widely used rodent model for the assessment of novel compounds and has face validity as

an assessment for the subjective effects of compounds in animals and humans (Appel and Cunningham, 1986; Bergman et al., 2000; Schuster and Johanson, 1988; Teuns et al., 2014). In this paradigm, rats are trained to discriminate a training drug from its vehicle based on the interoceptive cue elicited by the drug. The drug discrimination behavioral model can be employed to assess mechanisms of action of a novel compound as well as to assess abuse liability of new chemical compounds (Colpaert, 1999). To assess mechanisms of action, rats are trained to discriminate a training drug with a known mechanism of action (*e.g.*, 5-HT_{2C}R agonist). The rats are subsequently tested with the novel compound in substitution and combination tests to determine if the novel compound induces similar interoceptive cues recognized as similar to the training drug. Receptor involvement in the mechanism of action can thus be discerned based on rats performance on this task. Additionally, the abuse liability and similarity to an abused drug can be assessed using the drug discrimination model. For this assessment, rats are trained to discriminate a drug of abuse (*e.g.*, cocaine) and the ability of the novel compound to suppress the stimulus effects of the training drug are assessed. Since humans engage in the use of drugs of abuse to attain the subjective effects elicited by these drugs (e.g., euphoria, relaxation), the interoceptive cues elicited by drugs of abuse can promote the addiction cycle (Bubar and Cunningham, 2008). Notably, selective 5-HT_{2C}R agonists suppress the stimulus effects of cocaine (Callahan and Cunningham, 1995) as well as other addiction related behaviors (Bubar and Cunningham, 2008; Howell and Cunningham, 2015). Thus, in the present behavioral analyses, we explored the ability of **TAT-r3L4F** to affect the stimulus properties of selective 5-HT_{2C}R agonists or cocaine.

We show that **TAT-r3L4F** enhances the discriminative stimulus effects of the selective 5-HT_{2C}R agonists lorcaserin and WAY163909 in a potentially PLC_{β}-independent manner. Given that 5-HT_{2C}R agonists are known to suppress stimulus effects of the psychostimulant cocaine, we also tested **TAT-r3L4F** in a cocaine versus saline discrimination assay and show that treatment with **TAT-r3L4F** enhances the suppressive effects of 5-HT_{2C}R agonists. Thus, disruption of the 5-HT_{2C}R:PTEN complex potentiates 5-HT_{2C}R agonist-induced effects suggesting that this protein-protein interaction is a promising target to allosterically modulate the 5-HT_{2C}R.

An important disadvantage of peptide-based protein-protein interaction disrupters is their unfavorable pharmacokinetic properties (e.g., short half-life, low permeability, and poor absorption distribution, metabolism and excretion (ADME) properties) which is a concern for their therapeutic use (Adessi and Soto, 2002; Di, 2015). The unfavorable pharmacokinetic profiles of peptides are related to their generally high conformation instability which exposes the peptide bond to proteolytic attack, decreases cell penetration by increase backbone desolvation energy and facilitates renal clearance (Gongora-Benitez et al., 2014). Our chemistry efforts focused on the design and synthesis of novel constrained peptide derivatives based on the sequence of peptide **2** which are expected to improve upon the pharmacokinetic profiles of the original peptides **h3L4F** and **2**. An alanine scan was employed to determine which amino acids residues were critical for *in vitro* peptide activity. Based on those results, cyclized peptides and peptidomimetic derivatives were designed and synthesized and shown to retain *in vitro* potency and efficacy.

RESULTS AND DISCUSSION

We first investigated the effects of the parent peptide **TAT-r3L4F** (**3**; **Figure 2.1**) *in vivo* through the use of a two-lever, water-reinforced drug discrimination paradigm in three separate cohorts of rats. In the first set of experiments, rats were trained to discriminate lorcaserin or WAY163909 from saline to investigate the effects of **TAT-r3L4F** on the interoceptive cues elicited by the 5-HT_{2C}R agonists and to begin to elucidate the signal transduction pathways that are recruited for 3L4F-mediated effects. Note, the TAT-conjugated **TAT-r3L4F** peptide was employed in all rodent experiments.

Lorcaserin-Saline and WAY163909-Saline Discrimination

All rats in both cohorts acquired the discrimination within similar number of training sessions. Rats (n=14) acquired the discrimination of lorcaserin (0.75 mg/kg; 15 min pretreatment) versus saline within an average of 51 two-lever training sessions (range 33-71); response rates after lorcaserin (21.4 ± 0.74/min) were statistically lower than rates after saline (33.8 ± 1.2/min; $p<0.05$). Rats (n=12) acquired the discrimination of WAY163909 (0.75 mg/kg; 15 min pretreatment) versus saline within an average of 44 two-lever training sessions (range 39-57); response rates after WAY163909 (17.2 ± 1.44/min) were statistically lower than rates after saline (22.1 ± 1.3/min; $p<0.05$). Substitution tests indicated that saline (**Figure 2.2A, 2.2B**, SAL) engendered <10% drug-lever responding in both cohorts. Both lorcaserin and WAY163909 evoked similar dose-related (0.125 – 1 mg/kg) increases in drug-appropriate responding (**Figure 2.2A, 2.2B**) as well as suppression of response rates (**Figure 2.2C, 2.2D**). In the lorcaserin-saline trained rats, drug-appropriate lever responding after lorcaserin doses of 0.125, 0.25, 0.5, and 1 mg/kg were significantly different from the previous lorcaserin maintenance session ($p<0.05$;

Figure 2.2A). Doses of 0.125, 0.25 and 0.5 mg/kg produced response rates significantly elevated versus previous lorcaserin maintenance sessions ($p<0.05$; **Figure 2.2C**). Similarly, in the WAY163909 versus saline trained rats, drug-appropriate lever responding after WAY163909 (0.125, 0.25, 0.5 mg/kg) was significantly different from the previous WAY163909 maintenance session ($p<0.05$; **Figure 2.2B**), while response rates were different at 0.5 mg/kg ($p<0.05$; **Figure 2.2D**). The dose of lorcaserin predicted to elicit 50% lorcaserin-lever responding (ED_{50}) was 0.58 mg/kg (95% CL 0.51-0.64 mg/kg). The ED_{50} of WAY163909 was 0.60 mg/kg (95% CL 0.37-0.82 mg/kg).

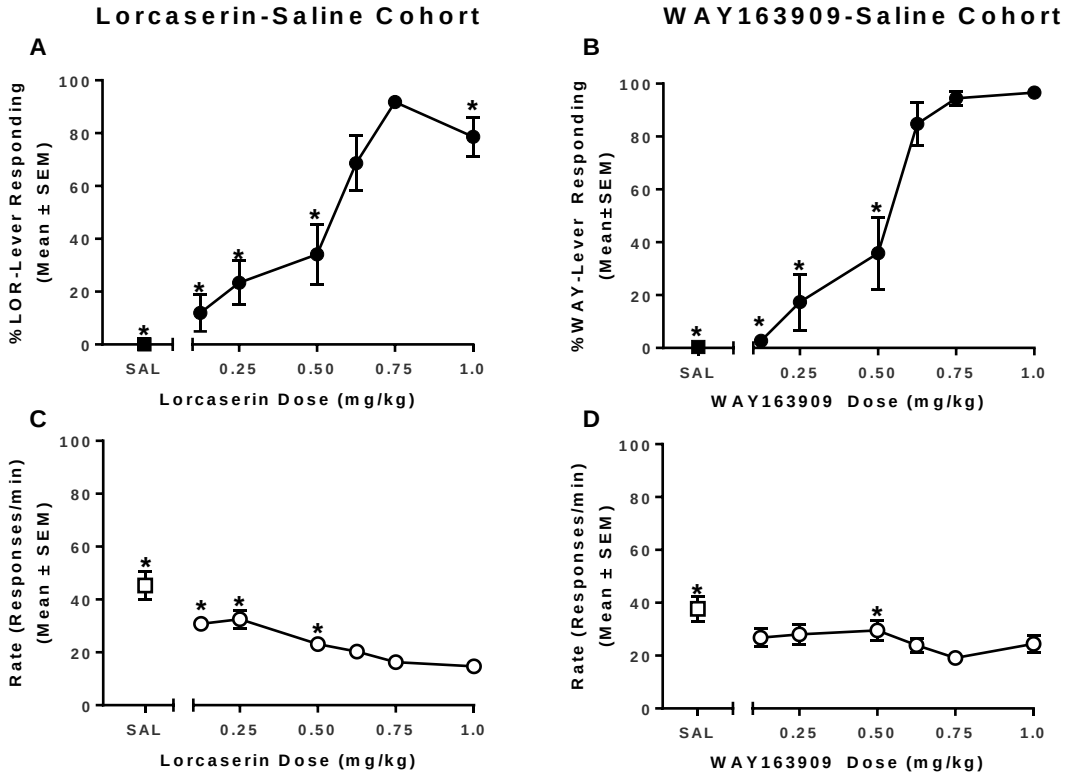


Figure 2.2: Lorcaserin and WAY163909 induce dose-dependent stimulus discrimination

Results of dose-response tests in (A) rats (n=14) trained to discriminate lorcaserin (LOR; 0.75 mg/kg) from saline, and (B) rats (n=12) trained to discriminate WAY163909 (WAY; 0.75 mg/kg) from saline. Closed symbols denote the mean (±SEM) percentage of drug-lever responding. C,D: open symbols denote the mean (±SEM) response rate per minute. For comparison, the percentage of drug-appropriate responding and response rate observed after saline tests are included (SAL: squares). *p<0.05 vs. previous drug maintenance session.

Substitution Tests in Lorcaserin-Saline and WAY163909-Saline Cohorts

We assessed whether **TAT-r3L4F** substitutes for either lorcaserin or WAY163909 as a measure of intrinsic 5-HT_{2C}R agonist activity *in vivo* (**Figure 2.3**). WAY163909 (0.75 mg/kg) induced a full substitution in the lorcaserin-saline trained cohort (**Figure 2.3A**) and suppressed response rates similar to the training drug (**Figure 2.3C**). Additionally, lorcaserin (1 mg/kg) induced a full substitution in the WAY163909-saline trained cohort (**Figure 2.3B**) and also suppressed response rates versus saline (**Figure 2.3D**). Given that lorcaserin and WAY163909 are similar in structure and have similar (but not identical) pharmacological properties (Dunlop et al., 2006; Thomsen et al., 2008), cross substitution analysis indicate that lorcaserin and WAY163909 induce similar interoceptive cues.

Pretreatment with **TAT-r3L4F** (1, 2 μmol/kg) resulted in <10% drug-like responding in either rats trained on lorcaserin (**Figure 2.3A**) or WAY163909 (**Figure 2.3B**), suggesting that **TAT-r3L4F** does not induce similar interoceptive cues associated with 5-HT_{2C}R agonists. Pretreatment with **TAT-r3L4F** (2 μmol/kg) resulted in response rates that were significantly higher than the rate on the previous lorcaserin maintenance session in the lorcaserin-saline cohort (with a trend for 1 μmol/kg **TAT-r3L4F**) while rates elicited by both tested doses of **TAT-r3L4F** were significantly higher than from the previous WAY163909 maintenance session in the WAY163909-saline cohort (**Figure 2.3C, 2.3D**). Thus, **TAT-r-3L4F** does not induce lorcaserin or WAY163909 stimulus generalization or reduction in response rates associated with the training drugs. These results are consistent with previously published cellular assays in which **h3L4F** lacks efficacy to induce 5-HT_{2C}R-associated intracellular signaling.

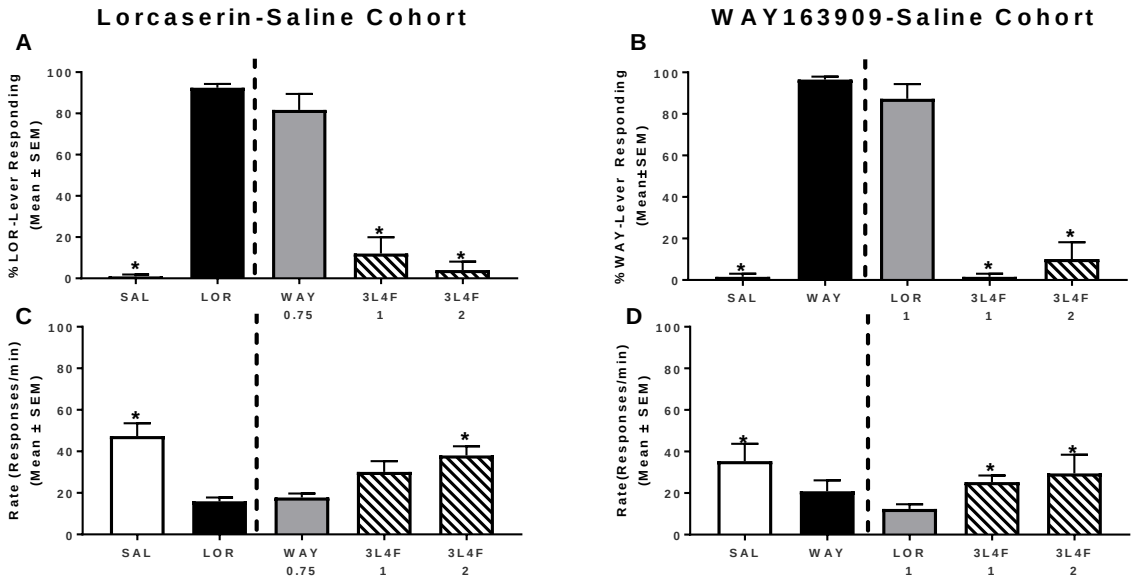


Figure 2.3: TAT-r3L4F does not induce lorcaserin- or WAY16390-associated interoceptive cues

Results of WAY163909 (WAY; 0.75 mg/kg), lorcaserin (LOR; 1 mg/kg) and TAT-r3L4F (3L4F; 1, 2 μmol/kg) substitution tests in lorcaserin-saline (A, C; n=13) and WAY163909-saline trained rats (B, D; n=10). A,B: Data represent the mean percentage of drug (A, lorcaserin: LOR; B, WAY163909: WAY)-lever responding (±SEM) observed during test sessions. C,D: Rate of response in tests depicted in Panels A and B. For comparison, the percentage of drug-appropriate responding and response rate observed after training drug (0.75 mg/kg; black bar) and saline (SAL; white bar) are presented left of the dashed line. *p<0.05 vs. previous drug maintenance session.

Combination Tests in Lorcaserin-Saline and WAY163909-Saline Cohorts

Combination tests were employed to assess the ability of **TAT-r3L4F** to potentiate 5-HT_{2C}R agonist-elicited interoceptive cues. The same doses of **TAT-r3L4F** (1, 2 μmol/kg) that did not substitute in either the lorcaserin, or WAY163909, versus saline discrimination, enhanced drug-lever responding when combined with lorcaserin (0.5 mg/kg; p<0.025; **Figure 2.4A**) or WAY163909 (0.5 mg/kg; p<0.025; **Figure 2.4B**) with no change in response rates (**Figure 2.4C, 2.4D**). These data suggest that disruption of the 5-HT_{2C}R:PTEN complex by **TAT-r3L4F** (Anastasio et al., 2013) enhances the interoceptive cues elicited by lorcaserin or WAY163909. These results are expected and

consistent with previous reports that **h3L4F** enhances 5-HT_{2C}R signaling *in vitro* and potentiates the behavioral effects of a selective 5-HT_{2C}R agonist *in vivo* (Anastasio et al., 2013; Ji et al., 2006).

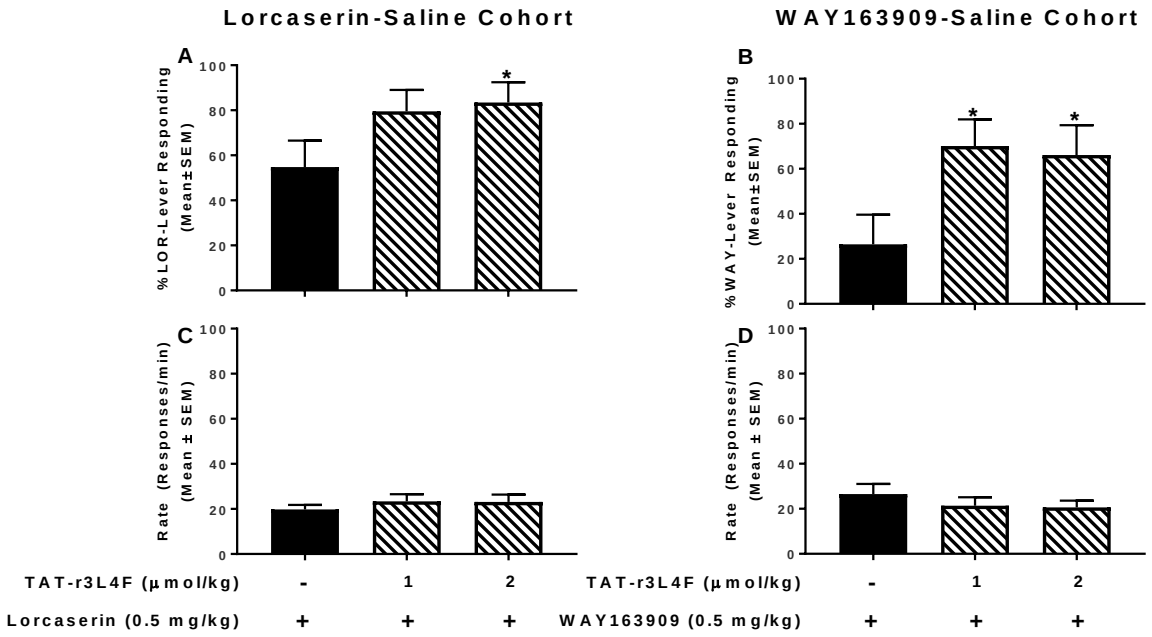


Figure 2.4: TAT-r3L4F potentiates lorcaserin and WAY163909 stimulus effects

Response on combination test of lorcaserin (0.5 mg/kg) and TAT-r3L4F (1, 2 µmol/kg) in lorcaserin-saline (A, C; n=14) and WAY163909-saline (B, D; n=11) trained rats. A,B: Data represent the mean percentage of drug (A, lorcaserin: LOR; B, WAY163909: WAY)-lever responding (±SEM) observed during test sessions. C,D: Rate of response in tests depicted in Panels A and B. **p*<0.025 vs. lorcaserin or WAY163909 (0.75 mg/kg; black bar).

This paradigm was then employed to explore the role of PLC_β in the effects of **TAT-r3L4F** to enhance the stimulus effects of 5-HT_{2C}R agonists. Stimulation of the 5-HT_{2C}R is known to recruit $G\alpha_q$-mediated signal transduction through PLC_β to result in Ca_i^{2+} release, but is also known to induce signaling in PLC_β-independent, and perhaps G protein-independent, mechanisms (Felder et al., 1990; Labasque et al., 2008). We hypothesized that lorcaserin and WAY163909, both of which are full agonists to induce Ca_i^{2+} release *in vitro*, may evoke their discrimination stimulus effects, in part, through PLC_β-dependent mechanisms. To test this hypothesis, we employed the PLC_β inhibitor U73122 which has

been shown to suppress PLC_{β}-mediated inositol phosphate and Ca_i^{2+} release in a variety of cells, including neurons (Jin et al., 1994), as well as suppress Ca_i^{2+} release elicited by 5-HT in 5-HT_{2C}R-expressing cells (Labasque et al., 2008). Additionally, systemic administration of U73122 in rats has been employed to assess the involvement of PLC_{β} in dopamine D_1 receptor locomotion in rats (Medvedev et al., 2013) Here, U73122 (0.5 mg/kg) evoked saline-appropriate responding in the lorcaserin and WAY163909 cohort (data not shown). In the lorcaserin-saline and the WAY163909-saline cohorts, U73122 (0.5 mg/kg) modestly, but not significantly, suppressed drug-lever responding evoked by the training dose (0.75 mg/kg; $p<0.05$; **Figure 2.5A,B**) with no change in response rate (data not shown). These results suggests that perhaps lorcaserin- and WAY163909-induced internal cues are mediated in part by PLC_{β}-dependent signaling, consistent with 5-HT_{2C}R actions through $G\alpha_q$ protein signaling *in vitro*. Higher doses of U73122 (0.75 and 1 mg/kg) dramatically reduced response rates when tested alone and in combination with lorcaserin (0.75 mg/kg) in the lorcaserin-saline trained cohort; thus, these doses were not pursued in subsequent tests (data not shown).

Interestingly, the full substitution elicited by **TAT-r3L4F** plus a low dose of lorcaserin in the lorcaserin-saline cohort (**Figure 2.5C**) and TAT-r3L4F plus a low dose of WAY163909 in the WAY163909-saline cohort (**Figure 2.5D**) is insensitive to PLC_{β} inhibition, unlike the full substitution elicit by the respective training drug alone. This finding may suggest that **TAT-r3L4F**-mediated potentiation of lorcaserin- or WAY163909-elicited interoceptive cues may involve PLC_{β}-, and perhaps $G\alpha_q$ protein-, independent mechanism, suggesting modulation of an intracellular signaling pathway by

this protein-protein interaction distinct from a full 5-HT_{2C}R agonist. Future studies *in vitro* and *in vivo* are needed to support this interpretation.

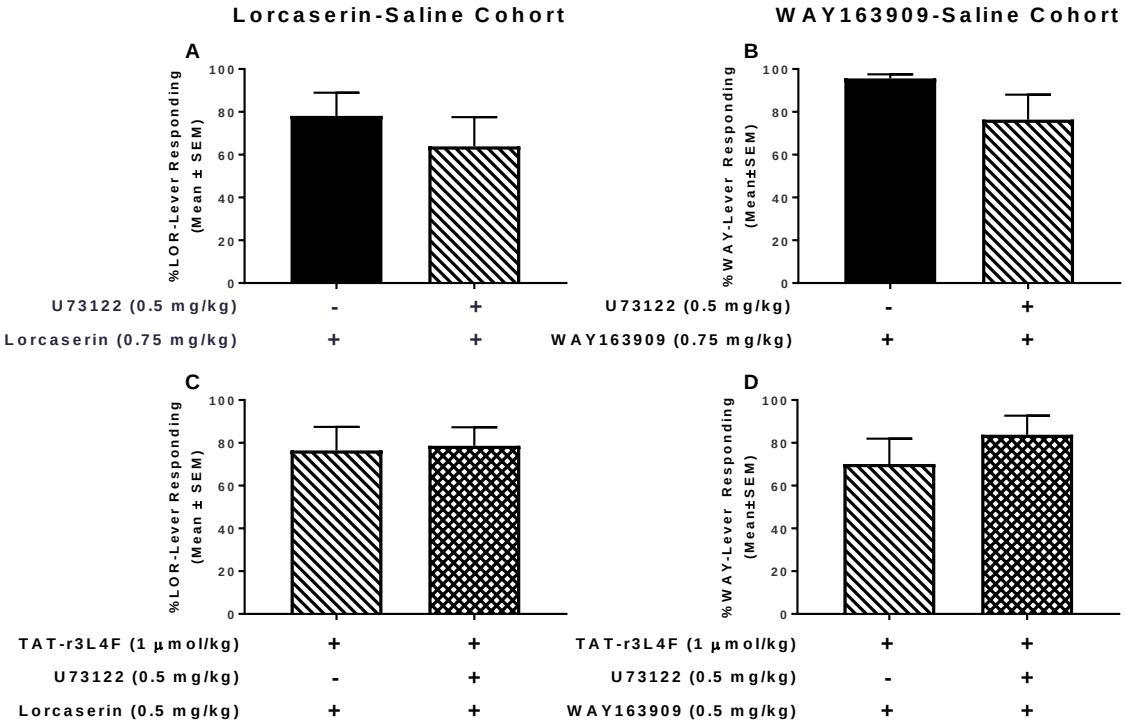


Figure 2.5: U73122 suppresses full substitution in the absence, but not presence, of TAT-r3L4F

Results of indicated combination tests in lorcaserin-saline (A, C; n=12) and WAY163909-saline (B, D, n=11) trained rats. Data represent the mean percentage of drug (A,C lorcaserin: LOR; B,D WAY163909: WAY)-lever responding (±SEM) observed during test sessions. Dashed line represents criterion for full substitution. *p<0.05 vs. lorcaserin (0.75 mg/kg). Response rates were not significantly different (data not shown)

Selective 5-HT_{2C}R agonists suppress behaviors associated with drugs of abuse (for review, (Cunningham and Anastasio, 2014). One of these behaviors is the suppression of the stimulus effects of the psychostimulant cocaine (Callahan and Cunningham, 1995; Filip and Cunningham, 2002; Frankel and Cunningham, 2004). Cocaine produces robust subjective effects that contribute to its abuse (Kleven et al., 1990) and, as such, therapeutics that suppress the stimulus effects of cocaine may have important clinical implications. Here, we hypothesize that **TAT-r3L4F** will potentiate selective 5-HT_{2C}R agonist-induced suppression of the stimulus effects of cocaine. To test this, we trained a third cohort of rats to discriminate cocaine (5 mg/kg) from saline.

Cocaine-Saline Discrimination

All rats (n=12) acquired the discrimination of cocaine (5 mg/kg; 15 min pretreatment) versus saline within an average of 36 two-lever training sessions (range 32-42); response rates after cocaine (25.6 ± 0.55/min) were not statistically different from rates after saline (27.2 ± 1.3/min). During dose-response tests, cocaine (0.313 – 5 mg/kg) produced a dose-dependent increase in cocaine-appropriate responding (**Figure 2.6A**). Saline (**Figure 2.6A**, SAL) engendered <10% cocaine-lever responding. The mean response rates after cocaine (0.313 – 5 mg/kg) did not differ significantly from those on the previous cocaine maintenance session (p>0.05; **Figure 2.6B**).

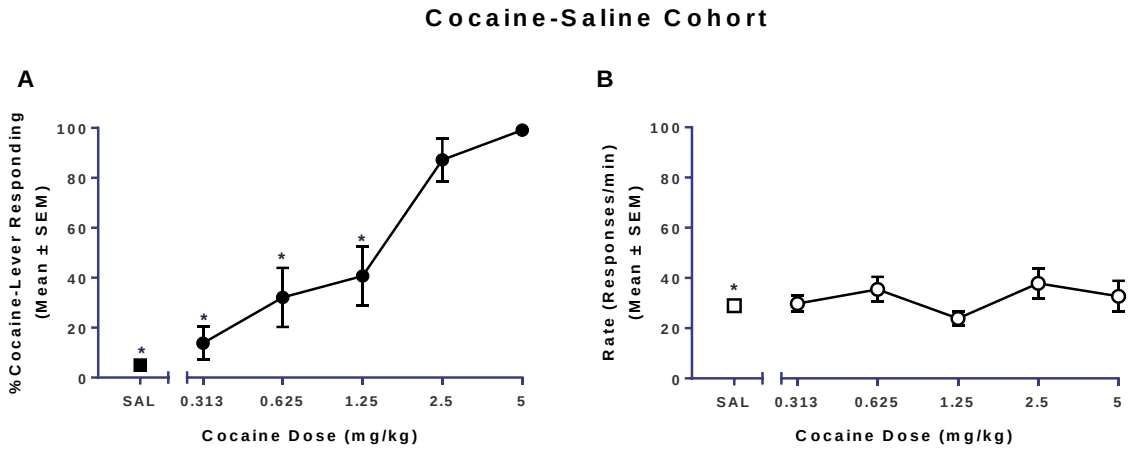


Figure 2.6: Cocaine induces dose-dependent stimulus discrimination

Results of dose-response tests in rats (n=16) trained to discriminate cocaine (COC; 5 mg/kg) from saline. (A) Closed symbols denote the mean (±SEM) percentage of cocaine-lever responding; (B) open symbols denote the mean (±SEM) response rate per minute. For comparison, the percentage of cocaine-appropriate responding and response rate observed after saline test are included (SAL: squares). *p<0.05 vs. previous cocaine maintenance session.

Substitution and Combination Tests in Cocaine-Saline Cohort

We confirmed previous studies that showed that 5-HT_{2C}R agonists suppress cocaine stimulus effects (Callahan and Cunningham, 1995; Filip and Cunningham, 2002; Frankel and Cunningham, 2004) by testing lorcaserin in the presence versus absence of selective 5-HT_{2C}R antagonist SB242084. As expected, both lorcaserin (1 mg/kg) and SB242084 (0.5 mg/kg) engendered primarily saline-lever responding indicating that neither drug substituted for cocaine (**Figure 2.7A**). Lorcaserin (1 mg/kg) produced a significant reduction in response rate versus previous cocaine maintenance session rates, while SB242084 (0.5 mg/kg) produced a slight, but significant increase in response rate ($p<0.05$; **Figure 2.7C**). Two rats failed to complete the FR20 schedule after administration of lorcaserin (1 mg/kg) alone, indicating that this dose induces behavioral disruption.

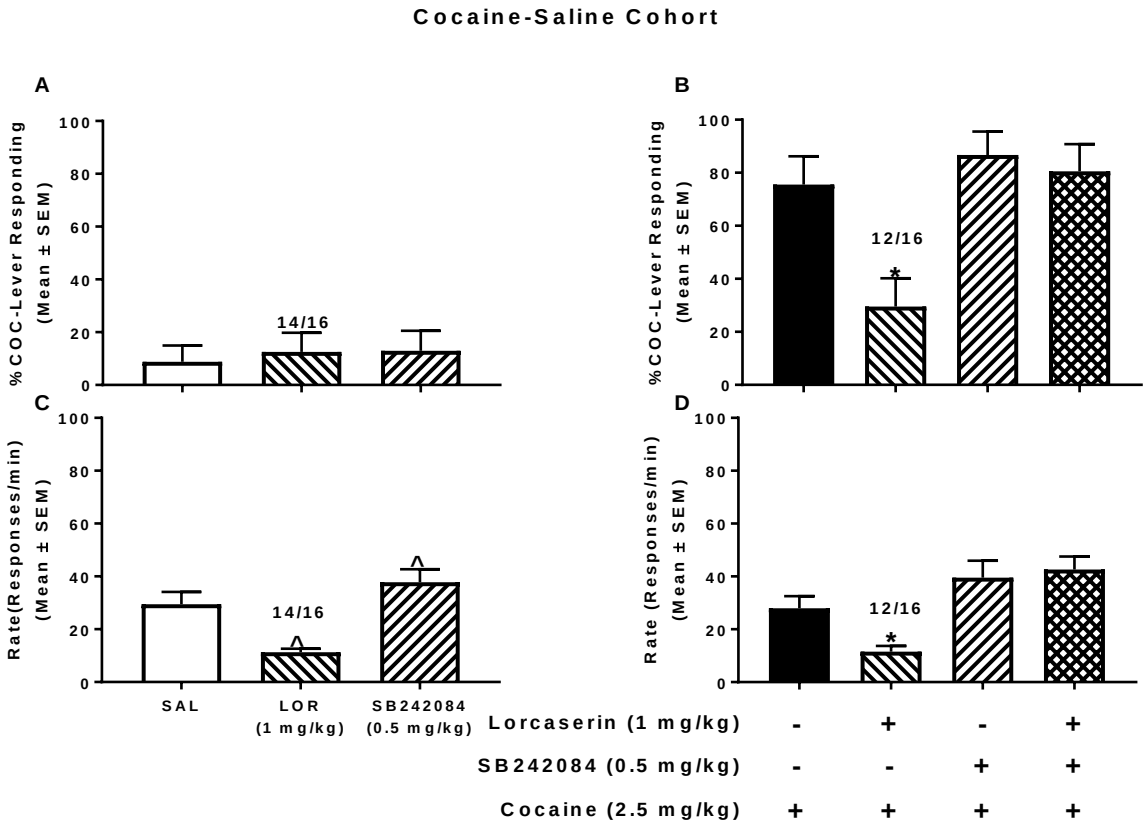


Figure 2.7: Lorcaserin suppresses cocaine stimulus effects which is reversed by SB242084 treatment

Results of substitution tests (A,C) with saline (1 ml/kg), lorcaserin (1 mg/kg), and SB242084 (0.5 mg/kg) and combination tests (B,D) with cocaine (2.5 mg/kg), lorcaserin (1 mg/kg) and SB242084 (0.5 mg/kg). A,B: Data represent the mean percentage of cocaine (COC)-lever responding (±SEM) observed during indicated test session. C,D: Rate of response following tests depicted in Panels A and B. *n/N* indicates the number of rats completing the fixed ratio 20 versus the number of rats tested. ^$p<0.05$ vs. previous cocaine maintenance session; *$p<0.017$ vs. cocaine (2.5 mg/kg); n=16 rats unless otherwise indicated.

A full substitution of 2.5 mg/kg of cocaine was observed (**Figure 2.7B**). Pretreatment with lorcaserin (1 mg/kg) significantly reduced cocaine (2.5 mg/kg) lever-appropriate responding ($p<0.017$; **Figure 2.7B**) and response rates ($p<0.017$; **Figure 2.7D**) consistent with previously reports that 5-HT_{2C}R agonists suppress the stimulus effects of cocaine (Callahan and Cunningham, 1995; Filip and Cunningham, 2002; Frankel and Cunningham, 2004). Pretreatment with SB242084 (0.5 mg/kg) did not alter cocaine (2.5

mg/kg) lever-responding (**Figure 2.7B**) or response rate (**Figure 2.7D**). The lorcaserin-mediated suppression of cocaine (2.5 mg/kg) of lever responding and response rate was fully recovered by SB242084 pretreatment (**Figure 2.7B, 2.7D**), further supporting that lorcaserin suppresses the stimulus effects of cocaine and response rate via 5-HT_{2C}R agonism.

We then investigated whether **TAT-r3L4F** pretreatment would synergize with an ineffective dose (0.5 mg/kg) of lorcaserin to suppress the stimulus effects of cocaine. Of note, in substitution tests, **TAT-r3L4F** (1, 2 μmol/kg), lorcaserin (0.5 mg/kg) and the combination of **TAT-r3L4F** (1, 2 μmol/kg) *plus* lorcaserin (0.5 mg/kg) evoked saline-appropriate responding and significant reduction in response rates versus the previous cocaine maintenance session (data not shown). Cocaine (2.5 mg/kg) evokes full substitution which is not suppressed by lorcaserin (0.5 mg/kg; **Figure 2.8A**) indicating that at this dose, lorcaserin does not suppress the stimulus effects of cocaine. Excitingly, pretreatment with the combination of **TAT-r3L4F** (1, 2 μmol/kg) with lorcaserin (0.5 mg/kg) significantly reduced cocaine-appropriate responding versus cocaine (2.5 mg/kg) alone ($p<0.01$; **Figure 2.8A**) suggesting that **TAT-r3L4F** enhances the efficacy of lorcaserin to suppress the stimulus effects of cocaine. However, the triple combination of **TAT-r3L4F** (1, 2 μmol/kg) *plus* lorcaserin (0.5 mg/kg) *plus* cocaine (2.5 mg/kg) is not statistically different from lorcaserin (0.5 mg/kg) *plus* cocaine (2.5 mg/kg). Lorcaserin (0.5 mg/kg) and the combination of **TAT-r3L4F** (1, 2 μmol/kg) *plus* lorcaserin (0.5 mg/kg) significantly suppressed the response rates associated with cocaine (2.5 mg/kg; $p<0.01$; **Figure 2.8C**). Of note, treatment with **TAT-r3L4F** (1 or 2 μmol/kg) alone did not alter the stimulus effects of cocaine (2.5 mg/kg; **Figure 2.8B**) or the associated response rate

(**Figure 2.8D**) in the absence of lorcaserin. The lack of effect with **TAT-r3L4F** treatment in the absence of lorcaserin is not unexpected given that **TAT-r3L4F** is not predicted to act as a 5-HT_{2C}R agonist, but rather potentiate the effects of 5-HT_{2C}R activation. However, given that cocaine enhances the levels of endogenous 5-HT, it could be hypothesized that **TAT-r3L4F** treatment might suppress the stimulus effects of cocaine by enhancing endogenous 5-HT-induced 5-HT_{2C}R signaling. One potential explanation for failure to see this outcome may be that the levels of endogenous 5-HT generated by cocaine exposure under the employed conditions are not sufficient for **TAT-r3L4F** to potentiate, which is consistent with **h3L4F** inability to potentiate low 5-HT concentrations in cellular assays (Anastasio et al., 2013) (and see **Figure 2.11**).

Overall, these experiments provide further evidence that **TAT-r3L4F** enhances 5-HT_{2C}R agonist-mediated effects *in vivo.* Although the magnitude of **TAT-r3L4F** effects in these data is modest, **TAT-r3L4F** effects are reproducible in rats trained to discriminate the interoceptive cues of two selective 5-HT_{2C}R agonists. The modest effect size may be attributable to poor bioavailability and peptide distribution and perhaps more robust effects could be attained with 5-HT_{2C}R:PTEN complex disrupters with more favorable pharmacokinetic properties. In an effort to achieve this goal, we next developed constrained peptide derivatives via cyclization of the peptide **2** sequence as well a replacement of peptide backbone with a rigid, non-peptide linker. These constrained peptide derivatives are expected to enhance pharmacodynamics profile of 3L4F by reducing the potential for peptide proteolysis, increase membrane penetration and decrease renal clearance (Adessi and Soto, 2002; Di, 2015; Gongora-Benitez et al., 2014). We began

with peptide **2** (**Figure 2.1**), an eight amino acid derivative of **h3L4F**, as the scaffold due to its retention of activity *in vitro* (Anastasio et al., 2013).

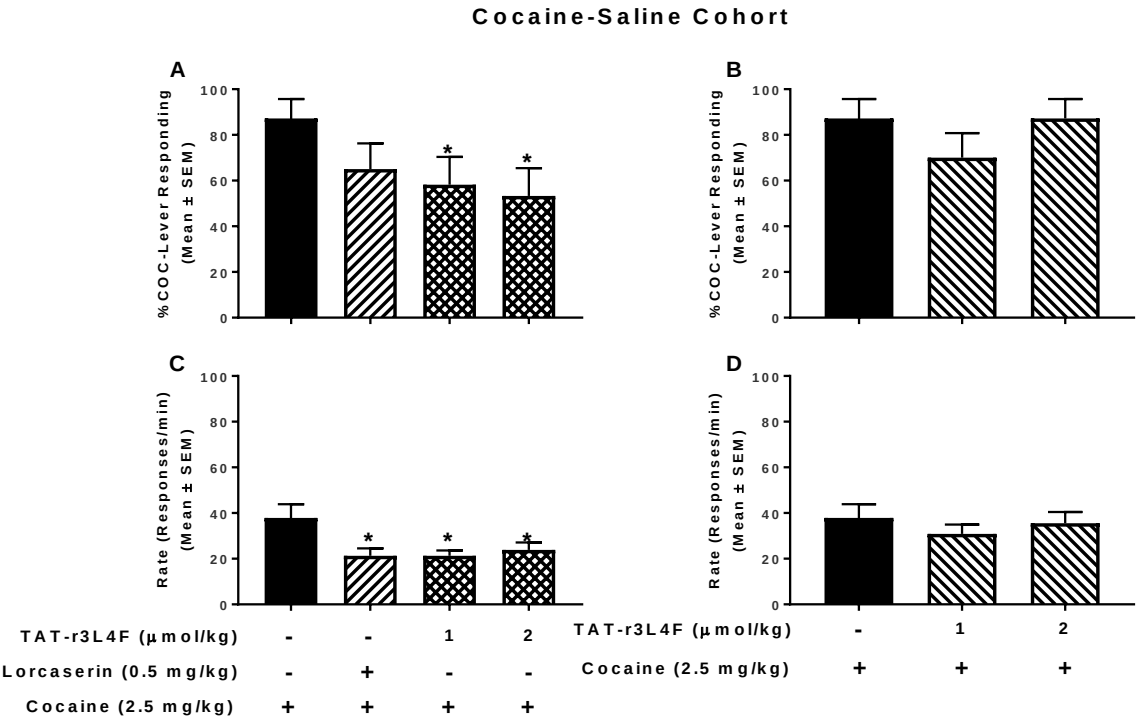


Figure2.8: TAT-r3L4F enhances lorcaserin-induced suppression of cocaine stimulus effects

Results of combination tests with cocaine (2.5 mg/kg), lorcaserin (0.5 mg/kg) and TAT-r3L4F (1, 2 µmol/kg). A,B: Data represent the mean percentage of cocaine (COC)-lever responding (±SEM) observed during indicated combination test session. C,D: Rate of response following combination tests depicted in Panels A and B. *p<0.01 vs. cocaine (2.5 mg/kg); n=16 rats.

Alanine Scan

The initial step in the generation of cyclized and peptidomimetic derivatives was to identify the amino acid residues that could be modified without loss of *in vitro* activity. Each amino acid in the peptide **2** sequence was sequentially replaced with an alanine which resulted in seven alanine peptide analogs, since amino acid 7 is already an alanine (**Figure 2.9A**). Alanine replacement was used to determine the amino acid side chains critical for the activity of the peptide.(Morrison and Weiss, 2001) Thus, by replacing a critical amino

acid with an alanine, the peptide analog is predicted to lose activity, while replacement of amino acids necessary to retain the conformation of the peptide will have little to no effect on the activity of the analog. The ability of these alanine analogs to potentiate 5-HT-induced intracellular calcium (Ca_i^{2+}) release was tested in h5-HT_{2C}R-CHO cells. Serotonin induces a concentration-dependent increase in Ca_i^{2+} release (pEC_{50} = 8.1 ± 0.1, EC_{50} = 7.8 nM). Pretreatment with **h3L4F** or peptide **2** elevates the maximum Ca_i^{2+} release induced by 5-HT by ~30% and ~20% respectively ($p<0.05$; **Figure 2.9B**; black bars), with no change in EC_{50} versus 5-HT alone (data not shown). As shown in **Figure 2.9B**, analogs with an alanine replacement in positions 3-6 retain significant potentiation of 5-HT-induced Ca_i^{2+} release ($p<0.05$; **Ala$_3$**, E_{MAX} = 118 ± 3.8; **Ala$_4$**, E_{MAX} = 121 ± 2.0, **Ala$_5$**, E_{MAX} = 122 ± 2.7; **Ala$_6$**, E_{MAX} = 132 ± 6.4) that is comparable to the parent peptides **h3L4F** (E_{MAX} = 131 ± 6.3) and **1** (E_{MAX} = 118 ± 4.0). This suggests that the Q-D-Q-N sequence in the middle portion of the peptide can be manipulated without loss of activity. Analogs **Ala$_1$** (E_{MAX} = 125 ± 9.3), **Ala$_2$** (E_{MAX} = 118 ± 6.8) and **Ala$_8$** (E_{MAX} = 131 ± 17) exhibited a marked increase in variability from assay to assay and did not significantly potentiate 5-HT-induced Ca_i^{2+} release ($p>0.05$). Based on these results, these residues were not manipulated in subsequent modifications.

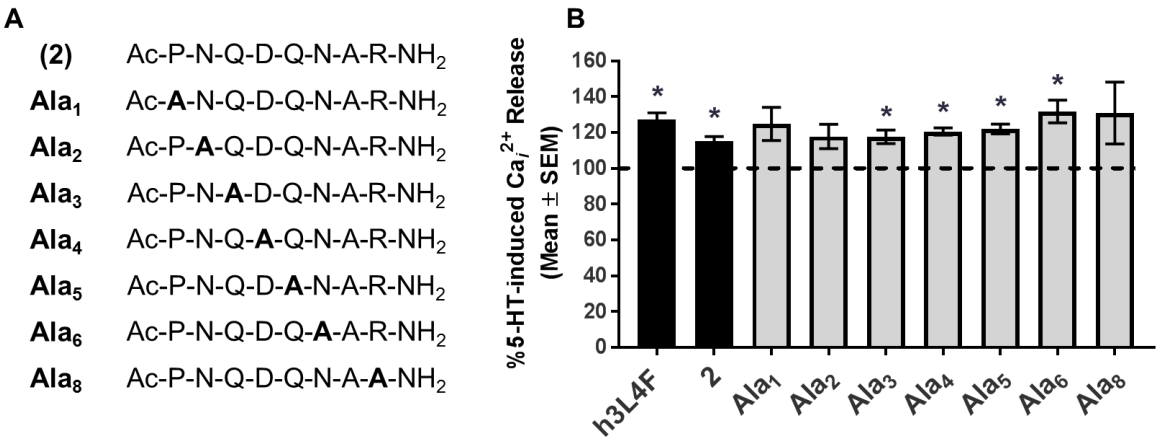


Figure 2.9: Results from alanine scan of peptide 2

Alanine derivatives of peptide 1 (A) were tested in Ca_i^{2+} release assay in live h5-HT_{2C}R-CHO cells in the presence of 5-HT (B). The maximum 5-HT-induced Ca_i^{2+} release in the absence of the compounds was set as 100% (dashed line). Bars represent the average E_{MAX} produced by 1 nM alanine derivative in the presence of 5-HT, shown as mean ± SEM of 4-5 biological replicates. *$p<0.05$ vs. 5-HT alone (dashed line).

Based on the results from the alanine screen, and previous work in which modeling of peptide **2** indicated a potential preference for a turn-type conformation,(Anastasio et al., 2013) peptide derivatives of **2** shown in **Figure 2.10** were synthesized. The head to tail cyclized versions (**4** and **5**) were designed to improve the stability of the molecules, limit their conformational flexibility and increase their potency over the linear peptide precursor.(Venkatesan and Kim, 2002) Peptide derivative **6** was cyclized through the side chains in an *i, i+2* orientation in order to mimic a turn-type conformation. Peptide derivative **7** was designed to be a peptide turn-mimic in which the pyrrolidine-3,4-dicarboxamide replaced the middle four amino acids, but retained the necessary orientation for the amino acids at the N and C terminal ends.(Whitby et al., 2011) In addition to increasing *in vitro* stability, these constraints should limit the number of peptide conformations available and may affect the activity of the peptide.

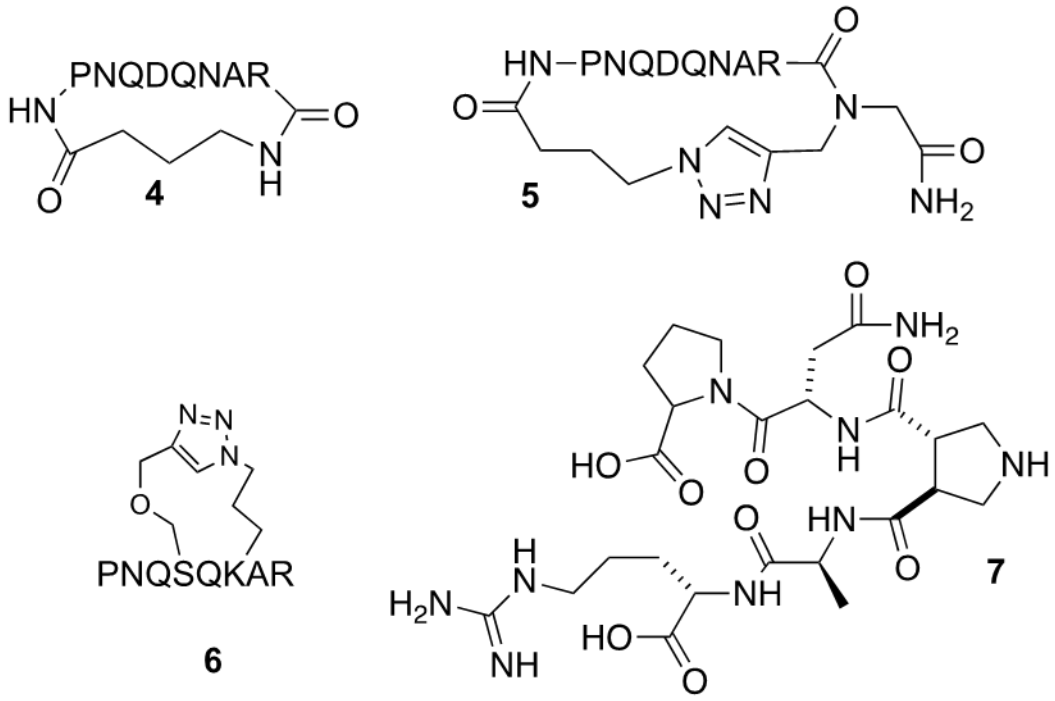
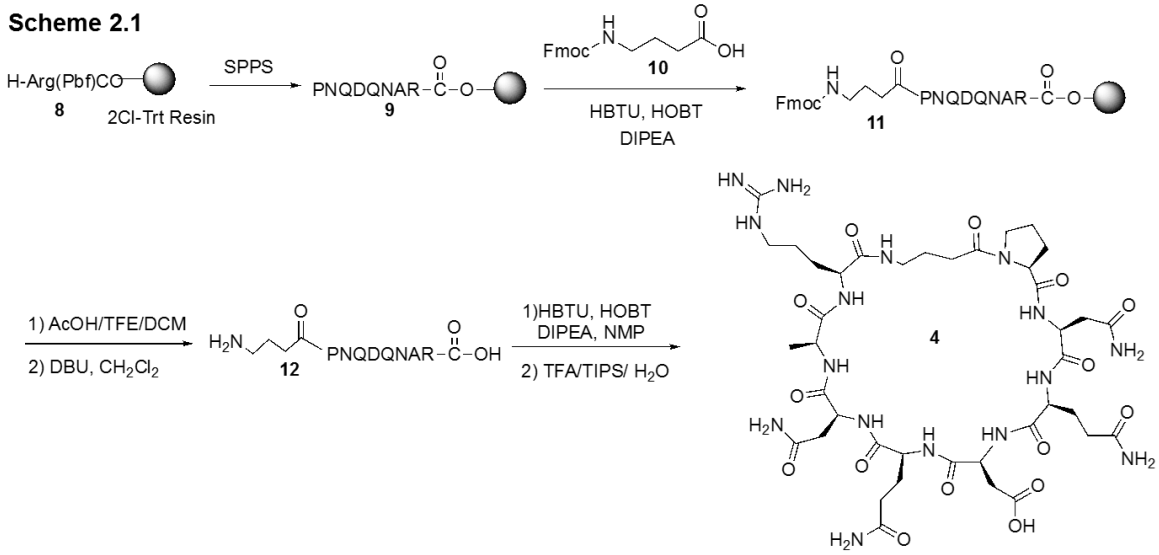


Figure 2.10: Modified derivatives of peptide 2

Synthesis of Peptide Derivatives 4, 5 and 6

The amide cyclized peptide derivative **4** was synthesized starting with a linear precursor with side chains protected but a free amine and acid in the N- and C-terminus (**Scheme 2.1**). The linear peptide was synthesized by using an arginine preloaded 2-chlorotrityl chloride (2-CTC) resin, which can be cleaved under mild acid conditions without deprotecting the peptide side chains. After standard peptide coupling to provide the sequence, Fmoc-4-amino butyric acid (**10**) was added to the amino end of the linear peptide. Cleavage from the polymer resin was accomplished by treating with 10% of AcOH in CH_2Cl_2 followed by the removal of Fmoc from the amino butyric acid end in the presence of DBU in CH_2Cl_2. The key intramolecular macrocyclization was performed under high dilution conditions using HBTU/HOBt as the coupling reagents and DIPEA as the base. The side chain protecting groups (Trt, ^{t}Bu, and Pbf groups) were then removed in a single step with TFA solution (TFA/TIPS/H_2O 95:2.5:2.5) to give cyclized peptide

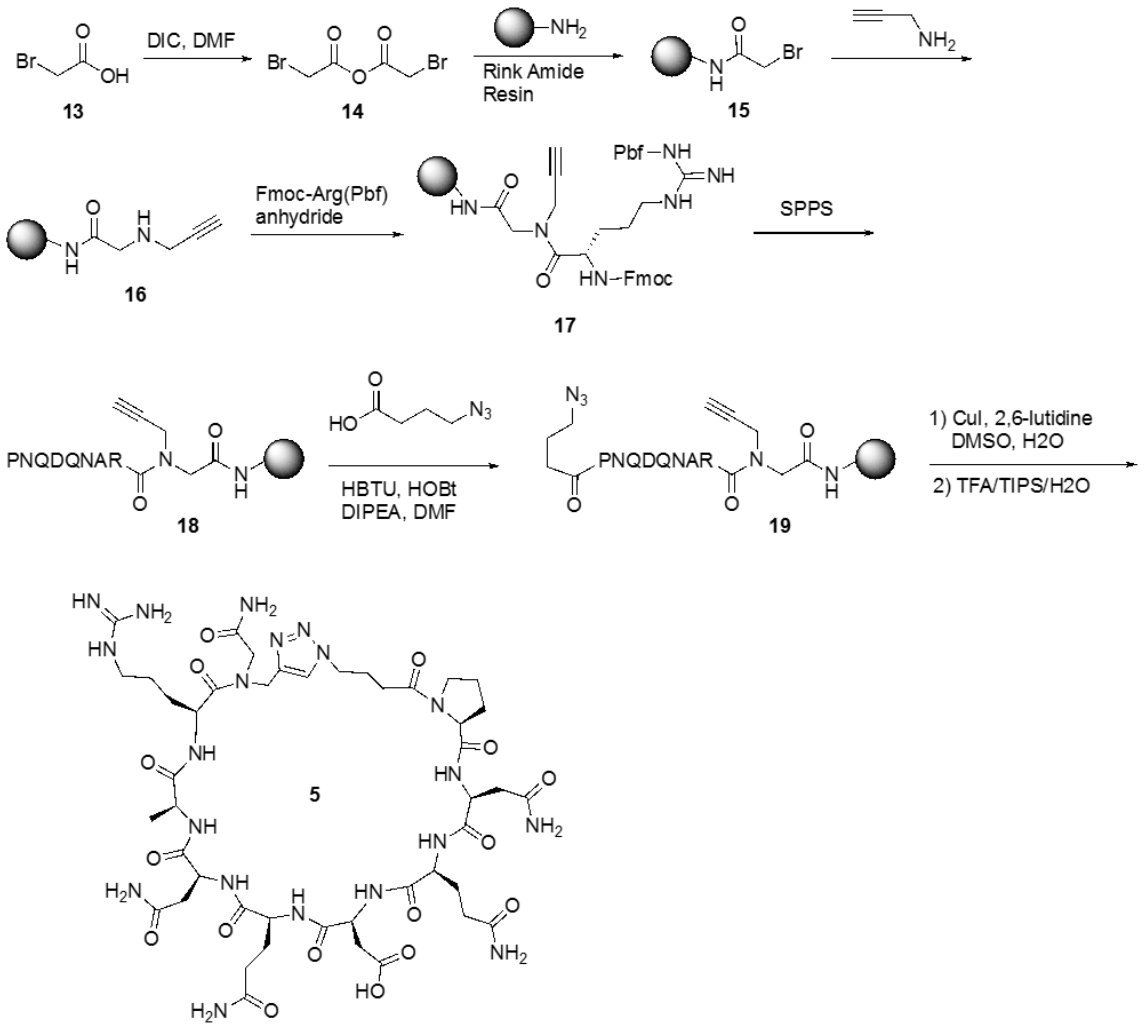
derivative **4**. The crude material was purified by reverse-phase HPLC to furnish pure cyclic peptide in 13% yield based on the initial resin loading.



Scheme 2.1: Synthesis of cyclic peptide derivative 4

Standard amide formation and copper catalyzed azide-alkyne cycloaddition (CuAAC) were used to perform cyclizations in the approach to **5** and **6**. The cyclic peptide **5** was cyclized by formation of a 1,2,3-triazole ring (**Scheme 2.2**) (Ingale and Dawson, 2011; Jagasia et al., 2009). The synthesis was started with the reaction of bromoacetic anhydride with Rink amide resin to afford **15**. The bromide was then substituted by propargyl amine to incorporate the necessary alkyne for triazole ring formation (**16**). The first amino acid, Arg, was coupled to the resultant secondary amine by using a presynthesized symmetrical anhydride of arginine. After the incorporation of the necessary amino acids by solid phase peptide synthesis, the azide was introduced by the attachment of 4-azidobutyric acid. The cyclization was carried out through an on-resin strategy in the presence of Cu(I) catalyst and 2,6-lutidine in DMSO. The cyclic peptide was then cleaved from the resin to give **5** in 40% yield, based on the initial loading of the resin.

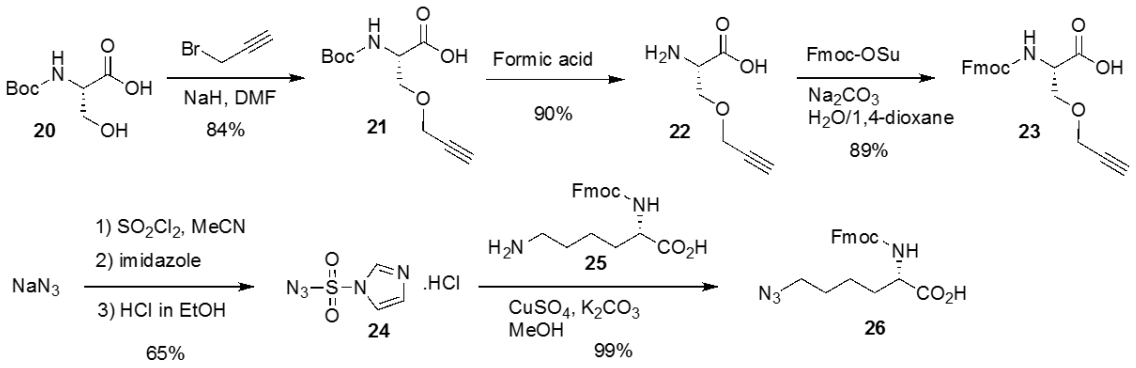
Scheme 2.2

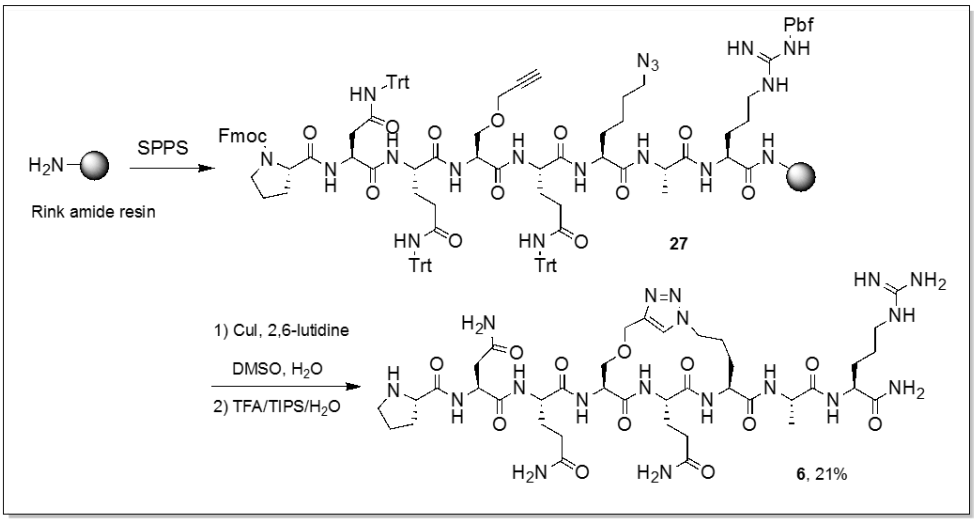


Scheme 2.2: Synthesis of cyclic peptide derivative 5

The synthesis of **6,** the peptide derivative linked through side chains in *i*, *i+2* positions, was perform by using similar procedure as utilized for **5**, with two presynthesized alkyne and azide moieties (**Scheme 2.3**) incorporated in the peptide sequence. After linear precursor **27** was generated, the cyclic peptide derivative **6** was obtained on the resin by Cu(I) catalyzed cycloaddition followed by subsequent TFA deprotection and cleavage (**Scheme 2.3**).

Scheme 2.3





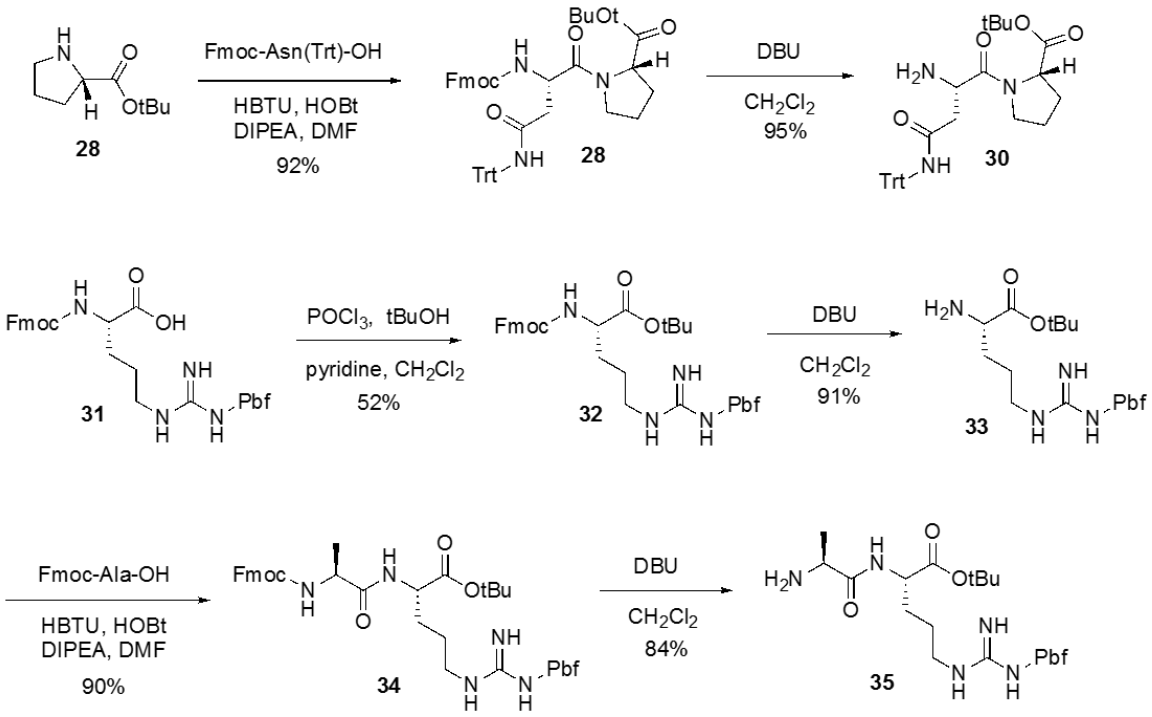
Scheme 2.3: Synthesis of side chain-to-side chain cyclized peptide derivative 6

Synthesis of Turn Mimic 7

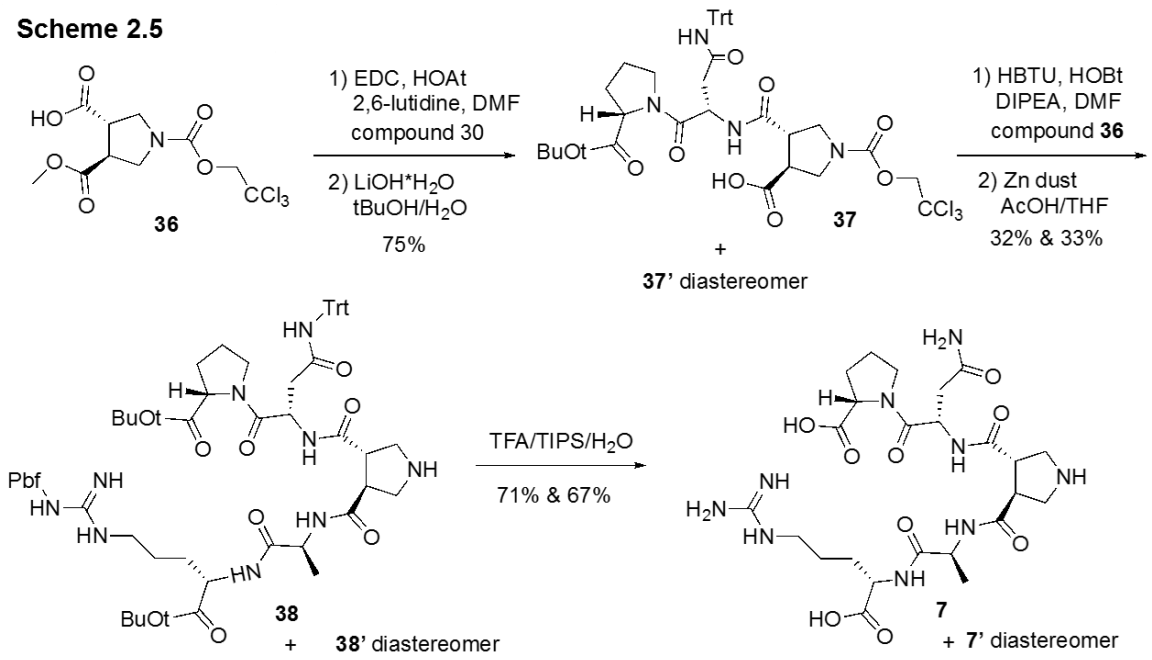
The synthesis of the pyrrolidine-3,4-dicarboxamide was carried out according to the report of Boger (Whitby et al., 2011). With this *β*-turn template **36** in hand, we moved our synthetic efforts to the preparation of peptide fragments, which were installed on the template. In order to achieve the maximum similarity of the 3L4F sequence, two amino acid residues of each (N- and C-) terminus were preserved in the synthesis (**Scheme 2.4**). The first fragment was synthesized by coupling the proline *tert*-butyl ester amine with Fmoc-Asn(Trt)-OH followed by removal of Fmoc to yield amine **30** in 87% yield. The fragment for the other position on the pyrrolidine was obtained by esterification of

commercial Fmoc-Arg(Pbf)-OH (**31**) with ^{t}BuOH in the presence of $POCl_3$ and pyridine (52% yield) followed by deprotection of N-terminal Fmoc with DBU (91% yield). Amide formation with alanine, and deprotection provided dipeptide **35** in 76% yield (**Scheme 2.4**). Dipeptide **30** was coupled to **36** (**Scheme 2.5**) by reaction with HOAt and the subsequent acid hydrolysis produced the diastereomers **37** and **37'** (75% yield). After addition of dipeptide **35** and separation of the diastereomers, the removal of Troc group was conducted via EDC/HOAt and zinc dust to give **38** and **38'** in 32% and 33% yield, respectively. A solution of TFA cocktail solution was used to remove all other protecting groups in one step to give the crude material by cold ether precipitation. Finally, the diastereomeric β-turn peptidomimetics **7** and **7'** were obtained (71% and 67% yields) after the preparative HPLC system purification.

Scheme 2.4



Scheme 2.4: Synthesis of intermediates for turn mimic peptide derivative 7



Scheme 2.5: Synthesis of turn mimic peptide derivative 7

We tested the ability of these molecules to potentiate 5-HT-induced 5-HT_{2C}R signaling in the Ca_i^{2+} release assay (**Table 2.1; Figure 2.11**). Derivative **4** (E_{MAX} = 128 ± 7.4; $p<0.05$), a head-to-tail cyclized version of **2**, results in potentiation of 5-HT induced Ca_i^{2+} release that is comparable to the parent peptides **h3L4F** (**1**) and peptide **2**. Interestingly, **5** (E_{MAX} = 107 ± 5.2), another head-to-tail cyclized peptide and its linear analog **19** (E_{MAX} = 109 ± 12), did not potentiate 5-HT induced Ca_i^{2+} release, suggesting that these modifications may not allow the peptide to disrupt the 5-HT_{2C}R:PTEN complex.

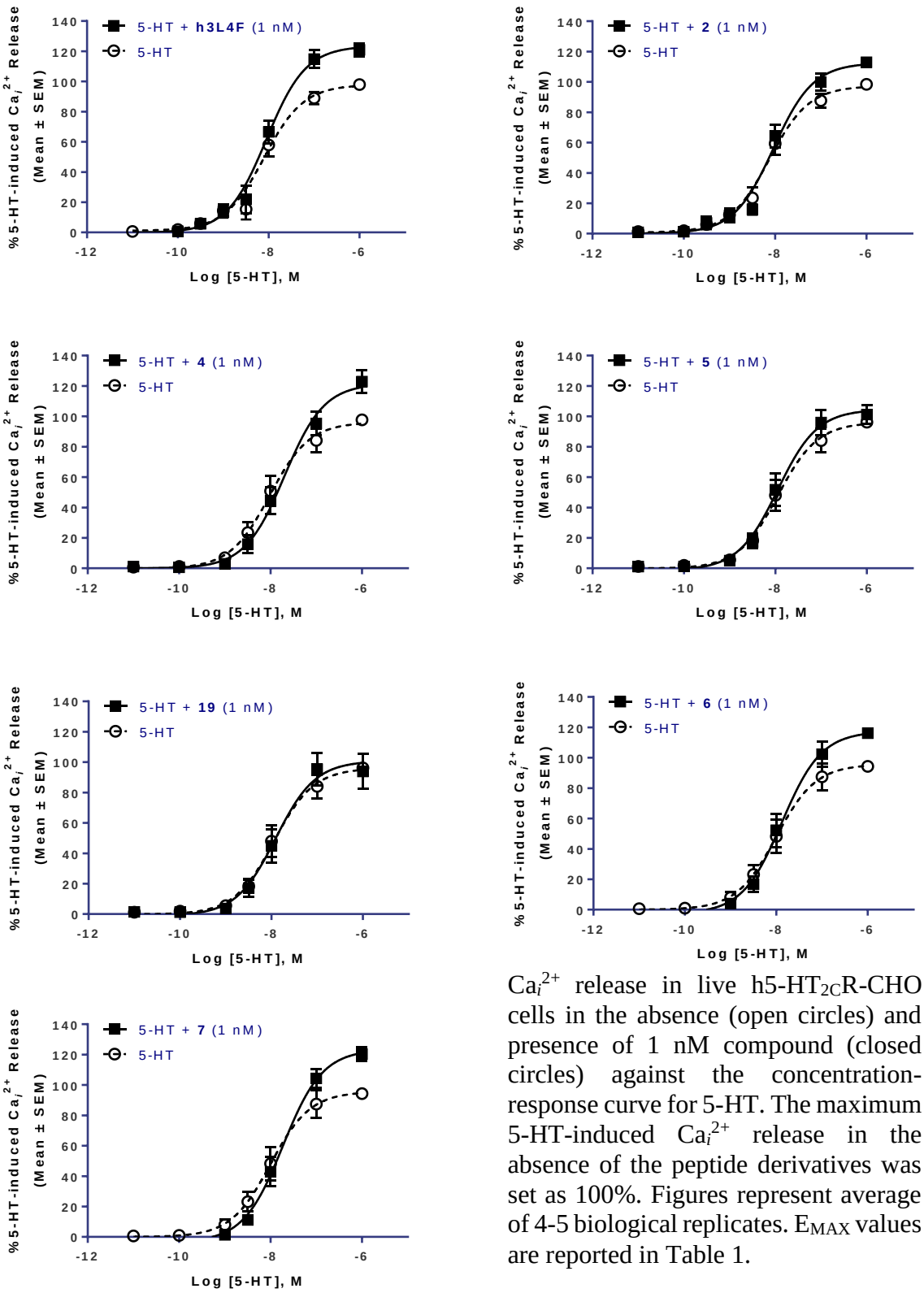
The side chain-to-side chain cyclized peptide derivative **6** (E_{MAX} = 120 ± 3.0; $p<0.05$) retained activity in the Ca_i^{2+} assay. The peptidomimetic derivative **7** (E_{MAX} = 125 ± 5.2; $p<0.05$) also potentiated 5-HT induced Ca_i^{2+} release which is consistent with the hypothesis from the alanine scan that amino acids 3-6 are not necessary for retention of peptide activity. Furthermore, activity of **6** and **7** suggests that a β-turn conformation in the middle portion of the peptide may facilitate disruption of the 5-HT_{2C}R:PTEN complex. Of

note, none of the peptides alter the 5-HT potency in this assay (**Figure 2.11**). Importantly, these results demonstrate that activity *in vitro* and, presumably, disruption of the 5-HT_{2C}R:PTEN complex is retained after peptide **2** modifications predicted to enhance peptide pharmacokinetic properties.

Table 2.1: Effects of h3L4F and peptide 2 derivatives on 5-HT-induced Ca_i^{2+} release in h5-HT_{2C}R-CHO cells

Peptide Derivative ID	E_{MAX} (%5-HT)[a]	p-Value
h-3L4F	131 ± 6.3%	p=0.038
2	118 ± 4.0%	p=0.012
4	128 ± 7.4%	p=0.020
5	107 ± 5.2%	p=0.281
19	109 ± 12%	p=0.497
6	120 ± 3.0%	p=0.003
7	125 ± 5.2%	p=0.008

[a] Maximum 5-HT-induced Ca_i^{2+} release (E_{MAX}) in the presence of 1 nM of test peptide derivative; the screen utilized concentrations of 5-HT (vehicle, [10^{-11}] to [10^{-6} M]) to establish the E_{MAX} of 5-HT in the presence of the test peptide derivative as described in methods. Statistical analyses were conducted using the unpaired t test with Welch's correction. The criterion for statistically significant difference was set at 0.05. Data represented as mean ± SEM of 4-5 biological replicates run in technical triplicates.



Ca_i^{2+} release in live h5-HT$_{2C}$R-CHO cells in the absence (open circles) and presence of 1 nM compound (closed circles) against the concentration-response curve for 5-HT. The maximum 5-HT-induced Ca_i^{2+} release in the absence of the peptide derivatives was set as 100%. Figures represent average of 4-5 biological replicates. E_{MAX} values are reported in Table 1.

Figure 2.11: Derivatives of peptide 2 potentiate Ca_i^{2+} release in h-5-HT$_{2C}$R expressing cells

Lastly, the ability of **h3L4F** (**1**), peptide **2** and derivatives **4-7** to suppress PTEN lipid phosphatase activity was assessed. PTEN, a dual phosphatase that contains distinct lipid and protein phosphatase functions, is proposed to regulate 5-HT_{2C}R signaling through the protein phosphatase activity (Ji et al., 2006) and thus these peptide derivatives are not expected to disrupt the lipid phosphatase activity of PTEN. However, it is essential to confirm that ligands predicted to interact with PTEN do not suppress the lipid phosphatase activity due to the potential for carcinogen properties. The lipid phosphatase function of PTEN is critical for the suppression of the AKT-mediated cell proliferation pathway by dephosphorylation PI(3,4,5)P_3 to PI(4,5)P_2 (Maehama and Dixon, 1998; Stambolic et al., 1998) and thus PI(4,5)P_2 was used as an output measure for the assessment of PTEN lipid phosphatase activity in a competitive ELISA-based assay. Recombinant PTEN (Echelon Biosciences) was incubated with PTEN inhibitor SF1670 (200 μM) or **h3L4F** and peptide derivatives **2, 4-7** (10 μM) followed by addition of PI(3,4,5)P_3 substrate. The absorbance, which is inversely related to the amount of generated PI(4,5)P_2, was assessed and compared to the absorbance generated by PTEN and PI(3,4,5)P_3 alone. There is a main effect of treatment [$F_{(8,9)} = 8.71$, $p < 0.05$; **Figure 2.12**]; and *a priori* comparisons show that, as expected, the PTEN inhibitor SF1670 suppresses PTEN lipid phosphatase activity. Conversely, peptides **h3L4F** (**1**), **2** and peptide derivatives of **2** do not suppress PTEN lipid phosphatase activity, which is an important necessity for the continued investigation of these derivatives.

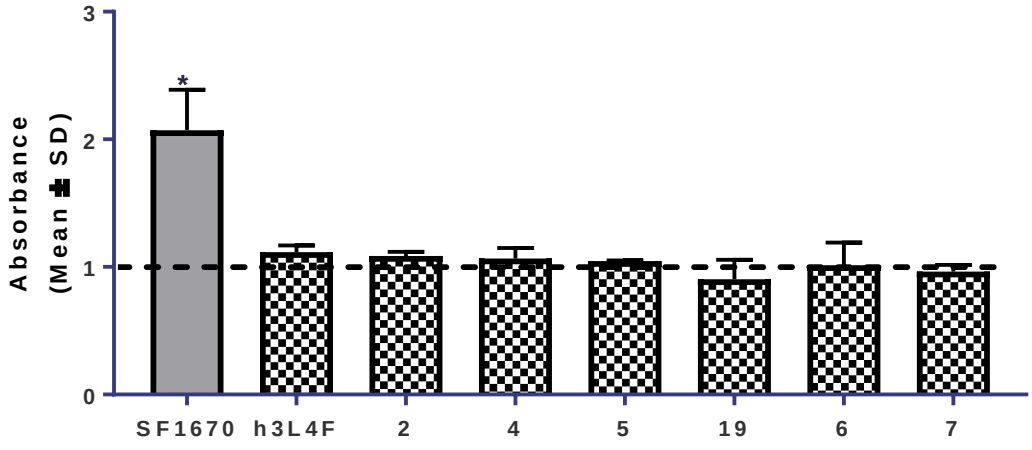


Figure 2.12: Derivatives of peptide 2 do not suppress lipid phosphatase activity of PTEN

Effects of peptide 1 derivatives on PTEN lipid phosphatase activity. Absorbance is inversely correlated to amount of PIP_2 generated in reaction of PTEN and PIP_3 in the absence of (dashed line) or presence (checkered bars) of peptide derivatives (10 μM). Thus, elevated absorbance indicates reduced PTEN lipid phosphatase activity. PTEN inhibitor, SF1670 (200 μM; gray bar) is included as a positive control. The bars represent mean absorbance fold change over PTEN + PIP3 alone (± SD) of 2 independent experiments run in triplicate. *$p < 0.05$ vs. PTEN + PIP_3 alone (dashed line).

In summary, the peptide **TAT-r3L4F** (**3**), which disrupts the 5-HT_{2C}R:PTEN complex (Anastasio et al., 2013; Ji et al., 2006), enhanced the interoceptive cues elicited by both selective 5-HT_{2C}R agonists lorcaserin and WAY163909. Pretreatment with **TAT-r3L4F** (**3**) also enhanced lorcaserin-induced suppression of the stimulus effects of cocaine, which solidifies the potential therapeutic use for ligands that disrupt the 5-HT_{2C}R:PTEN complex. Additionally, we have demonstrated that the sequence of peptide **2** can be modified to potentially increase drug-like properties and retain activity to potentiate 5-HT_{2C}R signaling *in vitro* while not disrupting the lipid phosphatase activity of PTEN. Together, these data suggest that disruption of the 5-HT_{2C}R:PTEN complex may have positive therapeutic implications and that the generation of bioavailable disrupters with efficacy is possible.

Signaling through GPCRs has been shown to occur through G protein-dependent and -independent mechanisms, which can impact overall signaling outcomes of receptor activation. Different ligands for the same receptor have been shown to induce certain intracellular signaling pathways over others, a concept known as biased signaling or functional selectivity (Kenakin and Christopoulos, 2013; Whalen et al., 2011). Our results suggest that **TAT-r3L4F** (**3**) may alter the signaling pathways induced by lorcaserin and WAY163909 *in vivo* which could suggest that disruption of the 5-HT_{2C}R:PTEN complex may alter recruitment of downstream effectors or stabilize different receptor conformations. Future studies should explore the potential of this protein-protein interaction to bias signaling of the 5-HT_{2C}R.

This is the first report, to our knowledge, that employs PLC_{β} inhibitor U73122 in the drug discrimination paradigm. Here, we show that systemic administration of U73122 can modestly, but not significantly, suppress the stimulus discrimination generated by CNS-located targets. Since PLC_{β} is a known downstream effector of $G\alpha_q$ protein-mediated signal transduction, this report provides preliminary evidence that the drug discrimination paradigm maybe useful in the elucidation of signal transduction pathways that are recruited for the generation of interoceptive cues associated with different receptor types. Through the use of PLC_{β} inhibitors, as well as other inhibitors downstream of G protein signal transduction, this rodent model may be useful to study the potential for biased signaling which has been notoriously difficult to study *in vivo* (Zhou and Bohn, 2014). It will be interesting to assess *in vitro* characterized biased ligands in this behavioral paradigm in future studies.

Finally, the present study demonstrates that modifications predicted to enhance peptide drug-like properties are able to retain *in vitro* activity. Future studies will explore *in vivo* absorption, distribution, metabolism and excretion properties to confirm that these modifications do in fact enhance the pharmacokinetic properties of the parent peptides (**Figure 2.1**).

Overall, the present study provides further evidence that 5-HT_{2C}R activity can be modulated through an allosteric protein-protein interaction. As such, this work provides the groundwork for the continued exploration of protein-protein interactions that can allosterically modulate this critical receptor and other important GPCRs for new therapeutic development through mechanisms that may have enhanced selectivity for targets of interest and thus enhanced clinical utility.

MATERIALS AND METHODS

Drug Discrimination Assays

ANIMALS. Experimentally naïve male Sprague-Dawley rats (n=47; Harlan, Inc.) weighing 300-325 g at the beginning of the experiment were housed two per cage in a temperature- (21-23°C) and humidity- (45-50%) controlled environment; lighting was maintained under a 12-h light-dark cycle (0700-1900 h). Rats were maintained at 80-90% of their free-feeding weights by restricting access to water. Rats received water during daily training sessions (5-6 ml/rat/session), in the afternoon several hours after training (20 min), and over the weekend (36 h). Experiments were conducted during the light phase of the light-dark cycle (between 0900 and 1200 h) and were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and with

the approval of the Institutional Animal Care and Use Committee at University of Texas Medical Branch.

Apparatus. The procedures were conducted in commercially available two-lever operant chambers (Med Associates, St. Albans, USA). Each chamber was equipped with a water-filled dispenser mounted equidistantly between two retractable response levers on the wall and housed in a light- and sound-proof cubicle. Illumination came from a 28-V house light; ventilation and masking noise were provided by a ventilation fan in the right side wall. A computer with Med-PC IV software was used to run programs and record all experimental events.

Drug Discrimination Procedure. Standard two-lever, water-reinforced drug discrimination procedures were used (Appel and Cunningham, 1986; Bergman et al., 2000; Callahan and Cunningham, 1994, 1995; Colpaert, 1999; Cunningham et al., 1985; Filip et al., 2006; Li et al., 2009). Three cohorts (n=15-16/cohort) were trained to discriminate an injection of a training drug from saline (1.0 ml/kg, i.p) administered 15 min before start of daily (Monday – Friday) training sessions. In one cohort (n=16), rats were trained to discriminate the training drug lorcaserin (0.75 mg/kg, 1.0 ml/kg, i.p) from saline. In a second cohort (n=15) rats were trained to discriminate the training drug WAY163909 (0.75 mg/kg, 1.0 ml/kg, i.p) from saline. In third cohort, rats (n=16) were trained to discriminate the training drug cocaine (5 mg/kg, 1.0 ml/kg, i.p) from saline. Of note, two rats in this lorcaserin-saline cohort were excluded from the present study due to loss of discrimination part of the way through completion of the presented tests. Additionally, rats in the WAY163909-saline cohort also participated in additional studies currently under submission (Wild et al., 2017, in preparation).

Errorless training. During this phase, only the stimulus-appropriate (drug or saline) lever was present. Training began under a fixed ratio 1 (FR 1) schedule of water reinforcement, and the FR requirement was incremented until all animals were responding reliably under an FR 20 schedule for each experimental condition. For half of the rats, left lever responses were reinforced after training drug administration, whereas right lever responses were reinforced after saline administration; conditions were reversed for the remaining animals. During this phase of training, drug and saline were administered irregularly with the restriction that neither condition prevailed for more than three consecutive sessions.

Discrimination training. After responding stabilized, both levers were presented simultaneously during 15-min training sessions. The rats were required to respond on the stimulus-appropriate (correct) lever to obtain water reinforcement. There were no programmed consequences for responding on the incorrect lever. This phase of training continued until the performance of all rats attained criterion (defined as mean accuracies of at least 80% stimulus-appropriate responding for ten consecutive sessions).

Test protocols. Test sessions were initiated and conducted once or twice per week following attainment of criterion. Training sessions were run during the intervening days to maintain discrimination accuracy. Rats were required to maintain accuracies of at least 80% correct for saline and training drug maintenance sessions which immediately preceded a test. During test sessions, animals were placed in the chambers and, upon completion of 20 responses on either lever, a single reinforcer was delivered and the houselights were turned off. The rat was removed from the chamber, returned to the colony, and allowed free access to water for 15 min beginning 2-3 hours after the end of each test. Test sessions

were terminated after 15 min if the rats did not complete 20 responses on either lever; only data from rats that accomplished the FR20 during test sessions within 15 minutes were employed in data analysis.

Two pharmacological test manipulations were performed during test sessions. In *substitution tests*, rats were administered various doses of the training drugs (lorcaserin-saline: 0.125-1.0 mg/kg lorcaserin; WAY163909-saline: 0.125-1.0 mg/kg WAY163909; cocaine-saline: 0.313-5 mg/kg cocaine), saline or test compounds. In *combination tests*, rats were tested for lever selection following intraperitoneal administration (unless otherwise indicated) of a fixed dose of a test compound, or compounds, prior to a dose of the training drug. In lorcaserin-saline trained rats, 3L4F (1, 2 μmol/kg) was given 30 min prior to testing, and PLCβ inhibitor U73122 (0.5 mg/kg), WAY163909 (0.75 mg/kg) and lorcaserin (0.5 or 0.75 mg/kg) were given 15 min prior to testing. In WAY163909-saline trained rats, 3L4F (1, 2 μmol/kg), PLCβ inhibitor U73122 (0.5 mg/kg), lorcaserin (1 mg/kg, subcutaneous) and WAY163909 (0.5 or 0.75 mg/kg) were given 15 min prior to testing. In cocaine-saline trained rats, 3L4F (1-2 μmol/kg) or SB242084 was given 45 min prior to testing, lorcaserin (0.5 or 1 mg/kg) was given 30 min prior to testing and cocaine (2.5 mg/kg) was given 15 min prior to testing. Full substitution was defined as ≥ 80% drug-appropriate responding and not statistically different from the training drug, and partial substitution as ≥ 40% and < 80% drug-appropriate responding.

DRUGS. **R-TAT-3L4F** (r-3L4F; Ac-YGRKKRRPNPDQKPRRKKKEKR-NH_2; pepMic Co., China), lorcaserin ([(1*R*)-8-chloro-2,3,4,5-tetrahydro-1-methyl-1*H*-3 benzazepine]; Hangzhou Trylead Chemical Technology Co., Ltd, Hangzhou, China), WAY163909 ([(7b-R,10a-R)-1,2,3,4,8,9,10,10a-octahydro-7bH-cyclopenta[b][1,4]

diazepino [6,7,1hi] indole]; gift from Pfizer, Inc., New York, NY) and (—)-Cocaine (National Institute on Drug Abuse, Research Triangle Park, NC, USA) were dissolved in 0.9% NaCl for *in vivo* studies. U73122 (Tocris, UK) was dissolved in 0.4% tween-20 in 0.9% NaCl. Due to solubility issues, U73122 first grinded in 100% tween-20 using a mortar and pestle until an opaque solution was obtained. Then 0.9% NaCl was added, 1 ml at a time, and mixed in mortar and pestle until the solution of required concentration was obtained.(Medvedev et al., 2013)

DATA ANALYSIS. Accuracy was defined as the percentage of correct responses to total responses before the delivery of the first reinforcer. During test sessions, performance was expressed as the percentage of drug-lever responses to total responses upon completion of an FR 20 on either lever. Response rates (responses per minute) were also evaluated during training and test sessions as a measure of behavioral disruption. The response rate (responses per minute) was calculated as the total number of responses emitted before completion of the first FR 20 divided by the number of minutes taken to complete the first ratio. For substitution tests, Student's *t*-test for repeated measures was used to compare the percentage of drug-lever responding and response rate during test sessions with the corresponding values for the previous drug maintenance session. Combination tests were analyzed by one-tailed Student's *t*-test for repeated measures with bonferroni correction for preplanned comparisons. All statistical analyses were conducted with an experimentwise error rate of α=0.05.

In vitro Screening

CELL LINES AND CELL CULTURE. Chinese hamster ovary K1 (CHO-K1) cells stably transfected with human unedited 5-HT_{2C}R (h5-HT_{2C}R-CHO cells) were a generous gift of

K. Berg and W. Clarke (University of Texas Health Science Center, San Antonio). The h5-HT_{2C}R-CHO cells express approximately 200 fmol/mg of 5-HT_{2C}R protein which approaches physiological levels in brain (Berg et al., 2001; Gavarini et al., 2006; Seitz et al., 2012; Stout et al., 2002). Cells were grown at 37°C, 5% CO_2 and 85% relative humidity environment in GlutaMax™-MEM medium (Invitrogen, Carlsbad CA) containing 5% fetal bovine serum (Atlanta Biologicals, Atlanta GA), and 100 µg/ml hygromycin (Mediatech, Manassas VA), and were passaged when they reached 80% confluence.

LIGANDS. Serotonin (5-HT; Acros Organics, ThermoFisher Scientific, Pittsburgh, PA) was dissolved in 1X Hank's balanced salt solution (HBSS; Cellgro, Invitrogen). Peptides (**h3L4F**, Ac-PNQDQNARRRKKKERR-NH_2; **1,** Ac-PNQDQNAR-NH_2) were dissolved in DMSO to a concentration of 10 mM working stock solutions for functional assays. PTEN inhibitor SF1670 (Echelon Biosciences, Salt Lake City, UT) was dissolved in DMSO to a working concentration of 5 mg/ml.

INTRACELLULAR CALCIUM ASSAY. The Ca_i^{2+} release assay was performed according to our recent publications with minor modifications (Anastasio et al., 2013; Chen et al., 2017; Ding et al., 2012; Seitz et al., 2012). Briefly, 150 µL cells from passage 6-16 were plated in black-walled flat clear bottom 96-well tissue culture plates at a density of 14,000-16,000 cells/well in serum-replete medium. Care was taken to ensure even plating of cells, including frequent agitation or trituration of cells in the source reservoir; plates were placed on a rotary shaker at low speed for 20 min. Approximately 24 h following plating, the medium was removed and cells were fed with serum-free (SF) GlutaMax™-MEM medium supplemented with 1 µM putrescine (Sigma-Aldrich, St. Louis, MO), 10 mM progesterone (Sigma-Aldrich), and 1:100 ITS (1000 mg/L human recombinant insulin,

550 mg/L human recombinant transferrin, 0.67 mg/L selenious acid; Corning Inc, Corning, NY) (SF+ medium). Following a 3 h incubation, SF+ medium was replaced with 40 µL Hank's balance saline solution (HBSS; without $CaCl_2$ or $MgCl_2$, pH 7.4) plus 40 µL Calcium 4 dye solution (FLIPR No-wash kit, Molecular Devices, Sunnyvale, CA, part # R8142) supplemented with 2.5 mM water-soluble probenicid (Sigma) to inhibit extracellular transport of the dye. Plates were incubated for 60 min at 37 °C followed by 15 min at RT in the dark. Calcium-induced fluorescence signal (λex = 485 nm, λem = 525 nm) was measured with a FlexStation 3 instrument (Molecular Devices). A baseline was established for each well during the initial segment of each run. Addition of 20 µL of 5× concentrated peptide derivatives or vehicle (HBSS) occurred at 17 s, and fluorescence was recorded every 5 s for 240 s to determine intrinsic agonist activity. Fifteen min later, following another 17 s baseline recording, 25 µL of 5× concentrated 5-HT was added and fluorescence was again measured every 1.7 s for 240 s. Maximum peak heights and area under the curve (AUC) of the Ca_i^{2+} transient were determined by the SoftMax software (Pro 5.4.5) for each well. After the final readings, cells were fixed in 2% paraformaldehyde (Sigma) overnight.

Data from each well were normalized to total cell mass as determined with crystal violet staining, a value proportional to cell mass that can be used as an estimate of cell number (Seitz et al., 2012). After fixation, cells were rinsed with water, air dried, and 50 µL of filtered crystal violet solution (0.1% in water) was added for 10 min at RT and the wells were rinsed again. Cell-adsorbed dye was extracted by the addition of 50 µl of 10% acetic acid (10 min, RT) and absorbance read at 590 nm. AUC of the Ca_i^{2+} transient was normalized to the crystal violet values for each well and then expressed as a percent of the

maximum and minimum Ca_i^{2+} response. The AUC of Ca_i^{2+} transients was utilized as this measure incorporates both information on the duration of the calcium signal as well as the maximum amount of ligand-evoked calcium release (King et al., 2015). The pIC_{50} and E_{MAX} values for Ca_i^{2+} assay were determined using 3-parameter nonlinear regression analysis (GraphPad Prism 7.02) and calculated from at least four independent experiments, each conducted in technical quadruplicates. Raw relative fluorescence units were then normalized to maximum Ca_i^{2+} release induced by 5-HT (100%) and are presented as the mean ± SEM% of 5-HT. An unpaired Welch's *t*-test was conducted to compare E_{MAX} of 5-HT in the presence and absence of peptide derivative (1 nM). All statistical analyses were conducted with an experimentwise error rate of α=0.05.

PTEN LIPID PHOSPHATASE ACTIVITY ASSAY. Lipid phosphatase activity of PTEN was quantified using a PTEN activity ELISA kit from Echelon Biosciences (K-4700, Echelon Biosciences, Salt Lake City, UT) following manufacture protocol. Briefly, 1 ng/µl PTEN enzyme (E-3000, Echelon Bioscience, Salt Lake City, UT) was incubated with PTEN inhibitor SF1670 (200 µM), **1**, **2**, and **2** derivatives (10 µM) for 15 min at 37°C. Then $PI(3,4,5)P_3$ substrate was added for a final concentration of 4 µM and the reaction was incubated at 37°C for 2.25 h. The lipid phosphatase activity of PTEN was quantified by measuring the amount of $PI(4,5)P_2$ by competitive ELISA in which final absorbance is inversely related to the amount of $PI(4,5)P_2$. The absorbance was measured by FlexStation3 at 450 nm (Molecular Devices). Results are represented as fold change over PTEN + $PI(3,4,5)P_3$ alone (mean ± SD) of two independent experiments run in technical triplicates and analyzed by one way ANOVA with *a priori* comparison to PTEN + $PI(3,4,5)P_3$ alone

(Dunnett's; GraphPad Prism 7.02). All statistical analyses were conducted with an experimentwise error rate of α=0.05

Chemistry

GENERAL. All starting material and reagents were purchased from Sigma-Aldrich, Acros, AstaTech and Aapptec and unless noted were used without further purification. The azide and alkyne functionalized where purchased or prepared by the methods of Pedersen.(Sminia and Pedersen, 2012) Thin layer chromatography (TLC) was performed on Silicycle glass backed plates (extra hard layer, 0.25 mm thick, 60 Å, with F-254 indicator) and components were visualized by UV light (254 nm) and/or *p*-anisaldehyde, basic permanganate ($KMnO_4$) solution, ninhydrin solution. Flash column chromatography was performed using Silicycle silica gel (particle size 40-63 □m, 230-400 mesh). NMR spectra were obtained using JEOL ECX-400 spectrometer (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR), JEOL ECA-500 spectrometer (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) or JEOL ECX-600 spectrometer (600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR). Chemical shifts were referenced to the residual chloroform-H peak at 7.26 ppm (^{1}H) and 77 ppm (^{13}C) in $CDCl_3$ or to DMSO-H peak at 2.5 ppm (^{1}H) and 39.5 ppm (^{13}C) in DMSO-d_6 or to D_2O at 4.79 ppm (^{1}H). Chemical shifts were reported in parts per million (ppm, δ). Multiplicity were indicated as s for singlet, d for doublet, t for triplet, q for quartet, m for multiplet, br for broad resonance and the coupling constants (*J*) were reported in Hz. High resolution mass spectra were recorded on an Agilent 6530 Accurate Mass Q-TOF LC-MS (high resolution ESI) from University of Texas at Austin, Mass Spectrometry Facility (MSF) of Department of Chemistry and Biochemistry. Low resolution mass

spectra were recorded on Thermo Scientific liquid chromatography mass spectrometry (LC-MS with low resolution ESI) in Gilbertson Laboratory.

Analytical and Preparative RP-HPLC. Analytical RP-HPLC was run on an HP1100 series instrument using A. Thermo Scientific BetaBasic column (C8, 100 x 4.6 mm, 5 μm particle size with a flow rate of 0.8 mL/min), B. Beckman Coulter column (C18, 250 x 4.6 mm, 5 μm particle size with a flow rate of 1.0 mL/min), or C. Grace Vydac column (C18, 250 x 4.6 mm, 5 μm particle size with a flow rate of 1.0 mL/min). The analyses were executed with the following solvent systems: 0.1% TFA in H_2O (A) and MeCN containing 0.1% TFA (B). Detection was performed with a photodiode array detector at a wavelength of λ=215 nm unless otherwise stated. Preparative purification was performed on a Gilson series instrument using a Grace Vydac protein & peptide C18 column (C18, 250 x 18 mm, 10 μm particle size). The analyses were executed with a flow rate of 8－12 mL/min and with the following solvent systems: H_2O containing 0.1% TFA (A) and MeCN (B).

General Method for Solid-Phase Peptide Synthesis (SPPS). Automated solid-phase peptide synthesis was performed at room temperature on Endeavor 90 III peptide synthesizer (AAPPTEC). All syntheses were executed using a standard Fmoc/^{t}Bu strategy. The resin (Rink amide resin or 2-Cl Trityl chloride (2-ClTrT) resin) was swollen in the solvent used in the reaction for 20 minutes prior to reaction. The first amino acid was attached to 2-ClTrT resin using the standard protocol provided in AAPPTEC technical support information bulletin 1027. The first amino acid attachment on Rink amine resin was introduced by standard coupling conditions. Briefly, the coupling reactions were carried out (for both Rink amide resin and 2-CTC resin) in NMP using 2 equiv of N-Fmoc

protected amino acid, HOBt (2 equiv), and HBTU (2 equiv) in the presents of DIPEA (5 equiv). The activated amino acid was then added to the resin and the vessel shaken for 25 min with nitrogen bubbling for the first 1 min, followed by draining of the solvent. The coupling procedure was performed twice. N-terminal Fmoc deprotection was achieved using 20% piperidine in DMF (vol/vol). The resin was washed with methanol, DMF, MeCN, and CH_2Cl_2 after every coupling and deprotection step. Peptide cleavage and side chain deprotection were carried out by agitating the crude peptide-loaded resin in TFA cocktail solution (95% TFA: 2.5% TIPS: 2.5% H_2O) for 3 h at room temperature or at 0°C. The ingredients were varied depending on the peptide sequence. The crude material was precipitated in cold diethyl ether, centrifuged, and purified by HPLC using 0－100% gradient in MeCN over 30 min. The purified aqueous solution was lyophilized to afford the final product. In the case of 2-ClTrT resin, peptide cleavage can be accomplished using AcOH/TFE/CH_2Cl_2 (1:1:8) solution to generate side chain protected peptide acid. The crude material was precipitated in 10－15 times the volume hexane. The solvent was removed under reduced pressure to afford the side chain protected peptide, unless noted was used without further purification.

Cyclic Peptide (4). 500 mg of H-Arg(bf)-2-CTC resin (0.29 mmole) was swollen in CH_2Cl_2 for 20 minutes prior to coupling. The attachment of the next 7 amino acid residues, Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Asp(tBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Pro-OH, and linker (Fmoc-GABA-OH) was conducted in an automated SPPS in which the iterative cycle (removal of the Fmoc group, wash, coupling of the next building block, wash) was performed consistently. The peptide loaded-2-ClTrT resin was treated with 5 mL of AcOH/TFE/CH_2Cl_2 (1:1:8) solution

for 30 min, filtered and washed the resin with 5 mL of 10% AcOH solution. 150 mL of hexane was added to the filtrate. Solvent was removed with a rotary evaporator (If the AcOH was not completely removed, more hexane can be added and continued evaporating the solvent until all the AcOH was removed) to give of crude peptide (591 mg, 0.26 mmol), which was dissolved in 3 mL of CH_2Cl_2, and DBU (79 mg, 0.52 mmol) was added. The reaction mixture was stirred for 30 min, and solvent was removed by rotary evaporator. The residue was purified by flash chromatography (using eluent gradient EtOAc to 1:9 methanol-dichloromethane) to afford peptide with desired free amine and carboxylic acid. The peptide (525 mg, 0.23 mmol), HBTU (95 mg, 0.25 mmol), and HOBt (38 mg, 0.25 mmol) was dissolved in 32 mL of DMF, and 10 min later DIPEA (119 mg, 0.68 mmol) was added. The resulting mixture was stirred overnight at room temperature. The reaction mixture was quenched by the addition of 320 mL of H_2O and was extracted with EtOAc (3x120 mL). The combined organic extracts were washed with 5% aqueous HCl (100 mL), sat. aqueous NaCl (100 mL), and H_2O (100 mL), dried over $MgSO_4$, filtered, and concentrated with rotary evaporator. The crude material was used without further purification. The crude material (500 mg) was treated with 20 mL of 95% TFA solution and stirred for 3 h at room temperature. The resulting solution was added to cold ether (400 mL), and the white precipitation was centrifuged, collected, dried under vacuum, and purified by RP-HPLC to give pure cyclic derivative **4** (38 mg) in 13% yield based on the initial resin loading. The NMR spectra were reported as a mixture of two conformations. ^{1}H NMR (500 MHz, D_2O) δ 4.70 (dd, *J* = 15.8, 9.2 Hz, 0.2H), 4.67–4.55 (m, 2.7H), 4.53 (dd, *J* = 8.7, 2.7 Hz, 0.2H), 4.37–4.28 (m, 2H), 4.28–4.15 (m, 2.7H), 3.69–3.51 (m, 1.8H), 3.47 (dd, *J* = 17.8, 9.8 Hz, 0.2H), 3.30–3.09 (m, 4H), 2.96 (dd, *J* = 17.1, 5.7 Hz, 1H), 2.93–

2.84 (m, 3H), 2.84–2.71 (m, 2H), 2.52–2.38 (m, 1.8H), 2.38–2.21 (m, 5.4H), 2.21–2.07 (m, 2.2H), 2.07–1.82 (m, 6.2H), 1.82–1.68 (m, 3H), 1.68–1.50 (m, 2H), 1.43–1.36 (m, 3H). ^{13}C NMR (126 MHz, D_2O) δ 177.82, 177.79, 176.04, 174.88, 174.77, 174.63, 174.53, 174.33, 174.10, 173.34, 173.12, 173.04, 172.59, 172.33, 171.89, 156.73, 131.47, 60.82, 53.95, 53.62, 53.41, 50.81, 50.73, 50.39, 47.99, 40.45, 38.86, 35.92, 35.50, 35.08, 31.71, 31.27, 31.13, 30.91, 29.59, 27.81, 27.51, 26.21, 25.92, 24.57, 24.43, 23.79, 22.38, 16.77, 16.48.

Cyclic Peptide (5). Bromoacetic acid anhydride (3.25 mmol), which was freshly generated by treating bromoacetic acid (903 mg, 6.50 mmol) with DIC (410 mg, 3.25 mmol) in DMF (13 mL), was added to the Rink amide resin (pre-generated free amine form, 1030 mg, 0.65 mmol, 0.63 mmol/g) and the reaction vessel was shaken for 80 min, followed by standard wash. DMF (13 mL) was added to the resulting resin followed by the addition of propargylamine and allowed it to shake for 20 h at room temperature. The first amino acid, arginine, was attached to the alkyl installed resin in a pre-generated anhydride (3.25 mmol) style. After the deprotection of Fmoc, the following 7 amino acid, Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Asp(tBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Pro-OH, and linker (4-azidobutyric acid) was attached by SPPS. A plastic vessel was charged with the peptide loaded-resin and 58 mL of DMSO (solvent was freshly deoxygenated by bubbling with nitrogen for 5 min) Cu(I)I (56 mg, 0.29 mmol) and 2,6-lutidine were added and the vessel was sealed and shaken for 48 h. After the reaction, the resin was then washed with DMF, MeCN, H_2O, sat. aqueous disodium EDTA solution (2x10 min), H_2O, MeCN, CH_2Cl_2, and ether. The peptide was cleaved from resin with 95% TFA via standard protocol. The crude material was purified by RP-HPLC to give pure cyclic peptide **5** (298 mg) in 40% yield based on the initial resin

loading. The NMR spectra were reported as a mixture of conformations. ^{1}H NMR (600 MHz, D_2O) δ 8.05 (s, 0.5H), 7.99 (s, 0.1H), 7.87 (s, 0.4H), 5.09 (d, *J* = 15.5 Hz, 0.3H), 5.03 (d, *J* = 15.3 Hz, 0.1H), 4.91 (m, 0.2H), 4.69 (m, 0.2H), 4.61 (ddd, *J* = 19.5, 12.5, 6.6 Hz, 2H), 4.57–4.51 (m, 1.3H), 4.50–4.34 (m, 2.6H), 4.33–4.28 (m, 0.5H), 4.25 (ddd, *J* = 16.4, 10.7, 6.3 Hz, 2H), 4.19–4.01 (m, 3.2H), 3.93 (d, *J* = 16.8 Hz, 0.1H), 3.59–3.36 (m, 2H), 3.12 (ddd, *J* = 20.6, 14.3, 6.9 Hz, 2H), 2.92 (dd, *J* = 17.1, 6.1 Hz, 0.9H), 2.86–2.76 (m, 2.7H), 2.75–2.69 (m, 2H), 2.50–2.41 (m, 0.5H), 2.41–2.16 (m, 6H), 2.16–2.02 (m, 4.2H), 2.02–1.83 (m, 5H), 1.76 (d, *J* = 6.8 Hz, 1H), 1.69 (dt, *J* = 14.3, 9.4 Hz, 1.6H), 1.59–1.51 (m, 1.8H), 1.48–1.34 (m, 0.7H), 1.25 (d, *J* = 7.2 Hz, 1.2H), 1.22 (d, *J* = 7.1 Hz, 1.5H), 1.19 (d, *J* = 7.2 Hz, 0.3H). ^{13}C NMR (151 MHz, D_2O) δ 177.66, 177.57, 177.53, 174.68, 174.62, 174.55, 174.43, 174.28, 174.17, 174.08, 173.97, 173.93, 173.74, 173.66, 173.51, 173.20, 173.01, 172.88, 172.55, 172.47, 172.32, 171.81, 156.67, 156.60, 143.04, 142.90, 60.73, 54.30, 54.09, 53.43, 51.11, 50.89, 50.51, 49.96, 49.70, 49.58, 49.49, 49.01, 47.90, 44.50, 40.55, 40.38, 36.16, 35.69, 35.21, 31.41, 30.98, 30.90, 30.49, 28.09, 26.02, 25.08, 24.80, 24.29, 16.59.

Side Chain-to-Side Chain Cyclic Peptide (6). A Rink Amide resin (300 mg, 0.189 mmol, 0.63 mmol/g)-bound desired peptide sequence was obtained by standard SPPS. The peptide loaded resin was swollen in CH_2Cl_2 for 10 min. After the resin was filtered, a solution of Cu(I)Br (27 mg, 0.189 mmol) in 9 mL of DMSO (freshly deoxygenated by bubbling with nitrogen for 5 min at least), a solution of sodium ascorbate (37 mg, 0.189 mmol) in H_2O (1.6 mL), 2,6-lutidine (203 mg, 1.89 mmol), and DIPEA (244 mg, 1.89 mmol) were added to resin in a plastic vessel. The mixture was purged with N_2 for 5 min and the vessel was sealed and shaken for 20 h at room temperature. The solvent

was drained and the resin was washed with DMF, MeCN, H_2O, sat. aqueous disodium EDTA solution (2x10 min), H_2O, MeCN, CH_2Cl_2, and ether. The resin was treated with 20% piperidine in DMF (2x10 mL) to deprotect the N-Fmoc. Finally, the peptide was cleaved from resin with 10 mL of TFA/TIPS/H_2O (95:2.5:2.5) solution via standard protocol. The crude material was purified by RP-HPLC to give pure cyclic peptide **6** (40 mg) in 21% yield based on the initial resin loading. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.22 (br, 1H), 8.75 (d, *J* = 7.4 Hz, 1H), 8.55 (br, *J* = 5.3 Hz, 1H), 8.22 (d, *J* = 7.8 Hz, 1H), 8.15–8.07 (m, 2H), 8.05 (dd, *J* = 7.3, 3.7 Hz, 2H), 7.95 (s, 1H), 7.84 (d, *J* = 8.0 Hz, 1H), 7.54 (br, 1H), 7.43 (s, 1H), 7.33 (s, 1H), 7.27 (d, *J* = 18.9 Hz, 2H), 7.13 (s, 1H), 6.99 (s, 1H), 6.80 (d, *J* = 18.5 Hz, 2H), 4.63 – 4.56 (m, 2H), 4.53 (dd, *J* = 12.9, 6.1 Hz, 1H), 4.48 (d, *J* = 12.4 Hz, 1H), 4.43–4.35 (m, 2H), 4.32–4.20 (m, 4H), 4.15 (dd, *J* = 13.6, 7.8 Hz, 2H), 3.51 (dd, *J* = 9.5, 5.7 Hz, 1H), 3.26–3.20 (m, 1H), 3.20–3.15 (m, 1H), 3.08 (dd, *J* = 12.9, 6.6 Hz, 2H), 2.59 (dd, *J* = 15.8, 4.7 Hz, 1H), 2.44 (dd, *J* = 15.8, 8.9 Hz, 1H), 2.28 (dd, *J* = 8.1, 5.5 Hz, 1H), 2.13–2.07 (m, 2H), 2.00 (t, *J* = 8.0 Hz, 2H), 1.94–1.82 (m, 5H), 1.79–1.63 (m, 6H), 1.55–1.36 (m, 4H), 1.22 (d, *J* = 7.2 Hz, 3H), 1.04 (d, *J* = 7.5 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 174.03, 173.97, 173.14, 172.07, 171.38, 171.05, 170.94, 170.75, 170.41, 169.32, 168.10, 156.64, 143.22, 123.99, 68.86, 62.91, 58.87, 52.11, 52.07, 51.92, 51.84, 51.66, 50.16, 49.43, 48.44, 45.81, 40.39, 40.03, 36.80, 31.82, 31.35, 29.51, 29.26, 28.86, 28.47, 28.15, 25.01, 23.45, 22.28, 17.84.

TURN MIMIC 7. (S)-1-((S)-4-amino-2-((3S,4S)-4-((S)-1-((S)-1-carboxy-4-guanidinobutylamino)-1-oxo-propan-2-ylcarbamoyl)pyrrolidine-3-carboxamido)-4-oxobutanoyl)pyrrolidine-2-carboxylic acid (turn mimic **7**)

N-Troc protected turn mimic (trans-pyrrolidine-3,4-dicarboxamide) was prepared by the procedure of Boger(Whitby et al., 2011) and was used for the preparation of **7** by standard peptide coupling methods. To a solution of the N-Troc protected turn mimic (120 mg, 0.09 mmol) in 1.8 mL of AcOH/THF (1:2) was added zinc dust (114 mg, 1.74 mmol). The resulting suspension was stirred at room temperature for 8 h, and was then filtered through celite to remove zinc. The filtrate was concentrated with a toluene azeotrope to afford crude 30 (93 mg), which was employed directly into the next reaction without further purification. 3 mL of TFA/TIPS/H_2O (95:2.5:2.5) solution was added to the crude product (93 mg) and the mixture was stirred at room temperature for 3 h. Cold ether (30 mL) was added to the reaction mixture and the resulting white precipitation was centrifuged, collected, dried under vacuum, and purified by RP-HPLC to give pure turn mimic **7** (33 mg, 71%). ^{1}H NMR (500 MHz, D_2O) δ 4.96 (dd, *J* = 10.2, 4.2 Hz, 1H), 4.40 (dd, *J* = 8.7, 4.9 Hz, 1H), 4.34 (dd, *J* = 9.1, 5.1 Hz, 1H), 4.30–4.20 (m, 1H), 3.79 (dt, *J* = 10.3, 6.6 Hz, 1H), 3.68 (dt, *J* = 10.0, 6.5 Hz, 1H), 3.62–3.50 (m, 4H), 3.36–3.25 (m, 2H), 3.19 (t, *J* = 6.9 Hz, 2H), 2.80 (dd, *J* = 15.7, 4.2 Hz, 1H), 2.56 (dd, *J* = 15.8, 10.2 Hz, 1H), 2.37–2.24 (m, 1H), 2.11–1.95 (m, 3H), 1.95–1.84 (m, 1H), 1.81–1.69 (m, 1H), 1.63 (tt, *J* = 13.5, 6.9 Hz, 2H), 1.37 (d, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.34, 173.28, 172.93, 170.48, 170.33, 170.21, 170.00, 156.88, 58.77, 51.95, 48.83, 48.57, 47.11, 46.88, 46.50, 46.11, 40.34, 35.85, 28.60, 28.04, 25.23, 24.56, 17.70.

Chapter 3: Novel Bivalent 5-HT_{2A} Receptor Antagonists Exhibit High Affinity and Potency *In Vitro* and Efficacy *In Vivo* (Soto et al.)

INTRODUCTION

Serotonin (5-HT) neurotransmission is critically involved in the regulation of normal behavior (e.g., cognition, mood, satiety, sexual behavior, sleep) and pathological disorders (e.g., anxiety, depression, schizophrenia, substance use disorder) and is therefore, an important medications target. Actions of 5-HT are mediated by at least 14 subtypes of 5-HT receptors, 13 of which are G protein coupled receptors (GPCRs) and are presently grouped into seven families (5-HT_1R – 5-HT_7R) according to their structural and functional characteristics (Hoyer et al., 2002). The metabotropic 5-HT_2R family (5-HT_{2A}R, 5-HT_{2B}R, 5-HT_{2C}R) plays an important role in the regulation of CNS function and dysfunction. The receptors in the 5-HT_2 family couple predominantly to $G\alpha_{q/11}$ proteins to activate phospholipase C_β (PLC_β) resulting in downstream intracellular calcium (Ca_i^{2+}) release and phosphorylation of $ERK_{1/2}$ ($pERK_{1/2}$) and can also induce arachidonic acid release through phospholipase A 2-dependent mechanism (Felder et al., 1990). Abnormalities of 5-HT_2R function have been implicated in several neuropsychological and neurological disorders (Bubar and Cunningham, 2008; Cunningham and Anastasio, 2014; Hoyer et al., 2002; Meltzer et al., 2010; Roth et al., 2004) and active initiatives are underway to develop novel 5-HT_2R ligands as therapies for such disorders (Howell and Cunningham, 2015).

The 5-HT_{2A}R is of particular interest as a key target of atypical antipsychotics which are thought to improve symptoms and cognitive functioning in schizophrenia due to potent 5-HT_{2A}R antagonist actions (Gray and Roth, 2007; Meltzer et al., 2012b; T., 2001). Other selective 5-HT_{2A}R antagonists show promise to improve symptomology in

preclinical models of psychostimulant substance use disorder (i.e., cocaine, nicotine) (Anastasio et al., 2011; Burmeister et al., 2004; Fletcher et al., 2012; Nic Dhonnchadha et al., 2009) anxiety,(Weisstaub et al., 2006), depression (Berg et al., 2008; Celada et al., 2004; Zaniewska et al., 2010), and sleep disorders (Landolt and Wehrle, 2009; Popa et al., 2005; Teegarden et al., 2008). Selective 5-HT_{2A}R antagonists have been in clinical trials for neurological and/or psychiatric disorders including volinanserin [MDL100907, M100907; (R)-(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperidinemethanol; Aventis Pharmaceuticals; compound **1**; **Figure 3.1**) (de, 2001; Maurel-Remy et al., 1995) and structurally similar 5-HT_{2A}R antagonists/inverse agonists for schizophrenia (Meltzer et al., 2004; Talvik-Lotfi et al., 2000) and sleep disorders (Al-Shamma et al., 2010; Ancoli-Israel et al., 2011). Of these, the 5-HT_{2A}R inverse agonist/antagonist pimavanserin (tradename NUPLAZID®; ACP-103; ACADIA Pharmaceuticals) has recently been approved by the FDA for Parkinson's Disease psychosis (Meltzer et al., 2010) and the 5-HT_{1A}R agonist/5-HT_{2A}R antagonist flibanserin (tradename Addyi®; BIMT 17; Sprout Pharmaceuticals) has been approved for the treatment of hypoactive sexual desire disorder in pre-menopausal women (Fisher and Pyke, 2017).

Traditional drug discovery efforts for GPCRs like the 5-HT_{2A}R have been designed to target monomeric receptors and conceptualized as the pharmacophore interacting at one receptor binding site (George et al., 2002). However, in recent years, the importance of GPCR dimerization and oligomerization on receptor signaling, trafficking and localization has been demonstrated for a variety of GPCRs, highlighting the need to investigate receptor-receptor interactions in disease pathology (for review, (George et al., 2002)).

Existence of homodimeric 5-HT_{2A}R:5-HT_{2A}R interactions is supported by co-immunoprecipitation and fluorescence resonance energy transfer (FRET) experiments(Brea et al., 2009) and 5-HT_{2A}R homodimers have been proposed as the minimum functioning unit of the PLCβ- and PLA_2-mediated signaling pathways induced by 5-HT and synthetic 5-HT_{2A}R agonists (Iglesias et al., 2016). Additionally, molecular dynamics modeling studies suggest that the putative 5-HT_{2A}R ligand binding sites displace differently in simulations of monomers versus homodimers, which suggests that receptor:receptor interactions may prefer different 5-HT_{2A}R ligands (Bruno et al., 2011). Therefore, tools to explore 5-HT_{2A}R homodimer relevance in signal transduction as well as behavioral outcomes are necessary.

One proposed approach to probe homodimer signaling and function is through the use of bivalent ligands. These ligands are comprised of two pharmacophores covalently tethered via a suitable spacer which are hypothesized to interact with a binding site on each receptor in the dimer pair (Shonberg et al.). Bivalent ligands have been synthesized to examine various neurotransmitter receptor systems (Portoghese, 2001), including 5-HT receptors (Bruno et al., 2009; Choi et al., 2008; Heinrich et al., 2004) such as the 5-HT_4R (Lezoualc'h et al., 2009; Soulier et al., 2005) and 5-HT_{1B}R (Decker and Lehmann, 2007; Halazy et al., 1996; Perez et al., 1998). Here, we characterize homobivalent ligands with the pharmacophore of the piperidine M100907 which may serve as future tools to pharmacologically probe 5-HT_{2A}R:5-HT_{2A}R homodimer biology. The active (+)-isomer of M100907 [(+)-M100907; compound **1**, **Figure 3.1**] binds the 5-HT_{2A}R with high affinity and has >100 fold selectivity over the 5-HT_{2B}R and 5-HT_{2C}R.(Herth et al., 2009; Knight et al., 2004) The pharmacology of M100907 has been demonstrated in a wide diversity of *in*

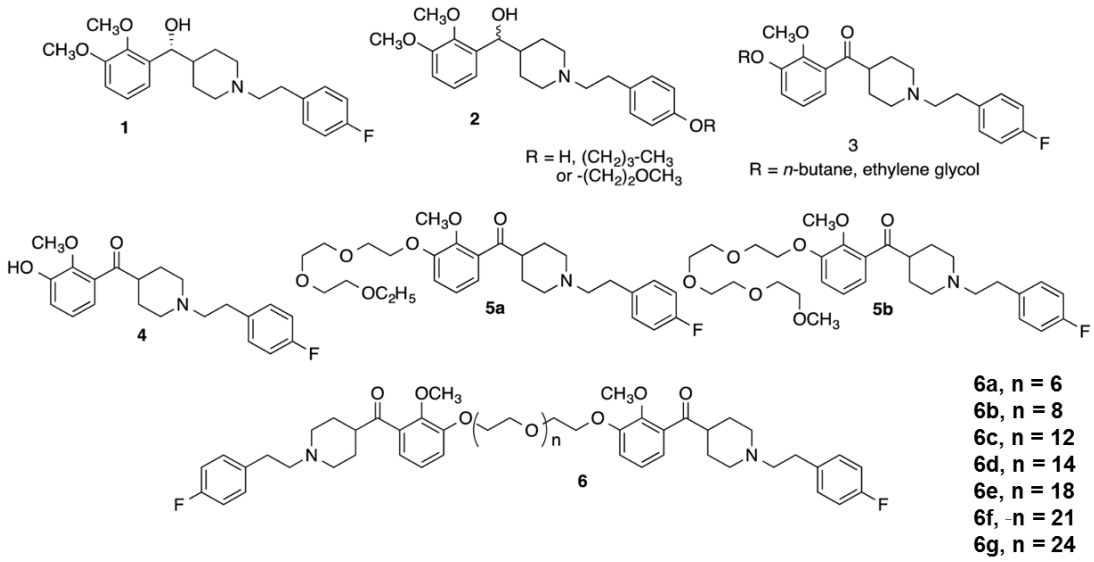
vitro (Seitz et al., 2012; Shashack et al., 2011) and *in vivo* (Anastasio et al., 2011; de; McMahon and Cunningham, 2001; Nic Dhonnchadha et al., 2009; Pockros et al., 2011) studies.

We have previously reported the synthesis and initial characterization of several bivalent analogues based on a modified structure of M100907 (compound **1, Figure 3.1**) (Shashack et al., 2011). To select the best location for the attachment of the tether used to link the molecules, derivatives with substitutions of the *p*-fluorine (compound **2**) or the methoxy group (compound **3**) were tested for their ability to inhibit 5-HT-stimulated Ca_i^{2+} release in a Chinese hamster ovary (CHO) cell line stably expressing the human 5-HT_{2A}R (h5-HT_{2A}R). The replacement of the fluorine with a hydroxyl or ether moiety (compound **2**) resulted in significant loss of antagonist potency to inhibit 5-HT-induced Ca_i^{2+} release (Shashack et al., 2011). Attachment of an ethylene glycol at the OH of the catechol (compound **3**) was found to retain significant activity (Shashack et al., 2011). Thus, this site was selected as the tether attachment site for synthesizing bivalent molecules. An active first pass metabolite of M100907 (des-3-methyl-ketone-M100907; compound **4**), which lacks a chiral center, also proved to be a potent (IC_{50} = 2.3 nM) 5-HT_{2A}R antagonist in the Ca_i^{2+} release bioassays (Shashack et al., 2011). This molecule was selected as the starting material for the synthesis of tethered analogs to avoid generation of diasteromeric intermediates and eliminate the need for chiral resolution of the molecules. Two versions of compound **4** were synthesized with ethylene glycol groups (**5a-b**) and tested in the Ca_i^{2+} bioassay to determine whether the polyether tether would have a deleterious effect on the activity of the ligand and found to retain nanomolar potency (Shashack et al., 2011). Finally, the desired bivalent molecules (**6a-g**) were synthesized and tested. The bivalent

ligands exhibited sub-micromolar potency to inhibit 5-HT-induced Ca_i^{2+} release demonstrating the retention of antagonist properties (Shashack et al., 2011). These studies suggested that intermediate tether lengths of 12-18 atoms in length are optimal for activity in Ca_i^{2+} assay (Shashack et al., 2011).

Figure 3.1: Chemical structures of (+)-M100907 derivatives

In the present report, we provide further functional characterization of these compounds. The compounds were additionally profiled through quantification of $ERK_{1/2}$



phosphorylation which represents a distal downstream signaling outcome of $5\text{-}HT_{2A}R$ activation. Phosphorylation of $ERK_{1/2}$ serves as an integration point of multiple upstream signaling pathways, including G protein dependent- and independent signal transduction (Hoyer et al., 2002; Kurrasch-Orbaugh et al., 2003; Raymond et al., 2001; Seitz et al., 2012). In addition, affinity of these compounds for the $5\text{-}HT_{2A}R$ and selectivity over the highly homologous $5\text{-}HT_{2B}R$ or $5\text{-}HT_{2C}R$ was determined. We found that the bivalent ligands (Compound **6** series) retained activity and selectivity similar to that of (+)-M100907 (Compound **1**) and that the optimal tether length is between 8- and 21- atom

linkers in the $ERK_{1/2}$ activation cellular assay. Thus, a homobivalent ligand with an intermediate tether length (compound **6c**) was selected for the first *in vivo* studies to evaluate the behavioral profile of a homobivalent 5-HT_{2A}R antagonist molecule. These studies open the door to the development of new bivalent molecules with the potential to elucidate the neurobiological role of 5-HT_{2A}R:5-HT_{2A}R homodimers in the CNS.

RESULTS AND DISCUSSION

The inhibitory potency of M100907 analogs (**Figure 3.1**) was evaluated in an $ERK_{1/2}$ activation assay in h5-HT_{2A}R-CHO cells (**Figure 3.2; Table 3.1**). Activation of $ERK_{1/2}$ occurs by phosphorylation of the kinase which, in turn, phosphorylates other downstream targets to regulate gene expression and a variety of cellular processes (for review, see (Roskoski, 2012)). Serotonin induces a concentration-dependent increase in phosphorylated $ERK_{1/2}$ ($pERK_{1/2}$) with an EC_{50} of ~72 nM (**Figure 3.2A**). Compound **1** and its analogs (**4-6g**; 10^{-10}–10^{-4} M) were examined for their ability to antagonized $ERK_{1/2}$ activation induced by a maximally effective concentration (1 μM) of 5-HT. The pIC_{50} and IC_{50} values are reported in **Table 3.1**. Compound **1**, the active isomer (+)-M100907 (Ullrich and Rice, 2000), and compound **4**, the des-3-methyl-ketone derivative starting material for synthesis of the bivalent ligands, displayed low nanomolar potency in inhibiting 5-HT-evoked $ERK_{1/2}$ activation (**Table 3.1**, **Figure 3.2B**).

The evaluation of inhibitory potency of compounds **5a** and **5b,** derivatives of compound **4** with 12- or 14-atom ethylene glycol linkers, revealed that the addition of the 12-atom linker (**5a**) retained comparable potency compared to the parent compounds, while addition of a 14-atom linker (**5b**) reduced the potency ~23-fold compared to the ketone starting material (**4**). This reduction in potency is not surprising since others (Shonberg et

al., 2011) have also noted that mere addition of the polyether tether diminishes the functional activity of the ligand. Although both **5a** and **5b** maintained sub-micromolar potency to inhibit 5-HT-mediated $ERK_{1/2}$ activation, these data suggest that the 12-atom chain may be of optimal length. The potency of the homobivalent ligands (**6a-g**) to inhibit 5-HT-mediated $pERK_{1/2}$ was diminished compared to the parent compounds and varied modestly as a function of linker length (**Table 3.1**). Interestingly, the homobivalent with the shortest (6 atoms; **6a**) and the longest (24 atoms; **6g**) linkers had the lowest potency which suggests that there is an optimal linker length for maintenance of antagonist activity. Comparison of the inhibitory potency of the **6c**, the 12-atom linked bivalent ligand, and **5a**, its respective 12-atom monovalent counterpart, show that the potency of **6c** is decreased by ~20-fold versus **5a**, suggesting that addition of a second pharmacophore decreased the functional activity (**Figure 3.2B**). On the contrary, comparison of the 14-atom linker bivalent ligand (**6d**) to its respective 14 atom monovalent counterpart (**5b**) resulted in a slight increased potency (**Table 3.1**). These findings are consistent with previously published effects of these compounds in the Ca_i^{2+} bioassay (Shashack et al., 2011) and, together, may suggest that linker length impacts the potency of these ligands and should be considered for future experiments.

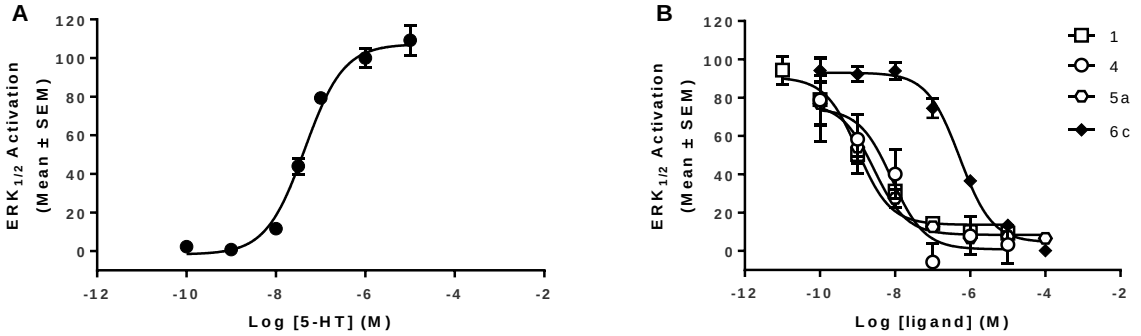


Figure 3.2: 5-HT$_{2A}$R antagonist homobivalent ligands suppress 5-HT-induced ERK$_{1/2}$ activation n h5-HT$_{2A}$R-CHO expressing cells

Representative ERK$_{1/2}$ activation response in h5-HT$_{2A}$R-CHO cells. (A) 5-HT evokes a concentration-dependent elevation of pERK$_{1/2}$ expression (pEC$_{50}$ = 7.14 ± 0.04; EC$_{50}$ = 72.4 nM) and 1 μM 5-HT induces maximal ERK$_{1/2}$ activation. (B) M100907 derivatives induces a concentration-dependent inhibition of 1 μM 5-HT. pIC$_{50}$ and IC$_{50}$ values are listed in Table 1.

Table 3.1: Potency of M100907derivatives on ERK$_{1/2}$ activation in h5-HT$_{2A}$R-CHO cells.

ID	Linker #	pIC$_{50}$ ± SEM[a]	IC$_{50}$ (nM)
1	—	8.29 ± 0.52	5.13
2	—	N.T.	N.T.
3	—	N.T.	N.T.
4	—	8.00 ± 0.08	10.1
5a	12	8.11 ± 0.05	7.76
5b	14	6.65 ± 0.07	224
6a	6	6.53 ± 0.41	295
6b	8	7.60 ± 0.53	25.1
6c	12	6.55 ± 0.19	179
6d	14	7.00 ± 0.13	100
6e	18	6.78 ± 0.39	166
6f	21	6.72 ± 0.14	191
6g	24	6.55 ± 0.12	284

[a]pIC$_{50}$ is presented as mean ± SEM (n = 3-6). IC$_{50}$ values were calculated from averaged pIC$_{50}$ values. "—" indicates that there is no atom linker on this compound. "NT" indicates compound has not been tested in this assay.

The affinity and selectivity of the synthesized M100907 derivatives for the 5-HT_{2A}R versus the highly homologous 5-HT_{2B}R and 5-HT_{2C}R, were assessed via radioligand binding assays at the Psychoactive Drug Screening Program (PSDP). The resultant K_i values for binding to 5-HT_{2A}R, 5-HT_{2B}R and 5-HT_{2C}R, as assessed by displacement of [^{3}H]-ketanserin (0.5 nM), [^{3}H]-LSD (1 nM) and [^{3}H]-mesulergine (0.5 nM), respectively, are shown in **Table 3.2**. All ligands displayed nanomolar affinity for the 5-HT_{2A}R, but K_i values were ~4-8 fold lower than that previously reported for the high affinity (+)-M100907 isomer (Compound **1**; K_i = 3 nM) (Kehne et al.). According to the PDSP, variations of this magnitude can be expected in K_i assessments, thus the differences in K_i values observed for 5-HT_{2A}R for most of the M100907 analogs versus the compound **1** were modest (**Table 3.2**). Only the bivalent ligand with the 24-atom linker (**6g**) exhibited a substantial decrease (~10 fold) in affinity for the (5-HT_{2A}R, K_i = 32 nM) which coincides with the decrease in potency observed for this molecule in the $ERK_{1/2}$ activation assay. In addition, the presence of the second pharmacophore in the 12- (**6c**) and 14-atom (**6d**) tethered bivalent ligands afforded no overt gain in affinity compared to their respective monovalent counterparts (**5a**, **5b**). These lack of differences may just be due to the inherent variability in K_i measurements, and/or the lack of measurement of actual association/dissociation constants of the ligands; the binding analysis was done at equilibrium, as opposed to during the dynamic state related to the functional endpoints (Christopoulos et al., 1999).

Table 3.2: Affinity profile of M100907 derivatives

ID	Linker #	5-HT$_{2A}$R		5-HT$_{2B}$R		5-HT$_{2C}$R	
		pK$_i$ [1]	K$_i$ (nM)	pK$_i$ [2]	K$_i$ (nM)	pK$_i$ [3]	K$_i$ (nM)
1	--	8.5 [4]	3.0	6.2	612	7.4	41
2	--	N.T.		N.T.		N.T.	
3	--	N.T.		N.T.		N.T.	
4	--	8.8	1.6	6.6	261	7.8	15
5a	12	8.3	5.4	6.2	701	6.5	312
5b	14	8.2	6.9	N.D. [5]		6.3	472
6a	6	8.5 [4]	3.1	6.7	202	7.6	27
6b	8	8.1 [4]	7.2	6.3	476	7.2	64
6c	12	8.2 [4]	5.9	6.9	126	7.1	79
6d	14	8.5 [4]	3.4	6.8	157	7.1	84
6e	18	8.5 [4]	3.0	6.6	267	7.3	50
6f	21	8.4 [4]	4.4	6.6	238	7.0	97
6g	24	7.5 [4]	32	5.9	1399	6.3	543

K$_i$ determinations were generously provided by the NIMH Psychoactive Drug Screening Program.
[1]Determined by displacement of [^{3}H] ketanserin (0.5 nM) relative to displacement by 10 µM clozapine; [2]Determined by displacement of [^{3}H] LSD (1.0 nM) relative to displacement by 10 µM methysergide; [3]Determined by displacement of [^{3}H] mesulergine (0.5 nM) relative to displacement by 10 µM chlorpromazine. [4]Determined in two independent assays; N.D. = Not determined; Displacement by 10 µM of methylsergide was < 50%. N.T.= Not tested in this assay

The inclusion of the second pharmacophore in the bivalent molecules did not afford any improvement in selectivity of the compounds for the 5-HT$_{2A}$R versus the 5-HT$_{2B}$R or 5-HT$_{2C}$R compared to the parent compounds. However, all the M100907 analogs maintained ~10-fold or greater affinity for the 5-HT$_{2A}$R over the 5-HT$_{2C}$R and >21-fold selectivity over the 5-HT$_{2B}$R. Due to the high degree of homology between these receptors, the selectivity of **6c** and **6d** bivalent compounds for the 5-HT$_{2A}$R was confirmed by assessment of these ligands in a functional assay of Ca_i^{2+} release, in 5-HT$_{2C}$R-expressing cells. Similar to compound **1**, compounds **6c** and **6d** had no effect upon 5-HT$_{2C}$R-mediated Ca_i^{2+} release evoked by 1 µM 5-HT at concentrations <10 µM (data not shown).

The bivalent ligand with an intermediate (12-atom) linker (**6c**) was selected for initial *in vivo* efficacy studies based upon the *in vitro* results described above. Previous studies have reported that (+)-M100907 (Compound **1**; 0.02-2 mg/kg) significantly suppresses cocaine-evoked, but not spontaneous, locomotor activity (McMahon and Cunningham, 2001; Szucs et al., 2005). Therefore, the effects of compound **6c** on spontaneous (saline) - versus cocaine-evoked locomotor activity was assessed in rats (**Figure 3.3**). Locomotor activity was measured after (intraperitoneal) injection with vehicle (1% Tween 80 with 2% ethanol in 0.9% NaCl) or bivalent ligand **6c** (2 mg/kg) 30 minutes prior to saline (1 ml/kg) or cocaine (15 mg/kg) injection. Data are presented as mean total horizontal ambulations summed for the entire 90 minute session (**Figure 3.3A**) or in 5-min bins (**Figure 3.3B**) and mean total vertical activity counts summed for the entire 90 min session (**Figure 3.3C**) or in 5-min bins (**Figure 3.3D**). A main effect of treatment was observed for both mean total horizontal ambulations [$F_{(3,27)} = 9.71$, $p<0.05$; **Figure 3.3A**] and mean total vertical activity counts in the 90-min session [$F_{(3,27)} = 13.90$, $p<0.05$; **Figure 3.3B**]. *A priori* comparisons indicated that bivalent ligand **6c** did not suppress saline-evoked horizontal ambulations or vertical activity counts in the 90-min session ($p<0.05$). Additionally, as expected, administration of vehicle plus cocaine (Veh + Coc) elevated total horizontal ambulations and vertical activity counts versus vehicle plus saline (Veh + Sal) administration for the entire 90-min session ($p<0.05$) which was similarly observed at all twelve 5-min intervals (**Figure 3.3B** and 3.**3D**). *A priori* comparisons showed that the bivalent ligand **6c** significantly suppressed cocaine-evoked horizontal ambulations ($p<0.05$; **Figure 3.3A**) and vertical activity counts ($p<0.05$; **Figure 3.3C**). Thus, similar to the previously published (+)-M100907 (McMahon and Cunningham,

2001), the bivalent ligand **6c** suppressed cocaine-evoked, but not spontaneous, locomotor activity. These data provide the first positive confirmation that a newly synthesized homobivalent ligand displays efficacy to produce behavioral effects similar to the parent 5-HT_{2A}R antagonist.

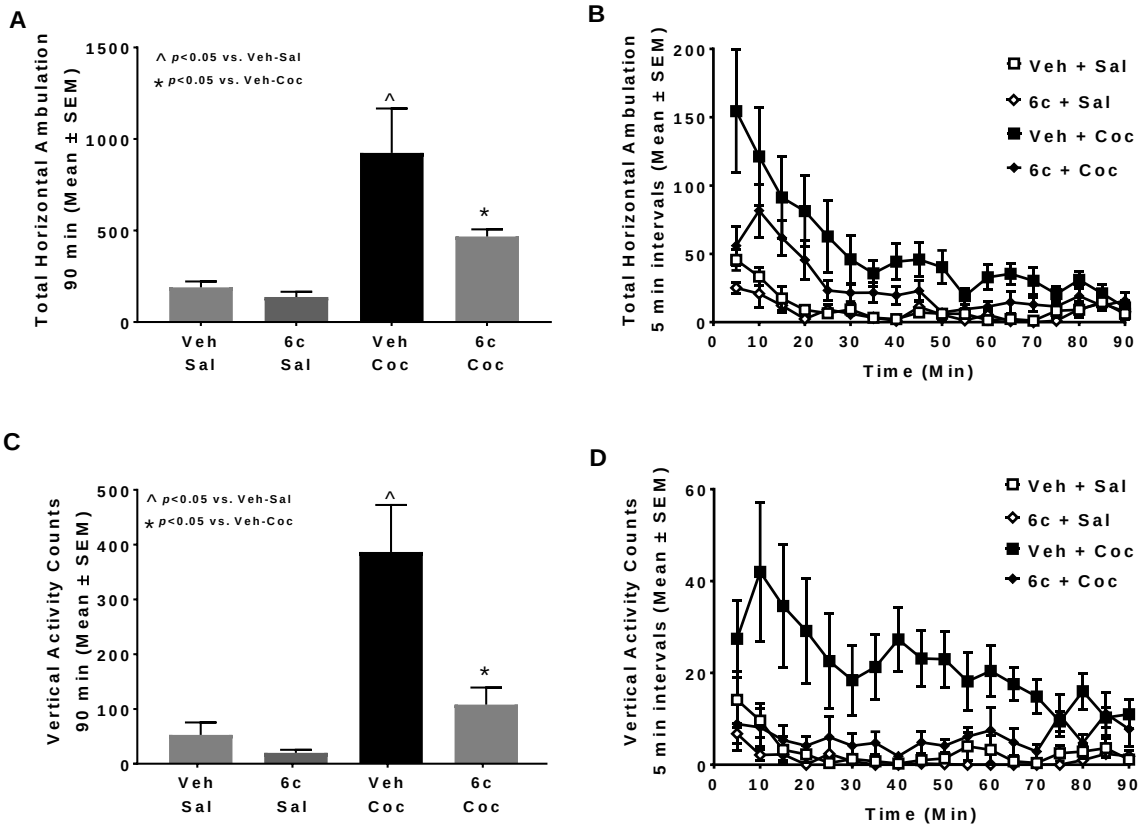


Figure 3.3: 5-HT_{2A}R antagonist homobivalent ligands suppresses cocaine-, but not saline-evoked locomotor activity

Effects of pretreatment with the bivalent derivative 6c. Rats (n=8/group) were treated (i.p.) with vehicle (Veh) or bivalent ligand 6c (2 mg/kg) 30 min prior to injection with saline (Sal; 1 ml/kg) or cocaine (Coc; 15 mg/kg) immediately before the test session commenced. Data are presented as mean (± SEM) total horizontal ambulation summed for the entire 90 min session (A) or in 5-min time bins (B) and vertical activity counts in summed for the entire 90 min session (C) or 5-min time bins (D).

The present data indicates that the series of covalently-linked homobivalent 5-HT_{2A}R antagonist ligands based on the structure of the potent 5-HT_{2A}R antagonist (+)-M100907 (Compound **1**) retain potency and efficacy *in vitro* and *in vivo*. These ligands

maintained nanomolar affinity for the 5-HT_{2A}R, selectivity over the 5-HT_{2B}R and 5-HT_{2C}R, and nanomolar potency as 5-HT_{2A}R antagonists in a cellular assay of $ERK_{1/2}$ activation. Furthermore, these results suggest that tether lengths of 8-21 atoms in length are optimal to suppress $ERK_{1/2}$ activation (present studies), which is consistent with our previous findings in the Ca_i^{2+} assay (Shashack et al., 2011). Encouragingly, we also demonstrated that the covalently-linked homobivalent ligand with optimal spacing suppresses cocaine-evoked behavior *in vivo*, reaching similar levels of efficacy as the well-characterized parent ligand (+)-M100907. Thus, we have generated homobivalent ligands that retain effects consistent with 5-HT_{2A}R antagonism both *in vitro* and *in vivo.* Future studies are required to explore whether the addition of a second pharmacophore results in a second binding site which could represent the active site of a second interacting receptor, as might be expected given that the 5-HT_{2A}R has been demonstrated to exist as a receptor homodimer (Brea et al., 2009; Bruno et al., 2011; Iglesias et al., 2016; Teitler and Klein, 2012). Thus, these newly-designed bivalent ligands may provide useful tools to further explore 5-HT_{2A}R function and homodimerization in relation to human disease and development of novel therapeutics.

MATERIALS AND METHODS

CELL LINES AND CELL CULTURE. A CHO-K1 cell line stably transfected with the 5-HT_{2A}R (5-HT_{2A}R-CHO cells; FA4 line) was a generous gift of K. Berg and W. Clarke (University of Texas Health Science Center at San Antonio). This line expresses transfected h5-HT_{2A}R in the p198-DHFR-Hygro vector containing a hygromycin resistance gene (Berg et al., 1994a). Reverse transcription of RNA followed by a quantitative real time PCR assay confirmed that 5-HT_{2A}R-CHO cells expressed 5-HT_{2A}R

mRNA (estimated to be approximately 3-4% of the mRNA level of the housekeeping gene cyclophilin; data not shown), but did not express either 5-HT_{2B}R or 5-HT_{2C}R mRNA (Berg et al., 2001); the parental CHO-K1 cell line did not express detectable amounts of any 5-HT_2R mRNAs (Seitz et al., 2012). Cells were grown at 37°C, 5% CO_2 and 85% relative humidity in GlutaMax α-MEM (Invitrogen, Carlsbad CA), 5% fetal bovine serum (Atlanta Biologicals, Atlanta GA), 100 µg/ml hygromycin (Mediatech, Manassas VA) and were passaged when they reached 80% confluence.

LIGANDS. Serotonin (5-HT; Acros Organics, ThermoFisher Scientific, Pittsburgh, PA) was dissolved in 1X Hank's balanced salt solution (HBSS; Cellgro, Invitrogen) for *in vitro* studies. The (+)-M100907 (**1**) [R-(+)-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidine-methanol] was synthesized in the Drug Design and Synthesis Section, National Institute on Drug Abuse (National Institutes of Health) as described (Ullrich and Rice, 2000), and was dissolved in 1X HBSS for *in vitro* studies. The (+)-M100907 (**1**), des-3-methyl-ketone-M100907 (**4**), and the monovalent and bivalent M100907 derivative compounds (**5, 6**; see **Fig. 1**.) were synthesized as described previously (Shashack et al., 2011) and dissolved in 1X HBSS for *in vitro* studies. Compound **6c** was dissolved in 1% Tween 80 + 2% ethanol in 0.9% NaCl for *in vivo* studies. (-)-Cocaine (National Institute on Drug Abuse, Research Triangle Park, NC, USA) was dissolved in 0.9% NaCl.

PLATE IMMUNOASSAY FOR ERK1/2 ACTIVATION. We adapted a previously developed plate immunoassay (Bulayeva et al., 2004), to measure levels of $pERK_{1/2}$ expression following ligand administration, with optimized fixation and antibody incubation conditions for use in this cell line (Seitz et al., 2012). Cells were plated in serum-

replete medium at 16-20K cells in 150 µl in clear-sided, clear bottom 96-well tissue culture plates. Cells were grown for 24 hr in serum-replete medium, and then shifted overnight to serum-free medium. The day of the experiment, cells were fed with 80 µl of serum-free medium and returned to the incubator for 1-2 hrs, as adding medium alone caused a measurable $pERK_{1/2}$ that subsided by 1 hr (data not shown), as seen by others (Kim et al., 2005).

Test compounds were added as 20 µl of a 5x stock concentration and plates were incubated at 37°C for 15 min. Cells were then stimulated with 1 µM of 5-HT (25 µl of a 5 µM stock) and incubated at 37°C for 5 min. Full concentration-response curves (10^{-11}-10^{-4} M) for 5-HT and each compound were performed in each experiment. Reactions were stopped by the addition of 2% paraformaldehyde (PFA) in phosphate buffered saline (PBS, pH 7.4). Cells were fixed for 45 min at room temperature (RT) then rinsed with PBS. Cells were then permeabilized with ice-cold methanol to ensure antibody access to intracellular antigens, washed with PBS and blocked for 45 min at RT with 0.1% fish gelatin (Sigma, St. Louis MO). Cells were then incubated with a 1:500 dilution of mouse monoclonal anti-$pERK_{1/2}$ (p44/42; Cell Signaling, Danvers MA; #9106) overnight at 4°C with gentle shaking. Background was determined in wells incubated with no primary antibody. After washing with PBS, biotin-conjugated secondary antibody (Vector Labs, Burlingame CA; # BA-9200, 1:500 diluted in blocking solution) was added and incubated for 1 hr at RT. Following washing, alkaline phosphatase (AP) complexed with avidin (Vector Labs, #AK5000) was prepared according to the manufacturer's directions, added to the wells and incubated for 1 hr at RT. After washing, 50 µl of the AP substrate para-nitrophenyl-phosphate (pNpp; Vector Labs, #SK-5900) with levamisole (an inhibitor of endogenous

phosphatases; Vector Labs, #SP-5000; two drops/10 ml), freshly prepared in 100 mM sodium bicarbonate was added, and the plate was incubated at 37°C for 30 min. The absorbance of the yellow product para-nitrophenol (pNp) was measured at 405 nm (A_{405}). Data were normalized to total cell mass in each well as measured by crystal violet staining (below) and expressed as A_{405}/A_{590}. The pIC_{50} values for $pERK_{1/2}$ expression were determined using 3-parameter nonlinear regression analysis (GraphPad Prism 7.02) and calculated from at least three independent experiments, each conducted in technical replicates of 3-8, and are presented as the mean ± SEM.

CRYSTAL-VIOLET STAINING. Total cell mass in each well was measured by crystal violet staining, a value proportional to cell number used to estimate cell number in each well. Upon completion of an experiment, wells were rinsed with water, air dried and 50 µl of crystal violet solution (0.1% in water) was added for 30 min at RT, followed by one additional rinse. Cell-absorbed dye was extracted by the addition of 10% acetic acid (30 min, RT) and absorbance read at 590 nm (A_{590}).

RADIOACTIVE BINDING ASSAYS. K_i determinations were generously provided by the NIMH Psychoactive Drug Screening Program (PDSP; http://pdsp.med.unc.edu/). Briefly, binding assays were performed using crude membrane fractions from cell lines transiently or stably transfected with the appropriate receptor. The 5-HT_{2A}R binding was determined by displacement of [^{3}H] ketanserin (0.5 nM) relative to displacement by 10 µM clozapine. The 5-HT_{2B}R binding was determined by displacement of [^{3}H] LSD (1.0 nM) relative to displacement by 10 µM methysergide. The 5-HT_{2C}R binding was determined by displacement of [^{3}H] mesulergine (0.5 nM) relative to displacement by 10 µM chlorpromazine. An initial screen measured net displacement of bound ligand by 10 µM of

each synthetic antagonist. K_i values then were determined on all compounds that yielded > 50% displacement by performing competitive binding curves using 11 concentrations spanning six orders of magnitude, with triplicate determinations for each concentration. Binding determinations were repeated for the racemic M100907 and for the bivalent molecules and the resultant K_i values are represented as mean ± SEM.

ANIMALS. A total of 32 male Sprague-Dawley rats (Harlan, Inc., Indianapolis, IN, USA) weighing 225-325 g at the start of the experiments were used. Rats were allowed to acclimate for 5-7 days in a colony room at a constant temperature (21-23°C) and humidity (45-50%) on a 12 hr light-dark cycle (lights on 0700-1900 hr). Rats were housed two rats per cage and food and water was available *ad libitum*. All experiments were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (2011) and with the approval of the UTMB Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques, when available.

IN VIVO BEHAVIORAL ASSESSMENT. Locomotor activity was monitored and quantified under low light conditions using a modified open field activity system (San Diego Instruments, San Diego, CA) according to previous publications with minor modifications (Anastasio et al., 2013; Cunningham et al., 2013; Cunningham et al., 2011; McMahon and Cunningham, 2001). Clear Plexiglass chambers (40 x 40 x 40 cm) were surrounded by a 4 x 4 photobeam matrix positioned 4 cm from the chamber floor. Consecutive photobeam breaks within the 16 x 16 cm of the activity monitor were recorded as central ambulation. Peripheral ambulation was counted as consecutive beam breaks in the surrounding perimeter. Central and peripheral ambulations were summed to provide a

measure of total horizontal ambulation. Vertical activity was quantified as the sum of the upper photobeam breaks that occurred within the activity monitor every 5 min. Rats were acclimated to the colony room and following 1 week of handling, rats were habituated to the activity monitors for 3 h/day for 2 days before the test day. Using a between-subjects design, rats (n=7-8/group) received vehicle (1% Tween 80 + 2% ethanol in 0.9% NaCl, 1 ml/kg, i.p.), or compound **6c** (2 mg/kg, i.p.), followed 30 minutes later by an injection of either saline (1 ml/kg, i.p.) or cocaine (15 mg/kg, i.p) and were immediately placed in activity monitors; locomotor activity was assessed for 90 min. Due to a misinjection, one rat was removed from vehicle plus cocaine group for a final n=7 in that group; all other groups were n=8.

Locomotor activity data are presented as mean total horizontal ambulation or vertical activity (± SEM) across the session the entire 90-min session or within 5 min time bins. The main effect of treatment on total horizontal ambulation and vertical activity were analyzed with a one-way ANOVA using the GLM procedure (SAS for Windows). Subsequent *a priori* comparisons between means for total horizontal ambulation and vertical activity were made using the Bonferroni correction. All statistical analyses were conducted with an experimentwise error rate of α=0.05.

Chapter 4: Discussion

This dissertation provides evidence that modulators proposed to disrupt (Chapter 2) or stabilize (Chapter 3) PPIs within the 5-HT_2 receptor family, can be developed to regulate signaling *in vitro* and alter behavior *in vivo*. As discussed, compounds such as the ones presented here may allow for more selective targeting of desired receptors over other highly homologous receptors. In the case of the 5-HT_{2C}R:PTEN complex, for example, PTEN has not been shown to interact with the 5-HT_{2A}R and is not expected to interact with the 5-HT_{2B}R (Anastasio et al., 2013). Thus, this new druggable interface allows for distinction between these three highly homologous receptors which is difficult to achieve via the orthosteric binding site. Discovery and targeting of GPCR PPIs is likely to yield many additional targets to consider in future drug discovery efforts.

Along with the opportunity for enhanced target selectivity, GPCR PPIs may also afford a variety of additional advantages that could positively impact drug discovery efforts. First, PPIs may allow for tissue- or region-specific targeting of GPCRs. Second, PPIs may also allow for induction of specific signal transduction mechanisms. Third, PPIs may allow for modulation of other related PPIs. These three advantages, which will be further discussed below, could overall allow for more detailed modulation of targeted GPCRs than that afforded by receptor binding sites.

Region and Tissue-Specific Selectivity

An important and exciting attribute of PPIs is not only their potential for enhanced receptor selectivity between other homologous receptors, but PPIs may also allow for tissue- or region-specific targeting. Orthosteric ligands are designed to bind to the GPCR

itself, and thus in whatever brain region or tissue the receptor is expressed, the orthosteric ligand is likely to bind and regulate receptor activity, assuming that the ligand is distributed within those regions. PPIs, on the other hand, depend on the interacting proteins being expressed in the same cell and within compartments that allow for the interaction to occur. This constraint could be harnessed for the development of tissue and region selective PPI modulators.

For example, disruption of the 5-HT_{2C}R:PTEN complex which enhances 5-HT_{2C}R agonist-induced signaling (Chapter 2) (Anastasio et al., 2013; Ji et al., 2006) may allow for brain region specific targeting of the 5-HT_{2C}R depending on the expression pattern of both 5-HT_{2C}R and PTEN. The 5-HT_{2C}R is highly expressed in various brain regions, including the lateral habenula (Clemett et al., 2000; Pompeiano et al., 1994). An interesting study by Han and colleagues implicates 5-HT_{2C}R localized to the lateral habenula modulates depression-like behaviors in the rat (Han et al., 2015). In this study, the authors infused the 5-HT_{2C}R agonist Ro-600175 directly into the lateral habenula. As a result, rats displayed a decrease in sucrose consumption in a sucrose preference test as well as increased immobility in the forced swim test, which are rodent models of depressive-like behaviors (Porsolt et al., 1977). Both findings were reversed by intra-lateral habenular administration of selective 5-HT_{2C}R antagonist SB242084, supporting the involvement of 5-HT_{2C}R agonism in these behaviors (Han et al., 2015). Based on these results, it could be hypothesized that that systemic 5-HT_{2C}R agonist administration, which is likely to activate the 5-HT_{2C}R localized to the lateral habenula, could lead to depressive-like symptoms in humans, which may thus limit clinical utility of 5-HT_{2C}R agonists. Interestingly, PTEN expression in the lateral habenula is very low in comparison to PTEN expression in other

brain regions (Cai et al., 2009). The differing expression patterns of 5-HT_{2C}R and PTEN could suggest that 5-HT_{2C}R:PTEN interaction may be lower or, perhaps, non-existent in the lateral habenula versus brain regions where both proteins are highly expressed (e.g., frontal cortex, ventral tegmental area) (Cai et al., 2009; Clemett et al., 2000; Pompeiano et al., 1994). Thus, one could hypothesize that 5-HT_{2C}R:PTEN complex disrupters may not enhance 5-HT_{2C}R signaling in the lateral habenula and, thus, not enhance 5-HT_{2C}R agonist-induction of depressive-like behaviors. Experiments must, of course, be conducted to test this hypothesis, but this example illustrates the important clinical implication that targeting GPCRs in a region/tissue specific manner could provide and which could be achieved through targeting GPCR PPIs.

Future studies should assess receptor interactomes in different tissues and compartment as an effort to identify novel druggable targets that may allow for region specific therapeutics. For example, proteomics could be conducted in various brain regions for CNS-localized GPCRs, like the 5-HT_{2C}R, and explore if different brain regions possess differing PPIs. This information could then be used to develop PPI modulators that may allow for the targeting of certain neurociruits over others and, as such, enhance the therapeutic efficacy of the developed modulator, as well as increase the understanding of the role of the targeted receptor in specific neurocircuits.

MODULATION OF SPECIFIC RECEPTOR SIGNAL TRANSDUCTION CASCADES

The GPCR PPIs may allow for modulation of specific receptor signal transduction mechanisms, a concept known as biased signaling or functional selectivity (Kenakin and Christopoulos, 2013; Whalen et al., 2011). Classically, GPCRs were considered to signal solely through G protein-dependent mechanisms, but recent transformative work has

demonstrated that other proteins, like β-arrestins (*i.e.*, β-arrestin$_1$ or β-arrestin$_2$), can mediate additional signaling pathways that is independent and distinguishable from G protein-dependent mechanisms (Lefkowitz and Shenoy, 2005). Additionally, ligands have been developed that can preferentially induce certain signaling pathways over others and display overall differing effects *in vitro* and *in vivo* (Allen et al., 2011; Bruchas and Chavkin, 2010; Gesty-Palmer et al., 2006; Lovell et al., 2015; Marti-Solano et al., 2015; Masri et al., 2008). To understand how GPCR PPIs may modulate biased signaling, it is important to understand the mechanisms that lead to bias which are related to the signal transduction cascades induced by receptor activation, as described below.

In the presence of an agonist, GPCRs assume an active state and catalyze the exchange of guanosine diphosphate for guanosine triphosphate on the Gα subunit of a G protein which then leads to dissociation and release of the heterotrimeric subunits and activation of G protein-dependent signaling (Ritter and Hall, 2009). The second messengers that are subsequently activated depend on the G protein that associates with the receptor. In the case of the 5-HT$_{2A}$R and 5-HT$_{2C}$R, agonist activation preferentially induces coupling of the G$_{q/11}$ family of G proteins that results in activation of phospholipase Cβ (PLCβ) which generates intracellular second messengers inositol-1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG). IP$_3$ interacts with the IP$_3$ receptor leading to increased intracellular calcium (Ca_i^{2+}) release (Hannon and Hoyer, 2008; Millan et al., 2008) and DAG activates protein kinase C (PKC) which then activates the MAP kinase cascade resulting in phosphorylation of ERK$_{1/2}$ (Werry et al., 2005). The 5-HT$_{2A}$R and 5-HT$_{2C}$R can also activate phospholipase A$_2$ (PLA$_2$) and generate arachidonic acid (AA) through an

(unidentified) pertussis toxin-sensitive G protein (Felder et al., 1990) as well as phospholipase D (PLD) via $G\alpha_{12/13}$ (McGrew et al., 2002; Moya et al., 2011).

Once the GPCR is in an active conformation, GPCR kinases (GRKs) and other protein kinases (e.g., PKA and PKC) phosphorylate the receptor at serine and threonine sites which then induces β-arrestin recruitment to the receptor (Gurevich and Gurevich, 2006). Importantly, an active receptor conformation *and* receptor phosphorylation are required for a high affinity interaction of β-arrestins with the receptor. The active receptor conformation exposes the β-$arrestin_2$ binding site and the receptor phosphorylation stabilizes a required β-arrestin conformation by disrupting the polar core within the β-arrestin protein (Gurevich and Gurevich, 2006). The β-arrestin interaction with the GPCR results in receptor desensitization by sterically hindering the G protein binding site, thus preventing further G protein-dependent signal transduction (Gurevich and Gurevich, 2006). β-arrestins are scaffolding proteins that are best known for arresting G protein-dependent signaling and leading to receptor internalization by facilitating receptor interaction with clathrin and endocytosis machinery (DeWire et al., 2007). However, recent work has also implicated β-arrestins in signal transduction by which β-arrestins serve as a scaffolding protein for signaling cascades including the MAP kinase pathway (Kholodenko et al., 2010).

Biased signaling ensues when an agonist or allosteric modulator differentially engage downstream signaling cascades by recruiting specific immediate effectors (e.g., G proteins vs. β-arrestins). Depending on the immediate effectors that are recruited, differing signal transduction patterns ensue. For example, G protein-dependent signaling pathways result in activation of specific downstream signaling effectors and kinases (e.g., Ca_i^{++}, $pERK_{1/2}$) while β-$arrestin_2$-dependent signaling can result in a different subset of downstream

effectors as well as different subcellular distribution of shared effectors (e.g., cytoplasmic vs. nuclear $pERK_{1/2}$). These signaling profiles can then lead to distinct overall effects of GPCR activation (Gesty-Palmer et al., 2006). Targeting G protein- vs. β-$arrestin_2$-dependent mechanisms can allow for the selective induction of certain outcomes of receptor activation and, therefore, reduce undesired side effects and minimize translational barriers to therapeutic development, an hypothesis which is supported by work in various GPCR systems (Allen et al., 2011; Bruchas and Chavkin, 2010; Gesty-Palmer et al., 2006; Lovell et al., 2015; Marti-Solano et al., 2015; Masri et al., 2008).

A recent publication has suggested that β-$arrestin_2$ involvement in β_2-adrenergic receptor (β_2-AR) signaling may relate specifically to β-$arrestin_2$ scaffolding of G protein-activated $pERK_{1/2}$ in specific cellular compartment, rather than β-$arrestin_2$ mediated signaling that is independent of the cognate G protein (O'Hayre et al., 2017). Previous work has implicated β-$arrestin_2$ in the phosphorylation of $pERK_{1/2}$ in G protein-independent mechanism (Lefkowitz and Shenoy, 2005), particularly for the angiotensin II receptor (Ahn et al., 2004), and, thus, the exact role of β-$arrestin_2$ in β_2-AR signaling and other GPCR signal transduction remains to be resolved.

The therapeutic potential of biased signaling has only recently begun to be harnessed. Important examples of enhanced therapeutic efficacy with reduced side effect profiles of both G protein and β-arrestin biased ligands have been demonstrated across all three classes of GPCRs (for review) (Rankovic et al., 2016). A prominent example is the class A G protein coupled μ-opioid receptor (μ-OR) which is an important target for the treatment of pain and a site of action for morphine. Morphine administration in β-$arrestin_2$ knockout mice results in enhanced antinociceptive effects versus wild type mice while

simultaneously produces a reduction in morphine-associated side effects (e.g., tolerance, constipation, colonic propulsion, respiratory suppression) (Bohn et al., 2000; Bohn et al., 1999; Raehal et al., 2005). These studies suggested that β-arrestin$_2$-dependent mechanisms contribute to the adverse effect of morphine and, as such, sparked an interest in the development of G protein biased ligands that has culminated in the development of TRV130 which is currently in clinical trials. In clinical trials, TRV130 produced greater categorical pain relief compared to morphine and displayed no serious adverse effects, including reduced respiratory suppression, which is the most detrimental side effect of morphine and generally the cause of death in cases of opiate overdose (Soergel et al., 2014; Viscusi et al., 2016).

Another important example of the therapeutic potential of biased ligands comes from the class B G protein coupled angiotensin II receptor 1 (AT1R) which is a target for the treatment of hypertension. Unbiased AT1R blockers (e.g., losartan) are clinically approved for the treatment of hypertension (Burnier, 2001). However, losartan, and other AT1R blockers, also suppress AT1R-mediated inotropy (force of contraction) which consequently reduces cardiac output and limits the clinical utility of these medications. Recent work has developed the β-arrestin$_2$ biased agonist TRV027 which retained the therapeutic benefit of losartan without significant adverse effects in humans and has recently proceeded to phase 2b clinical studies (Violin et al., 2014).

As evidenced by these two examples, both G protein and β-arrestin biased ligands have shown therapeutic benefit over non-biased ligands, and, thus, approaches to bias receptor signaling are attractive avenues of investigation. However, like most GPCR drug discovery efforts, the main focus for development of bias signaling modulators currently underway

focuses on the orthosteric binding site (Rankovic et al., 2016). There are some examples in which researchers are focused on allosteric receptor binding sites for the development biased signaling modulators, but this is a newer approach (Sengmany et al., 2017). As discussed in Chapter 1, targeting the orthosteric site can be challenging and thus alternative approaches may have important clinical impact.

Biased signaling is induced by differential recruitment of immediate effectors (i.e., G proteins, β-arrestins) and secondary messengers (i.e., cAMP, Ca_i^{2+}, $pERK_{1/2}$). Thus, approaches that impact the recruitment of these signaling molecules may induce bias. One approach is through the stabilization of certain receptor conformations that "open" binding sites for certain effectors over others, which is the main concept of current bias signaling development via orthosteric and allosteric biased ligands (Whalen et al., 2011). However, GPCRs interact with a variety of other protein partners, some of which, as shown for the 5-HT_{2C}R (Chapter 2) can regulate receptor signaling. It could be conceived that PPIs may impact the differential recruitment of downstream effectors and thus modulate biased signaling.

Biased signaling of the 5-HT_{2C}R may be modulated by PTEN through the regulation of receptor phosphorylation, which is one of the triggers for β-$arrestin_2$ recruitment. Ji and colleagues conducted phospho-band shift Western blot analyses to suggest that PTEN dephosphorylates the 5-HT_{2C}R in cellular models (Ji et al., 2006). Furthermore, the dephosphorylation of 5-HT_{2C}R is suppressed by treatment with 3L4F, suggesting that disruption of the 5-HT_{2C}R:PTEN complex allows the receptor to be phosphorylated (Ji et al., 2006). Since receptor phosphorylation is one of the triggers of β-$arrestin_2$ recruitment, disruption of the 5-HT_{2C}R:PTEN complex may enhance recruitment of β-$arrestin_2$ by

allowing the receptor to remain phosphorylated and as such may induce β-arrestin$_2$-dependent signaling. Preliminary evidence to support this hypothesized change in signal transduction comes from the lorcaserin-saline drug discrimination experiments (Chapter 2). In these experiments, rats were trained to discriminate the interoceptive cue elicited by the selective 5-HT$_{2C}$R agonist lorcaserin from saline in a two-lever drug discrimination model. We demonstrate that the full substitution of the training dose of lorcaserin (0.75 mg/kg) was suppressed by treatment with PLC$_\beta$ inhibitor U73122, suggesting that PLC$_\beta$-mediated signaling is involved in the generation of the stimulus discrimination. Excitingly, the full substitution elicited by a combination of a low dose of the training drug *plus* 3L4F was not sensitive to the PLC$_\beta$ inhibitor, suggesting that 3L4F may enhance the stimulus effects of the 5-HT$_{2C}$R agonists in a PLC$_\beta$-independent mechanism. While these results alone do not imply that 3L4F induces β-arrestin$_2$-mediated signal transduction, these outcomes support the interpretation that disruption of the 5-HT$_{2C}$R:PTEN complex may shift signaling away from PLC$_\beta$-, and thus Gα_q-dependent mechanisms and as such may suggest that this PPI modulates biased signaling of the 5-HT$_{2C}$R. Future studies should be conducted to investigate β-arrestin$_2$ recruitment and probe a variety of intracellular effectors following administration of 5-HT$_{2C}$R:PTEN complex disruptors to determine if disruption of this PPI alters the signal transduction mechanisms elicited by 5-HT and synthetic 5-HT$_{2C}$R agonists. As illustrated by this example, it is likely that receptor-receptor and receptor-accessory protein interactions impact the recruitment of G proteins and β-arrestins by regulating receptor phosphorylation states and conformations (e.g., through steric hindrance of intracellular loops) and may, thus, provide a new loci for the development of biased GPCR ligands.

REGULATION OF RELATED PPIS

A third potential advantage of targeting GPCR PPIs is the potential to influence various receptor interaction through the stabilization or disruption of one PPI. For example, stabilization of 5-HT_{2A}R:5-HT_{2A}R homodimers through the use of homobivalent ligands or other PPI stabilizers, could alter 5-HT_{2A}R heterodimerization with other proposed GPCRs [i.e., mGluR2 (Gonzalez-Maeso et al., 2008); 5-HT_{2C}R (Moutkine et al., 2017)]. The 5-HT_{2A}R interaction with mGluR2 is suggested to be involved in the psychosis-like behavior effect of 5-HT_{2A}R hallucinogenic agonists in rodent models (Gonzalez-Maeso et al., 2008; Moreno et al., 2011; Moreno et al., 2012). Additionally, the 5-HT_{2A}R:mGluR2 interaction may be dysregulated in schizophrenic patients and may play a role in the positive symptoms of the disorder (Moreno et al., 2012). Overall, available data on the 5-HT_{2A}R:mGluR2 interaction suggest that disrupting this interaction suppresses the psychosis-like behaviors of 5-HT_{2A}R agonists and, as such, may be a target in the treatment of schizophrenia (Moreno et al., 2012).

One potential approach to disrupt the 5-HT_{2A}R:mGluR2 interaction could be to sequester the 5-HT_{2A}R in homodimers, thereby reducing the available 5-HT_{2A}R to interact with mGluR2. Thus, PPI modulators that stabilize the 5-HT_{2A}R:5-HT_{2A}R homodimer may also indirectly reduce the 5-HT_{2A}R:mGluR2 heterodimer. It yet remains to be determined whether disruption of 5-HT_{2A}R:mGluR2 interaction will have clinical implications for the treatment of schizophrenia or other disorders. However, if disruption of 5-HT_{2A}R:mGluR2 interaction proves to be beneficial, 5-HT_{2A}R antagonist homobivalent ligands could provide enhanced therapeutic benefit over a single-pharmacophore 5-HT_{2A}R antagonist by

suppressing 5-HT_{2A}R signaling (Chapter 3) (Shashack et al., 2011) while at the same time potentially decreasing the 5-HT_{2A}R:mGluR2 interaction thought to contribute to hallucinogenic properties of 5-HT_{2A}R agonism. As illustrated by this example, PPI modulators could provide an approach to potentially impact associated PPIs that could be related to outcomes of interest.

Overall, PPIs remain an under-explored attribute of GPCRs in the drug discovery arena (George et al., 2002). As this work suggests, PPI have potential as targets for the discovery of highly selective drugs for GPCRs that have historically been difficult to manipulate. Although consideration of PPIs, both receptor-receptor and receptor–accessory proteins, complicates the GPCR drug discovery field, these interesting interactions should be considered, studied and exploited for their potential clinical implications.

References

Adessi, C., and Soto, C. (2002). Converting a peptide into a drug: strategies to improve stability and bioavailability. Curr. Med. Chem. *9*, 963-978.

Ahn, S., Shenoy, S.K., Wei, H., and Lefkowitz, R.J. (2004). Differential kinetic and spatial patterns of beta-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. J. Biol. Chem. *279*, 35518-35525.

Al-Shamma, H.A., Anderson, C., Chuang, E., Luthringer, R., Grottick, A.J., Hauser, E., Morgan, M., Shanahan, W., Teegarden, B.R., Thomsen, W.J., *et al.* (2010). Nelotanserin, a novel selective human 5-hydroxytryptamine2A inverse agonist for the treatment of insomnia. J.Pharmacol.Exp.Ther. *332*, 281-290.

Allen, J.A., Yost, J.M., Setola, V., Chen, X., Sassano, M.F., Chen, M., Peterson, S., Yadav, P.N., Huang, X.P., Feng, B., *et al.* (2011). Discovery of beta-arrestin-biased dopamine D2 ligands for probing signal transduction pathways essential for antipsychotic efficacy. Proc. Natl. Acad. Sci. U. S. A. *108*, 18488-18493.

Anastasio, N.C., Gilbertson, S.R., Bubar, M.J., Agarkov, A., Stutz, S.J., Jeng, Y.J., Bremer, N.M., Smith, T.D., Fox, R.G., Swinford, S.E., *et al.* (2013). Peptide inhibitors disrupt the serotonin 5-HT_{2C} receptor interaction with phosphatase and tensin homolog to allosterically modulate cellular signaling and behavior. J. Neurosci. *33*, 1615-1630.

Anastasio, N.C., Stoffel, E.C., Fox, R.G., Bubar, M.J., Rice, K.C., Moeller, F.G., and Cunningham, K.A. (2011). Serotonin (5-hydroxytryptamine) 5-HT_{2A} receptor: Association with inherent and cocaine-evoked behavioral disinhibition in rats. Behav. Pharmacol. *22*, 248-261.

Anastasio, N.C., Stutz, S.J., Fox, R.G., Sears, R.M., Emeson, R.B., DiLeone, R.J., O'Neil, R.T., Fink, L.H., Li, D., Green, T.A., *et al.* (2014). Functional status of the serotonin 5-HT_{2C} receptor (5-HT_{2C}R) drives interlocked phenotypes that precipitate relapse-like behaviors in cocaine dependence. Neuropsychopharmacology *39*, 370-382.

Ancoli-Israel, S., Vanover, K.E., Weiner, D.M., Davis, R.E., and Van Kammen, D.P. (2011). Pimavanserin tartrate, a 5-HT(2A) receptor inverse agonist, increases slow wave sleep as measured by polysomnography in healthy adult volunteers. Sleep Med. *12*, 134-141.

Appel, J.B., and Cunningham, K.A. (1986). The use of drug discrimination procedures to characterize hallucinogenic drug action. Psychopharmacol.Bull. *22*, 959-967.

Arkin, M.R., and Wells, J.A. (2004). Small-molecule inhibitors of protein-protein interactions: progressing towards the dream. Nat. Rev. Drug Discov. *3*, 301-317.

Barnes, N.M., and Sharp, T. (1999). A review of central 5-HT receptors and their function. Neuropharmacology *38*, 1083-1152.

Berg, K.A., Clarke, W.P., Chen, Y., Ebersole, B.J., McKay, R.D.G., and Maayani, S. (1994a). 5-hydroxytryptamine type 2A receptors regulate cyclic AMP accumulation in a neuronal cell line by protein kinase C- dependent and calcium/calmodulin-dependent mechanisms. Mol.Pharmacol. *45*, 826-836.

Berg, K.A., Clarke, W.P., Sailstad, C., Saltzman, A., and Maayani, S. (1994b). Signal transduction differences between 5-hydroxytryptamine type 2A and type 2C receptor systems. Mol. Pharmacol. *46*, 477-484.

Berg, K.A., Harvey, J.A., Spampinato, U., and Clarke, W.P. (2008). Physiological and therapeutic relevance of constitutive activity of 5-HT 2A and 5-HT 2C receptors for the treatment of depression. Prog.Brain Res *172*, 287-305.

Berg, K.A., Stout, B.D., Maayani, S., and Clarke, W.P. (2001). Differences in rapid desensitization of 5-hydroxytryptamine2A and 5-hydroxytryptamine2C receptor-mediated phospholipase C activation. J Pharmacol Exp.Ther. *299*, 593-602.

Bergman, J., France, C.P., Holtzman, S.G., Katz, J.L., Koek, W., and Stephens, D.N. (2000). Agonist efficacy, drug dependence, and medications development: preclinical evaluation of opioid, dopaminergic, and GABAA-ergic ligands. Psychopharmacology (Berl.) *153*, 67-84.

Blair, D.T., and Dauner, A. (1992). Extrapyramidal symptoms are serious side-effects of antipsychotic and other drugs. Nurse Pract. *17*, 56, 62-54, 67.

Bohn, L.M., Gainetdinov, R.R., Lin, F.T., Lefkowitz, R.J., and Caron, M.G. (2000). Mu-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. Nature *408*, 720-723.

Bohn, L.M., Lefkowitz, R.J., Gainetdinov, R.R., Peppel, K., Caron, M.G., and Lin, F.T. (1999). Enhanced morphine analgesia in mice lacking beta-arrestin 2. Science *286*, 2495-2498.

Bortolato, A., Dore, A.S., Hollenstein, K., Tehan, B.G., Mason, J.S., and Marshall, F.H. (2014). Structure of Class B GPCRs: new horizons for drug discovery. Br. J. Pharmacol. *171*, 3132-3145.

Brady, A.E., and Limbird, L.E. (2002). G protein-coupled receptor interacting proteins: emerging roles in localization and signal transduction. Cell. Signal. *14*, 297-309.

Brea, J., Castro, M., Giraldo, J., Lopez-Gimenez, J.F., Padin, J.F., Quintian, F., Cadavid, M.I., Vilaro, M.T., Mengod, G., Berg, K.A., *et al.* (2009). Evidence for distinct antagonist-revealed functional states of 5-hydroxytryptamine(2A) receptor homodimers. Mol.Pharmacol *75*, 1380-1391.

Brogi, S., Tafi, A., Desaubry, L., and Nebigil, C.G. (2014). Discovery of GPCR ligands for probing signal transduction pathways. Front. Pharmacol. *5*, 255.

Bruchas, M.R., and Chavkin, C. (2010). Kinase cascades and ligand-directed signaling at the kappa opioid receptor. Psychopharmacology (Berl.) *210*, 137-147.

Bruno, A., Beato, C., and Costantino, G. (2011). Molecular dynamics simulations and docking studies on 3D models of the heterodimeric and homodimeric 5-HT(2A) receptor subtype. Future Med. Chem. *3*, 665-681.

Bruno, A., Guadix, A.E., and Costantino, G. (2009). Molecular dynamics simulation of the heterodimeric mGluR2/5HT(2A) complex. An atomistic resolution study of a potential new target in psychiatric conditions. J.Chem.Inf.Model. *49*, 1602-1616.

Bubar, M.J., and Cunningham, K.A. (2008). Prospects for serotonin 5-HT_2R pharmacotherapy in psychostimulant abuse. Prog. Brain Res. *172*, 319-346.

Bulayeva, N.N., Gametchu, B., and Watson, C.S. (2004). Quantitative measurement of estrogen-induced ERK 1 and 2 activation via multiple membrane-initiated signaling pathways. Steroids *69*, 181-192.

Bunnage, M.E. (2011). Getting pharmaceutical R&D back on target. Nat. Chem. Biol. *7*, 335-339.

Burmeister, J.J., Lungren, E.M., Kirschner, K.F., and Neisewander, J.L. (2004). Differential roles of 5-HT receptor subtypes in cue and cocaine reinstatement of cocaine-seeking behavior in rats. Neuropsychopharmacology *29*, 660-668.

Burnier, M. (2001). Angiotensin II type 1 receptor blockers. Circulation *103*, 904-912.

Cai, Q.Y., Chen, X.S., Zhong, S.C., Luo, X., and Yao, Z.X. (2009). Differential expression of PTEN in normal adult rat brain and upregulation of PTEN and p-Akt in the ischemic cerebral cortex. Anat.Rec.(Hoboken.) *292*, 498-512.

Callahan, P.M., and Cunningham, K.A. (1994). Involvement of 5-HT_{2C} receptors in mediating the discriminative stimulus properties of *m*-chlorphenylpiperazine (MCPP). Eur.J.Pharmacol. *257*, 27-38.

Callahan, P.M., and Cunningham, K.A. (1995). Modulation of the discriminative stimulus properties of cocaine by 5-HT_{1B} and 5-HT_{2C} receptors. J.Pharmacol.Exp.Ther. *274*, 1414-1424.

Capriotti, T. (1998). In search of a 'magic' pill to treat obesity: the rise and fall of 'Fen-Phen'. Medsurg Nurs. *7*, 52-54.

Carr, G.V., and Lucki, I. (2011). The role of serotonin receptor subtypes in treating depression: a review of animal studies. Psychopharmacology (Berl.) *213*, 265-287.

Celada, P., Puig, M., margos-Bosch, M., Adell, A., and Artigas, F. (2004). The therapeutic role of 5-HT1A and 5-HT2A receptors in depression. J.Psychiatry Neurosci. *29*, 252-265.

Chan, W.Y., McKinzie, D.L., Bose, S., Mitchell, S.N., Witkin, J.M., Thompson, R.C., Christopoulos, A., Lazareno, S., Birdsall, N.J., Bymaster, F.P., *et al.* (2008). Allosteric modulation of the muscarinic M4 receptor as an approach to treating schizophrenia. Proc. Natl. Acad. Sci. U. S. A. *105*, 10978-10983.

Chen, C., Li, J.G., Chen, Y., Huang, P., Wang, Y., and Liu-Chen, L.Y. (2006). GEC1 interacts with the kappa opioid receptor and enhances expression of the receptor. J. Biol. Chem. *281*, 7983-7993.

Chen, Y.C., Hartley, R.M., Anastasio, N.C., Cunningham, K.A., and Gilbertson, S.R. (2017). Synthesis and structure-activity relationships of tool compounds based on WAY163909, a 5-HT_{2C} receptor agonist. ACS Chem. Neurosci. *8*, 1004-1010.

Choi, S.K., Green, D., Ho, A., Klein, U., Marquess, D., Taylor, R., and Turner, S.D. (2008). Designing selective, high affinity ligands of 5-HT1D receptor by covalent dimerization of 5-HT1F ligands derived from 4-fluoro-N-[3-(1-methyl-4-piperidinyl)-1H-indol-5-yl]benzamide. J.Med.Chem. *51*, 3609-3616.

Christopoulos, A., and Kenakin, T. (2002). G protein-coupled receptor allosterism and complexing. Pharmacol. Rev. *54*, 323-374.

Christopoulos, A., Parsons, A.M., Lew, M.J., and El-Fakahany, E.E. (1999). The assessment of antagonist potency under conditions of transient response kinetics. Eur. J. Pharmacol. *382*, 217-227.

Clemett, D.A., Punhani, T., Duxon, M.S., Blackburn, T.P., and Fone, K.C. (2000). Immunohistochemical localisation of the 5-HT2C receptor protein in the rat CNS. Neuropharmacology *39*, 123-132.

Colpaert, F.C. (1999). Drug discrimination in neurobiology. Pharmacol. Biochem. Behav. *64*, 337-345.

Conn, P.J., Christopoulos, A., and Lindsley, C.W. (2009). Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. Nat. Rev. Drug Discov. *8*, 41-54.

Connolly, H.M., Crary, J.L., McGoon, M.D., Hensrud, D.D., Edwards, B.S., Edwards, W.D., and Schaff, H.V. (1997). Valvular heart disease associated with fenfluramine-phentermine. N. Engl. J. Med. *337*, 581-588.

Cunningham, K.A., and Anastasio, N.C. (2014). Serotonin at the nexus of impulsivity and cue reactivity in cocaine addiction. Neuropharmacology *76 Pt B*, 460-478.

Cunningham, K.A., Anastasio, N.C., Fox, R.G., Stutz, S.J., Bubar, M.J., Swinford, S.E., Watson, C.S., Gilbertson, S.R., Rice , K.C., Rosenzweig-Lipson, S., *et al.* (2013). Synergism between a serotonin 5-HT_{2A} receptor (5-HT_{2A}R) antagonist and 5-HT_{2C}R agonist suggests new pharmacotherapeutics for cocaine addiction. ACS Chem. Neurosci. *4*, 110-121.

Cunningham, K.A., Callahan, P.M., and Appel, J.B. (1985). Discriminative stimulus properties of clonidine: Substitution by ergot derivatives. Eur.J.Pharmacol. *119*, 225-229.

Cunningham, K.A., Fox, R.G., Anastasio, N.C., Bubar, M.J., Stutz, S.J., Moeller, F.G., Gilbertson, S.R., and Rosenzweig-Lipson, S. (2011). Selective serotonin 5-HT2C receptor activation suppresses the reinforcing efficacy of cocaine and sucrose but differentially affects the incentive-salience value of cocaine- vs. sucrose-associated cues. Neuropharmacology *61*, 513-523.

Daniels, D.J., Lenard, N.R., Etienne, C.L., Law, P.Y., Roerig, S.C., and Portoghese, P.S. (2005). Opioid-induced tolerance and dependence in mice is modulated by the distance between pharmacophores in a bivalent ligand series. Proc.Natl.Acad.Sci.U.S.A *102*, 19208-19213.

de, P.T. (2001). M-100907 (Aventis). Curr.Opin.Investig.Drugs *2*, 123-132.

Decker, M., and Lehmann, J. (2007). Agonistic and antagonistic bivalent ligands for serotonin and dopamine receptors including their transporters. Curr.Top.Med.Chem. *7*, 347-353.

DeWire, S.M., Ahn, S., Lefkowitz, R.J., and Shenoy, S.K. (2007). Beta-arrestins and cell signaling. Annu. Rev. Physiol. *69*, 483-510.

Di Giovanni, G., and De Deurwaerdere, P. (2016a). New therapeutic opportunities for 5-HT2C receptor ligands in neuropsychiatric disorders. Pharmacol. Ther. *157*, 125-162.

Di Giovanni, G., and De Deurwaerdere, P. (2016b). New therapeutic opportunities for 5-HT receptor ligands in neuropsychiatric disorders. Pharmacol. Ther. *157* 125-162.

Di, L. (2015). Strategic approaches to optimizing peptide ADME properties. AAPS J *17*, 134-143.

Ding, C., Bremer, N.M., Smith, T.D., Seitz, P.K., Anastasio, N.C., Cunningham, K.A., and Zhou, J. (2012). Exploration of synthetic approaches and pharmacological evaluation of PNU-69176E and its stereoisomer as 5-HT_{2C} receptor allosteric modulators. ACS Chem. Neurosci. *3*, 538-545.

Dorr, P., Westby, M., Dobbs, S., Griffin, P., Irvine, B., Macartney, M., Mori, J., Rickett, G., Smith-Burchnell, C., Napier, C., *et al.* (2005). Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. Antimicrob. Agents Chemother. *49*, 4721-4732.

Dunlop, J., Marquis, K.L., Lim, H.K., Leung, L., Kao, J., Cheesman, C., and Rosenzweig-Lipson, S. (2006). Pharmacological profile of the 5-HT(2C) receptor agonist WAY-163909; therapeutic potential in multiple indications. CNS Drug Rev *12*, 167-177.

Felder, C.C., Kanterman, R.Y., Ma, A.L., and Axelrod, J. (1990). Serotonin stimulates phospholipase A2 and the release of arachidonic acid in hippocampal neurons by a type 2 serotonin receptor that is independent of inositolphospholipid hydrolysis. Proc. Natl. Acad. Sci. U. S. A. *87*, 2187-2191.

Filip, M., Bubar, M.J., and Cunningham, K.A. (2006). Contribution of serotonin (5-HT) 5-HT2 receptor subtypes to the discriminative stimulus effects of cocaine in rats. Psychopharmacology (Berl.) *183*, 482-489.

Filip, M., and Cunningham, K.A. (2002). Serotonin 5-HT(2C) receptors in nucleus accumbens regulate expression of the hyperlocomotive and discriminative stimulus effects of cocaine. Pharmacol. Biochem. Behav. *71*, 745-756.

Fink, L.H., Anastasio, N.C., Fox, R.G., Rice, K.C., Moeller, F.G., and Cunningham, K.A. (2015). Individual differences in impulsive action reflect variation in the cortical serotonin 5-HT2A receptor system. Neuropsychopharmacology *40*, 1957-1968.

Fisher, W.A., and Pyke, R.E. (2017). Flibanserin Efficacy and Safety in Premenopausal Women With Generalized Acquired Hypoactive Sexual Desire Disorder. Sex Med Rev.

Fitzgerald, L.W., Burn, T.C., Brown, B.S., Patterson, J.P., Corjay, M.H., Valentine, P.A., Sun, J.H., Link, J.R., Abbaszade, I., Hollis, J.M., *et al.* (2000). Possible role of valvular serotonin 5-HT(2B) receptors in the cardiopathy associated with fenfluramine. Mol. Pharmacol. *57*, 75-81.

Fletcher, P.J., Chintoh, A.F., Sinyard, J., and Higgins, G.A. (2004). Injection of the 5-HT2C receptor agonist Ro60-0175 into the ventral tegmental area reduces cocaine-induced locomotor activity and cocaine self-administration. Neuropsychopharmacology *29*, 308-318.

Fletcher, P.J., Grottick, A.J., and Higgins, G.A. (2002). Differential effects of the 5-HT2A receptor antagonist M100,907 and the 5-HT2C receptor antagonist SB242,084 on cocaine-induced locomotor activity, cocaine self-administration and cocaine-induced reinstatement of responding. Neuropsychopharmacology *27*, 576-586.

Fletcher, P.J., Rizos, Z., Noble, K., Soko, A.D., Silenieks, L.B., Le, A.D., and Higgins, G.A. (2012). Effects of the 5-HT2C receptor agonist Ro60-0175 and the 5-HT2A receptor antagonist M100907 on nicotine self-administration and reinstatement. Neuropharmacology *62*, 2288-2298.

Fletcher, P.J., Tampakeras, M., Sinyard, J., and Higgins, G.A. (2007). Opposing effects of 5-HT(2A) and 5-HT(2C) receptor antagonists in the rat and mouse on premature responding in the five-choice serial reaction time test. Psychopharmacology (Berl.) *195*, 223-234.

Fletcher, P.J., Tampakeras, M., Sinyard, J., Slassi, A., Isaac, M., and Higgins, G.A. (2009). Characterizing the effects of 5-HT(2C) receptor ligands on motor activity and feeding behaviour in 5-HT(2C) receptor knockout mice. Neuropharmacology *57*, 259-267.

Fontaine, K.R., Heo, M., Harrigan, E.P., Shear, C.L., Lakshminarayanan, M., Casey, D.E., and Allison, D.B. (2001). Estimating the consequences of anti-psychotic induced weight gain on health and mortality rate. Psychiatry Res. *101*, 277-288.

Frankel, P.S., and Cunningham, K.A. (2004). m-Chlorophenylpiperazine (mCPP) modulates the discriminative stimulus effects of cocaine through actions at the 5-HT2C receptor. Behav.Neurosci *118*, 157-162.

Gavarini, S., Becamel, C., Altier, C., Lory, P., Poncet, J., Wijnholds, J., Bockaert, J., and Marin, P. (2006). Opposite effects of PSD-95 and MPP3 PDZ proteins on serotonin 5-hydroxytryptamine2C receptor desensitization and membrane stability. Mol. Biol. Cell *17*, 4619-4631.

George, S.R., O'Dowd, B.F., and Lee, S.P. (2002). G-protein-coupled receptor oligomerization and its potential for drug discovery. Nat. Rev. Drug Discov. *1*, 808-820.

Georgoussi, Z., Georganta, E.M., and Milligan, G. (2012). The other side of opioid receptor signalling: regulation by protein-protein interaction. Curr. Drug Targets *13*, 80-102.

Gesty-Palmer, D., Chen, M., Reiter, E., Ahn, S., Nelson, C.D., Wang, S., Eckhardt, A.E., Cowan, C.L., Spurney, R.F., Luttrell, L.M., *et al.* (2006). Distinct beta-arrestin- and G protein-dependent pathways for parathyroid hormone receptor-stimulated ERK1/2 activation. J. Biol. Chem. *281*, 10856-10864.

Gether, U. (2000). Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. Endocr. Rev. *21*, 90-113.

Gongora-Benitez, M., Tulla-Puche, J., and Albericio, F. (2014). Multifaceted roles of disulfide bonds. Peptides as therapeutics. Chem. Rev. *114*, 901-926.

Gonzalez-Maeso, J., Ang, R.L., Yuen, T., Chan, P., Weisstaub, N.V., Lopez-Gimenez, J.F., Zhou, M., Okawa, Y., Callado, L.F., Milligan, G., *et al.* (2008). Identification of a serotonin/glutamate receptor complex implicated in psychosis. Nature *452*, 93-97.

Gray, J.A., and Roth, B.L. (2007). Molecular targets for treating cognitive dysfunction in schizophrenia. Schizophr.Bull. *33*, 1100-1119.

Green, A.I., Patel, J.K., Goisman, R.M., Allison, D.B., and Blackburn, G. (2000). Weight gain from novel antipsychotic drugs: need for action. Gen. Hosp. Psychiatry *22*, 224-235.

Grottick, A.J., Fletcher, P.J., and Higgins, G.A. (2000). Studies to investigate the role of 5-HT(2C) receptors on cocaine- and food-maintained behavior. J. Pharmacol. Exp. Ther. *295*, 1183-1191.

Gurevich, V.V., and Gurevich, E.V. (2006). The structural basis of arrestin-mediated regulation of G-protein-coupled receptors. Pharmacol. Ther. *110*, 465-502.

Gustafson, A., King, C., and Rey, J.A. (2013). Lorcaserin (Belviq): A Selective Serotonin 5-HT2C Agonist In the Treatment of Obesity. P T *38*, 525-534.

Gustafsson, M., Jensen, J., Bertozzi, S.M., Currier, E.A., Ma, J.N., Burstein, E.S., and Olsson, R. (2010). Discovery of a class of calcium sensing receptor positive allosteric modulators; 1-(benzothiazol-2-yl)-1-phenylethanols. Bioorg.Med.Chem.Lett. *20*, 5918-5921.

Halazy, S., Perez, M., Fourrier, C., Pallard, I., Pauwels, P.J., Palmier, C., John, G.W., Valentin, J.P., Bonnafous, R., and Martinez, J. (1996). Serotonin dimers: Application of the bivalent ligand approach to the design of new potent and selective 5-$HT_{1B/1D}$ agonists. J. Med. Chem. *39*, 4920-4927.

Halford, J.C., Lawton, C.L., and Blundell, J.E. (1997). The 5-HT2 receptor agonist MK-212 reduces food intake and increases resting but prevents the behavioural satiety sequence. Pharmacol. Biochem. Behav. *56*, 41-46.

Han, L.N., Zhang, L., Li, L.B., Sun, Y.N., Wang, Y., Chen, L., Guo, Y., Zhang, Y.M., Zhang, Q.J., and Liu, J. (2015). Activation of serotonin(2C) receptors in the lateral habenular nucleus increases the expression of depression-related behaviors in the hemiparkinsonian rat. Neuropharmacology *93*, 68-79.

Hannon, J., and Hoyer, D. (2008). Molecular biology of 5-HT receptors. Behav. Brain Res. *195*, 198-213.

Heinrich, T., Bottcher, H., Schiemann, K., Holzemann, G., Schwarz, M., Bartoszyk, G.D., van Amsterdam, C., Greiner, H.E., and Seyfried, C.A. (2004). Dual 5-HT1A agonists and 5-HT re-uptake inhibitors by combination of indole-butyl-amine and chromenonyl-piperazine structural elements in a single molecular entity. Bioorg.Med.Chem. *12*, 4843-4852.

Heisler, L.K., Cowley, M.A., Kishi, T., Tecott, L.H., Fan, W., Low, M.J., Smart, J.L., Rubinstein, M., Tatro, J.B., Zigman, J.M., *et al.* (2003). Central serotonin and melanocortin pathways regulating energy homeostasis. Ann.N.Y.Acad.Sci. *994*, 169-174.

Herth, M.M., Kramer, V., Piel, M., Palner, M., Riss, P.J., Knudsen, G.M., and Rosch, F. (2009). Synthesis and in vitro affinities of various MDL 100907 derivatives as potential 18F-radioligands for 5-HT2A receptor imaging with PET. Bioorg.Med.Chem. *17*, 2989-3002.

Higgins, G.A., Silenieks, L.B., Rossmann, A., Rizos, Z., Noble, K., Soko, A.D., and Fletcher, P.J. (2012). The 5-HT_{2C} receptor agonist lorcaserin reduces nicotine self-administration, discrimination, and reinstatement: relationship to feeding behavior and impulse control. Neuropsychopharmacology *37*, 1177-1191.

Hiller, C., Kuhhorn, J., and Gmeiner, P. (2013). Class A G-protein-coupled receptor (GPCR) dimers and bivalent ligands. J. Med. Chem. *56*, 6542-6559.

Holshoe, J.M. (2009). Antidepressants and sleep: a review. Perspect.Psychiatr.Care *45*, 191-197.

Hopkins, C.R., Lindsley, C.W., and Niswender, C.M. (2009). mGluR4-positive allosteric modulation as potential treatment for Parkinson's disease. Future Med. Chem. *1*, 501-513.

Howell, L.L., and Cunningham, K.A. (2015). Serotonin 5-HT2 receptor interactions with dopamine function: implications for therapeutics in cocaine use disorder. Pharmacol. Rev. *67*, 176-197.

Hoyer, D., Hannon, J.P., and Martin, G.R. (2002). Molecular, pharmacological and functional diversity of 5-HT receptors. Pharmacol. Biochem. Behav. *71*, 533-554.

Iglesias, A., Lage, S., Cadavid, M.I., Loza, M.I., and Brea, J. (2016). Development of a Multiplex Assay for Studying Functional Selectivity of Human Serotonin 5-HT2A Receptors and Identification of Active Compounds by High-Throughput Screening. J Biomol Screen *21*, 816-823.

Im, W.B., Chio, C.L., Alberts, G.L., and Dinh, D.M. (2003). Positive allosteric modulator of the human 5-HT_{2C} receptor. Mol. Pharmacol. *64*, 78-84.

Ingale, S., and Dawson, P.E. (2011). On resin side-chain cyclization of complex peptides using CuAAC. Org Lett *13*, 2822-2825.

Jacoby, E., Bouhelal, R., Gerspacher, M., and Seuwen, K. (2006). The 7 TM G-protein-coupled receptor target family. ChemMedChem *1*, 761-782.

Jagasia, R., Holub, J.M., Bollinger, M., Kirshenbaum, K., and Finn, M.G. (2009). Peptide cyclization and cyclodimerization by Cu(I)-mediated azide-alkyne cycloaddition. J. Org. Chem. *74*, 2964-2974.

Ji, S.P., Zhang, Y., Van, C.J., Jiang, W., Liao, M., Li, L., Wan, Q., Backstrom, J.R., and Zhang, X. (2006). Disruption of PTEN coupling with 5-HT2C receptors suppresses behavioral responses induced by drugs of abuse. Nat. Med. *12*, 324-329.

Jin, W., Lo, T.M., Loh, H.H., and Thayer, S.A. (1994). U73122 inhibits phospholipase C-dependent calcium mobilization in neuronal cells. Brain Res. *642*, 237-243.

Kasper, J., Tikamdas, R., Kim, M.S., Macfadyen, K., Aramini, R., Ladd, J., Bisceglia, S., Booth, R., and Peris, J. (2013). The serotonin-2 receptor modulator, (-)-trans-PAT, decreases voluntary ethanol consumption in rats. Eur. J. Pharmacol. *718*, 98-104.

Kehne, J.H., Baron, B.M., Carr, A.A., Chaney, S.F., Elands, J., Feldman, D.J., Frank, R.A., van Giersbergen, P.L., McCloskey, T.C., Johnson, M.P., *et al.* (1996). Preclinical characterization of the potential of the putative atypical antipsychotic MDL 100,907 as a potent 5-HT2A antagonist with a favorable CNS safety profile. J.Pharmacol Exp.Ther. *277*, 968-981.

Kenakin, T., and Christopoulos, A. (2013). Signalling bias in new drug discovery: detection, quantification and therapeutic impact. Nat. Rev. Drug Discov. *12*, 205-216.

Kenakin, T., and Miller, L.J. (2010). Seven transmembrane receptors as shapeshifting proteins: the impact of allosteric modulation and functional selectivity on new drug discovery. Pharmacol. Rev. *62*, 265-304.

Kenakin, T.P. (2009). 7TM receptor allostery: putting numbers to shapeshifting proteins. Trends Pharmacol. Sci. *30*, 460-469.

Kholodenko, B.N., Hancock, J.F., and Kolch, W. (2010). Signalling ballet in space and time. Nat. Rev. Mol. Cell Biol. *11*, 414-426.

Kim, M.J., Dunah, A.W., Wang, Y.T., and Sheng, M. (2005). Differential roles of NR2A- and NR2B-Containing NMDA Receptors in Ras-ERK Signaling and AMPA Receptor Trafficking. Neuron *46*, 745-760.

King, J.R., Nordman, J.C., Bridges, S.P., Lin, M.K., and Kabbani, N. (2015). Identification and characterization of a G protein-binding cluster in alpha7 nicotinic acetylcholine receptors. J. Biol. Chem. *290*, 20060-20070.

Kleven, M.S., Anthony, E.W., and Woolverton, W.L. (1990). Pharmacological characterization of the discriminative stimulus effects of cocaine in rhesus monkeys. J.Pharmacol.Exp.Ther. *254*, 312-317.

Knight, A.R., Misra, A., Quirk, K., Benwell, K.R., Revell, D.F., Kennett, G.A., and Bickerdike, M.J. (2004). Pharmacological characterisation of the agonist radioligand binding site of 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors. Naunyn Schmiedebergs Arch. Pharmacol. *370*, 114-123.

Kroeze, W.K., Hufeisen, S.J., Popadak, B.A., Renock, S.M., Steinberg, S., Ernsberger, P., Jayathilake, K., Meltzer, H.Y., and Roth, B.L. (2003). H1-histamine receptor affinity predicts short-term weight gain for typical and atypical antipsychotic drugs. Neuropsychopharmacology *28*, 519-526.

Kurrasch-Orbaugh, D.M., Parrish, J.C., Watts, V.J., and Nichols, D.E. (2003). A complex signaling cascade links the serotonin2A receptor to phospholipase A2 activation: the involvement of MAP kinases. J.Neurochem. *86*, 980-991.

Labasque, M., Reiter, E., Becamel, C., Bockaert, J., and Marin, P. (2008). Physical interaction of calmodulin with the 5-hydroxytryptamine2C receptor C-terminus is essential for G protein-independent, arrestin-dependent receptor signaling. Mol. Biol. Cell *19*, 4640-4650.

Landolt, H.P., and Wehrle, R. (2009). Antagonism of serotonergic 5-HT2A/2C receptors: mutual improvement of sleep, cognition and mood? Eur.J.Neurosci. *29*, 1795-1809.

Lee, J.O., Yang, H., Georgescu, M.M., Di, C.A., Maehama, T., Shi, Y., Dixon, J.E., Pandolfi, P., and Pavletich, N.P. (1999). Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association. Cell *99*, 323-334.

Lefkowitz, R.J., and Shenoy, S.K. (2005). Transduction of receptor signals by beta-arrestins. Science *308*, 512-517.

Lezoualc'h, F., Jockers, R., and Berque-Bestel, I. (2009). Multivalent-based drug design applied to serotonin 5-HT(4) receptor oligomers. Curr.Pharm.Des *15*, 719-729.

Li, J.X., Unzeitig, A., Javors, M.A., Rice, K.C., Koek, W., and France, C.P. (2009). Discriminative stimulus effects of 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane (DOM), ketanserin, and (R)-(+)-{alpha}-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-pipidi nemethanol (MDL100907) in rats. J.Pharmacol.Exp.Ther. *331*, 671-679.

Lovell, K.M., Frankowski, K.J., Stahl, E.L., Slauson, S.R., Yoo, E., Prisinzano, T.E., Aube, J., and Bohn, L.M. (2015). Structure-activity relationship studies of functionally selective kappa opioid receptor agonists that modulate ERK 1/2 phosphorylation while preserving G protein over beta-arrestin$_2$ signaling bias. ACS Chem. Neurosci. *6*, 1411-1419.

Maehama, T., and Dixon, J.E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J. Biol. Chem. *273*, 13375-13378.

Maillet, J.C., Zhang, Y., Li, X., and Zhang, X. (2008). PTEN-5-HT2C coupling: a new target for treating drug addiction. Prog. Brain Res. *172*, 407-420.

Marti-Solano, M., Iglesias, A., de Fabritiis, G., Sanz, F., Brea, J., Loza, M.I., Pastor, M., and Selent, J. (2015). Detection of new biased agonists for the serotonin 5-HT2A receptor: modeling and experimental validation. Mol. Pharmacol. *87*, 740-746.

Masand, P.S. (1999). Relative weight gain among antipsychotics. J. Clin. Psychiatry *60*, 706-708.

Masri, B., Salahpour, A., Didriksen, M., Ghisi, V., Beaulieu, J.M., Gainetdinov, R.R., and Caron, M.G. (2008). Antagonism of dopamine D2 receptor/beta-arrestin 2 interaction is a common property of clinically effective antipsychotics. Proc. Natl. Acad. Sci. U. S. A. *105*, 13656-13661.

Maurel-Remy, S., Bervoets, K., and Millan, M.J. (1995). Blockade of phencyclidine-induced hyperlocomotion by clozapine and MDL 100,907 in rats reflects antagonism of 5-HT2A receptors. Eur.J Pharmacol. *280*, R9-11.

May, L.T., Leach, K., Sexton, P.M., and Christopoulos, A. (2007). Allosteric modulation of G protein-coupled receptors. Annu. Rev. Pharmacol. Toxicol. *47*, 1-51.

McGrew, L., Chang, M.S., and Sanders-Bush, E. (2002). Phospholipase D activation by endogenous 5-hydroxytryptamine 2C receptors is mediated by Galpha13 and pertussis toxin-insensitive Gbetagamma subunits. Mol. Pharmacol. *62*, 1339-1343.

McMahon, L.R., and Cunningham, K.A. (2001). Antagonism of 5-hydroxytryptamine(2a) receptors attenuates the behavioral effects of cocaine in rats. J.Pharmacol.Exp.Ther. *297*, 357-363.

Medvedev, I.O., Ramsey, A.J., Masoud, S.T., Bermejo, M.K., Urs, N., Sotnikova, T.D., Beaulieu, J.M., Gainetdinov, R.R., and Salahpour, A. (2013). D1 dopamine receptor coupling to PLCbeta regulates forward locomotion in mice. J. Neurosci. *33*, 18125-18133.

Melancon, B.J., Hopkins, C.R., Wood, M.R., Emmitte, K.A., Niswender, C.M., Christopoulos, A., Conn, P.J., and Lindsley, C.W. (2012). Allosteric modulation of seven transmembrane spanning receptors: theory, practice, and opportunities for central nervous system drug discovery. J.Med.Chem. *55*, 1445-1464.

Meltzer, H.Y., Arvanitis, L., Bauer, D., Rein, W., and Meta-Trial Study Group (2004). Placebo-Controlled Evaluation of Four Novel Compounds for the Treatment of Schizophrenia and Schizoaffective Disorder. Am. J. Psychiatry *161*, 975-984.

Meltzer, H.Y., Elkis, H., Vanover, K., Weiner, D.M., Van Kammen, D.P., Peters, P., and Hacksell, U. (2012a). Pimavanserin, a selective serotonin (5-HT)2A-inverse agonist, enhances the efficacy and safety of risperidone, 2mg/day, but does not enhance efficacy of haloperidol, 2mg/day: comparison with reference dose risperidone, 6mg/day. Schizophr.Res. *141*, 144-152.

Meltzer, H.Y., Massey, B.W., and Horiguchi, M. (2012b). Serotonin receptors as targets for drugs useful to treat psychosis and cognitive impairment in schizophrenia. Curr.Pharm.Biotechnol. *13*, 1572-1586.

Meltzer, H.Y., Matsubara, S., and Lee, J.C. (1989). Classification of typical and atypical antipsychotic drugs on the basis of dopamine D-1, D-2 and serotonin2 pKi values. J. Pharmacol. Exp. Ther. *251*, 238-246.

Meltzer, H.Y., Mills, R., Revell, S., Williams, H., Johnson, A., Bahr, D., and Friedman, J.H. (2010). Pimavanserin, a serotonin(2A) receptor inverse agonist, for the treatment of parkinson's disease psychosis. Neuropsychopharmacology *35*, 881-892.

Meltzer, H.Y., and Roth, B.L. (2013). Lorcaserin and pimavanserin: emerging selectivity of serotonin receptor subtype-targeted drugs. J. Clin. Invest. *123*, 4986-4991.

Menniti, F.S., Lindsley, C.W., Conn, P.J., Pandit, J., Zagouras, P., and Volkmann, R.A. (2013). Allosteric modulators for the treatment of schizophrenia: targeting glutamatergic networks. Curr. Top. Med. Chem. *13*, 26-54.

Millan, M.J., Marin, P., Bockaert, J., and Mannoury la Cour, C. (2008). Signaling at G-protein-coupled serotonin receptors: recent advances and future research directions. Trends Pharmacol. Sci. *29*, 454-464.

Miller, K.J. (2005). Serotonin 5-HT2C receptor agonists: potential for the treatment of obesity. Mol. Interv. *5*, 282-291.

Milligan, G., and White, J.H. (2001). Protein-protein interactions at G-protein-coupled receptors. Trends Pharmacol. Sci. *22*, 513-518.

Moreno, J.L., Holloway, T., Albizu, L., Sealfon, S.C., and Gonzalez-Maeso, J. (2011). Metabotropic glutamate mGlu2 receptor is necessary for the pharmacological and behavioral effects induced by hallucinogenic 5-HT2A receptor agonists. Neurosci.Lett. *493*, 76-79.

Moreno, J.L., Muguruza, C., Umali, A., Mortillo, S., Holloway, T., Pilar-Cuellar, F., Mocci, G., Seto, J., Callado, L.F., Neve, R.L., *et al.* (2012). Identification of three residues essential for 5-hydroxytryptamine 2A-metabotropic glutamate 2 (5-HT2A.mGlu2) receptor heteromerization and its psychoactive behavioral function. J. Biol. Chem. *287*, 44301-44319.

Morrison, K.L., and Weiss, G.A. (2001). Combinatorial alanine-scanning. Curr. Opin. Chem. Biol. *5*, 302-307.

Moutkine, I., Quentin, E., Guiard, B.P., Maroteaux, L., and Doly, S. (2017). Heterodimers of serotonin receptor subtypes 2 are driven by 5-HT2C protomers. J. Biol. Chem. *292*, 6352-6368.

Moya, P.R., Fox, M.A., Jensen, C.L., Laporte, J.L., French, H.T., Wendland, J.R., and Murphy, D.L. (2011). Altered 5-HT2C receptor agonist-induced responses and 5-HT2C receptor RNA editing in the amygdala of serotonin transporter knockout mice. BMC Pharmacol. *11*, 3.

Navarra, R., Comery, T.A., Graf, R., Rosenzweig-Lipson, S., and Day, M. (2008). The 5-HT(2C) receptor agonist WAY-163909 decreases impulsivity in the 5-choice serial reaction time test. Behav. Brain Res. *188*, 412-415.

Neelakantan, H., Holliday, E.D., Fox, R.G., Stutz, S.J., Comer, S.D., Haney, M., Anastasio, N.C., Moeller, F.G., and Cunningham, K.A. (2017). Lorcaserin Suppresses Oxycodone Self-Administration and Relapse Vulnerability in Rats. ACS Chem. Neurosci. *8*, 1065-1073.

Neisewander, J.L., and Acosta, J.I. (2007). Stimulation of 5-HT2C receptors attenuates cue and cocaine-primed reinstatement of cocaine-seeking behavior in rats. Behav. Pharmacol. *18*, 791-800.

Ng, G.Y., Clark, J., Coulombe, N., Ethier, N., Hebert, T.E., Sullivan, R., Kargman, S., Chateauneuf, A., Tsukamoto, N., McDonald, T., *et al.* (1999). Identification of a GABAB receptor subunit, gb2, required for functional GABAB receptor activity. J. Biol. Chem. *274*, 7607-7610.

Nic Dhonnchadha, B.A., and Cunningham, K.A. (2008). Serotonergic mechanisms in addiction-related memories. Behav. Brain Res. *195*, 39-53.

Nic Dhonnchadha, B.A., Fox, R.G., Stutz, S.J., Rice, K.C., and Cunningham, K.A. (2009). Blockade of the serotonin 5-HT_{2A} receptor suppresses cue-evoked reinstatement of cocaine-seeking behavior in a rat self-administration model. Behav Neurosci. *123*, 382-396.

Nichols, D.E. (2004). Hallucinogens. Pharmacol. Ther. *101*, 131-181.

Ning, K., Pei, L., Liao, M., Liu, B., Zhang, Y., Jiang, W., Mielke, J.G., Li, L., Chen, Y., El-Hayek, Y.H., *et al.* (2004). Dual neuroprotective signaling mediated by downregulating two distinct phosphatase activities of PTEN. J.Neurosci. *24*, 4052-4060.

O'Hayre, M., Eichel, K., Avino, S., Zhao, X., Steffen, D.J., Feng, X., Kawakami, K., Aoki, J., Messer, K., Sunahara, R., *et al.* (2017). Genetic evidence that beta-arrestins are dispensable for the initiation of beta2-adrenergic receptor signaling to ERK. Sci Signal *10*.

Perez, M., Jorand-Lebrun, C., Pauwels, P.J., Pallard, I., and Halazy, S. (1998). Dimers of 5HT1 ligands preferentially bind to 5HT1B/1D receptor subtypes. Bioorg.Med.Chem.Lett. *8*, 1407-1412.

Pharmaceuticals, A. (2007). Arena Pharmaceuticals Initiates Second and Third Pivotal Trials Evaluating Lorcaserin for the Treatment of Obesity. In Arena Pharmaceuticals, Inc.

Pillay, N.S., and Stein, D.J. (2007). Emerging anxiolytics. Expert.Opin.Emerg.Drugs *12*, 541-554.

Pockros, L.A., Pentkowski, N.S., Swinford, S.E., and Neisewander, J.L. (2011). Blockade of 5-HT2A receptors in the medial prefrontal cortex attenuates reinstatement of cue-elicited cocaine-seeking behavior in rats. Psychopharmacology (Berl.) *213*, 307-320.

Pompeiano, M., Palacios, J.M., and Mengod, G. (1994). Distribution of the serotonin 5-HT_2 receptor family mRNAs: Comparison between 5-HT_{2A} and 5-HT_{2C} receptors. Molecular Brain Research *23*, 163-178.

Popa, D., Lena, C., Fabre, V., Prenat, C., Gingrich, J., Escourrou, P., Hamon, M., and Adrien, J. (2005). Contribution of 5-HT2 receptor subtypes to sleep-wakefulness and respiratory control, and functional adaptations in knock-out mice lacking 5-HT2A receptors. J. Neurosci. *25*, 11231-11238.

Porsolt, R.D., Le, P.M., and Jalfre, M. (1977). Depression: a new animal model sensitive to antidepressant treatments. Nature *266*, 730-732.

Portoghese, P.S. (2001). From models to molecules: opioid receptor dimers, bivalent ligands, and selective opioid receptor probes. J.Med.Chem. *44*, 2259-2269.

Pytliak, M., Vargova, V., Mechirova, V., and Felsoci, M. (2011). Serotonin receptors - from molecular biology to clinical applications. Physiol. Res. *60*, 15-25.

Raehal, K.M., Walker, J.K., and Bohn, L.M. (2005). Morphine side effects in beta-arrestin 2 knockout mice. J. Pharmacol. Exp. Ther. *314*, 1195-1201.

Rajagopal, S., Rajagopal, K., and Lefkowitz, R.J. (2010). Teaching old receptors new tricks: biasing seven-transmembrane receptors. Nat. Rev. Drug Discov. *9*, 373-386.

Rankovic, Z., Brust, T.F., and Bohn, L.M. (2016). Biased agonism: An emerging paradigm in GPCR drug discovery. Bioorg. Med. Chem. Lett. *26*, 241-250.

Raymond, J.R., Mukhin, Y.V., Gelasco, A., Turner, J., Collinsworth, G., Gettys, T.W., Grewal, J.S., and Garnovskaya, M.N. (2001). Multiplicity of mechanisms of serotonin receptor signal transduction. Pharmacol. Ther. *92*, 179-212.

Reiter, E., and Lefkowitz, R.J. (2006). GRKs and beta-arrestins: roles in receptor silencing, trafficking and signaling. Trends Endocrinol. Metab. *17*, 159-165.

Ritter, S.L., and Hall, R.A. (2009). Fine-tuning of GPCR activity by receptor-interacting proteins. Nat. Rev. Mol. Cell Biol. *10*, 819-830.

Roskoski, R., Jr. (2012). ERK1/2 MAP kinases: structure, function, and regulation. Pharmacol. Res. *66*, 105-143.

Roth, B.L., Hanizavareh, S.M., and Blum, A.E. (2004). Serotonin receptors represent highly favorable molecular targets for cognitive enhancement in schizophrenia and other disorders. Psychopharmacology (Berl.) *174*, 17-24.

Rothman, R.B., Baumann, M.H., Savage, J.E., Rauser, L., McBride, A., Hufeisen, S.J., and Roth, B.L. (2000). Evidence for possible involvement of 5-HT2B receptors in the cardiac valvulopathy associated with fenfluramine and other serotonergic medications. Circulation *102*, 2836-2841.

Schuster, C.R., and Johanson, C.-E. (1988). Relationship between the discriminative stimulus properties and subjective effects of drugs. Psychopharmacology (Berl.) *4*, 161-175.

Seitz, P.K., Bremer, N.M., McGinnis, A.G., Cunningham, K.A., and Watson, C.S. (2012). Quantitative changes in intracellular calcium and extracellular-regulated kinase activation measured in parallel in CHO cells stably expressing serotonin (5-HT) 5-HT2A or 5-HT2C receptors. BMC Neurosci. *13*, 25.

Sengmany, K., Singh, J., Stewart, G.D., Conn, P.J., Christopoulos, A., and Gregory, K.J. (2017). Biased allosteric agonism and modulation of metabotropic glutamate receptor 5: Implications for optimizing preclinical neuroscience drug discovery. Neuropharmacology *115*, 60-72.

Shashack, M.J., Cunningham, K.A., Seitz, P.K., McGinnis, A., Smith, T.D., Watson, C.S., and Gilbertson, S.R. (2011). Synthesis and Evaluation of Dimeric Derivatives of 5-HT2A Receptor (5-HT2AR) Antagonist M-100907. ACS Chem. Neurosci. *2*, 640-644.

Shonberg, J., Scammells, P.J., and Capuano, B. (2011). Design strategies for bivalent ligands targeting GPCRs. ChemMedChem. *6*, 963-974.

Shram, M.J., Schoedel, K.A., Bartlett, C., Shazer, R.L., Anderson, C.M., and Sellers, E.M. (2011). Evaluation of the abuse potential of lorcaserin, a serotonin 2C (5-HT2C) receptor agonist, in recreational polydrug users. Clin.Pharmacol.Ther. *89*, 683-692.

Sminia, T.J., and Pedersen, D.S. (2012). Azide- and Alkyne-Functionalised α- and β3-Amino Acids. Synlett *23*, 2643-2646.

Smith, B.M., Smith, J.M., Tsai, J.H., Schultz, J.A., Gilson, C.A., Estrada, S.A., Chen, R.R., Park, D.M., Prieto, E.B., Gallardo, C.S., *et al.* (2008). Discovery and structure-activity relationship of (1R)-8-chloro-2,3,4,5-tetrahydro-1-methyl-1H-3-benzazepine (Lorcaserin),

a selective serotonin 5-HT_{2C} receptor agonist for the treatment of obesity. J Med.Chem. *51*, 305-313.

Soergel, D.G., Subach, R.A., Sadler, B., Connell, J., Marion, A.S., Cowan, C.L., Violin, J.D., and Lark, M.W. (2014). First clinical experience with TRV130: pharmacokinetics and pharmacodynamics in healthy volunteers. J. Clin. Pharmacol. *54*, 351-357.

Soto, C.A., Huang-Chi, D., Fox, R.G., Hartley, R.M., Anastasio, N.C., Gilbertson, S.R., and Cunningham, K.A. *In vivo* and *in vitro* analyses of novel peptidomimetic disruptors for the serotonin 5-HT_{2C} receptor interaction with phosphatase and tensin homolog (PTEN). Submitted.

Soto, C.S., Shashack, M.J., Fox, R.G., Bubar, M.J., Rice, K.C., Watson, C.S., Cunningham, K.A., Gilbertson, S.R., and Anastasio, N.C. Novel Bivalent 5-HT_{2A} Receptor antagonists exhibit high affinity and potency *in vitro* and efficacy *in vivo*. Submitted.

Soulier, J.L., Russo, O., Giner, M., Rivail, L., Berthouze, M., Ongeri, S., Maigret, B., Fischmeister, R., Lezoualc'h, F., Sicsic, S., *et al.* (2005). Design and synthesis of specific probes for human 5-HT4 receptor dimerization studies. J.Med.Chem. *48*, 6220-6228.

Stambolic, V., Suzuki, A., de la Pompa, J.L., Brothers, G.M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J.M., Siderovski, D.P., and Mak, T.W. (1998). Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell *95*, 29-39.

Stout, B.D., Clarke, W.P., and Berg, K.A. (2002). Rapid desensitization of the serotonin(2C) receptor system: effector pathway and agonist dependence. J Pharmacol Exp.Ther. *302*, 957-962.

Swinford-Jackson, S.E., Anastasio, N.C., Fox, R.G., Stutz, S.J., and Cunningham, K.A. (2016). Incubation of cocaine cue reactivity associates with neuroadaptations in the cortical serotonin (5-HT) 5-HT2C receptor (5-HT2CR) system. Neuroscience *324*, 50-61.

Szucs, R.P., Frankel, P.S., McMahon, L.R., and Cunningham, K.A. (2005). Relationship of cocaine-induced c-Fos expression to behaviors and the role of serotonin 5-HT2A receptors in cocaine-induced c-Fos expression. Behav.Neurosci. *119*, 1173-1183.

T., d.P. (2001). M-100907 (Aventis). Curr.Opin.Investig.Drugs *2*, 123-132.

Talvik-Lotfi, M., Nyberg, S., Nordstrom, A.L., Ito, H., Halldin, C., Brunner, F., and Farde, L. (2000). High 5HT2A receptor occupancy in M100907-treated schizophrenic patients. Psychopharmacology (Berl.) *148*, 400-403.

Tecott, L.H., Sun, L.M., Akana, S.F., Strack, A.M., Lowenstein, D.H., Dallman, M.F., and Julius, D. (1995). Eating disorder and epilepsy in mice lacking 5-HT2c serotonin receptors. Nature *374*, 542-546.

Teegarden, B.R., Al, S.H., and Xiong, Y. (2008). 5-HT(2A) inverse-agonists for the treatment of insomnia. Curr.Top.Med.Chem. *8*, 969-976.

Teitler, M., and Klein, M.T. (2012). A new approach for studying GPCR dimers: drug-induced inactivation and reactivation to reveal GPCR dimer function in vitro, in primary culture, and in vivo. Pharmacol. Ther. *133*, 205-217.

Teuns, G.B., Geys, H.M., Geuens, S.M., Stinissen, P., and Meert, T.F. (2014). Abuse liability assessment in preclinical drug development: Predictivity of a translational approach for abuse liability testing using methylphenidate in four standardized preclinical study models. J. Pharmacol. Toxicol. Methods.

Thangudu, R.R., Bryant, S.H., Panchenko, A.R., and Madej, T. (2012). Modulating protein-protein interactions with small molecules: the importance of binding hotspots. J. Mol. Biol. *415*, 443-453.

Thiel, P., Kaiser, M., and Ottmann, C. (2012). Small-molecule stabilization of protein-protein interactions: an underestimated concept in drug discovery? Angew.Chem.Int.Ed Engl. *51*, 2012-2018.

Thomsen, W.J., Grottick, A.J., Menzaghi, F., Reyes-Saldana, H., Espitia, S., Yuskin, D., Whelan, K., Martin, M., Morgan, M., Chen, W., *et al.* (2008). Lorcaserin, a novel selective human 5-hydroxytryptamine2C agonist: in vitro and in vivo pharmacological characterization. J. Pharmacol. Exp. Ther. *325*, 577-587.

Ullrich, T., and Rice, K.C. (2000). A practical synthesis of the serotonin 5-HT2A receptor antagonist MDL 100907, its enantiomer and their 3-phenolic derivatives as precursors for [11C]labeled PET ligands. Bioorg.Med.Chem. *8*, 2427-2432.

Umbricht, D.S., Pollack, S., and Kane, J.M. (1994). Clozapine and weight gain. J. Clin. Psychiatry *55 Suppl B*, 157-160.

Vanover, K.E., Weiner, D.M., Makhay, M., Veinbergs, I., Gardell, L.R., Lameh, J., Del Tredici, A.L., Piu, F., Schiffer, H.H., Ott, T.R., *et al.* (2006). Pharmacological and behavioral profile of N-(4-fluorophenylmethyl)-N-(1-methylpiperidin-4-yl)-N'-(4-(2-methylpropylo xy)phenylmethyl) carbamide (2R,3R)-dihydroxybutanedioate (2:1) (ACP-103), a novel 5-hydroxytryptamine(2A) receptor inverse agonist. J Pharmacol.Exp.Ther. *317*, 910-918.

Venkatesan, N., and Kim, B.H. (2002). Synthesis and enzyme inhibitory activities of novel peptide isosteres. Curr.Med.Chem. *9*, 2243-2270.

Violin, J.D., Crombie, A.L., Soergel, D.G., and Lark, M.W. (2014). Biased ligands at G-protein-coupled receptors: promise and progress. Trends Pharmacol. Sci. *35*, 308-316.

Viscusi, E.R., Webster, L., Kuss, M., Daniels, S., Bolognese, J.A., Zuckerman, S., Soergel, D.G., Subach, R.A., Cook, E., and Skobieranda, F. (2016). A randomized, phase 2 study investigating TRV130, a biased ligand of the mu-opioid receptor, for the intravenous treatment of acute pain. Pain *157*, 264-272.

Vives, E., Brodin, P., and Lebleu, B. (1997). A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. J. Biol. Chem. *272*, 16010-16017.

Weintraub, M., Sundaresan, P.R., Schuster, B., Moscucci, M., and Stein, E.C. (1992). Long-term weight control study. III (weeks 104 to 156). An open-label study of dose adjustment of fenfluramine and phentermine. Clin. Pharmacol. Ther. *51*, 602-607.

Weisstaub, N.V., Zhou, M., Lira, A., Lambe, E., Gonzalez-Maeso, J., Hornung, J.P., Sibille, E., Underwood, M., Itohara, S., Dauer, W.T., *et al.* (2006). Cortical 5-HT2A receptor signaling modulates anxiety-like behaviors in mice. Science *313*, 536-540.

Werry, T.D., Gregory, K.J., Sexton, P.M., and Christopoulos, A. (2005). Characterization of serotonin 5-HT2C receptor signaling to extracellular signal-regulated kinases 1 and 2. J. Neurochem. *93*, 1603-1615.

Whalen, E.J., Rajagopal, S., and Lefkowitz, R.J. (2011). Therapeutic potential of beta-arrestin- and G protein-biased agonists. Trends Mol. Med. *17*, 126-139.

Whitby, L.R., Ando, Y., Setola, V., Vogt, P.K., Roth, B.L., and Boger, D.L. (2011). Design, synthesis, and validation of a beta-turn mimetic library targeting protein-protein and peptide-receptor interactions. J. Am. Chem. Soc. *133*, 10184-10194.

Wild, C., Cunningham, K.A., and Zhou, J. (2014). Allosteric Modulation of G Protein-Coupled Receptors: An Emerging Approach of Drug Discovery. Austin J Pharmacol Ther. *2*, 1-3.

Winstanley, C.A., Theobald, D.E., Dalley, J.W., Glennon, J.C., and Robbins, T.W. (2004). 5-HT2A and 5-HT2C receptor antagonists have opposing effects on a measure of impulsivity: interactions with global 5-HT depletion. Psychopharmacology (Berl.) *176*, 376-385.

Zaniewska, M., McCreary, A.C., Wydra, K., and Filip, M. (2010). Effects of serotonin (5-HT)2 receptor ligands on depression-like behavior during nicotine withdrawal. Neuropharmacology *58*, 1140-1146.

Zhou, L., and Bohn, L.M. (2014). Functional selectivity of GPCR signaling in animals. Curr. Opin. Cell Biol. *27*, 102-108.

Zinzalla, G., and Thurston, D.E. (2009). Targeting protein-protein interactions for therapeutic intervention: a challenge for the future. Future Med. Chem. *1*, 65-93.

Vita

Claudia A. Soto was born on September 18, 1990 in Santiago Chile to Soledad De la Cerda and Claudio Soto. Ms. Soto attended Rice University in Houston, Texas where she obtained a Bachelor of Science degree in Biochemistry and Cell Biology (2012). Subsequently, Ms. Soto matriculated to UTMB in the Human Pathophysiology and Translation Medicine graduate program and joined the laboratory of Dr. Kathryn A. Cunningham to conduct her dissertation research. She has been an active participant in the HPTM graduate program student association, serving as Curriculum Advisory Committee Head as well as pioneered the restructuring of the HPTM student seminar series. During her tenure, Ms. Soto has received a number of awards, including a T32 trainee position through the National Institute on Drug Abuse and several travel awards. Ms. Soto has presented her research at ten national conferences and is primary author or co-author on four manuscripts in preparation. Chapters 2 and 3 have resulted in two co-author publications (listed below) which are currently in preparation (Chapter 2) or submitted (Chapter 3).

Awards

E. Fitzgerald Sporar Endowment Travel Award (2017)
Lynch Addiction Research Fund Travel Award (2016)
CPDD 2016 Primm-Singleton Minority Travel Award (2016)
David V. Herin, PhD Addiction Pioneer Endowment Travel Award (2015)

Manuscripts in preparation/submitted

Soto, CA, Shashack MJ, Fox RG, Bubar MJ, Rice KC, Watson CS, Cunningham KA, Gilbertson SR, Anastasio NC. Novel bivalent 5-HT_{2A} receptor antagonists exhibit high affinity and potency in vitro and efficacy in vivo. *Submitted to ACS Chem Neuroscience; 08/2017* **(Chapter 3)**

Soto CA, Du HC, Fox RG, Hartley RM, Zhang GZ, Anastasio NC, Gilbertson SR, Cunningham KA. In vivo and in vitro analyses of novel peptidomimetic disruptors for the serotonin 5-HT_{2C} receptor interaction with phosphatase and tensin homolog (PTEN). *In preparation.* ***(Chapter 2)***

Wild CT, Miszkiel JM, Wold EA, Soto CA, Ding C, Hartley RM, White MA, Anastasio NC, Cunningham KA, Zhou J. Design, Synthesis, and Characterization of 4-Undecylpiperidine-2-Carboxamides as Positive Allosteric Modulators of the Serotonin (5-HT) 5-HT2C Receptor. *In preparation.*

Gilbertson SR, Chen YC, Soto CA, Cunningham KA, Anastasio NC. Synthesis and structure activity relationships of functionalizable derivatives of the serotonin (5-HT) 2A receptor (5-HT_{2A}R) M100907. *In preparation.*

Permanent address: 2403 Pine Dr., Friendswood TX 77546

This dissertation was typed by Claudia A. Soto