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Strategies to Identify Therapeutic Opportunities in the Treatment of Addictive Disorders

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Strategies to Identify Therapeutic Opportunities in the Treatment of Addictive Disorders

by

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Dedication

For my parents, who instilled in me a love for knowledge.

For my husband, who has supported me throughout.

And for my children, who inspire me every day.

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Strategies to Identify Therapeutic Opportunities in the Treatment of Addictive Disorders

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Addictive disorders are a collection of maladaptive behaviors characterized by the uncontrolled use of a rewarding substance. These include substance use disorders, binge eating disorder, certain subtypes of obesity, gambling disorder, and internet gaming disorder. Each of these disorders share similar behavioral characteristics that may be motivated by common neural substrates and molecular mechanisms. This dissertation aims to elucidate some of the drivers of addictive behaviors and proposes four strategies to identify therapeutic opportunities in the treatment of addictive disorders, with a special emphasis on binge eating disorder. The current work demonstrates an association between high fat food binge intake and cue reactivity, both of which are modulated by insula activity. Further, the clinically-approved serotonin (5-HT) 5-HT_{2A} receptor (5-HT_{2A}R) antagonist/inverse agonist pimavanserin and 5-HT_{2C}R agonist lorcaserin used alone or in combination demonstrate efficacy in suppressing measures of binge eating. Pharmacological studies further show that activation of the 5-HT_{2C}R may suppress binge eating by decreasing the reinforcing and motivational properties of high fat food. Finally, a possible role for a 5-HT_{2A}R:5-HT_{2C}R protein interaction in the protection against addictive behaviors is proposed. This dissertation concludes by discussing possible routes of implementation of the present findings into clinical practice. The strategies discussed –

neuronal modulation, behavioral-guided therapy, drug repurposing, and combined therapeutic approaches – offer great possibilities in the development of new therapeutic approaches in the treatment of addictive disorders.

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5-HT	serotonin
5-HT_{2A}R	serotonin 2A receptor
5-HT_{2C}R	serotonin 2C receptor
ACC	anterior cingulate cortex
ANOVA	analysis of variance
BED	binge eating disorder
BMI	body mass index
CES	cranial electrostimulation
CNO	clozapine *N*-oxide
DAPI	4′,6-diamidino-2-phenylindole
DBS	deep brain stimulation
DREADD	designer receptors exclusively activated by designer drugs
DSLR	digital single-lens reflex
DSM-5	Diagnostic and Statistical Manual of Mental Disorders-V
ECT	electroconvulsive therapy
FR	fixed ratio
GABA	γ-aminobutyric acid
HFF	high fat food
IL	infralimbic cortex
i.m.	intramuscular
insula_{ant}	anterior insula
i.p.	intraperitoneal
LSD	lysergic acid diethylamide
MDMA	methylenedioxymethamphetamine
mPFC	medial prefrontal cortex
PBS	phosphate buffered saline
pcFos	phosphorylated cFos
PCP	phencyclidine
PFC	prefrontal cortex
PL	prelimbic cortex
PLA	proximity ligation assay
PR	progressive ratio
RASSL	receptors activated solely by synthetic ligands
rTMS	repetitive transcranial magnetic stimulation
s.c.	subcutaneous
SEM	standard error of the mean
SF	standard food
SUD	substance use disorder
TBS	theta-burst stimulation
tDCS	transcranial direct current stimulation
TMS	transcranial magnetic stimulation
VEH	vehicle

Chapter 1: Introduction

INTRODUCTION

The 2016 Surgeon General's Report on Alcohol, Drugs, and Health defines addiction as "the most severe form of substance use disorder (SUD), associated with compulsive or uncontrolled use of one or more substances." The report continues, "addiction is a chronic brain disease that has the potential for both recurrence (relapse) and recovery" (1). Yet when the term "abused substance" is expanded to describe non-drug reinforcers (e.g., food, money), the similarities between SUD and other disorders emerge. These other disorders include binge eating disorder (BED) and some forms of obesity when the abused reinforcer is food, while gambling and internet gaming disorder fit the definition when the abused reinforcer is money. Thus, this dissertation will refer to this broader, collective group of dysfunctional behaviors, in which controlled substance use shifts towards compulsive or uncontrolled use, as "addictive disorders."

Nora Volkow, the current Director for the National Institute on Drug Abuse, published a provocative article in 2013 in which she argues that there are many commonalities between the neural processes that underlie SUD and some forms of obesity (2). She terms these similarities the "addictive dimensionality" of obesity and proposes that our current knowledge of SUD may be instrumental in identifying new therapeutic options in obesity. The work in this dissertation is primarily focused on interrogating the behavioral, neural, and molecular mechanisms of BED. However, the rationale for the experimental approaches used is based highly upon a foundation of knowledge gained in the drug abuse field. This multi-disease approach has resulted in the establishment of four strategies to identify therapeutic opportunities in the treatment of addictive disorders.

MAPPING DIAGNOSTIC CRITERIA OF SUD ONTO OTHER ADDICTIVE DISORDERS

The Diagnostic and Statistical Manual of Mental Disorders-5 (DSM-5) diagnostic criteria for SUD is often mapped onto other addictive disorders to demonstrate the commonalities between the diseases. **Table 1.1** summarizes the key diagnostic criteria for SUD, using stimulant use disorder as the specific subtype (3). In sum, the criteria illustrate the compromised control (criteria 1-4), social impairment (criteria 5-7), risky use (criteria 8-9), and biological changes (criteria 10-11) that occur with SUD. These criteria can be applied across drug classes [e.g., a person with opioid use disorder who takes increasing doses of Oxycontin® to medicate chronic pain despite continued efforts to quit (criteria 1, 2, 8, 9, 10, and 11) and a person with stimulant use disorder who has lost his/her job, friends, and home because of chronic methamphetamine use and craving (criteria 3, 4, 5, 6, 7, and 9)]. Likewise, fulfillment of these criteria can be applied across addictive disorders, such as BED, which is defined by the DSM-5 as recurring episodes of uncontrollable excessive intake of food (3). This is illustrated by a patient who craves and then eats excessive amounts of food despite being on a diet because he/she is overweight and can no longer engage in moderate physical activities (criteria 1, 2, 4, 6, 7, and 10). When assessing these behaviors more broadly, the criteria describing SUD can be applied to other disorders with an “addictive dimensionality”.

STRATEGIES TO IDENTIFY THERAPEUTIC OPPORTUNITIES IN THE TREATMENT OF ADDICTIVE DISORDERS

One issue that plagues the treatment of addictive disorders is the few options available to healthcare providers to help patients. The current FDA-approved pharmacological options for select addictive disorders are listed in **Table 1.2**. Many of these drugs exhibit limited effectiveness (see **Table 1.2**) and pharmacological discontinuation may result in relapse. Of note, there are no medications approved for the treatment of multiple SUDs [including cocaine, methamphetamine, marijuana, synthetic cannabinoids, ketamine, lysergic acid diethylamide (LSD), phencyclidine (PCP), or

methylenedioxy-methamphetamine (MDMA)], gambling disorder, or internet gaming disorder. Behavioral therapies such as cognitive-behavioral therapy, contingency management, community reinforcement approaches, family therapies, motivational enhancement therapy, the matrix model, and twelve-step facilitation therapy have also proven effective but several can be inaccessible to certain populations (1). Thus, it is imperative that the scientific and medical community discover new therapeutic opportunities for the treatment of addictive disorders. This dissertation explores four strategies to broaden the treatment arsenal – neuronal modulation (**Chapters 2 and 5**), behavior-guided therapy (**Chapters 2, 3, and 4**), drug repurposing (**Chapters 3 and 4**), and combined therapeutic approaches (**Chapters 4 and 5**). Together, these four strategies offer the opportunity to identify new therapeutic approaches in the treatment of addictive disorders.

Table 1.1: Criteria for stimulant use disorder.

DSM-5 Criteria for Stimulant Use Disorder†
1. Substance is taken in larger amounts or over a longer period than was intended.
2. There is a persistent desire or unsuccessful effort to cut down or control substance use.
3. A great deal of time is spent in activities necessary to obtain the substance, use the substance, or recover from its effects.
4. Craving, or a strong desire or urge to use the substance.
5. Recurrent substance use resulting in a failure to fulfill major role obligations at work, school, or home.
6. Continued substance use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of the substance.
7. Important social, occupational, or recreational activities are given up or reduced because of substance use.
8. Recurrent substance use in situations in which it is physically hazardous.
9. Substance use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance.
10. Tolerance, as defined by either of the following: a. A need for markedly increased amounts of the substance to achieve intoxication or desired effect. b. A markedly diminished effect with continued use of the same amount of the substance.
11. Withdrawal, as manifested by either of the following: a. The characteristic withdrawal syndrome for the substance. b. The substance (or a closely related substance) is taken to relieve or avoid withdrawal symptoms.

† “Stimulant” is changed to “substance” to illustrate application of these criteria across addictive disorders.

Table 1.2: Current FDA-approved pharmacological options for treating select addictive disorders.

Drug	Disorder	Mechanism	Effectiveness	Ref.
Acamprosate (Campral®)	Alcohol use disorder	NMDAR antagonist and $GABA_AR$ positive allosteric modulator	Risk difference for return to any drinking -0.14 – -0.04	(4)
Disulfiram (Antabuse®, Antabus®)	Alcohol use disorder	Aldehyde dehydrogenase inhibitor	Risk difference for return to any drinking -0.11 – 0.03	(4)
Naltrexone (Vivitrol®, ReVia®)	Alcohol use disorder	Opioid receptor antagonist	Risk difference for return to any drinking (injection) -0.10 – 0.03 (50 mg) -0.10 – 0 (100 mg) -0.08 – 0.02	(4)
Bupropion (Wellbutrin®, Zyban®)	Nicotine use disorder	Dopamine and noradrenaline reuptake inhibitor	Odds ratio of abstinence 2.06	(5)
Nicotine replacement therapy (Nicoderm®, Nicorette®, Nicotrol®)	Nicotine use disorder	Stimulates nicotinic acetylcholine receptors	Odds ratio of abstinence 1.77	(5)
Varenicline (Chantix®, Champix®)	Nicotine use disorder	Partial α4β2 nicotinic acetylcholine receptor agonist	Odds ratio of abstinence 3.85 (12 weeks), 2.66-3.09 (1 year)	(5)
Buprenorphine (Subutex®, Suboxone®)	Opioid use disorder	Opioid receptor partial agonist	25% greater opioid free vs. placebo	(6)
Methadone (Dolophine®, Methadose™)	Opioid use disorder	Opioid receptor agonist	30% greater opioid free vs. placebo	(7)
Naltrexone (Vivitrol®, ReVia®)	Opioid use disorder	Opioid receptor antagonist	13% greater opioid free vs. placebo	(7)
Lisdexamfetamine (Vyvanse®, Venvanse®)	Binge eating disorder	Blocks reuptake of norepinephrine and dopamine	Relative risk for greater abstinence than placebo 2.61	(8)
Liraglutide (Saxenda®, Victoza®)	Obesity	GLP-1 receptor agonist	2.1-6.1% greater body weight reduction vs. placebo	(9)
Lorcaserin (Belviq®)	Obesity	Selective $5\text{-}HT_{2C}R$ agonist	1.8-3.6% greater body weight reduction vs. placebo	(9)
Naltrexone/bupropion (Contrave®)	Obesity	Opioid receptor antagonist + dopamine and noradrenaline reuptake inhibitor	3.7-4.8% greater body weight reduction vs. placebo	(9)
Orlistat (Xenical®, Alli®)	Obesity	Triacylglycerol lipase inhibitor	2.9% greater body weight reduction vs. placebo	(9)
Phentermine (Adipex-p®, Duromine™, Suprenza™)	Obesity	Noradrenergic sympathomimetic amine	0.6-6.0 kg greater weight reduction vs. placebo	(10)
Phentermine/topiramate (Qsymia®)	Obesity	Noradrenergic sympathomimetic amine + attenuation of GABA receptors for anorexigenic signaling	3.5-9.3% greater body weight reduction vs. placebo	(9)

Neuronal Modulation

Addictive disorders are characterized by cycles of binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation that are each primarily controlled by discrete neurocircuitry domains (11). The binge/intoxication state is primarily driven by the ventral tegmental area, nucleus accumbens, and dorsal striatum via regulation of reward and habit pathways. The withdrawal/negative affect state is controlled by the extended amygdala and habenula, regions known to regulate negative emotional states and stress. Finally, the preoccupation/anticipation state is modulated by cortical regions including the insula, prefrontal cortex (PFC), anterior cingulate cortex (ACC), and orbitofrontal cortex, which together regulate various components of craving, impulsivity, compulsivity, and executive function. While each of these neurocircuitry domains is important in the lifecycle of addictive disorders, this dissertation will focus on understanding the mechanisms guiding the preoccupation/anticipation stage by investigating specific cortical regions in rats.

The preoccupation/anticipation stage is thought to be the primary driver of relapse in addictive disorders due to its predominant role in executive function, impulsivity, compulsivity, and craving (especially cue-induced) (11). The insula is one component of this neurocircuitry domain that has an established, but not well-understood, role in cue-induced craving. The insula is a key interpreter of interoceptive information, which is thought to be the basis for its role in addictive disorders (12). During reward processes, hedonic experiences reach the insula via interoceptive signaling where the subject becomes aware of the positive reinforcement and commits this experience to memory, along with associated cues and context (13). This experience contributes to cue reactivity, or increased attentiveness to stimuli previously associated with the rewarding process (14). Increased levels of activity in the insula have been associated with cue-induced craving (15, 16), thus poising the insula as a critical neural node in the regulation of cue

reactivity. The causal relationship between insula activity and high fat food (HFF) binge intake and cue reactivity is explored in **Chapter 2**.

The preoccupation/anticipation stage of the addictive cycle is also regulated in part by the medial PFC (mPFC), which is defined in rats as the ACC, prelimbic cortex (PL), and infralimbic cortex (IL), moving in a ventral direction (17). The mPFC controls executive function via a "Go" system and a "Stop" system that is mediated by excitatory and inhibitory balance conferred by glutamate and γ-aminobutyric acid (GABA) signaling, respectively. The "Go" system is primarily mediated by the dorsal mPFC (i.e., ACC and PL), while the "Stop" system is regulated by the more ventral areas (i.e., IL). Compromised executive function (e.g., over-activation of the "Go" system or under-activation of the "Stop" system) can promote addiction-related behaviors such as cue reactivity and impulsivity, which is defined as a predisposition toward rapid unplanned reactions to stimuli without regard to the negative consequences (18-21). This is illustrated by the unplanned decision to engage in addictive behaviors (e.g., a person with SUD smoking marijuana at a party despite knowing this could initiate relapse or a person with BED ordering three desserts while on a cruise without considering the negative consequences). **Chapter 5** will discuss how interactions between two receptors in the mPFC (see **Drug Repurposing**) may modulate addiction-related behaviors via regulation of glutamatergic and GABAergic signaling, with a special emphasis on cue reactivity and impulsivity.

Behavior-Guided Therapy

This dissertation uses the term "behavior-guided therapy" to describe two strategies that emphasize the utility of understanding behaviors associated with disease to identify new therapeutic opportunities. The first behavior-guided therapy strategy is to exploit knowledge about behaviors associated with different stages within the addictive cycle to identify treatments that will be effective in clinical populations (i.e., behavior as a therapeutic target). For example, suppression of the binge/intoxication stage may be

achieved via suppression of behaviors exhibited during the preoccupation/anticipation stage such as cue reactivity or impulsivity (11). Thus, a therapeutic approach that suppresses these behaviors may be predictive of the efficacy to treat an addictive disorder in a clinical population. This is particularly important since even though animal studies give us great insight into how humans may react to a similar therapeutic approach, they are limited by what aspects of an addictive disorder we can model in our paradigms. For example, it is very difficult to model in animals the same types of negative effects of reinforcer use that humans face. While many studies have tried to model negative consequences associated with drug-taking by using physical punishment (e.g., foot shock) (22, 23), these negative consequences are not completely analogous to the consequences humans face when they lose their jobs or damage relationships with loved ones due to an addictive disorder. Negative consequences of reinforcer use are especially difficult to model in preclinical paradigms of BED (24). Thus, this dissertation proposes that we should employ behaviors associated with addictive disorders as a primary endpoint when deciding if a treatment approach is ready for testing in clinical populations rather than only focusing on more traditional endpoints such as reinforcer use (e.g., identify therapies that suppress cue reactivity or impulsivity rather than only drug taking or binge eating in rodent models). This approach is discussed in **Chapter 2** where we examine the consequences of the relationship between HFF binge intake and cue reactivity.

The second behavior-guided therapy strategy is to use behaviors as predictive biomarkers when deciding between therapeutic options. A predictive biomarker is one that can be used to identify those patients who are most likely to have a robust clinical response to a therapy (25). While many medical specialties already use biomarkers in practice, this method is rarely used in the treatment of addictive disorders due to a lack of identified predictive biomarkers (26). Current studies aim to adapt this method in the treatment of psychiatric disorders by searching for predictive biomarkers using techniques

including genetic screening, neuroimaging, and behavioral assessments (26). This dissertation will focus on the potential utility of behavior as a predictive biomarker in treatment response for addictive disorders. **Chapter 3** will consider the potential of a clinically available anti-obesity agent to exhibit differences in efficacy in patients with obesity with and without co-morbid BED. **Chapter 4** will then discuss specific behavioral profiles that may be more responsive to one therapeutic approach over another when treating patients with BED.

Drug Repurposing

The process of drug development is long, expensive, and often unsuccessful. Recently, a report was published indicating the average total expected cost of phase I ($25.3 million), phase II ($34.9 million), and phase III ($54.0 million) clinical trials was $114.2 million. However, only 11.83% of drugs are estimated to successfully transition from phase I of clinical testing to agency approval, thus resulting in an estimated $965 million cost for clinical trials when accounting for attrition rates (27). In addition to clinical trial testing, pre-human research and development costs are estimated at $430 million per approved drug. Together, this results in an average cost for research and development per approved drug of $1.395 billion (27). The drug approval process is also long; the mean time from phase I to phase II is 19.8 months, from phase II to phase III is 30.3 months, and from phase III to submission of a new drug application is 30.7 months. With an average approval phase of 16 months, this brings the total time from the start of clinical testing to marketing approval to 96.8 months or about eight years (27). In addition to these hurdles, pharmaceutical companies are only guaranteed 3-7 years of patent exclusivity which allows the company to recover expenditures and make a profit on drug development (https://www.fda.gov/Drugs/DevelopmentApprovalProcess/ucm079031.htm). Because of these hurdles, development of novel medications that treat addictive disorders has not kept pace with public health needs.

Drug repurposing or repositioning, defined as the process of identifying new indications for existing drug compounds, is often quicker and less expensive than traditional drug discovery because initial screening, chemical optimization, toxicology studies, and formulation development have already been finalized (28). This approach is especially enticing for diseases that are not common within populations (e.g., rare genetic mutation-causing disease) or for treatments that have low earning potential (e.g., one-time-use curative drugs) (29). Thus, drug repurposing is beneficial financially, ethically, and scientifically.

There are multiple situations in which a drug can be repurposed (29). Drugs already in clinical development can undergo testing for two indications simultaneously. This approach was used with the nonselective serotonin reuptake inhibitor duloxetine, which simultaneously underwent the clinical development process for treatment of both depression and stress urinary incontinence (29). Drugs that were deemed safe but not efficacious for the previously-tested indication can also be repurposed. Studies have estimated that over 2,000 compounds have been shelved for failing phase II or phase III clinical trials for their primary indication, which may include a number of drugs that could be repurposed for other indications (29). Other possibilities for repurposing include medications that have been discontinued for commercial reasons, drugs with patents near expiry, drugs with generic alternatives, drugs not currently marketed within the same geographic location, and drugs only partially developed in academic institutions or public-sector laboratories that cannot attain the resources necessary for the full development process.

One way to identify potential candidates for drug repurposing to treat addictive disorders is to identify medications that act on reward-related behaviors. This could be accomplished by targeting systems responsible for modulating the preoccupation/anticipation stage of the addictive cycle. For example, regulating glutamate and GABA signaling in the mPFC may suppress behaviors such as cue reactivity and

impulsivity, and thus prevent the binge/intoxication stage of the addictive cycle. Serotonin (5-HT) is capable of modulating both glutamatergic and GABAergic signaling within the PFC via its cognate 5-HT_{2A} receptor (5-HT_{2A}R) and 5-HT_{2C}R (14). Interestingly, these receptors have oppositional roles in regulating addictive behaviors even though both receptors primarily signal through $G_{\alpha q/11}$. Specifically, agonism at the 5-HT_{2C}R suppresses drug taking, cue reactivity, and impulsivity, while antagonism promotes these behaviors (for reviews, (14, 30). Conversely, the 5-HT_{2A}R plays a more permissive role in addiction-related behaviors in that antagonism of the 5-HT_{2A}R suppresses both cue reactivity and impulsivity (for review, (14). The exact mechanisms driving these oppositional effects are not fully understood (see **Chapter 5**. Fortunately, there are investigational ligands that allow researchers to probe the function of these receptors [e.g., the selective 5-HT_{2A}R antagonist M100907 (volinanserin) and selective 5-HT_{2C}R agonist WAY163909] in addition to FDA-approved drugs which have the potential to be repurposed for the treatment of addictive disorders. The 5-HT_{2A}R antagonist pimavanserin (Nuplazid®) is approved for the treatment of Parkinson's disease psychosis, while the first-in-class 5-HT_{2C}R agonist lorcaserin (Belviq®) is approved for weight loss. **Chapter 4** assesses the potential for repurposing pimavanserin and lorcaserin to treat BED.

Combined Therapeutic Approaches

Targeting multiple molecular mechanisms in the treatment of disease is not a novel idea. In fact, some of the most effective drugs in psychiatry are labeled as "dirty" because they have actions at multiple receptors in the brain. For example, clozapine, the only FDA-approved drug for refractory schizophrenia, has less than 10 nm affinity for the serotonin 5-HT_{2A}R, 5-HT_{2B}R, 5-HT_{2C}R, adrenergic α1, histamine H1, and muscarinic M1 receptors in addition to its actions on dopamine receptors (31). This results in high levels of efficacy (i.e., 60-70% of patients with refractory schizophrenia respond to clozapine) (32), but also numerous adverse effects including weight gain, metabolic dysfunction, sedation,

constipation, hypersalivation, and agranulocytosis (33). Adverse effects such as these can often be attributed directly to actions on non-targeted receptors (e.g., weight gain seen in most second-generation antipsychotics is likely due to blockade of the 5-HT_{2C}R) (34). Thus, for combination therapies to be most effective and safe, they must have great specificity for only the desired targets.

Previous studies have demonstrated that the 5-HT_{2A}R and 5-HT_{2C}R may work in concert to regulate addiction-related behaviors. For example, combined administration of ineffective doses of a 5-HT_{2A}R antagonist plus a 5-HT_{2C}R agonist suppresses cocaine cue reactivity, inherent and cocaine-evoked impulsivity, and cocaine-induced hyperactivity (35, 36). Further, decreased expression of one receptor can alter the functioning of the second receptor (37, 38). Recent studies have identified the occurrence of a 5-HT_{2A}R:5-HT_{2C}R heteromer that may mediate some of these effects (39). This dissertation assesses both the functional and physical interaction between the 5-HT_{2A}R and 5-HT_{2C}R. **Chapter 4** evaluates the efficacy of pimavanserin and lorcaserin to suppress binge eating when used in combination while **Chapter 5** characterizes the presence of a physical interaction between the 5-HT_{2A}R and 5-HT_{2C}R in the PFC that may regulate addiction-related behaviors.

CONCLUSIONS

Overall, this dissertation discusses four strategies that can be used to identify new therapeutic approaches in the treatment of addictive disorders. Within each chapter, multiple strategies will be analyzed. The final chapter will discuss how to implement the findings within each chapter using currently-available, and sometimes theoretical, technology.

Chapter 2: Anterior Insula Activity Regulates the Associated Behaviors of High Fat Food Binge Intake and Cue Reactivity[1]

INTRODUCTION

Binge eating is defined by uncontrolled excessive intake of food. It is the characteristic behavior underlying BED and is also associated with obesity (3, 40). Multiple psychological processes, including reactivity to food cues and craving, are proposed to drive binge eating (41). These behaviors are tightly interwoven in that exposure to food cues produces craving (41). Of note, binge eaters experience a higher level of cue-induced food craving which further associates with elevated food consumption relative to non-binge eaters (42). Thus, binge eating and cue reactivity may be interlocked behaviors.

The insula is a multimodal integration center for interoceptive, perceptive, motivational, and gustatory information that is poised to regulate binge eating (12, 43). Food cue exposure is associated with insula activation in healthy individuals and binge eaters (16). However, only subjects who engage in dysregulated eating exhibit altered functional connectivity between the insula and ACC and striatum upon food cue exposure, which may represent disruption in salience neurocircuitry (43, 44). Although these findings suggest that altered insular outflow of information may be responsible for some forms of dysregulated eating, it is unclear if insula activity, specifically via neurons in the anterior output subregion ($insula_{ant}$), causally regulates binge eating and cue reactivity. In the present study, we examined the relationship between binge eating and cue reactivity and further established the role of the $insula_{ant}$ on these behaviors in rats.

[1] The work in this chapter has been published in a modified form. It has been reprinted with permission from Price AE, Stutz SJ, Hommel JD, Anastasio NC, Cunningham KA. Anterior insula activity regulates the associated behaviors of high fat food binge intake and cue reactivity in male rats. *Appetite*. 2019;133:231-9.

METHODS

Animals: Male, outbred Sprague-Dawley rats (n=92; Envigo, Haslett, MI, USA and Envigo, Indianapolis, IN, USA) weighing 250-275 g at arrival were housed two per cage (except where noted below) under a 12-hour light-dark cycle (lights on between 0600-1800h) with controlled temperature (21-23°C) and humidity (40-50%). Animals were acclimated for one week to the colony room before the start of handling and experimental procedures. Standard food (SF) and water were available to rats *ad libitum* except during daily operant sessions and where noted below. Sample sizes were determined based on a power analysis (G*Power, Germany) (45) of pilot data collected from previous cohorts. All experiments were conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (2011) and with the University of Texas Medical Branch Institutional Animal Care and Use Committee approval.

Food: SF (LM-485 Mouse/Rat Sterilizable Diet; Teklad Diets, Madison, WI, USA; 3.1 kcal/g) consisted of 25% protein, 58% carbohydrate, and 17% fat (by kcal). HFF (D12451, Research Diets, New Brunswick, NJ, USA; 4.73 kcal/g) contained 20% protein, 35% carbohydrate, and 45% fat (by kcal). HFF pellets used in operant conditioning assays (#F06162, BioServ, Flemington, NJ, USA; 4.60 kcal/g) consisted of 16% protein, 38% carbohydrate, and 46% fat (by kcal).

Operant Conditioning for HFF Self-Administration: Self-administration studies took place between 1000-1500h in standard operant chambers housed within ventilated and sound-attenuated chambers; each chamber is equipped with two retractable levers, a stimulus light above each lever, and a houselight opposite the levers (MedAssociates, Georgia, VT, USA). HFF self-administration studies consisted of daily 30-minute sessions during which rats were trained to lever press for a HFF pellet. Rats were food restricted to 85-90% of free-feeding levels of SF for four days until acquisition of a fixed ratio (FR) FR1 schedule of reinforcement at which point they were provided with *ad libitum* access to SF.

Rats remained on an FR1 schedule of reinforcement for three additional days before moving to an FR5 schedule of reinforcement, which was maintained for the duration of testing except on progressive ratio (PR) and cue reactivity test days. Schedule completion on the active lever resulted in HFF pellet delivery and presentation of the discrete cue complex, which consisted of visual (flashing light), olfactory (scent of HFF pellet), and auditory (sound of pellet delivery) cues. There were no scheduled consequences for lever presses on the inactive lever. Rats were required to display stability prior to being assessed in test sessions. The criterion for stability on the FR schedule was less than 25% variability in the number of pellets earned over three consecutive days. Motivation for HFF was assessed using a PR schedule of reinforcement. In this session, the number of active lever presses required to earn the subsequent reinforcer progressively increased (1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95) (46, 47). The PR session ended after the rat had not earned a reinforcer in 10 minutes. HFF cue reactivity was assessed in a single test session. During this session, responding on the previously-active lever resulted in delivery of the discrete cue complex on an FR1 schedule of reinforcement; however, no pellets were delivered as they were diverted outside the chamber.

Association Between HFF Binge Intake and Cue Reactivity: Binge intake of HFF in Cohort 1 was assessed as described previously using an intermittent HFF access model (48, 49). Single-housed rats (n=12) received exclusive access to HFF for one week which was then replaced by exclusive access to SF. At the beginning of the dark cycle (1800h) one week after the last exposure to HFF, SF was replaced with 40 g of HFF. At the end of two hours, the HFF was removed and weighed to determine binge intake, and rats were given *ad libitum* access to SF. Rats were subsequently acclimated to pair housing and trained to self-administer HFF pellets using operant conditioning. After 29 days of self-administration, rats were subjected to a HFF cue reactivity session in which

schedule completion resulted in delivery of the discrete cue complex, but no pellet delivery. Rats which did not meet stability criteria on day 29 of self-administration (n=5) were excluded from analyses.

Viral-Mediated Gene Transfer: Rats (n=58) were anesthetized intramuscularly (i.m.) with a cocktail containing xylazine (8.6 mg/kg), acepromazine (1.5 mg/kg), and ketamine (43 mg/kg) in bacteriostatic saline and placed in a stereotaxic apparatus with the upper incisor bar at -3.8 mm below the interaural line. Two microsyringes (28 gauge, Hamilton Company, Reno, NV, USA) were lowered bilaterally to target the $insula_{ant}$; the coordinates targeted were anteroposterior +3.0 mm, mediolateral +3.9 mm, and dorsoventral -5.6 mm from skull in relation to bregma (50). The AAV8-CAMKIIa-hM3D(G_q)-mCherry excitatory DREADD (Designer Receptors Exclusively Activated by Designer Drugs; UNC Vector Core, Chapel Hill, NC, USA; lot #AV5361h, 1.8 x 10^{-12} vg/mL) was infused bilaterally at 0.1 µL/min over eight minutes for a total of 0.8 µL per infusion. The CAMKIIα promoter was employed to enrich hM3D expression in $insula_{ant}$ glutamatergic efferent neurons (51). A subset of rats (n=10) were used in initial pilot behavioral studies and to biochemically validate the hM3D system. The remainder of rats were divided into two cohorts (n=24/cohort). Cohort 2 was designated for SF and HFF intake studies while HFF self-administration and cue reactivity were assessed in Cohort 3. Activation of hM3D receptors was achieved via 1 mL/kg intraperitoneal (i.p.) injection of clozapine N-oxide (CNO; BML-NS105, Enzo Life Sciences, Farmingdale, NY, USA) dissolved in saline at a concentration of 2 mg/mL (for a final concentration of 2 mg/kg) 20 minutes prior to testing.

Validation of Cellular Activation by CNO: The ability of CNO to elicit cellular activation of the $insula_{ant}$ was assessed in a pilot cohort of rats receiving viral-mediated transfer of the hM3D system (n=10). This group underwent an extensive behavioral battery of tests that included cocaine self-administration, HFF binge intake, SF non-binge intake, novel environment-induced hypophagia, and elevated plus maze testing over a period of

three months. Twenty minutes prior to the final behavioral test (elevated plus maze) rats received i.p. injections of 1 mL/kg vehicle (VEH, 0.9% NaCl) or 2 mg/kg CNO. Upon completion of behavioral testing, rats were returned to the home cage. Ninety minutes after injection of VEH or CNO, rats were anesthetized (400 mg/kg of chloral hydrate, i.p.) and decapitated. Brains were extracted and sliced into 1 mm thick coronal sections to determine hM3D placement via visualization of mCherry expression. A DFP-1 Dual Fluorescent Protein Flashlight and VG2 barrier filter glasses were used to visualize red fluorescence (Nightsea, Bedford, MA, USA). Photographs were taken with a Canon Rebel XSi digital single lens reflex (DSLR) camera equipped with a macro lens and red filter. The $insula_{ant}$ was sub-dissected from rats displaying bilateral expression of mCherry (n=6), flash frozen in liquid nitrogen, and stored at -80°C for subsequent protein extraction.

Dissected tissue was extracted by homogenizing samples in 10 mM HEPES (pH 7.4) and 320 mM sucrose. Total homogenate was centrifuged at 1000 x g for 10 minutes at 4°C. The supernatant was removed and the pellet (containing the nuclear-enriched fraction) was resuspended in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 10 µl/mL protease inhibitor and phosphatase inhibitor 2 and 3 cocktails (Sigma-Aldrich, St. Louis, MO, USA). Lysates were probed for specific protein expression using the Wes™ automated western blotting system (ProteinSimple, San Jose, CA, USA), which utilizes capillary electrophoresis-based immunodetection for higher resolution, sensitivity, and reproducibility (even at low sample concentrations) relative to traditional immunoblotting techniques (38). Wes™ reagents (biotinylated molecular weight marker, streptavidin-HRP fluorescent standards, luminol-S, hydrogen peroxide, sample buffer, DTT, stacking matrix, separation matrix, running buffer, wash buffer, matrix removal buffer, secondary antibodies, antibody diluent, and capillaries) were obtained from the manufacturer (ProteinSimple) and used according to the manufacturer's recommendations. Levels of

phosphorylated c-Fos (pcFos) expression were assessed using a monoclonal rabbit antibody directed against pcFos Ser32 (D82C12, #5384, Cell Signaling Technology, Danvers, MA, USA) at a concentration of 1:50 which was multiplexed to a monoclonal rabbit antibody directed against the loading control, β-actin (D6A8, #8457, Cell Signaling Technology), at a concentration of 1:50.

Equal amounts of protein (4 µg) were combined with 0.1X sample buffer and 5X master mix (200 mM DTT, 5X sample buffer, 5X fluorescent standards), gently mixed, and then denatured at 90°C for 10 minutes. The denatured samples, biotinylated ladder, antibody diluent, primary antibodies, HRP-conjugated secondary antibodies, chemiluminescent substrate, and wash buffer were dispensed to designated wells in a pre-filled microplate (ProteinSimple). Separation electrophoresis (375 volts, 31 minutes, 25°C) and immunodetection in the capillaries were fully automated using the following settings: separation matrix load for 200 seconds, stacking matrix load for 14 seconds, sample load for 7 seconds, antibody diluent for 30 minutes, primary antibody incubation for 60 minutes, secondary antibody incubation for 30 minutes, and chemiluminescent signal exposure for 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 seconds. Data analyses were performed using the Compass Software (ProteinSimple). Relative pcFos expression in the $insula_{ant}$ after VEH or CNO administration was normalized to β-actin.

$Insula_{ant}$ Activation Effect on SF and HFF Intake: Two weeks after surgical implantation of the AAV8-CAMKIIa-hM3D(G_q)-mCherry excitatory DREADD, rats in Cohort 2 were single-housed and given exclusive access to HFF for 1 week to reduce food neophobia (acclimation), followed by SF access for the remainder of the study except during 2-hour HFF intake test sessions and during the 24 hours preceding intake test sessions under the food-restricted condition. Rats underwent HFF or SF intake testing in the home cage every week for two hours at the beginning of the dark cycle (1800-2000h) starting one week after HFF acclimation. Tests were conducted under both the freely-fed

and food-restricted condition. Rats received both VEH and CNO (in a counterbalanced manner) prior to the tests listed in the following order: HFF freely-fed intake (i.e., binge intake), HFF food-restricted intake, SF freely-fed intake, and SF food-restricted intake. Under freely-fed conditions, rats were injected i.p. with 1 mL/kg VEH or 2 mg/kg CNO at 1740h (20 minutes prior to the intake test). At 1800h, the SF in the home cage was replaced with 40 grams of HFF or SF. At 2000h, the remaining food was removed and weighed to determine 2-hour intake. For food-restricted conditions, SF was removed from the home cage 24 hours prior to testing. Rats were injected i.p. with 1 mL/kg VEH or 2 mg/kg CNO at 1740h the following day (20 minutes prior to the intake test). At 1800h, investigators administered 40 grams of HFF or SF to the home cages. At 2000h, the remaining food was removed and weighed to determine 2-hour intake. Following all tests, SF was then returned to the home cage for *ad libitum* feeding.

One week after completion of intake studies, rats were anesthetized (400 mg/kg of chloral hydrate, i.p.) and decapitated. Brains were extracted and sliced into 1 mm thick coronal sections to determine hM3D placement via visualization of mCherry expression. A DFP-1 Dual Fluorescent Protein Flashlight and VG2 barrier filter glasses (38, 52) were used to visualize red fluorescence. Photographs were taken with a Canon Rebel XSi DSLR camera equipped with a macro lens and red filter. Rats with bilateral expression of mCherry in the $insula_{ant}$, without spread to adjacent cortical regions, were included in the data analysis as judged by a blinded experimenter (n=9). Rats which exhibited no evidence of mCherry expression, unilateral mCherry expression, or inappropriately localized mCherry expression were excluded from behavioral analyses (n=14). An additional rat was excluded due to surgical complications resulting in early euthanasia.

To assess CNO specificity to the hM3D system in HFF binge intake, 1 mL/kg VEH or 2 mg/kg CNO was administered i.p. to a separate group of surgically naïve rats (n=11/group) 20 minutes prior to HFF binge intake testing (1740h). At 1800h, the SF in

the home cage was replaced with 40 grams of HFF. At 2000h, the remaining food was removed and weighed to determine 2-hour HFF intake, and SF was returned to the home cage for *ad libitum* feeding.

Insula$_{ant}$ Activation Effect on HFF Self-Administration and Cue Reactivity: Two weeks after surgical implantation of the AAV8-CAMKIIa-hM3D(G_q)-mCherry excitatory DREADD in Cohort 3, rats were trained to self-administer HFF as described above. Rats were injected i.p. with 1 mL/kg VEH 20 minutes prior to each FR5 session unless being tested with CNO. Once rats demonstrated stability on HFF self-administration, FR5 testing occurred. Rats were injected i.p. with 2 mg/kg CNO 20 minutes prior to an FR5 session. Responses after insula$_{ant}$ activation were compared to the prior VEH-treated FR5 session. Rats were restabilized on FR5 responding and then tested in two PR test sessions. Rats were injected i.p. with 1 mL/kg VEH or 2 mg/kg CNO 20 minutes prior to PR testing in a counterbalanced manner and were required to exhibit stable responding between test sessions. Rats were restabilized on FR5 responding and then tested in a cue test 20 minutes after i.p. injection of 1 mL/kg VEH or 2 mg/kg CNO. Treatments were counterbalanced so that the average lifetime pellets earned in the VEH and CNO groups were not significantly different.

One week after completion of cue testing, most rats (n=15) were anesthetized (400 mg/kg of chloral hydrate, i.p.) and decapitated. Brains were extracted and sliced into 1 mm thick coronal sections to determine hM3D placement via visualization of mCherry expression. A DFP-1 Dual Fluorescent Protein Flashlight and VG2 barrier filter glasses were used to visualize red fluorescence. Photographs were taken with a Canon Rebel XSi DSLR camera equipped with a macro lens and red filter. A subset of rats (n=8) were anesthetized (100 mg/kg sodium pentobarbital, i.p.) and transcardially perfused with 3% paraformaldehyde for visualization of mCherry using fluorescent microscopy. Brains were removed, post-fixed in 3% paraformaldehyde for four hours at 4°C, cryoprotected in 30%

sucrose for 72 hours at 4°C, and stored at -80°C until further use. Coronal sections (30 μm) at the level of the $insula_{ant}$ were mounted, and slides were coverslipped with Vectashield fluorescent mounting media with 4′,6-diamidino-2-phenylindole (DAPI) to stain cellular nuclei for fluorescence microscopy (Vector Laboratories, Burlingame, CA, USA). Visualization of mCherry was achieved using a Leica DFC3000 wide field camera and Leica Application Suite (Leica Microsystems, Wetzlar, Germany). Rats with bilateral expression of mCherry in the $insula_{ant}$, without spread to adjacent cortical regions as visualized using either a DFP-1 Dual Fluorescent Protein Flashlight or fluorescent microscopy, were included in the data analysis as judged by a blinded experimenter (n=7). Rats which exhibited no evidence of mCherry expression (n=8) were analyzed separately as a control group to assess specificity of CNO to the hM3D system. (This same control analysis was not completed in Cohort 2 because only two rats total displayed no evidence of mCherry expression; thus, surgically naïve rats were used instead.) Rats which exhibited unilateral mCherry expression or inappropriately localized mCherry expression were excluded from data analyses (n=8). An additional rat was excluded due to surgical complications resulting in early euthanasia.

We completed qualitative assessment of pcFos expression 90 minutes after administration of VEH or CNO in rats from Cohort 3 who underwent transcardial perfusion and demonstrated hM3D expression in the $insula_{ant}$ (n=2). Free-floating coronal sections (30 μm) at the level of the $insula_{ant}$ were washed with phosphate-buffered saline (PBS), permeabilized with 0.4% Triton PBS for 60 minutes at room temperature, blocked with 3% normal donkey serum in 0.4% Triton PBS for 60 minutes at room temperature, and incubated with primary antibody (rabbit x pcFos Ser32; D82C12, #5384, Cell Signaling Technology) 1:800 in 0.4% Triton PBS overnight at 4°C. The next day, sections were rinsed 3X with PBS for 10 minutes each, incubated in secondary antibody (Alexa Fluor 488 donkey anti-rabbit; A21206 Life Technologies, Carlsbad, CA) 1:2000 in 0.4% Triton

PBS for 2 hours at 4°C, and washed 3X with PBS for 5 minutes each. Sections were mounted, and slides were coverslipped with Vectashield fluorescent mounting media with DAPI (Vector Laboratories). Images were acquired using a Leica DFC3000 wide field camera and Leica Application Suite (Leica Mircosystems). Because one goal of the study was to enrich expression of hM3D in excitatory output neurons originating in the $insula_{ant}$, we were unable to conduct pcFos analyses on a negative control brain region since glutamatergic neurons originating in the $insula_{ant}$ innervate a large majority of the brain (53). Further, activation of hM3D on glutamatergic neurons should cause the release of glutamate in these down-stream regions which we suspect would cause increased pcFos expression globally. Thus, we believe the best control to assess the specificity of CNO to the hM3D system is through behavioral, rather than biochemical, analyses.

Statistical Analyses: The relationship between HFF binge intake and cue reactivity was assessed using Pearson's correlation. Comparison of pcFos expression 90 minutes after VEH or CNO administration was completed using a two-tailed unpaired Student's *t*-test. A two-way, repeated measures analysis of variance (ANOVA) was used to assess SF and HFF intake under freely-fed and food-restricted conditions. Planned comparisons were conducted using Bonferroni's multiple comparisons test. The specificity of CNO to the hM3D system on HFF binge intake was analyzed using a two-tailed unpaired Student's *t*-test. HFF cue reactivity was assessed using a two-tailed unpaired Student's *t*-test. FR and PR responding for HFF pellets was assessed using a two-tailed paired Student's *t*-test. All statistical analyses were performed in GraphPad Prism (Version 7) with an experimentwise error rate of α=0.05.

RESULTS

Association Between HFF Binge Intake and Cue Reactivity: The relationship between HFF binge intake and cue reactivity was assessed in Cohort 1. Pearson's

correlation revealed a positive correlation between HFF binge intake and cue reactivity (r=0.8719; p=0.0105; **Figure 2.1**), indicating that these behaviors are associated.

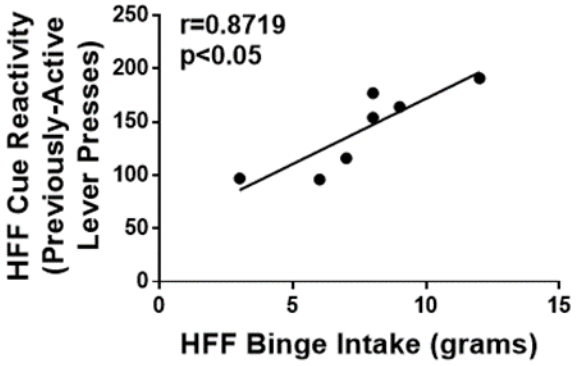

Figure 2.1: HFF binge intake and cue reactivity are positively correlated.

Cohort 1 rats underwent sequential HFF binge intake and cue reactivity assessments. Pearson's correlation revealed a positive correlation between HFF binge intake and cue reactivity (r=0.8719, p<0.05; Pearson's correlation).

Localization of hM3D within the Insula$_{ant}$: Accurate localization of hM3D within the insula$_{ant}$ was determined via visualization of mCherry using either a DFP-1 Dual Fluorescent Protein Flashlight (**Figure 2.2A**) or fluorescent microscopy (**Figure 2.2B**). Rats in Cohort 2 and Cohort 3 exhibiting bilateral mCherry expression exclusively in the insula$_{ant}$ (n=16) were included in data analyses (**Figure 2.2C**).

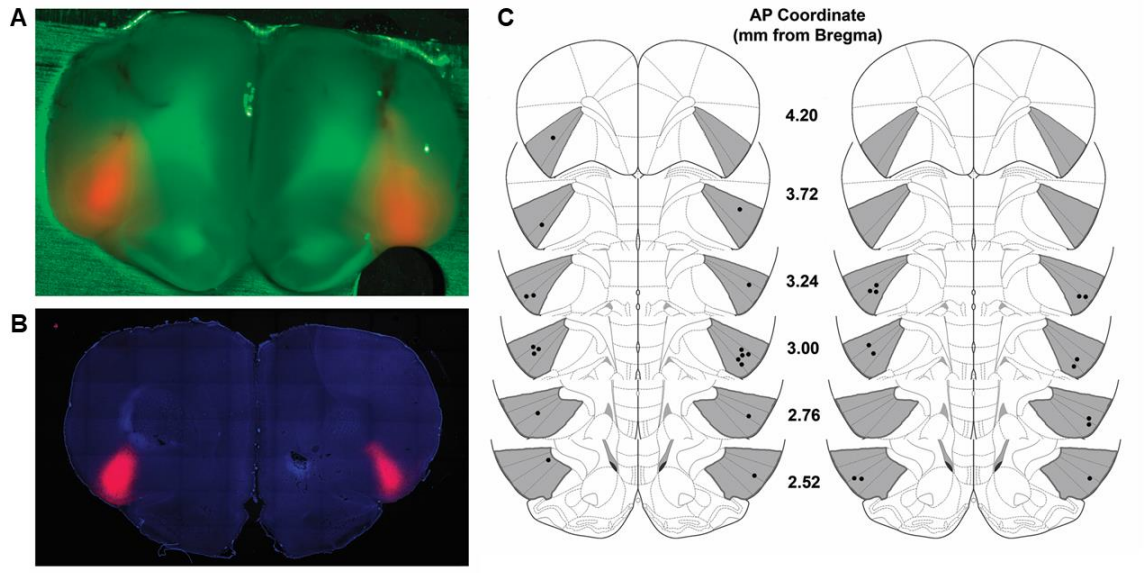

Figure 2.2: hM3D is accurately localized within the insula$_{ant}$.

Representative images of accurate hM3D expression in the insula$_{ant}$ determined by visualization of mCherry using a DFP-1 Dual Fluorescent Protein Flashlight (**Figure 2.2A**) or fluorescent microscopy (**Figure 2.2B**). Histologically verified hM3D placements of included rats from Cohort 2 (n=9; **Figure 2.2C, left**) and Cohort 3 (n=7; **Figure 2.2C, right**) using templates from Paxinos and Watson 2005 (50) to depict the central point of expression (insula$_{ant}$ depicted in gray).

Validation of Cellular Activation by CNO: The ability of CNO to induce activation of insula$_{ant}$ was assessed in a pilot study assessing the behavioral and biochemical effects of CNO in rats exhibiting accurate localization of hM3D in the insula$_{ant}$. A two-tailed unpaired Student's *t*-test demonstrated administration of CNO (2 mg/kg, i.p.) 90 minutes prior to euthanasia and insula$_{ant}$ dissection resulted in a significantly higher level of pcFos protein expression normalized to β-actin compared to VEH administration (1 mL/kg, i.p.) on western blot analysis ($p=0.0378$; **Figure 2.3A**). Immunohistochemical qualitative analyses also indicated greater evidence of pcFos expression in rats from Cohort 3 that exhibited accurate localization of hM3D in the insula$_{ant}$ who were treated with CNO (2

mg/kg, i.p.) 90 minutes prior to transcardial perfusion compared to VEH (1 mL/kg, i.p.) administration (**Figure 2.3B**).

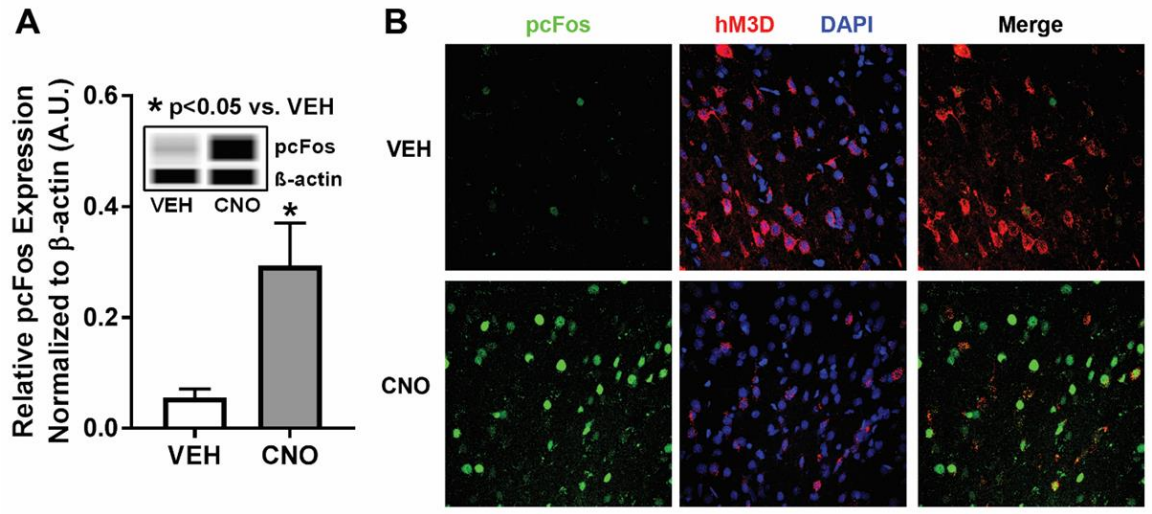

Figure 2.3: Insula$_{ant}$ activation induces pcFos expression.

Administration of CNO (2 mg/kg, i.p.) 90 minutes prior to insula$_{ant}$ dissection demonstrated a significantly higher level of pcFos protein expression normalized to β-actin compared to VEH (1 mL/kg, i.p.) administration (**Figure 2.3A**). Immunohistochemical qualitative analyses indicated greater evidence of pcFos expression in rats treated with CNO (2 mg/kg, i.p.) 90 minutes prior to transcardial perfusion compared to VEH (1 mL/kg, i.p.) administration (**Figure 2.3B**). Composite data are represented as mean +/- standard error of the mean (SEM). *p<0.05 vs. VEH (Student's *t*-test).

Insula$_{ant}$ Activation Effect on SF and HFF Intake: The role of insula$_{ant}$ activation on HFF and SF 2-hour intake was assessed under freely-fed and food-restricted conditions in Cohort 2. HFF intake in the freely-fed condition represented binge intake. Effects on caloric intake were assessed in a two-way repeated measures ANOVA for each feeding condition. A main effect of food ($F_{1,8}$=144.9, p<0.0001) and treatment ($F_{1,8}$=9.282, p=0.0159), but no food x treatment interaction ($F_{1,8}$=2.086, p=0.1867), was observed in

the freely-fed condition (**Figure 2.4A**). Bonferroni's multiple comparisons test showed $insula_{ant}$ activation did not alter SF intake (p=0.4673), but significantly suppressed HFF intake (p=0.0207), suggesting that the $insula_{ant}$ regulates hedonic feeding. A main effect of food ($F_{1,8}$=96.22, p<0.0001), but not of treatment ($F_{1,8}$=2.407, p=0.1594) or a food x treatment interaction ($F_{1,8}$=0.02894, p=0.8691), was observed in the food-restricted condition (**Figure 2.4B**), suggesting the $insula_{ant}$ does not modulate homeostatic feeding. To demonstrate CNO specificity for the hM3D system, the effects of CNO administration (2 mg/kg, i.p.) on binge intake were compared to VEH administration in a group of surgically naïve rats. A two-tailed unpaired Student's *t*-test indicated no difference between VEH and CNO administration (p=0.4735; **Figure 2.4C**), thus supporting that CNO specifically suppresses HFF binge intake via activation of hM3D.

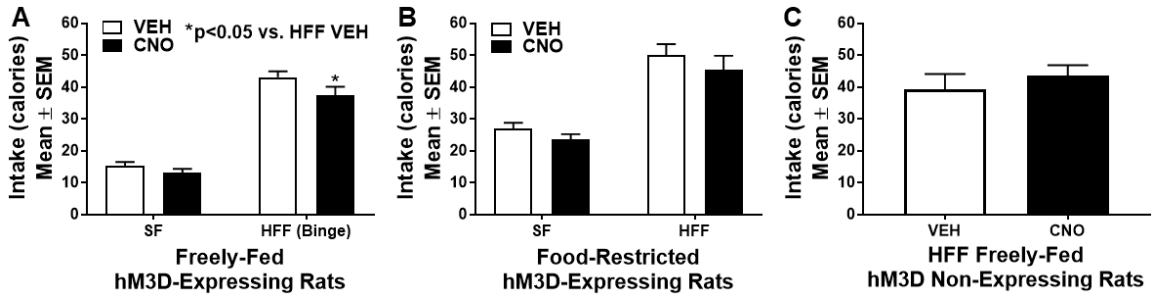

Figure 2.4: $Insula_{ant}$ activation suppresses HFF binge intake.

Cohort 2 rats underwent SF and HFF intake testing under either a freely-fed or food-restricted condition after VEH or CNO (2 mg/kg) administration. A two-way repeated measures ANOVA followed by Bonferroni's multiple comparisons test revealed $insula_{ant}$ activation suppressed HFF (i.e., binge eating; p<0.05), but not SF, intake in freely-fed rats (**Figure 2.5A**). $Insula_{ant}$ activation did not alter SF or HFF intake in food-restricted rats (**Figure 2.5B**). There was no effect of 2 mg/kg CNO in surgically naïve rats on HFF binge intake (**Figure 2.5C**). Composite data are represented as mean +/- SEM. *p<0.05 vs. HFF VEH (Bonferroni's multiple comparisons test).

Insula$_{ant}$ Activation Effect on HFF Cue Reactivity: The role of insula$_{ant}$ activation on HFF cue reactivity was assessed in Cohort 3. An unpaired Student's *t*-test demonstrated insula$_{ant}$ activation suppressed previously-active lever responding (p=0.0304; **Figure 2.5A**), but not inactive lever responding (p=0.1551), suggesting that the insula$_{ant}$ modulates HFF cue reactivity. This effect was not seen in rats exhibiting no evidence of hM3D expression (**Figure 2.5B**), thus suggesting the effects of CNO on cue reactivity are specific to hM3D activation.

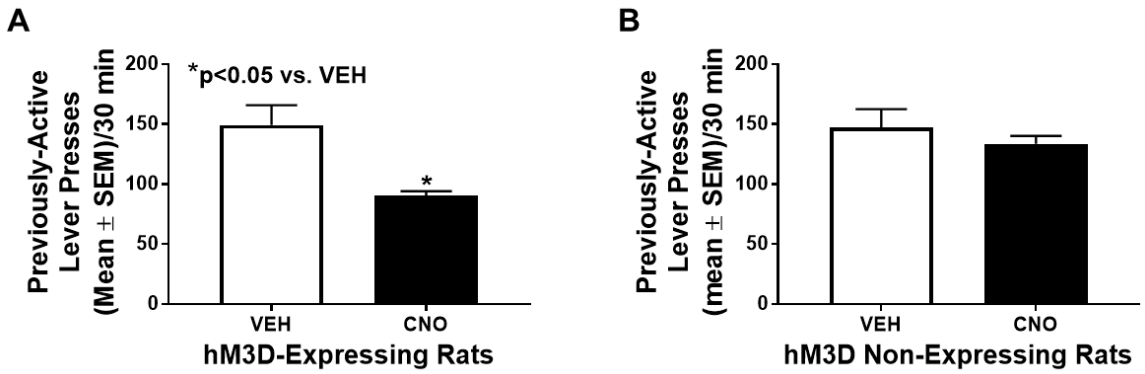

Figure 2.5: Insula$_{ant}$ activation suppresses HFF cue reactivity.

The role of insula$_{ant}$ activation on HFF cue reactivity was assessed in Cohort 3. An unpaired Student's *t*-test revealed insula$_{ant}$ activation suppressed HFF cue reactivity (p<0.05; **Figure 2.5A**). There was no effect of 2 mg/kg CNO on HFF cue reactivity in rats from Cohort 3 that demonstrated no evidence of hM3D expression (**Figure 2.5B**). Composite data are represented as mean +/- SEM. *p<0.05 vs. VEH (unpaired Student's *t*-test).

Insula$_{ant}$ Activation Effect on FR Responding for HFF: The role of insula$_{ant}$ activation on FR responding for HFF pellets was assessed in Cohort 3. A paired Student's *t*-test indicated a trend to increase FR active lever responding (p=0.054; **Figure 2.6A**), but no effect on inactive lever responding (p=0.3144) after insula$_{ant}$ activation, suggesting that

insula$_{ant}$ activation does not decrease HFF-reward efficacy, as a decrease in FR responding is often interpreted as a decrease in the perceived reinforcing efficacy (54). A two-tailed paired Student's *t*-test demonstrated no difference between VEH and CNO administration on FR responding for HFF pellets in rats exhibiting no evidence of hM3D expression (p=0.5144; **Figure 2.6B**).

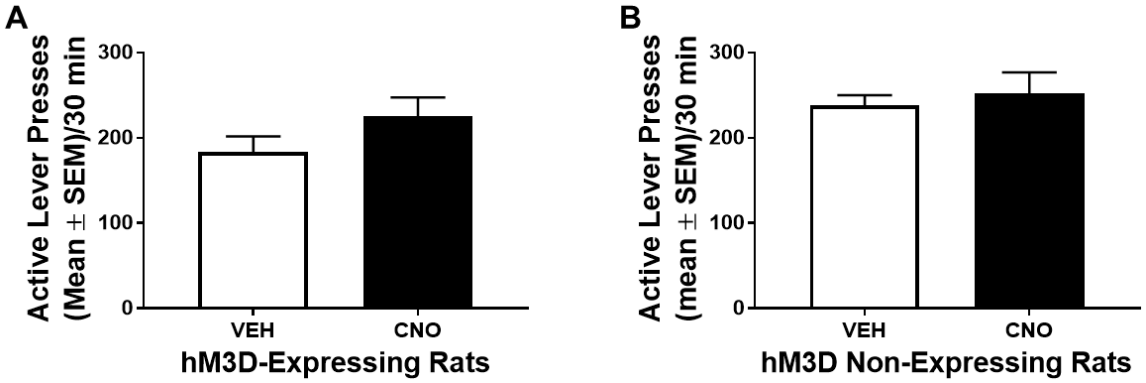

Figure 2.6: Insula$_{ant}$ activation does not suppress HFF-reward efficacy.

The role of insula$_{ant}$ activation on FR responding for HFF pellets was assessed in Cohort 3. A paired Student's *t*-test revealed insula$_{ant}$ activation did not alter FR responding for HFF pellets (**Figure 2.6A**). There was also no effect of 2 mg/kg CNO on FR responding in rats from Cohort 3 that demonstrated no evidence of hM3D expression (**Figure 2.6B**). Composite data are represented as mean +/- SEM.

Insula$_{ant}$ Activation Effect on PR Responding for HFF: A paired Student's *t*-test indicated insula$_{ant}$ activation did not alter PR active lever responding (p=0.8745), inactive lever responding (p=0.3352), or breakpoint (p=0.7882; **Figure 2.7A**). These data, together with no change in homeostatic food intake observed in Cohort 2, indicate insula$_{ant}$ activation does not disrupt global motivation. A two-tailed paired Student's *t*-test also demonstrated no difference between VEH and CNO administration on PR responding for HFF pellets in rats exhibiting no evidence of hM3D expression (p=0.5281; **Figure 2.7B**).

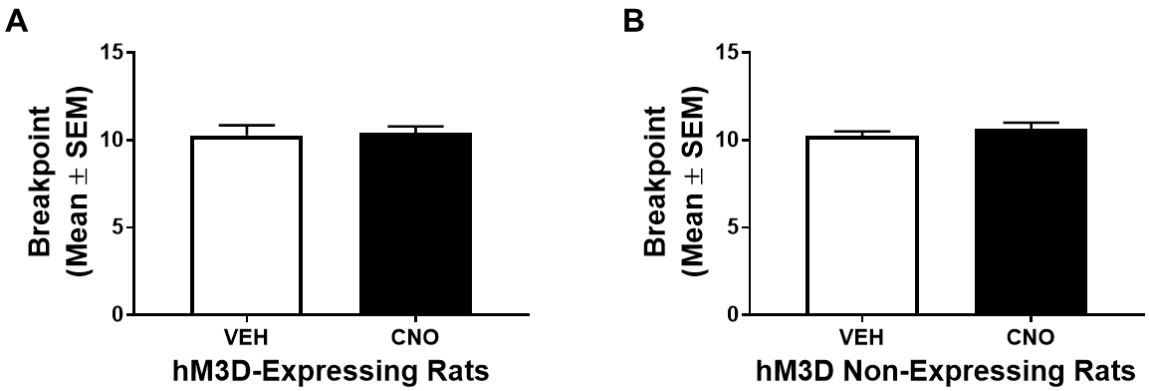

Figure 2.7: Insula$_{ant}$ activation does not suppress motivation for HFF.

The role of insula$_{ant}$ activation on PR responding for HFF pellets was assessed in Cohort 3. A paired Student's *t*-test revealed insula$_{ant}$ activation did not alter PR responding for HFF (**Figure 2.7A**). There was also no effect of 2 mg/kg CNO on PR responding in rats from Cohort 3 that demonstrated no evidence of hM3D expression (**Figure 2.7B**). Composite data are represented as mean +/- SEM.

DISCUSSION

The present work established a positive association between HFF binge intake and cue reactivity, similar to studies demonstrating higher cue reactivity levels in patients with dysregulated eating (55). While traditional therapeutic approaches to suppress binge eating have aimed to suppress episode frequency and severity, the observation of interlocked binge and cue behaviors substantiates the alternative goal of indirectly suppressing binge eating via attenuation of cue reactivity. This approach would broaden the treatment arsenal for binge eating, as medications such as *N*-acetylcysteine (56) and varenicline (57) have been shown to suppress cue-induced food- or drug-seeking in preclinical or clinical studies. Non-pharmacological approaches such as transcranial magnetic stimulation (TMS) can also successfully attenuate cue-induced food craving in humans (58). Evidence that the weight loss drug *D*-fenfluramine may suppress food intake

via suppression of cue-induced food-seeking further supports the goal to effectively suppress food cue reactivity as a treatment modality in binge eating (59).

We hypothesized $insula_{ant}$ activation would alter HFF binge intake and cue reactivity through changes in internal and external cue interpretation. The $insula_{ant}$ is highly implicated in interoceptive processing (e.g., recognition of satiety signals), which is believed to be disrupted in dysregulated eating (43). Thus, $insula_{ant}$ activation may restore interoceptive capabilities, evoking greater awareness of satiety. Our findings suggesting that the $insula_{ant}$ is a mediator of hedonic, but not homeostatic, feeding align with a previous study that demonstrated inactivation of the $insula_{ant}$ does not alter food-seeking behavior in food-restricted rats (60). Interestingly, studies investigating the role of the mid-insula have demonstrated opposite effects from the present study (i.e., mid-insula *inactivation* suppresses cue-driven food intake and palatable feeding) (61, 62), stressing the variability in function of different insula subregions (e.g., posterior regions perceive somatosensation which is not integrated with emotional and cognitive information until reaching the $insula_{ant}$) (43). Admittedly, the suppression of HFF binge intake seen upon $insula_{ant}$ activation was modest. However, it is impossible to determine if this effect size would be clinically relevant given the additional motivational factors that drive decision-making in people with obesity that cannot be modeled in preclinical paradigms (e.g., awareness of negative health effects, social stigma, etc.). Thus, it is possible that this seemingly small effect may be just enough to assist a patient who binge eats achieve behavioral modification.

The $insula_{ant}$ also regulates processing of external cues associated with hedonic eating, perhaps through outflow networks (43). Subjects with dysregulated eating demonstrate disrupted functional connectivity of the $insula_{ant}$ with various brain regions (i.e., connectivity is lower in the resting-state but higher after food cue exposure) (43, 63). In the present study, $insula_{ant}$ activation occurred prior to cue exposure, suggesting that

increasing $insula_{ant}$ activity before cue exposure (as in the resting-state) may be protective in suppressing cue reactivity. Thus, normalization of $insula_{ant}$ function may be necessary to prevent cue-induced food-seeking in individuals who binge eat.

In conclusion, this study offers compelling evidence to approach treatment of binge eating in novel ways such as aiming to decrease binge eating indirectly via suppression of food-associated cue reactivity. Furthermore, we have demonstrated that the $insula_{ant}$, a region amenable to activity modulation via modalities such as TMS, exerts a regulatory role over both HFF binge intake and cue reactivity, thus offering a new therapeutic target in the treatment of dysregulated eating.

Chapter 3: 5-HT_{2C}R Activation Suppresses Binge Intake and the Reinforcing and Motivational Properties of High Fat Food[2]

INTRODUCTION

BED is characterized by uncontrollable, recurrent episodes of excessive intake of food (40) that is often driven by hedonic rather than homeostatic mechanisms (i.e., food intake driven by wanting and liking factors but not necessary for energy balance) (64-66). Patients with BED deem high fat foods more rewarding than people without BED, which may motivate them to consume palatable food (67), and also exhibit disruptions in reward neurocircuitry (68). Current treatments for BED are comprised of behavioral therapy, off-label use of pharmacotherapies, and lisdexamfetamine, the only clinically-approved drug in the treatment of BED (69). One avenue to identify novel treatment approaches in BED is to explore targets known to alter both food intake and reward-related behaviors. The 5-HT_{2C}R fulfills these criteria in that previous studies have demonstrated that 5-HT_{2C}R activation suppresses feeding behavior (70-72) via promotion of satiety (for review, (73), which in part led to the development and subsequent FDA-approval of the weight loss drug lorcaserin (Belviq®), a first-in-class selective 5-HT_{2C}R agonist (74-77). In addition, preclinical studies demonstrate that 5-HT_{2C}R activation also regulates the hedonic properties of rewarding substances such as food and drugs of abuse (for reviews, (14, 78-80). Thus, suppression of the reinforcing and motivational properties of palatable food via 5-HT_{2C}R activation may be one mechanism to decrease hedonic eating and ultimately binge intake.

The investigational compound WAY163909 is a high affinity, full efficacy 5-HT_{2C}R agonist relative to the homologous 5-HT_{2A}R and 5-HT_{2B}R (81). WAY163909 suppresses

[2] The work in this chapter has been published in a modified form. It has been reprinted with permission from Price AE, Anastasio NC, Stutz SJ, Hommel JD, Cunningham KA. Serotonin 5-HT_{2C} receptor activation suppresses binge intake and the reinforcing and motivational properties of high-fat food. *Frontiers in Pharmacology*. 2018;9:821.

food intake in Sprague-Dawley rats, obese Zucker rats, and diet-induced obese mice, effects that are completely reversed by a selective 5-HT_{2C}R antagonist (81). Furthermore, WAY163909 decreases intake of both drug and sucrose reinforcers (54, 82-84). To our knowledge, WAY163909 has not been previously tested for efficacy in preclinical binge eating models. Herein, we tested the hypothesis that WAY163909 would suppress binge intake of HFF in an intermittent access model at doses that do not interrupt SF non-binge intake in adult male Sprague-Dawley rats. We further explored if WAY163909 alters hedonic eating via suppression of the reinforcing and motivational properties of HFF in two operant conditioning paradigms. These studies provide valuable insight into the potential therapeutic applicability of 5-HT_{2C}R activation in BED.

METHODS

Animals: Naïve male, outbred Sprague-Dawley rats (n=42; Harlan) weighing 200-225 g at arrival were housed two per cage (except where noted below) under a 12-h light-dark cycle (lights on between 0600-1800h) with controlled temperature (21-23°C) and humidity (40-50%). Animals were acclimated for seven days to the colony room prior to handling and experimental procedures. SF and water were available to rats *ad libitum* except during daily operant sessions and where noted below. All experiments were conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (2011) and with the University of Texas Medical Branch Institutional Animal Care and Use Committee approval.

Food: SF available *ad libitum* and used in non-binge intake studies consisted of 25% protein, 58% carbohydrate, and 17% fat (by kcal; LM-485 Mouse/Rat Sterilizable Diet; Teklad Diets; 3.1 kcal/g). HFF employed for binge intake studies contained 20% protein, 35% carbohydrate, and 45% fat (by kcal; D12451, Research Diets; 4.73 kcal/g). HFF pellets used in operant assays consisted of 16% protein, 38% carbohydrate, and 46% fat (by kcal; #F06162, BioServ, 45 mg/pellet; 4.60 kcal/g).

Drugs: WAY163909 [(7b-R,10a-R)-1,2,3,4,8,9,10,10a-octahydro-7bH-cyclopenta[b][1,4] diazepino [6,7,1hi]indole] was a gift from Pfizer, Inc. (New York, NY, USA) and was dissolved in 0.9% NaCl (VEH). WAY163909 was tested at a dose range (0-2 mg/kg) that did not alter total horizontal ambulation in a motor activity monitor but dose-dependently suppressed operant responding for self-administered sucrose pellets (54). These effects are completely blocked following pretreatment with the selective 5-HT_{2C}R antagonist SB242084 (54, 81). All injections were administered i.p. in a volume of 1 ml/kg.

WAY163909 Effect on HFF Binge Intake: Binge intake of HFF was assessed as described previously in a HFF intermittent access model (48). Briefly, single-housed rats (n=9) were acclimated to exclusive *ad libitum* access to HFF for one week to prevent food neophobia. Following this acclimation timeframe, rats were provided with exclusive *ad libitum* access to SF except during binge intake testing. The effects of 5-HT_{2C}R activation on binge intake were determined after i.p. injection of VEH or 0.5, 1, or 2 mg/kg WAY163909 15 minutes prior to the beginning of the dark cycle (1745h). At 1800h, SF was removed and 40 g of HFF was added to the home cage. HFF was removed two hours later (2000h) and weighed to determine binge intake. Rats were then provided *ad libitum* access to SF. All rats received these four pharmacological treatments prior to HFF binge intake testing in a randomized manner with testing spaced at least one week apart. Previously published results using this paradigm demonstrate that rats receiving continuous access to HFF eat an average of 5 grams of HFF in two hours whereas rats subjected to intermittent access to HFF eat an average of 7 grams of HFF in two hours (i.e., binge intake) (48). Thus, rats which consumed <5 grams HFF in two hours after VEH pretreatment were excluded for not exhibiting HFF binge intake (n=2). An additional rat was excluded as an outlier (i.e., intake greater than two standard deviations from the mean).

WAY163909 Effect on SF Non-Binge Intake: The effects of 5-HT_{2C}R activation on non-binge SF intake was also assessed in single-housed rats (n=8) that were injected i.p. with VEH or 0.5, 1, or 2 mg/kg WAY163909 15 minutes prior to the beginning of the dark cycle (1745h). At 1800h, all but 40 g of SF was removed from the home cage. At the end of two hours (2000h), SF was removed and weighed to determine non-binge intake, and rats were allowed *ad libitum* access to SF. Rats received all four treatments prior to non-binge intake testing in a randomized manner at least one week apart.

Operant Conditioning for HFF Self-Administration: Rats were trained to self-administer HFF pellets via an operant conditioning paradigm. Operant studies took place between 0900-1200h in standard operant chambers housed within a ventilated and sound-attenuated chamber and equipped with two retractable levers (MedAssociates). Operant studies consisted of 30-minute sessions (5 days/week) during which rats were trained to lever press for a HFF pellet. Completion of the FR or PR schedule of reinforcement on the active lever resulted in delivery of the reinforcer (one HFF pellet); on the FR schedule, pellet delivery was paired with a discrete, flashing light. There were no scheduled consequences for lever presses on the inactive lever.

WAY163909 Effect on FR Responding for HFF: Rats (n=9) were SF restricted to 85-90% of free-feeding levels for the first three days of operant conditioning to facilitate acquisition of HFF self-administration and then provided with *ad libitum* access to SF while in the home cage for the remainder of the study. Rats were trained on an FR1 schedule of reinforcement for HFF pellets for five days, moved to an FR3 schedule of reinforcement for two days, and maintained on an FR5 schedule of reinforcement for the remainder of the study. The criterion for stable FR acquisition and responding (<25% variability in the number of HFF pellets earned over three consecutive FR5 training sessions) was achieved prior to initiation of test sessions. Once stable, rats underwent two consecutive days of testing in which they received VEH on one day and 1 mg/kg WAY163909 the following day. After allowing at least three days for washout of drug and re-establishment

of stability (54), rats underwent two additional consecutive days of testing in which they received VEH on one day and 0.3 mg/kg WAY163909 the following day. All injections were administered i.p. 15 minutes prior to the beginning of the operant session. One rat was removed from the study for failure to achieve the stability criterion.

WAY163909 Effect on PR Responding for HFF: Rats (n=16) were SF restricted to 85-90% of free-feeding levels for the first three days of operant conditioning to facilitate acquisition of HFF self-administration and then provided with *ad libitum* access to SF while in the home cage for the remainder of the study. Rats were trained on an FR1 schedule of reinforcement for HFF pellets for five days, moved to an FR3 schedule of reinforcement for two days, moved to an FR5 schedule of reinforcement for two days, and then maintained on a PR schedule of reinforcement for the remainder of the study. The PR schedule of reinforcement (1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95) required rats to progressively increase the number of active lever presses needed to receive a single HFF reinforcer (46, 47). PR sessions ended 10 minutes after the last reinforcer was received. The criterion for stable PR acquisition and responding (<25% variability in the number of HFF pellets earned over three consecutive PR training sessions) was achieved prior to initiation of test sessions. Following achievement of stability, rats were tested with 1 mg/kg of WAY163909, 0.3 mg/kg of WAY163909, and VEH with at least three days between tests to allow for washout of drug and re-establishment of stability (54). All injections were administered i.p. 15 minutes prior to the beginning of the operant session. Four rats were excluded for not achieving stability, and an additional rat was removed from the study due to an equipment malfunction.

Statistical Analyses: A repeated measures, one-way ANOVA was employed to assess the main effect of WAY163909 treatment on HFF binge intake, 2-hour SF non-binge intake, and measures of operant responding for self-administration of HFF pellets (i.e., active and inactive lever presses, pellets earned, breakpoint, and latency to first reinforcer) (54). Subsequent *a priori* comparisons to VEH were analyzed using a one-

tailed Dunnett's procedure. A paired Student's *t*-test was employed to assure consistent baseline responding between FR VEH test days and between the days preceding the first and last PR tests. All statistical analyses were conducted with an experimentwise error rate of α=0.05 in SAS for Windows 9.4.

RESULTS

WAY163909 Effect on HFF Binge Intake: The effects of the 5-HT_{2C}R agonist WAY163909 on HFF binge intake were assessed in an intermittent HFF access model. A repeated measures, one-way ANOVA revealed a main effect of treatment ($F_{3,15}$=4.79; p=0.0155). *A priori* comparisons demonstrated that 1 mg/kg and 2 mg/kg of WAY163909 significantly suppressed binge intake compared to VEH treatment (p<0.05; **Figure 3.1A**). These data indicate that activation of 5-HT_{2C}R signaling suppresses binge eating.

WAY163909 Effect on SF Non-Binge Intake: The effects of WAY163909 on 2-hour, non-binge SF intake were also assessed. A repeated measures one-way ANOVA revealed no main effect of treatment ($F_{3,21}$=1.12; p=0.3642; **Figure 3.1B**). These results are consistent with published results indicating the ED_{50} for WAY163909-induced suppression of 2-hour food intake in 24-hour fasted Sprague-Dawley rats is 2.93 mg/kg (81). Together, these data indicate that lower doses of WAY163909 preferentially suppress HFF binge intake over non-binge intake of SF.

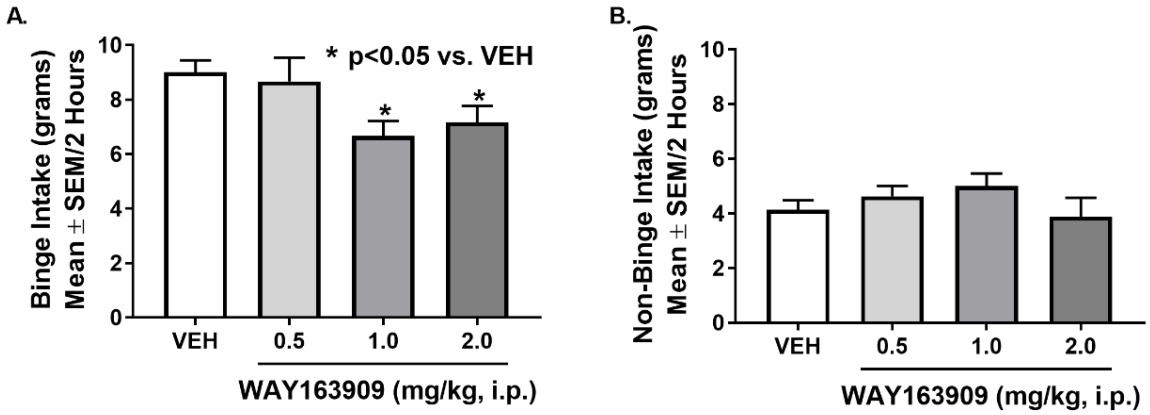

Figure 3.1: WAY163909 preferentially suppresses HFF binge intake over SF non-binge intake.

WAY163909 (1.0, 2.0 mg/kg) suppressed HFF binge intake in an intermittent access binge intake paradigm (n=6; **Figure 3.1A**). WAY163909 did not alter SF non-binge intake (n=8; **Figure 3.1B**). Data represented as mean +/- SEM. *p<0.05 vs. VEH (Dunnett's test).

WAY163909 Effect on FR Responding for HFF: The effect of WAY163909 on the reinforcing value of HFF was assessed using FR responding for HFF pellets in freely-fed rats. A paired Student's *t*-test between the first and second VEH test indicated there were no significant differences in active lever presses (p=0.934), inactive lever presses (p=0.621), pellets earned (p=0.605), or latency to first reinforcer (p=0.089); thus, the average VEH response was used as control for analysis of the WAY163909 dose-response relationship. A repeated measures, one-way ANOVA revealed a main effect of treatment on active lever presses ($F_{2,14}$=47.54; p<0.0001) and pellets earned ($F_{2,14}$=42.08; p<0.0001), but not for inactive lever presses ($F_{2,14}$=0.34; p=0.7185) or latency to first reinforcer ($F_{2,14}$=1.39; p=0.2819). *A priori* comparisons indicated that 1 mg/kg of WAY163909 significantly suppressed active lever presses (p<0.05) and pellets earned (p<0.05) compared to VEH (**Figure 3.2**). These data indicate that activation of the 5-HT_{2C}R suppresses the reinforcing value of HFF, a finding which coalesces with previously published results demonstrating that WAY163909 also dose-dependently suppresses operant responding for self-administration of sucrose pellets (54).

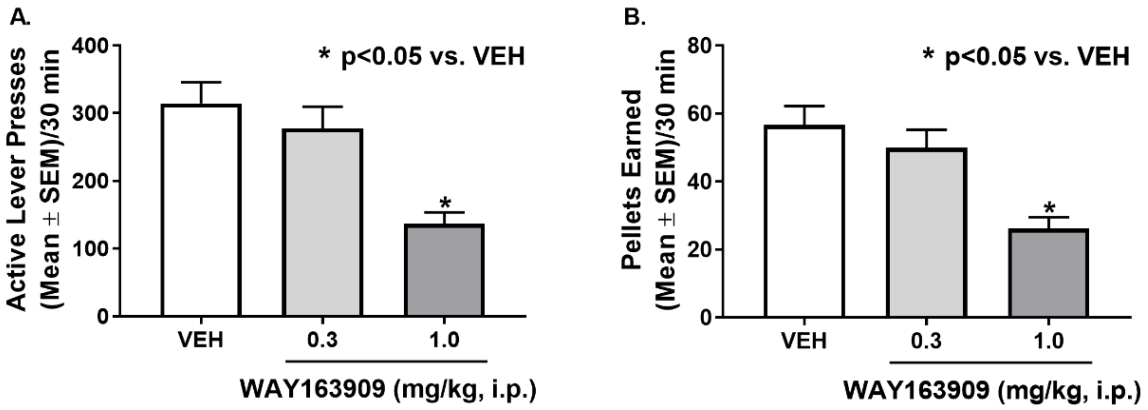

Figure 3.2. WAY163909 suppresses the reinforcing efficacy of HFF.

WAY163909 (1.0 mg/kg) suppressed (**Figure 3.2A**) active lever presses and (**Figure 3.2B**) pellets earned during FR responding for HFF (n=8). Data represented as mean +/- SEM. *p<0.05 vs. VEH (Dunnett's test).

WAY163909 Effect on PR Responding for HFF: The effects of WAY163909 on the motivational value of HFF was assessed using PR responding for HFF pellets in freely-fed rats. A paired Student's *t*-test was used to assess consistent baseline responding between the days preceding the first and last test sessions; analyses indicated there was no difference in active lever presses (p=0.819) or pellets earned (p=0.714). A repeated measures one-way ANOVA revealed a main effect of treatment on active lever presses ($F_{2,20}$=9.12; p=0.0015) and breakpoint ($F_{2,20}$=19.11; p<0.0001), but not inactive lever presses ($F_{2,20}$=2.59; p=0.0998) or latency to first reinforcer ($F_{2,20}$=1.81; p=0.1888). *A priori* comparisons indicated that 1 mg/kg of WAY163909 significantly suppressed active lever presses (p<0.05) and breakpoint (p<0.05) compared to VEH treatment (**Figure 3.3**). These data indicate that 5-HT_{2C}R activation suppresses the motivational value of HFF.

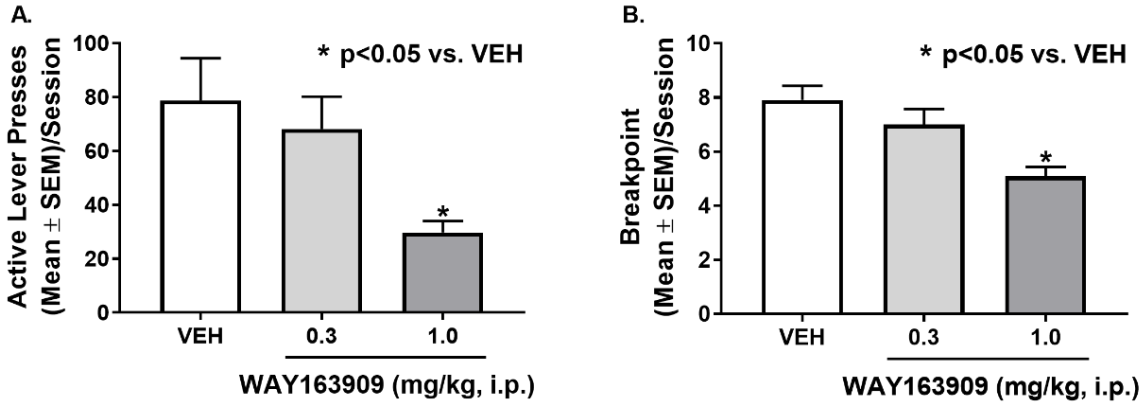

Figure 3.3. WAY163909 suppresses the motivational value of HFF.

WAY163909 (1.0 mg/kg) suppressed active lever presses (**Figure 3.3A**) and breakpoint (**Figure 3.3B**) during PR responding for HFF (n=11). Data represented as mean +/- SEM. *p<0.05 vs. VEH (Dunnett's test).

DISCUSSION

Food intake can be described as homeostatic (intake necessary to maintain energy balance) or hedonic (intake driven by reward-related factors) (85). Hedonic processes are postulated as an important component of binge eating episodes seen in disorders such as BED (66). To model binge eating in rodents, we gave rats *ad libitum* access to standard chow along with intermittent access to a highly palatable food (48, 86, 87). The palatable food used in this study was a HFF chow, which is nutritionally representative of foods that patients with BED may eat in excess during a binge episode (86). Administration of 1 mg/kg of WAY163909 significantly suppressed HFF binge intake in rodents, suggesting that 5-HT_{2C}R activation may be a viable therapeutic approach to suppress binge eating episodes in patients with BED. This same dose of WAY163909 did not alter non-binge intake, consistent with previous literature that demonstrated that higher doses of WAY163909 (i.e., 3-10 mg/kg) are required to suppress 2-hour SF intake in 24-hour fasted male Sprague-Dawley rats (81). This suggests that 5-HT_{2C}R activation preferentially suppresses hedonic intake of food at lower doses but attenuates both hedonic and homeostatic intake of food at higher doses. We then demonstrated that 1 mg/kg of WAY163909 suppressed both FR and PR responding, suggesting that 5-HT_{2C}R activation attenuates the reinforcing efficacy and motivational properties of HFF, respectively. These findings are congruent with literature demonstrating 5-HT_{2C}R activation decreases intake of other types of palatable food, such as those high in carbohydrates (78, 88, 89).

Activation of the 5-HT_{2C}R is postulated to primarily suppress feeding by increasing production of α-melanocyte stimulating hormone which acts on melanocortin 4 receptors in the paraventricular nucleus of the hypothalamus to promote satiety (90-92). However, 5-HT_{2C}R activation-induced suppression of operant responding for food in self-administration studies suggests that the 5-HT_{2C}R regulates intake of food in additional ways since total food intake in these paradigms is often not enough to promote satiety

(93). A recent study demonstrated that the clinically-approved 5-HT_{2C}R agonist lorcaserin suppressed both binge-like eating and hunger-driven feeding in wildtype mice (94). Interestingly, lorcaserin-induced suppression of binge-like eating in mice is dependent upon 5-HT_{2C}R expression on dopaminergic neurons in the ventral tegmental area, cells highly implicated in reward-related behaviors (94). In mice with selective knockout of 5-HT_{2C}R in dopaminergic neurons, lorcaserin is unable to suppress binge-like eating, suggesting that the 5-HT_{2C}R is mediating its effects on hedonic feeding through mechanisms beyond hypothalamus-dependent promotion of satiety (94). Indeed, lorcaserin also suppresses PR responding for chocolate pellets via activation of 5-HT_{2C}R in the ventral tegmental area (95), further supporting a role for mesolimbic 5-HT_{2C}R in the control of palatable food intake.

Elevations in body weight are seen in approximately 70% of patients with BED (96). Thus, a medication that effectively suppresses both binge magnitude and overall food intake at therapeutic doses would be highly beneficial in the treatment of comorbid BED and obesity. The FDA-approved selective 5-HT_{2C}R agonist lorcaserin may be of benefit for this population of patients. Interestingly, clinical trials for lorcaserin showed that while, on average, treatment produced modest effects (about 3% weight loss when accounting for effects of placebo), lorcaserin treatment resulted in 5% or even 10% body weight loss in certain subpopulations, a phenomenon occurring at twice the frequency in the lorcaserin group compared to the placebo group (74). Our findings combined with previous literature suggest that 5-HT_{2C}R activation suppresses hedonic intake of food in addition to homeostatic intake of food; thus, lorcaserin may show higher efficacy in patients seeking weight loss treatment with co-morbid BED compared to patients without BED. To our knowledge, a behavioral subtyping of the individuals who exhibit the greatest weight loss upon lorcaserin treatment has not yet been reported. The present study suggests that analyses assessing the efficacy of lorcaserin in different behavioral subtypes of obesity is warranted.

This study supports 5-HT_{2C}R activation as a novel therapeutic target to suppress hedonic food intake in patients with BED. Future studies should assess the viability of repurposing the weight loss drug, lorcaserin, in the treatment of BED. These findings, in combination with the larger body of literature surrounding the role of 5-HT_{2C}R in food intake, suggest that 5-HT_{2C}R activation may be especially helpful in the treatment of co-morbid BED and obesity.

Chapter 4: Pimavanserin and Lorcaserin Suppress Measures of Binge Eating in Male Sprague-Dawley Rats[3]

INTRODUCTION

BED is defined by repeated binge eating episodes that are characterized by uncontrollable, excessive intake of food (40). These episodes are driven by hedonic eating, which can be described as food intake beyond what is physiologically necessary to maintain energy balance (i.e., homeostatic intake of food), and may be caused by disruptions in reward circuitry (85). Current BED treatments in the United States are limited to lisdexamfetamine (Vyvanse®), the only FDA-approved pharmaceutical treatment, in addition to behavioral therapy and off-label use of other pharmacological agents (69). We propose that repurposing clinically-available drugs that alter both food intake and reward-related behaviors may represent new therapeutic options in the treatment of BED.

Lorcaserin (Belviq®) is currently FDA-approved for weight loss in patients with an elevated body mass index (BMI) and is a strong candidate for drug repurposing in the treatment of BED. Lorcaserin alters both food intake and reward-related processes via activation of the 5-HT_{2C}R (for reviews, (79, 93). Activation of the 5-HT_{2C}R suppresses food intake via production of α-melanocyte stimulating hormone, which acts on melanocortin 4 receptors in the paraventricular nucleus of the hypothalamus to promote satiety (90-92). Patients with BED are thought to consume excessive amounts of food in part due to disrupted satiety signals (97), suggesting that satiety signal restoration via administration of a 5-HT_{2C}R agonist may decrease food intake during a binge episode. In addition to dysregulated food consumption, people with BED also deem palatable foods more rewarding and exhibit greater motivation to consume these substances compared

[3] The work in this chapter has been published in a modified form. It has been reprinted with permission from Price AE, Brehm VD, Hommel JD, Anastasio NC, Cunningham KA. Pimavanserin and lorcaserin attenuate measures of binge eating in male Sprague-Dawley rats. *Frontiers in Pharmacology*. 2018;9:1424.

to people without BED (65-67). Activation of the 5-HT_{2C}R regulates reward-related processes (for reviews, (14, 78-80) and is therefore likely to suppress hedonic food intake via stabilization of reward-related behaviors.

The closely-related 5-HT_{2A}R also serves as an intriguing target for the treatment of BED. Both the 5-HT_{2A}R and 5-HT_{2C}R are G-protein coupled receptors that primarily exert effects via $G_{\alpha q}$ signaling pathways. However, these two receptors regulate reward-related behaviors in opposing ways. Specifically, 5-HT_{2C}R agonists and 5-HT_{2A}R antagonists suppress reward-related behaviors, while 5-HT_{2C}R antagonists promote reward-related behaviors (for review, (14). Preclinical studies have also indicated that the 5-HT_{2A}R is implicated in regulation of feeding behavior. The non-specific 5-HT receptor antagonist metitepine exerts anorectic effects via the 5-HT_{2A}R (98), while systemic administration of non-specific 5-HT_{2A}R antagonists inhibits overfeeding and obesity in obese A(y) mice and food reinforced operant behavior in fasted Sprague-Dawley rats (99, 100). Further, diet-induced obese rats display elevated 5-HT_{2A}R binding in the lateral hypothalamus and arcuate nucleus, which regulate feeding, (vs. chow fed controls) (101) as well as within the nucleus accumbens shell and olfactory nucleus, which mediate rewarding effects of food (vs. diet-resistant rats or mice) (102, 103). Human studies have also demonstrated that BMI positively correlates with *in vivo* cerebral 5-HT_{2A}R binding (104). Together, these data suggest that 5-HT_{2A}R systems are engaged in processes related to food intake. Excitingly, the selective 5-HT_{2A}R antagonist/inverse agonist pimavanserin (Nuplazid®) is clinically approved for treating psychosis in Parkinson's Disease and therefore has potential to be repurposed for the treatment of BED.

The present study tested the hypothesis that the clinically-available 5-HT_{2A}R antagonist/inverse agonist pimavanserin and 5-HT_{2C}R agonist lorcaserin suppress measures of binge eating. We assessed the effects of both drugs in an intermittent-access HFF binge eating model in adult male Sprague-Dawley rats on the measures of binge episode occurrence, binge intake, and weight gain associated with HFF exposure. We

further tested the hypothesis that combined administration of pimavanserin plus lorcaserin would be more effective in suppressing measures of binge eating than single administration of either drug alone. The results offer exciting new possibilities in the treatment of BED.

METHODS

Animals: Outbred, male Sprague-Dawley rats (n=64, Envigo, Haslett, MI, USA) weighing 225-250 g at arrival were single-housed under a 12-h light-dark cycle (lights on between 0600-1800h) with controlled temperature (21-23°C) and humidity (40-50%). SF and water were available *ad libitum* except where noted below. Animals were acclimated to the colony room for 7-9 days prior to handling and experimentation. All experiments were conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (2011) and with the University of Texas Medical Branch Institutional Animal Care and Use Committee approval.

Food: SF (LM-485 Mouse/Rat Sterilizable Diet; Teklad Diets; 3.1 kcal/g) consisted of 25% protein, 58% carbohydrate, and 17% fat (by kcal). HFF (D12451, Research Diets; 4.73 kcal/g) contained 20% protein, 35% carbohydrate, and 45% fat (by kcal).

Drugs: Pimavanserin (Hangzhou Trylead Chemical Technology Co., Ltd., Hangzhou, China) was dissolved in acidic 0.9% NaCl, then brought to a final pH of ~6.0 using NaOH. Lorcaserin (Hangzhou Trylead Chemical Technology Co., Ltd.) was dissolved in 0.9% NaCl.

Binge Eating Paradigm: Rats were acclimated to HFF for seven days to avert food neophobia then returned to *ad libitum* SF access (48). On the sixth day of SF access, 2-hour HFF intake was measured in a subset of rats (n=32) in the home cage from 1800-2000h (beginning of the dark cycle) to determine intake during continuous access conditions. Measures of binge eating were assessed once a week following pharmacological treatment with pimavanserin and/or lorcaserin starting one week after

completion of the acclimation period. On test days, rats received free access to 40 g of HFF in the home cage from 1800-2000h. At 2000h, the remaining HFF was weighed, and rats received *ad libitum* access to SF again.

Three measures of binge eating were used to assess the effects of drug administration:

BINGE EPISODE OCCURRENCE: Binge episode occurrence was assessed to determine if drug treatment could prevent the occurrence of binge eating. The average 2-hour HFF intake in continuous access conditions (i.e., on day six of the acclimation period) was set as the minimum intake necessary to constitute a binge episode since continuous access to HFF does not result in binge eating (48). This criterion was set using intake as a percent of body weight to control for weight gain throughout the study. Thus, HFF intake more than this percentage during a test session was classified as a binge episode. Rats were dichotomized as exhibiting binge episode occurrence (yes) or not exhibiting binge episode occurrence (no).

BINGE INTAKE: Binge intake was assessed to determine if drug treatment could suppress the magnitude of food consumed during a binge episode. Only rats exhibiting binge episode occurrence after VEH administration were used to assess this measure. Binge intake was measured in grams of HFF consumed during 2-hour access.

WEIGHT GAIN ASSOCIATED WITH HFF EXPOSURE: Weight gain during a 22-hour period encompassing drug administration and HFF exposure was analyzed to determine if drug treatment could suppress weight gain associated with exposure to HFF. Rats were weighed at 1400h on the day of the binge, treated with drug between 1730-1745h, given access to HFF from 1800-2000h, then weighed again at 1200h the following day. Weight gain was recorded as the difference in body weight in grams from the beginning to the end of this 22-hour period.

Pharmacological Testing: Four cohorts of rats were used for pharmacological testing. Initial testing was conducted in Cohorts 1 and 2, and Cohorts 3 and 4 were used

to independently replicate the observed findings. Cohorts 1 (n=16) and 3 (n=16) were injected with VEH, 0.3 mg/kg, 1.0 mg/kg, or 3.0 mg/kg pimavanserin subcutaneously (s.c.) 30 minutes prior to the start of the 2-hour HFF intake session (1730h). Cohorts 2 (n=16) and 4 (n=16) were injected with VEH, 0.25 mg/kg, 0.5 mg/kg, or 1.0 mg/kg lorcaserin s.c. 15 minutes prior to the start of the 2-hour HFF intake session (1745h). Each rat received each dose of the assigned drug in a counterbalanced manner.

After dose response testing was completed, Cohorts 3 and 4 were used to assess the effects of combined administration of pimavanserin plus lorcaserin on 2-hour HFF intake using the lowest dose of each drug shown to suppress binge intake. Rats were injected with either VEH or 0.3 mg/kg pimavanserin s.c. 30 minutes prior to the start of the 2-hour HFF intake session (1730h) plus VEH or 1.0 mg/kg lorcaserin s.c. 15 minutes prior to the start of the 2-hour HFF intake session (1745h). Each rat in Cohorts 3 and 4 received each combination of injections in a counterbalanced manner.

Statistical Analyses: Three measures were used to assess binge eating. For *binge episode occurrence*, a Cochran's *Q* test (a non-parametric test that compares differences between three or more sets of binary responses) was used to determine significant differences between drug treatments (105, 106). *A priori* comparisons were analyzed using multiple McNemar's tests with a Bonferroni corrected α value of 0.0167 (107). A Chi-square test was used to determine significant differences in binge episode occurrence after VEH administration between the four cohorts. Statistical analyses were conducted with an experimentwise error rate of α=0.05 in SPSS Statistics Version 24. For *binge intake*, a repeated measures one-way ANOVA was used to determine significant differences between drug treatments. Subsequent *a priori* comparisons to VEH were analyzed using Dunnett's multiple comparisons test. A repeated measures two-way ANOVA was used to assess interactions between treatment with pimavanserin and lorcaserin in the combination study. Statistical analyses were conducted with an experimentwise error rate of α=0.05 in GraphPad Prism 7. For *weight gain associated with*

exposure to HFF, a repeated measures one-way ANOVA was used to determine significant differences between drug treatments. Subsequent *a priori* comparisons to VEH were analyzed using Dunnett's multiple comparisons test. Statistical analyses were conducted with an experimentwise error rate of α=0.05 in GraphPad Prism 7.

RESULTS:

Classifying Binge Episode Occurrence: The average 2-hour HFF intake after continuous access was 1.47 +/- 0.063% of body weight. Thus, HFF intake of >1.47% of body weight during test sessions was classified as a binge episode occurrence.

Effect of Pimavanserin on Binge Episode Occurrence, Binge Intake, and Weight Gain Associated with HFF Exposure: The dose response for pimavanserin on binge episode occurrence, binge intake, and weight gain associated with HFF exposure were assessed in Cohorts 1 and 3. All statistical analyses and results, including from individual and combined cohorts, can be found in **Table 4.1**. Cochran's *Q* test demonstrated no statistically significant difference in binge episode occurrence in the combined Cohort 1 (n=16) and Cohort 3 (n=16) analyses (χ^2_3=3.923; p=0.270; **Figure 4.1A**). Rats exhibiting binge episode occurrence after VEH administration in Cohorts 1 (n=15) and 3 (n=14) were collapsed into one group; a repeated measures one-way ANOVA revealed a main effect of pimavanserin dose on binge intake ($F_{3,84}$=12.6; p<0.001). Dunnett's multiple comparisons test demonstrated pimavanserin suppressed binge intake at 0.3 mg/kg (p<0.001), 1.0 mg/kg (p<0.001), and 3.0 mg/kg (p<0.001) compared to VEH administration (**Figure 4.1B**). This lack of dose-dependent responding is consistent with other studies that have demonstrated that 5-HT_{2A}R antagonists often exhibit flat or very narrow dose-response curves on behavioral analyses (108-110). Finally, a repeated measures one-way ANOVA demonstrated a main effect of pimavanserin on weight gain associated with HFF exposure in the combined analyses of Cohort 1 (n=16) and Cohort 3 (n=16) ($F_{3,93}$=12.37; p<0.001). Dunnett's multiple

comparisons test demonstrated that both 1.0 mg/kg ($p<0.001$) and 3.0 mg/kg ($p<0.001$) but not 0.3 mg/kg ($p=0.412$) pimavanserin significantly suppressed weight gain associated with HFF exposure (**Figure 4.1C**). Together, these data suggest that pimavanserin is effective at suppressing the magnitude, but not the occurrence, of binge episodes. The data further suggest that suppression of binge intake alone is not sufficient to suppress weight gain associated with HFF exposure since 0.3 mg/kg pimavanserin is only effective at suppressing the former. Thus, higher doses of pimavanserin (1.0 mg/kg and 3.0 mg/kg) may suppress weight gain associated with HFF exposure through alternative mechanisms such as suppression of SF intake. However, this effect is unlikely to be a result of suppression of general motor activity given that the chosen dose range did not alter locomotor activity in the same strain of rats (111).

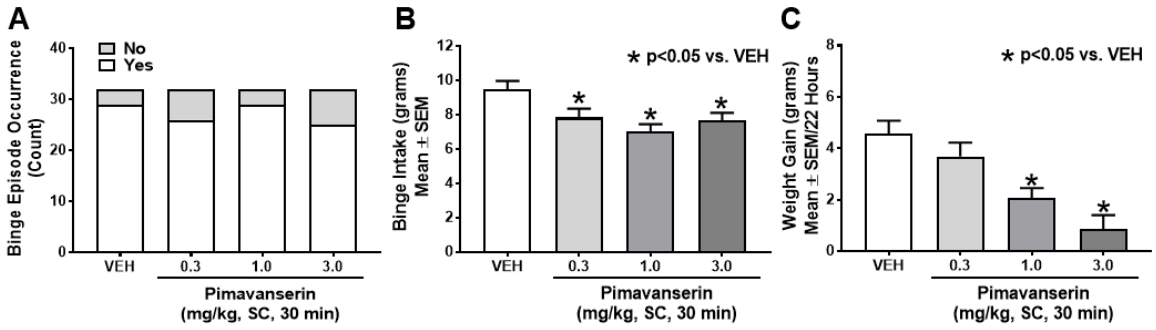

Figure 4.1: Pimavanserin suppresses binge intake and weight gain associated with HFF exposure but not binge episode occurrence.

Pimavanserin (0.3, 1.0, and 3.0 mg/kg) did not alter binge episode occurrence (**Figure 4.1A**, n=32), but did suppress binge intake (**Figure 4.1B**, n=29). Pimavanserin (1.0 and 3.0 mg/kg) suppressed weight gain associated with HFF exposure in a dose-related manner (**Figure 4.1C**, n=32). Composite data are represented as mean +/- SEM. *p<0.05 vs. VEH (Dunnett's test).

Effect of Lorcaserin on Binge Episode Occurrence, Binge Intake, and Weight Gain Associated with HFF Exposure: The dose response of lorcaserin on binge episode occurrence, binge intake, and weight gain associated with HFF exposure were assessed in Cohorts 2 and 4. All statistical analyses and results, including from individual and combined cohorts, can be found in **Table 4.2**. Cochran's *Q* test identified a statistically significant difference in binge episode occurrence in the combined Cohort 2 (n=16) and Cohort 4 (n=16) analyses (χ^2_3=8.510; p=0.037; **Figure 4.2A**). McNemar's test was used to identify differences between VEH and dose using a Bonferroni corrected significant α value of 0.0167. A significant difference between VEH and 0.5 mg/kg lorcaserin (p=0.016) was identified; however, the analysis indicated that 0.5 mg/kg lorcaserin resulted in a significant *increase* in binge episode occurrence compared to VEH. This may be due to the low percentage of rats exhibiting binge episode occurrence after VEH administration in Cohort 4; a Chi-square test demonstrated a significant difference in binge episode occurrence after VEH administration between the four cohorts (χ^2_3=8.012; p=0.046). In Cohorts 1, 2, and 3, greater than 80% of VEH-treated rats exhibited binge episode occurrence, whereas less than 60% of VEH-treated rats in Cohort 4 exhibited binge episode occurrence (**Tables 4.1** and **4.2**), which may be attributable to environmental and/or genetic factors that contribute to individual differences between outbred rats. Rats exhibiting binge episode occurrence after VEH administration in Cohorts 2 (n=13) and Cohorts 4 (n=9) were collapsed into one group; a repeated measures one-way ANOVA revealed a main effect of lorcaserin dose on binge intake ($F_{3,63}$=10.24; p<0.001). Dunnett's multiple comparisons test demonstrated lorcaserin suppressed binge intake at 1.0 mg/kg (p<0.001) compared to VEH treatment (**Figure 4.2B**). Finally, a repeated measures one-way ANOVA demonstrated no main effect of lorcaserin on weight gain associated with HFF exposure when Cohort 2 (n=16) and Cohort 4 (n=16) were combined for analyses ($F_{3,93}$=0.9926; p=0.400; **Figure 4.2C**). Together, these results suggest that lorcaserin is

effective at suppressing the magnitude of binge episodes, but not the occurrence of binge episodes or weight gain associated with HFF exposure.

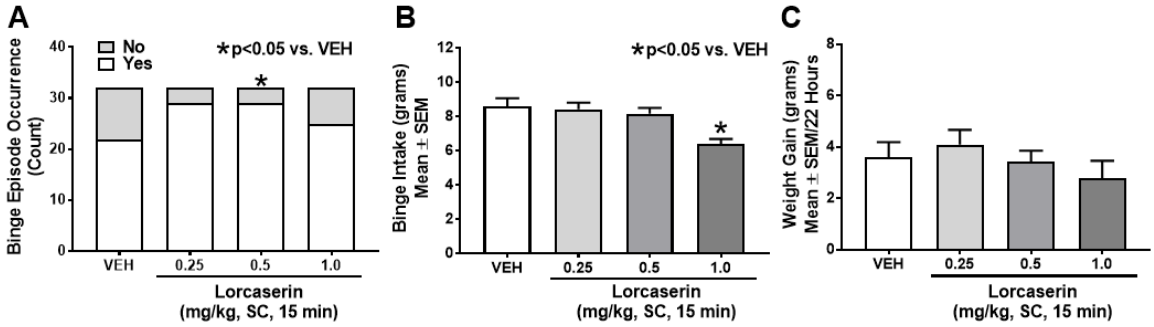

Figure 4.2: Lorcaserin suppresses binge intake but not binge episode occurrence or weight gain associated with HFF exposure.

Lorcaserin (1.0 mg/kg) did not suppress binge episode occurrence (**Figure 4.2A**, n=32), but did suppress binge intake (**Figure 4.2B**, n=22). Lorcaserin did not alter weight gain associated with HFF exposure (**Figure 4.2C**, n=32). Composite data are represented as mean +/- SEM. *p<0.05 vs. VEH (McNemar's test or Dunnett's test).

Effect of Combined Pimavanserin Plus Lorcaserin on Binge Episode Occurrence, Binge Intake, and Weight Gain Associated with HFF Exposure: Combined administration of effective doses of pimavanserin (0.3 mg/kg) plus lorcaserin (1.0 mg/kg) on binge episode occurrence, binge intake, and weight gain associated with HFF exposure were assessed in Cohorts 3 and 4. All statistical analyses and results, including from individual and combined cohorts, can be found in **Table 4.3**. Cochran's *Q* test demonstrated a statistically significant difference in binge episode occurrence in the combined analyses of Cohort 3 (n=16) and Cohort 4 (n=16) (χ^2_3=19.412; p<0.001; **Figure 4.3A**). McNemar's test (with a Bonferroni corrected significant α value of 0.0167) demonstrated a significant difference in binge episode occurrence after administration of lorcaserin alone (p=0.001) and pimavanserin plus lorcaserin (p<0.001) but only a trend for

pimavanserin alone (p=0.039). Rats exhibiting binge episode occurrence after VEH administration in Cohorts 3 (n=14) and Cohorts 4 (n=16) were combined for analyses; a repeated measures one-way ANOVA revealed a main effect of treatment on binge intake ($F_{3,87}$=11.55; p<0.001). Dunnett's multiple comparisons test demonstrated a significant suppression of binge intake after administration of lorcaserin alone (p<0.001) and pimavanserin plus lorcaserin (p<0.001) but not after administration of pimavanserin alone (p=0.2344; **Figure 4.3B**). Finally, a repeated measures one-way ANOVA demonstrated a main effect of treatment on weight gain associated with HFF exposure in combined analyses of Cohort 3 (n=16) and Cohort 4 (n=16) ($F_{3,93}$=9.81; p<0.0001). Dunnett's multiple comparisons test demonstrated a suppression of weight gain associated with HFF exposure following treatment with lorcaserin (p=0.002) and after pimavanserin plus lorcaserin (p<0.001; **Figure 4.3C**), but not after pimavanserin alone (p=0.133). Together, these results suggest combined administration of pimavanserin and lorcaserin can suppress both the occurrence and magnitude of binge episodes as well as weight gain associated with HFF exposure.

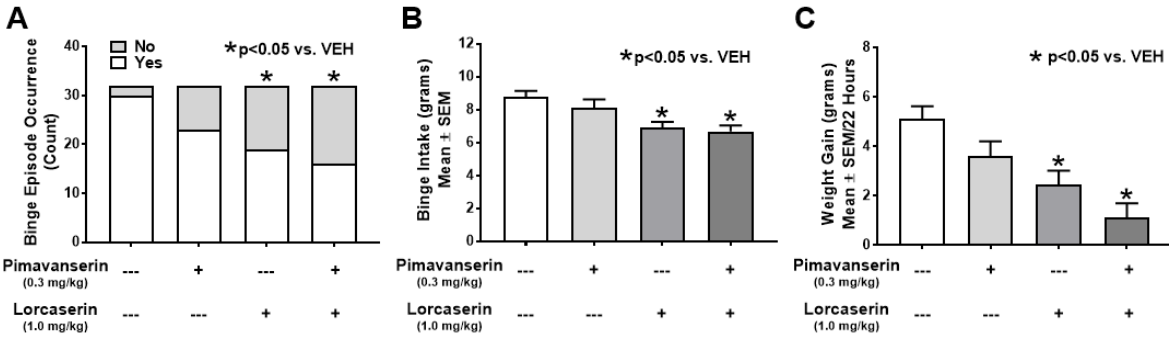

Figure 4.3: Combined pimavanserin plus lorcaserin suppresses binge episode occurrence, binge intake, and weight gain associated with HFF exposure.

Both lorcaserin (1.0 mg/kg) alone and pimavanserin (0.3 mg/kg) plus lorcaserin (1.0 mg/kg) suppressed binge episode occurrence (**Figure 4.3A**, n=32), binge intake (**Figure 4.3B**, n=25), and weight gain associated with HFF exposure (**Figure 4.3C**, n=32).

Composite data are represented as mean +/- SEM. *p<0.05 vs. VEH (McNemar's test or Dunnett's test).

The goal of the combination study was to determine if combined administration of pimavanserin plus lorcaserin was more effective at suppressing measures of binge eating than administration of pimavanserin or lorcaserin alone. Interestingly, 0.3 mg/kg pimavanserin significantly suppressed binge intake in the single drug dose-response study, but not in the combination study. Furthermore, 1.0 mg/kg lorcaserin significantly suppressed both binge episode occurrence and weight gain associated with HFF exposure in the combination study, but not in the single drug dose-response study. These results were surprising since the single drug dose-response assessments demonstrated consistent results across two independent cohorts. We suspected a possible interaction between drug treatments, so we further analyzed the data in a repeated measures two-way ANOVA using the factors of treatment 1 (pimavanserin) and treatment 2 (lorcaserin). There was a main effect of lorcaserin ($F_{1,29}$=28.14; p<0.001), but not of pimavanserin ($F_{1,29}$=2.44; p=0.129), or a pimavanserin x lorcaserin interaction ($F_{1,29}$=0.6671; p=0.421). While these data suggest there is no interaction between pimavanserin and lorcaserin when given concurrently, the possibility that previous exposure to one drug affects subsequent response to the second drug remains. Other potential explanations for the seemingly discrepant results include altered responses due to repeated exposure of the drug and differences in basal behavior prior to HFF access (e.g., higher stress levels of the rats due to increased number of injections).

DISCUSSION

The present study demonstrated that the selective 5-HT_{2A}R antagonist/inverse agonist pimavanserin and selective 5-HT_{2C}R agonist lorcaserin are effective at decreasing the magnitude, but not the occurrence, of binge episodes in adult male Sprague-Dawley

rats. Pimavanserin, but not lorcaserin, was also effective at suppressing weight gain associated with HFF exposure but only at higher doses, thus suggesting suppression of binge intake alone is not sufficient to suppress weight gain associated with HFF exposure. Excitingly, combined administration of pimavanserin plus lorcaserin was effective at suppressing both the occurrence and magnitude of binge episodes in addition to weight gain associated with HFF exposure. These data support future studies assessing the repurposing of these medications for treatment of BED.

Activation of the 5-HT_{2C}R has been established to suppress food intake and reward-related behaviors, which, in part, led to the approval of lorcaserin for weight loss. Our finding that this 5-HT_{2C}R agonist suppresses binge intake coalesces with our previous results that demonstrated the selective 5-HT_{2C}R agonist WAY163909 suppresses binge intake in adult male Sprague-Dawley rats in an intermittent access model via suppression of the reinforcing and motivational properties of HFF (**Chapter 3**). These findings also align with a recent study demonstrating that lorcaserin suppresses binge-like eating in mice via activation of the 5-HT_{2C}R localized in dopaminergic neurons (94). Since an estimated 70% of people with BED also have elevated BMIs (96), identification of a clinically-available drug that both suppresses binge eating and promotes weight loss would be extremely valuable in this patient population. The anti-obesity medication lorcaserin provides this opportunity as it is currently approved for weight loss, and cumulative evidence suggests its efficacy in treating BED.

The role of the 5-HT_{2A}R in feeding-related behaviors is less clear than the role of the 5-HT_{2C}R. The present findings agree with previous studies that demonstrated systemic administration of non-specific 5-HT_{2A}R antagonists suppress feeding (98-100). Other studies have suggested that 5-HT_{2A}R DNA hypermethylation, which would be predicted to result in gene inactivation, associates with obesity-related measures (112) while 5-HT_{2A}R agonist administration into the hypothalamus attenuates feeding (113, 114), suggesting that a number of pharmacological, genetic, and biochemical factors may contribute to 5-

HT_{2A}R-mediated feeding-related behaviors. One proposed hypothesis for these discordant findings is that peripherally- and centrally-expressed 5-HT_{2A}R regulate food intake differently (115), although to our knowledge this hypothesis has not been directly explored. Alternatively, centrally-expressed 5-HT_{2A}R may mediate feeding behaviors differently when activated or antagonized in various brain regions. Conversely, the role of the 5-HT_{2A}R in reward-related behaviors has been well-studied in the drug addiction field. For example, 5-HT_{2A}R blockade reduces reward-seeking behaviors for cocaine, nicotine, and MDMA [(116, 117), and (for review, (14)]. However, 5-HT_{2A}R blockade is not effective in reducing self-administration of cocaine or nicotine [(110, 116, 118) and (for review, (14)], suggesting that the 5-HT_{2A}R is not directly responsible for mediating drug reward-taking behaviors. While studies have demonstrated overlapping neural mechanisms responsible for driving food-reward and drug-reward behaviors (119), to our knowledge, blockade of the 5-HT_{2A}R has not been assessed in a HFF self-administration operant conditioning paradigm. Thus, future studies are necessary to determine if pimavanserin specifically suppresses binge intake via the role of the 5-HT_{2A}R in feeding-mediated, reward-seeking, and/or reward-taking behaviors.

Recent studies demonstrate that combinations of pharmacotherapies may be more effective at treating dysregulated eating (e.g., patients who are overweight or obese who are attempting to lose weight) than monotherapy alone. For example, the weight loss drug Contrave® (a combined formulation of naltrexone and extended-release bupropion) results in weight loss greater than either drug alone (120). While both single and combined administration of pimavanserin and/or lorcaserin suppressed the magnitude of binge episodes, the occurrence of binge episodes was decreased only in the combination study. Thus, while single treatments may reduce food consumption during a binge episode in BED, combined therapy may be necessary to prevent the occurrence of binge episodes. This finding is in line with previous preclinical studies that have demonstrated that 5-HT_{2A}R antagonists/inverse agonists and 5-HT_{2C}R agonists can have additive or even synergistic

effects on impulsivity and reward-related behaviors (35). The present study further supports at least an additive effect of these drugs on food-reward behaviors and supports the use of dual therapy or development of combined formulations or heterobivalent ligands to alter reward-related or feeding behaviors.

The different response profiles of pimavanserin, lorcaserin, and the combination of drugs offers the opportunity for individualized treatment for patients with BED. For example, healthy-weight patients who engage in infrequent, but severe, binge episodes may benefit from low-dose pimavanserin since this drug is effective in suppressing the magnitude of a binge episode but does not affect weight change. Conversely, a patient with obesity who also engages in infrequent, but very severe, binge episodes and displays dysregulated eating behaviors beyond BED may benefit most from treatment with lorcaserin since this drug suppresses binge magnitude and is also clinically-approved for weight loss. Finally, a patient with obesity who experiences binge episodes that are both severe and frequent may benefit most from combined administration of pimavanserin plus lorcaserin since this approach prevents binge episode occurrence and suppresses both binge magnitude and weight gain associated with exposure to HFF.

Herein, we described two clinically-available drugs that have the potential to be successfully repurposed for treatment of BED. Future studies should assess the efficacy of pimavanserin and lorcaserin in the treatment of BED in clinical populations in addition to the safety of combination therapy with these two medications.

Table 4.1: Pimavanserin effects on binge eating measures.

	Cohort	Main Effect	Vehicle	0.3 mg/kg	1.0 mg/kg	3.0 mg/kg
Binge Episode Occurrence†	1 (n=16)	X^2_3=1.737; p=0.629	15:1	13:3	15:1	14:2
	3 (n=16)	X^2_3=3.600; p=0.308	14:2	13:3	14:2	11:5
	1+3 (n=32)	X^2_3=3.923; p=0.270	29:3	26:6	29:3	25:7
Binge Intake‡	1 (n=15)	$F_{3,42}$=4.998; p=0.005*	10.1 ± 2.7	8.4 ± 0.9; p=0.026*	7.8 ± 0.7; p=0.002*	8.5 ± 0.6; p=0.039*
	3 (n=14)	$F_{3,39}$=7.867; p<0.001*	8.9 ± 0.7	7.3 ± 0.5; p=0.020*	6.3 ± 0.4; p<0.001*	6.8 ± 0.6; p=0.002*
	1+3 (n=29)	$F_{3,84}$=12.6; p<0.001*	9.5 ± 0.5	7.8 ± 0.5; p<0.001*	7.0 ± 0.4; p<0.001*	7.7 ± 0.4; p<0.001*
Weight Gain Associated with HFF Exposure‡	1 (n=16)	$F_{3,45}$=7.542; p<0.001*	3.8 ± 0.5	3.3 ± 0.8; p=0.878	1.2 ± 0.5; p=0.008*	0.5 ± 0.6; p<0.001*
	3 (n=16)	$F_{3,45}$=5.362; p=0.003*	5.3 ± 0.8	4.1 ± 0.7; p=0.502	2.9 ± 0.5; p=0.075	1.2 ± 0.9; p=0.001*
	1+3 (n=32)	$F_{3,93}$=12.37; p<0.001*	4.6 ± 0.5	3.7 ± 0.5; p=0.412	2.1 ± 0.4; p<0.001*	0.9 ± 0.5; p<0.001*

†Data are represented as the ratio of binge episode occurrence to non-occurrence and were analyzed using a Cochran's Q test followed by McNemar's test with a Bonferroni corrected significant α level of 0.0167 (p values are versus vehicle; *significant).

‡Data are represented as mean ± SEM and were analyzed using a repeated measures one-way ANOVA followed by Dunnett's multiple comparisons test (p values are versus vehicle; *significant).

Table 4.2: Lorcaserin effects on binge eating measures.

	Cohort	Main Effect	Vehicle	0.25 mg/kg	0.5 mg/kg	1.0 mg/kg
Binge Episode Occurrence†	2 (n=16)	X^2_3=2.182; p=0.536	13:3	15:1	15:1	13:3
	4 (n=16)	X^2_3=7.444; p=0.059	9:7	14:2	14:2	12:4
	2+4 (n=32)	X^2_3=8.510; p=0.037*	22:10	29:3; p=0.039	29:3; p=0.016*	25:7; p=0.549
Binge Intake‡	2 (n=13)	$F_{3,36}$=5.351; p=0.004*	8.7 ± 0.6	8.0 ± 0.5; p=0.477	7.8 ± 0.3; p=0.248	6.4 ± 0.4; p=0.001*
	4 (n=9)	$F_{3,24}$=6.575; p=0.002*	8.4 ± 0.8	9.0 ± 0.6; p=0.626	8.8 ± 0.7; p=0.872	6.4 ± 0.3; p=0.017*
	2+4 (n=22)	$F_{3,63}$=10.24; p<0.001*	8.6 ± 0.5	8.4 ± 0.4; p=0.967	8.2 ± 0.3; p=0.667	6.4 ± 0.3; p<0.001*
Weight Gain Associated with HFF Exposure‡	2 (n=16)	$F_{3,45}$=0.7968; p=0.502	4.1 ± 0.8	4.1 ± 0.5	3.3 ± 0.6	2.9 ± 1.0
	4 (n=16)	$F_{3,45}$=0.5066; p=0.680	3.1 ± 0.9	4.1 ± 1.0	3.6 ± 0.6	2.6 ± 1.0
	2+4 (n=32)	$F_{3,93}$=0.9926; p=0.400	3.6 ± 0.6	4.1 ± 0.6	3.4 ± 0.4	2.8 ± 0.7

†Data are represented as the ratio of binge episode occurrence to non-occurrence and were analyzed using a Cochran's Q test followed by McNemar's test with a Bonferroni corrected significant α level of 0.0167 (p values are versus vehicle; *significant).

‡Data are represented as mean ± SEM and were analyzed using a repeated measures one-way ANOVA followed by Dunnett's multiple comparisons test (p values are versus vehicle; *significant).

Table 4.3: Combined pimavanserin plus lorcaserin effects on binge eating measures.

	Cohort	Main Effect	Vehicle Vehicle	0.3 mg/kg Pimavanserin Vehicle	Vehicle 1.0 mg/kg Lorcaserin	0.3 mg/kg Pimavanserin 1.0 mg/kg Lorcaserin
Binge Episode Occurrence†	3 (n=16)	X^2_3=7.258; p=0.064	16:0	11:5	11:5	11:5
	4 (n=16)	X^2_3=15.811; p=0.001*	14:2	12:4; p=0.625	8:8; p=0.031	5:11; p=0.004*
	3+4 (n=32)	X^2_3=19.412; p<0.001*	30:2	23:9; p=0.039	19:13; p=0.001*	16:16; p<0.001*
Binge Intake‡	3 (n=14)	$F_{3,45}$=4.168; p=0.011*	8.7 ± 0.5	8.5 ± 0.8; p=0.969	7.0 ± 0.3; p=0.017*	7.2 ± 2.3; p=0.051
	4 (n=16)	$F_{3,39}$=9.746; p<0.001*	8.9 ± 0.5	7.7 ± 0.6; p=0.080	6.9 ± 0.6; p=0.002*	6.0 ± 0.4; p<0.001*
	3+4 (n=30)	$F_{3,87}$=11.55; p<0.001*	8.8 ± 0.4	8.1 ± 0.5; p=0.234	6.9 ± 0.3; p<0.001*	6.7 ± 0.4; p<0.001*
Weight Gain Associated with HFF Exposure‡	3 (n=16)	$F_{3,45}$=4.545; p=0.007*	5.1 ± 0.7	4.0 ± 0.9; p=0.598	2.5 ± 0.8; p=0.050*	1.4 ± 0.9; p=0.004*
	4 (n=16)	$F_{3,45}$=5.085; p=0.004*	5.1 ± 0.7	3.2 ± 0.8; p=0.239	2.4 ± 0.8; p=0.055	0.8 ± 0.7; p=0.001*
	3+4 (n=32)	$F_{3,93}$=9.81; p<0.001*	5.1 ± 0.5	3.6 ± 0.6; p=0.133	2.5 ± 0.6; p=0.002*	1.1 ± 0.6; p<0.001*

†Data are represented as the ratio of binge episode occurrence to non-occurrence and were analyzed using a Cochran's Q test followed by McNemar's test with a Bonferroni corrected significant α level of 0.0167 (p values are versus vehicle; *significant).

‡Data are represented as mean ± SEM and were analyzed using a repeated measures one-way ANOVA followed by Dunnett's multiple comparisons test (p values are versus vehicle; *significant).

Chapter 5: *Ex Vivo* Identification of the Serotonin 5-HT$_{2A}$:5-HT$_{2C}$ Receptor Protein Complex: A Theoretical Construct for Implications in Addictive Disorders[4]

INTRODUCTION

Addictive behaviors are characterized by recurrent cycles of binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation (11). Preoccupation and anticipation are modulated via executive control over addiction-related behaviors including cue reactivity, the heightened sensitivity to stimuli previously associated with a reinforcer (121-123), and impulsivity, action without sufficient forethought (124). The PFC is particularly important in executive control and decision-making, and heightened cue reactivity and impulsivity signal a failure of "top-down" executive control by the PFC over connectivity with basal ganglia control of reward responsivity (11). Thus, targeting the preoccupation/anticipation stage through interventions that suppress cue reactivity and impulsivity offers new therapeutic opportunities in the treatment of addictive disorders.

Disruptions in both excitatory glutamate and inhibitory GABA neurotransmission within cortical regions are thought to dictate some of the behavioral consequences observed in the preoccupation/anticipation stage. For example, evidence from rodent studies suggests that increased glutamate activity in cortical regions elicits craving-like behavior and cue reactivity while disrupted GABA activity in cortical regions is thought to contribute to dysregulated inhibitory control and increased impulsivity (for review, (11). Essentially, altered neurotransmission in cortical regions leads to an overactive "Go"

[4] The work in this chapter has been published in a modified form. It has been reprinted with permission from Price AE, Sholler DJ, Stutz SJ, Anastasio NC, Cunningham KA. Endogenous serotonin 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors associate in the medial prefrontal cortex. *ACS Chemical Neuroscience*. 2019;10(7):3241-8. Copyright 2019 American Chemical Society.

system and an underactive “Stop” system, which together promote addiction-related behaviors.

Serotonin can regulate activity of both cortical glutamate and GABA neurons via actions through its cognate receptors. In particular, the 5-HT_{2A}R and 5-HT_{2C}R have the potential to profoundly modulate the progressive, preoccupation/anticipation stage of addiction by altering cue reactivity or impulsivity through regulation of cortical balance (for review, (14). Specifically, systemic administration of 5-HT_{2A}R antagonists and 5-HT_{2C}R agonists suppress cue reactivity and impulsivity (35, 54, 83, 109, 110, 125-133). The mPFC, defined here as the region spanning the medial pole of the PFC which includes the ACC, PL, and IL in rats, is critical in mediating these behaviors. Localized infusion of a 5-HT_{2A}R antagonist or 5-HT_{2C}R agonist into specific subregions of the mPFC suppresses impulsive action (134) and cue reactivity (135), respectively, while intra-mPFC 5-HT_{2C}R knockdown elevates both impulsive action and cue reactivity (52). This dichotomy is particularly interesting since both receptors are known to primarily signal through the same $G_{\alpha q}$ pathway and receptor expression has been identified within the same cells (136).

One intriguing explanation for the divergent behavioral effects driven by the 5-HT_{2A}R and 5-HT_{2C}R is altered signaling mediated by a 5-HT_{2A}R:5-HT_{2C}R protein:protein interaction (38). This is evidenced by numerous studies demonstrating that altered expression or activity of one receptor causes compensatory changes to the other. For example, 5-HT_{2A}R knockout increases 5-HT_{2C}R control of pyramidal neuronal excitability within the mPFC (37) while intra-mPFC 5-HT_{2C}R knockdown increases local 5-HT_{2A}R expression and the potency of a 5-HT_{2A}R antagonist to suppress impulsive action (38). Further, lower levels of synaptosomal 5-HT_{2A}R:5-HT_{2C}R protein complex in the mPFC, as assessed through co-immunoprecipitation studies, are observed in phenotypically-identified high impulsive rats compared to low impulsive rats (38). Previous work has also demonstrated that combined administration of subthreshold doses of a 5-HT_{2A}R

antagonist and a 5-HT_{2C}R agonist results in behavioral modification that is greater than what is achieved with single drug administration (35, 36). Most recently, a study was published that described the formation of a heterodimer between 5-HT_{2A}R and 5-HT_{2C}R that preferentially shifted signaling towards the 5-HT_{2C}R (39). Taken together, these data suggest that complex formation between 5-HT_{2A}R and 5-HT_{2C}R may act as a protective force in addictive behaviors by enhancing the effects of 5-HT_{2C}R signaling while blunting the effects of 5-HT_{2A}R signaling.

This study investigated the pattern of 5-HT_{2A}R and 5-HT_{2C}R localization and colocalization within subregions of the mPFC, a key region in which the 5-HT_{2A}R and 5-HT_{2C}R may interact to elicit protection against addictive behaviors. We first employed immunohistochemical methods to characterize expression of these receptors within the layers of the ACC, PL, and IL subregions of mPFC. Next, we used a proximity ligation assay (PLA) to identify specific regions in which the 5-HT_{2A}R:5-HT_{2C}R complex is expressed. Herein, we discuss our findings in context with previous behavioral findings and speculate a possible role for 5-HT_{2A}R:5-HT_{2C}R complex expression in addictive disorders.

METHODS

Animals: Male, outbred Sprague-Dawley rats (n=6; Envigo, Haslett, MI, USA) weighing 175-199 g at arrival were housed two per cage under a 12-hour light-dark cycle (lights on between 0600-1800h) with controlled temperature (21-23°C) and humidity (40-50%). Animals were acclimated for one week to the colony room before transcardial perfusion. SF and water were available to rats *ad libitum*. All experiments were conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (2011) and with the University of Texas Medical Branch Institutional Animal Care and Use Committee approval.

Transcardial Perfusion: Rats were anesthetized (100 mg/kg sodium pentobarbital, i.p.) and transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were removed, post-fixed in 4% paraformaldehyde for four hours at 4°C, cryoprotected in 30% sucrose for 72 hours at 4°C, and stored at -80°C until further use.

Immunohistochemical Staining for 5-HT_{2A}R and 5-HT_{2C}R: Free-floating coronal sections (30 µm) at the level of the ACC, PL, and IL (approximately 3.5-4.0 mm anterior to bregma, **Figure 5.1**) (50) were washed with PBS, permeabilized with 0.4% Triton-X 100 in PBS for 60 minutes at room temperature, blocked with 5% normal donkey serum, 0.4% Triton-X 100, 0.1% glycine, and 0.1% lysine in PBS for two hours at room temperature, and incubated with primary antibodies in 5% normal donkey serum and 0.4% Triton-X 100 in PBS overnight at 4°C. The primary antibodies used were a polyclonal rabbit anti-5-HT_{2A}R (RA24288, Neuromics, Edina, MN, USA) at a concentration of 1:500 and a monoclonal mouse anti-5-HT_{2C}R (sc17797, Santa Cruz Biotechnology, Dallas, TX, USA) at a concentration of 1:50. Both antibodies have been extensively characterized in our laboratory and others (52, 82, 83, 136-140). The next day, sections were rinsed 6X with PBS for 10 minutes each and incubated in secondary antibodies for 60 minutes at room temperature. The secondary antibodies used were a donkey anti-rabbit with a conjugated 594 nm fluorophore (711-585-152, Jackson ImmunoResearch, West Grove, PA, USA) at a concentration of 1:500 and a donkey anti-mouse with a conjugated 488 nm fluorophore (A21202, Invitrogen, Carlsbad, CA, USA) at a concentration of 1:200. Slices were then washed 3X with 0.4% Triton-X 100 in PBS for five minutes each and then rinsed in PBS. Sections were mounted, and slides were coverslipped with Vectashield fluorescent mounting media with DAPI to stain cellular nuclei for fluorescence microscopy (Vector Laboratories).

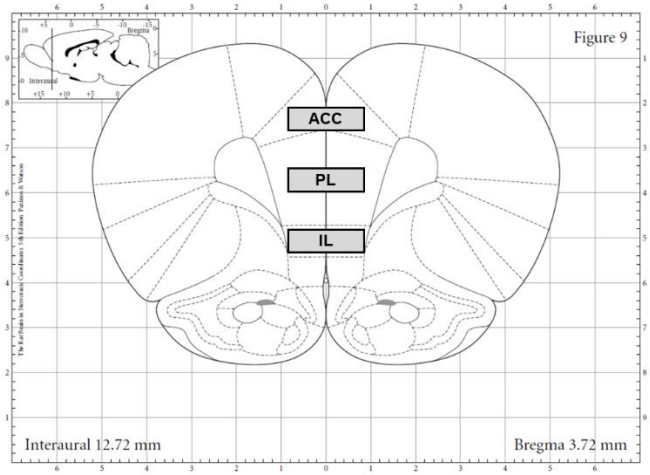

Figure 5.1: Cartoon representing the specific location of the ACC, PL, and IL that was assessed throughout this study.

Tilescan images encompassing all layers of either the ACC, PL, or IL (three fields of view in the dorsal-ventral direction by six fields of view in the medial-lateral direction) were acquired using a 40X objective on a Leica DFC3000 wide field camera (Leica Microsystems) (for a final magnification of 400X) and automatically stitched together using the Leica Application Suite. Each hemisphere of one brain section represented one technical replicate (n=4 per one biological replicate). Each technical replicate was imaged three times (i.e., tilescans of each region were acquired). Individual channel threshold values were set at the lowest value at which a signal was not visualized on the negative control (secondary antibody only) for each technical replicate. Expression of 5-HT_{2A}R and 5-HT_{2C}R in the ACC, PL, and IL spanning all layers of the cortex was determined using mean signal intensity analyzed using the Open Source software Fiji (141). A secondary analysis was also completed to assess expression in specific layers (I, II/III, V, and VI) of these regions. All expression analyses were carried out using region of interest boxes with predefined dimensions. The technical replicate most representative of an individual biological replicate was used for statistical analyses and represented within each graph. The final values used in graphical representation and statistical analyses are the mean +/-

SEM for all biological replicates combined (n=3). Like all antibody-based assays, absolute expression levels of a protein cannot be compared without establishment of a calibration curve (142). Since this technique was not utilized, expression levels are represented in a relative manner and therefore can be compared within the same antibody-based protein assessment, but not between different antibody-based protein assessments (e.g., 5-HT_{2A}R expression can be compared between regions and layers of the PFC but cannot be compared directly to 5-HT_{2C}R expression) (143).

PLA for 5-HT_{2A}R:5-HT_{2C}R Complex Identification: A commercial PLA was employed to assess the 5-HT_{2A}R:5-HT_{2C}R complex in rat mPFC *ex vivo* (Duolink® PLA, Sigma-Aldrich). Coronal sections (10 µm) at the level of the ACC, PL, and IL (approximately 3.5-4.0 mm anterior to bregma, **Figure 5.1**) (50) were thaw-mounted directly to glass slides and allowed to dry for two hours at room temperature. Sections were rehydrated in PBS for 30 minutes at room temperature, and antigen retrieval was performed by incubating sections in citric acid (pH 6.0) at 90°C for 20 minutes then allowing the sections to cool to room temperature for 20 minutes. Sections were rinsed in PBS 3X for three minutes each and incubated in 50 mM ammonium chloride for 20 minutes at room temperature to decrease auto-fluorescence. Sections were rinsed 5X for three minutes at room temperature in PBS and blocked for two hours at room temperature in 5% normal donkey serum and 0.4% Triton-X 100 in PBS. Sections were incubated with primary antibodies in 5% normal donkey serum and 0.4% Triton-X 100 in PBS overnight at 4°C. The polyclonal rabbit anti-5-HT_{2A}R (RA24288, Neuromics) was employed at a concentration of 1:500. The monoclonal mouse anti-5-HT_{2C}R employed in immunohistochemical studies (above) was not compatible with the PLA reagents; thus, a previously validated (144) polyclonal goat anti-5-HT_{2C}R (ab32887, Abcam, Cambridge, MA, USA) was used in the PLA at a concentration of 1:100. The next day, sections were rinsed 5X with PBS for 10 minutes each and incubated in Duolink® secondary antibodies/probes for 60 minutes at 37°C. The secondary antibodies/probes used were

donkey anti-rabbit PLUS (DUO92002), donkey anti-rabbit MINUS (DUO92005), donkey anti-goat PLUS (DUO92003) and donkey anti-goat MINUS (DUO92006) and were diluted in 5% normal donkey serum and 0.4% Triton-X 100 in PBS per manufacturer instructions. Following incubation, sections were washed in PBS for five minutes followed by 2X Wash Buffer A (DUO82049) for five minutes each at room temperature. Sections were then incubated with ligation reagents for 30 minutes at 37°C, washed with 2X Wash Buffer A for two minutes at room temperature, and incubated with amplification reagents for 100 minutes at 37°C. Ligation and amplification solutions (DUO92008) were made according to manufacturer instructions. Sections were then washed in 3X Wash Buffer B (DUO82049) for 10 minutes each at room temperature and rinsed with 0.01% Wash Buffer B. Sections were allowed to dry overnight. Slides were coverslipped using Duolink® *in situ* mounting medium with DAPI (DUO82040).

Tilescan images encompassing all layers of either the ACC, PL, or IL (three fields of view in the dorsal-ventral direction by six fields of view in the medial-lateral direction) were acquired using a 40X objective on a Leica DFC3000 wide field camera (for a final magnification of 400X) and automatically stitched together using the Leica Application Suite. Each brain section represented one technical replicate (n=3 within one biological replicate). Each technical replicate was imaged six times (i.e., tilescans of each region were acquired from both hemispheres). This process was completed for single-labeled 5-HT_{2A}R, single-labeled 5-HT_{2C}R, and dual-labeled 5-HT_{2A}R:5-HT_{2C}R sections. Three non-overlapping region of interest boxes spanning all layers of the cortex (100 µm in the dorsal-ventral direction by 600 µm in the medial-lateral direction) were then applied to each tilescan image for quantification of the PLA signal. The PLA signal was quantified using automatic threshold and classification based on intensity and size of the signal (Duolink® ImageTool, Sigma-Aldrich). A corrected total signal was calculated by subtracting signal from the nuclear and immediate perinuclear regions from the total signal. Technical replicates were averaged to determine the mean expression within a biological replicate.

The technical replicate most representative of an individual biological replicate was used for statistical analyses and represented within each graph. The average corrected total signal of the negative control images (secondary antibody/probe only) was subtracted from the average corrected total signal of the experimental images (primary antibody plus secondary antibody/probe) to determine the expression of 5-HT_{2A}R, 5-HT_{2C}R, or 5-HT_{2A}R:5-HT_{2C}R for each biological replicate. The final values presented are the mean ± SEM for all biological replicates combined (n=3). Final values are relative and not absolute.

Statistical Analyses: A repeated measures one-way ANOVA followed by Tukey's multiple comparisons test was used to assess regional differences in 5-HT_{2A}R expression (immunohistochemical and PLA experiments), 5-HT_{2C}R expression (immunohistochemical and PLA experiments), and 5-HT_{2A}R:5-HT_{2C}R complex expression (PLA experiments). A repeated measures two-way ANOVA followed by Tukey's multiple comparisons test was used to analyze expression patterns of 5-HT_{2A}R and 5-HT_{2C}R using the factors of region (ACC, PL, IL) and layer (layers I, II/III, V, and VI).

RESULTS

Immunohistochemical Characterization of 5-HT_{2A}R Expression: A representative image of 5-HT_{2A}R expression in the ACC, PL, and IL regions is shown in **Figure 5.2A**. A repeated measures one-way ANOVA demonstrated a main effect of region on 5-HT_{2A}R expression ($F_{2,4}$=14.99; p=0.0139). Tukey's multiple comparisons test indicated a significant difference between the ACC and IL (p=0.0190) and between the PL and IL (p=0.0203), but not between the ACC and PL (p=0.9958; **Figure 5.2B**). Further analyses assessed differences in 5-HT_{2A}R expression between layers within the ACC, PL, and IL (**Figure 5.2C**). A repeated measures two-way ANOVA indicated a main effect of region ($F_{2,4}$=9.416; p=0.0307), layer ($F_{3,6}$=54.35, p<0.0001), and a region x layer interaction ($F_{6,12}$=11.02; p=0.0003). Tukey's multiple comparisons test demonstrated 5-

$HT_{2A}R$ expression was highest in layer V of all regions (**Table 5.1, Figure 5.2C**). These results demonstrate higher levels of 5-$HT_{2A}R$ expression in the dorsal regions of the cortex (ACC and PL) that is also layer dependent, which is consistent with previously published literature (136).

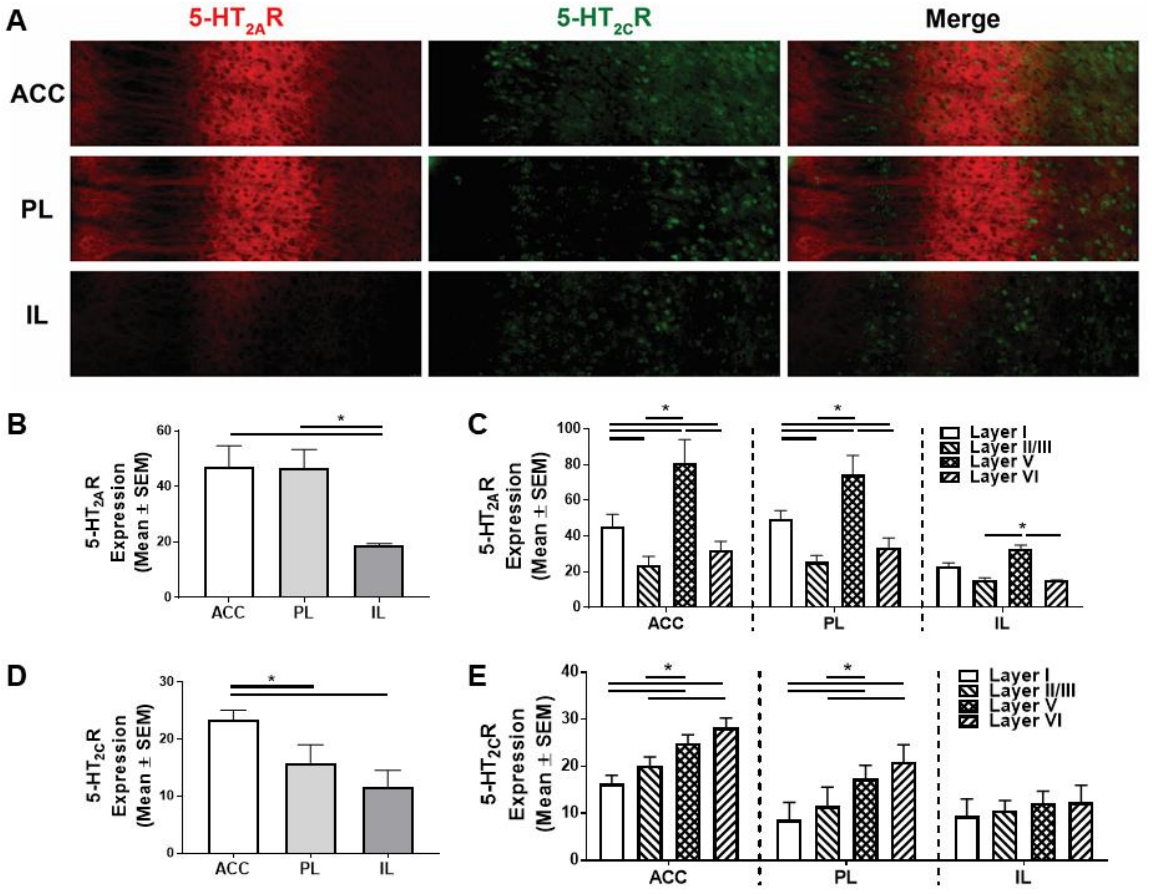

Figure 5.2: Immunohistochemical characterization of 5-$HT_{2A}R$ and 5-$HT_{2C}R$ expression.

Figure 5.2A illustrates representative 5-$HT_{2A}R$ and 5-$HT_{2C}R$ expression in the ACC, PL, and IL. Significant differences in 5-$HT_{2A}R$ expression between regions (**Figure 5.2B**) and between layers within regions (**Figure 5.2C**) were observed. Significant differences in 5-$HT_{2C}R$ expression between regions (**Figure 5.2D**) and between layers within regions (**Figure 5.2E**) were also observed. Composite data are represented as mean +/- SEM for biological replicates (n=3). *p<0.05

Table 5.1: Comparison of 5-HT_{2A}R expression between layers within regions.

ACC			
Layer	**I**	**II/III**	**V**
II/III	p=0.0006*		
V	p<0.0001*	p<0.0001*	
VI	p=0.0240*	p=0.1898	p<0.0001*
PL			
Layer	**I**	**II/III**	**V**
II/III	p=0.0002*		
V	p=0.0002*	p<0.0001*	
VI	p=0.0069*	p=0.2111	p<0.0001*
IL			
Layer	**I**	**II/III**	**V**
II/III	p=0.2642		
V	p=0.0965	p=0.0034*	
VI	p=0.2380	p=0.9999	p=0.0030*

Immunohistochemical Characterization of 5-HT_{2C}R Expression: A representative image of 5-HT_{2C}R expression in the ACC, PL, and IL regions is shown in **Figure 5.2A**. A repeated measures one-way ANOVA demonstrated a main effect of region on 5-HT_{2C}R expression ($F_{2,4}$=23; p=0.0064). Tukey's multiple comparisons test indicated a significant difference between the ACC and PL (p=0.0254) and between the ACC and IL (p=0.0058), but not between the PL and IL (p=0.1712; **Figure 5.2D**). Further analyses assessed differences in 5-HT_{2C}R expression between layers within the ACC, PL, and IL (**Figure 5.2E**). A repeated measures two-way ANOVA indicated a main effect of region ($F_{2,4}$=14.01; p=0.0156), layer ($F_{3,6}$=37.75, p=0.0003), and a region x layer interaction ($F_{6,12}$=5.902; p=0.0045). Tukey's multiple comparisons test was used to compare 5-HT_{2C}R expression between layers within each region (**Table 5.2 and Figure 5.2E**). Together, these data indicate a decreasing gradient of 5-HT_{2C}R expression moving from dorsal to ventral cortical regions with an increasing gradient of 5-HT_{2C}R expression moving from superficial to deeper layers, consistent with previously published literature (136, 145).

Table 5.2: Comparisons of 5-HT_{2C}R expression between layers within regions.

ACC			
Layer	**I**	**II/III**	**V**
II/III	p=0.0676		
V	p=0.0002*	p=0.0211*	
VI	p<0.0001*	p=0.0003*	p=0.1144
PL			
Layer	**I**	**II/III**	**V**
II/III	p=0.1921		
V	p=0.0002*	p=0.0055*	
VI	p<0.0001*	p<0.0001*	p=0.1014
IL			
Layer	**I**	**II/III**	**V**
II/III	p=0.8578		
V	p=0.2540	p=0.6531	
VI	p=0.1994	p=0.5581	p=0.9984

PLA Characterization of 5-HT_{2A}R Expression: We used a single-label PLA to validate that 5-HT_{2A}R expression could be appropriately quantified using the PLA assay. A representative image of 5-HT_{2A}R expression in the ACC, PL, and IL regions is shown in **Figure 5.3A**. A repeated measures one-way ANOVA demonstrated a main effect of region ($F_{2,4}$=10.39; p=0.0260) on 5-HT_{2A}R PLA signal (**Figure 5.3B**). Tukey's multiple comparisons test demonstrated a significant difference in 5-HT_{2A}R PLA signal between ACC and IL (p=0.0373) and PL and IL (p=0.0350), but not between ACC and PL (p=0.9966). These results were consistent with the results from immunohistochemical analyses, indicating the PLA can be used to reliably assess 5-HT_{2A}R expression.

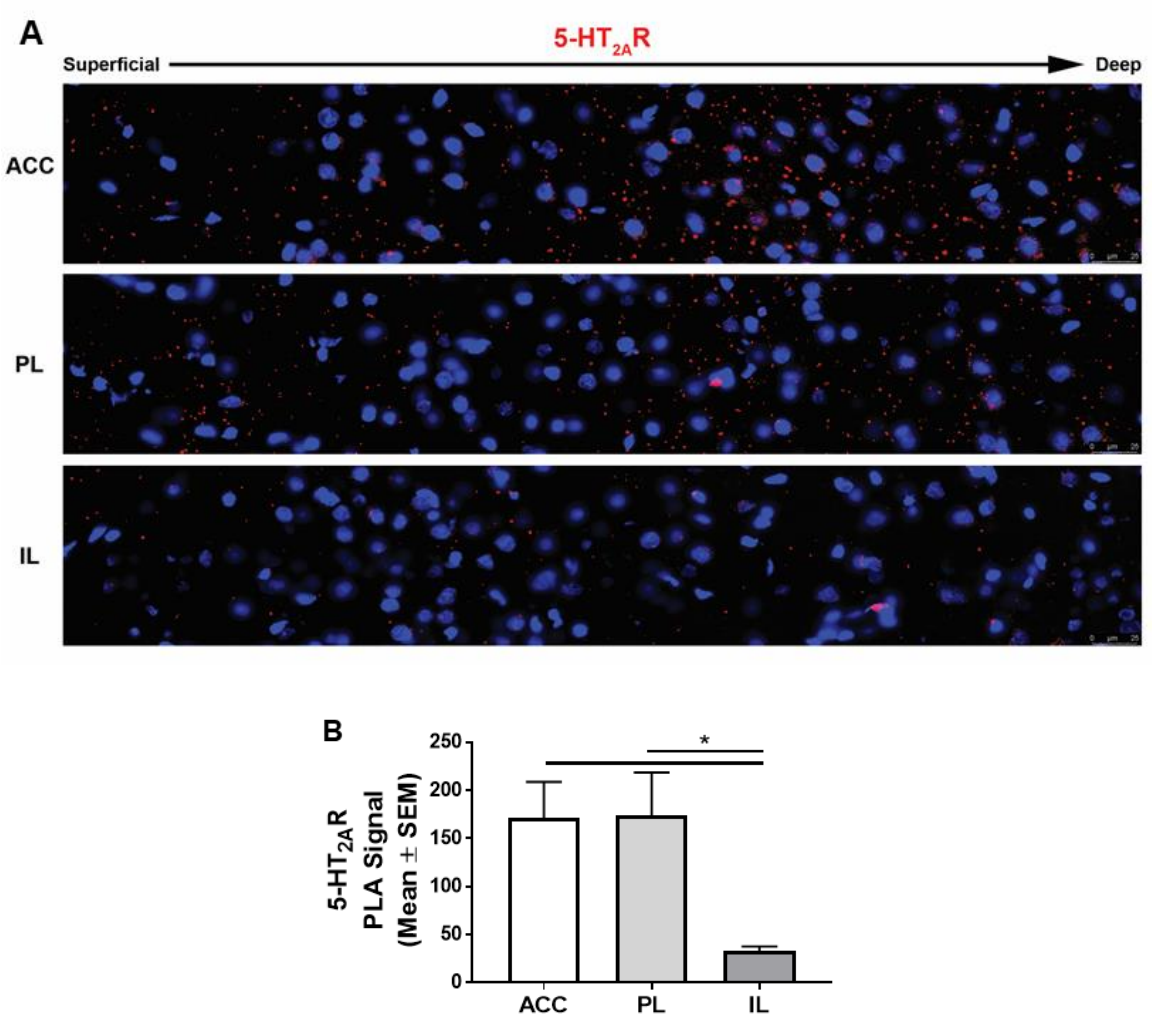

Figure 5.3: PLA characterization of 5-HT_{2A}R expression.

Figure 5.3A illustrates representative 5-HT_{2A}R expression in the ACC, PL, and IL. Red puncta represent PLA signal while DAPI-staining is visualized in blue. Significant differences in PLA puncta between brain regions were observed (**Figure 5.3B**). Composite data are represented as mean +/- SEM for biological replicates (n=3). *p<0.05

PLA Characterization of 5-HT_{2C}R Expression: We used a single-label PLA to validate that 5-HT_{2C}R expression could be appropriately quantified with this assay. A representative image of 5-HT_{2C}R expression in the ACC, PL, and IL regions is shown in **Figure 5.4A**. A repeated measures one-way ANOVA demonstrated no main effect of

region ($F_{2,4}$=6.095; p=0.0610) on 5-HT_{2C}R PLA signal (**Figure 5.4B**). Although not significant (probably due to the large variance in the ACC), each individual rat demonstrated higher amounts of 5-HT_{2C}R PLA puncta in the more dorsal regions of the mPFC compared to the IL, which is consistent with the immunohistochemical findings.

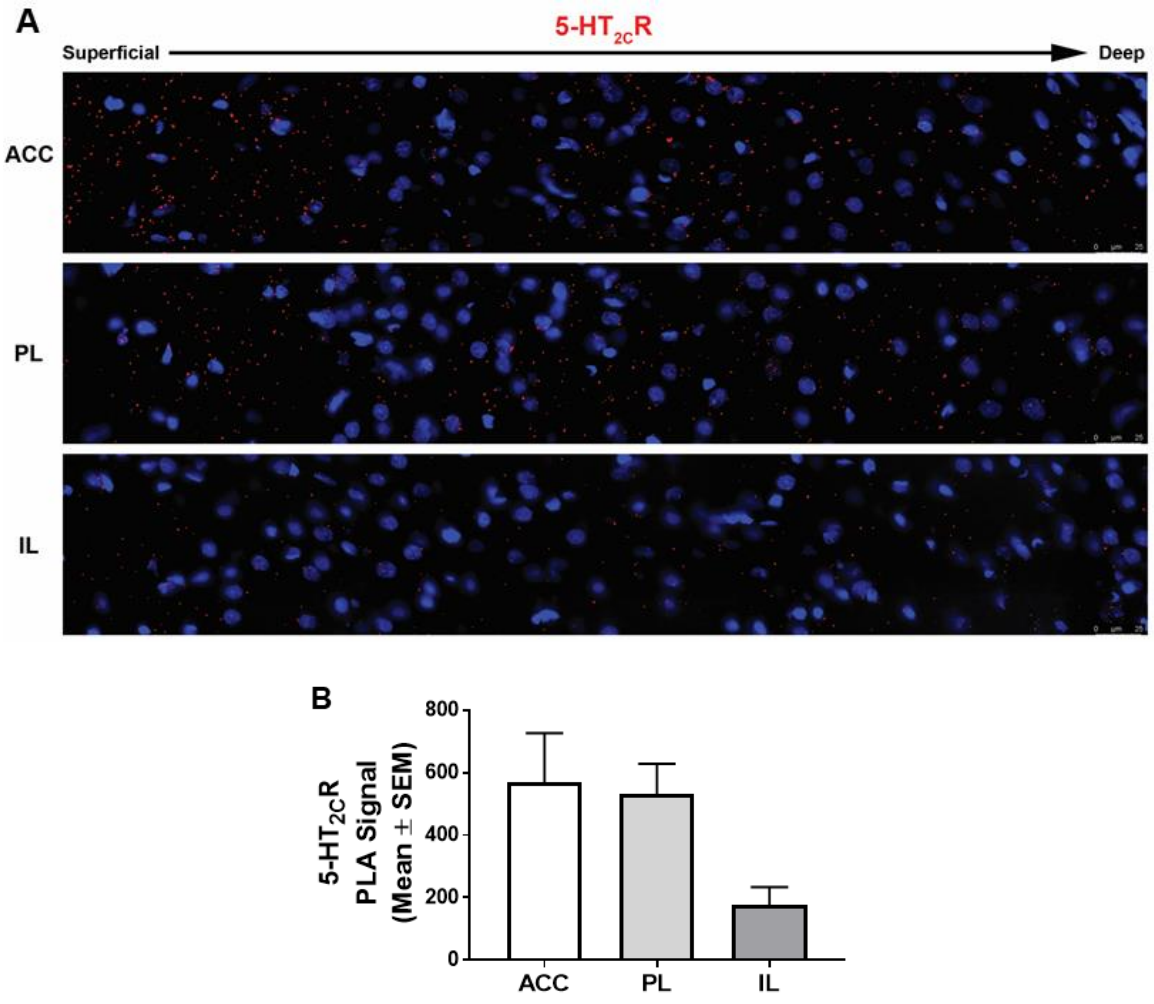

Figure 5.4: PLA characterization of 5-HT_{2C}R expression.

Figure 5.4A illustrates representative 5-HT_{2A}R expression in the ACC, PL, and IL. Red puncta represent the PLA signal while DAPI-staining is visualized in blue. There were no significant differences in PLA puncta between brain regions (**Figure 5.4B**). Composite data are represented as mean +/- SEM for biological replicates (n=3).

PLA Characterization of 5-HT_{2A}R:5-HT_{2C}R Complex Expression: We used a dual-label PLA to quantify the expression of a 5-HT_{2A}R:5-HT_{2C}R complex within the ACC, PL, and IL. The presence of a 5-HT_{2A}R:5-HT_{2C}R interaction within 40 nm is required for expression of a PLA signal. A representative image of 5-HT_{2A}R:5-HT_{2C}R complex expression in the ACC, PL, and IL regions is shown in **Figure 5.5A**. A repeated measures one-way ANOVA demonstrated a main effect of region ($F_{2,4}$=39.71; p=0.0023) on 5-HT_{2A}R:5-HT_{2C}R PLA signal (**Figure 5.5B**). Tukey's multiple comparisons test demonstrated a significant difference in 5-HT_{2A}R PLA signal between ACC and PL (p=0.0472), ACC and IL (p=0.0020) and PL and IL (p=0.0139). These results suggest a dorsal-ventral gradient of 5-HT_{2A}R:5-HT_{2C}R complex expression in the mPFC.

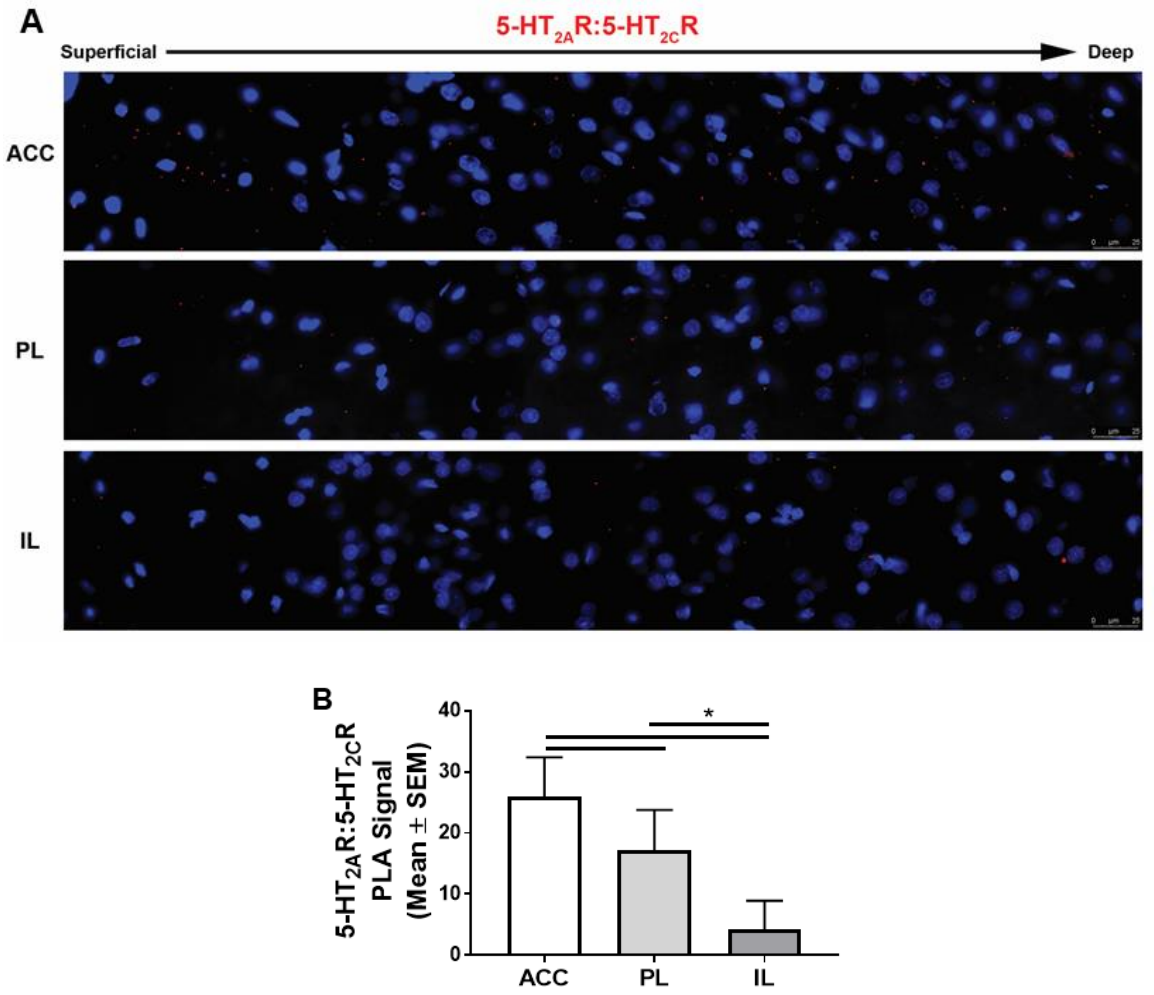

Figure 5.5: PLA characterization of 5-HT_{2A}R:5-HT_{2C}R complex expression.

Figure 5.5A illustrates representative 5-HT_{2A}R:5-HT_{2C}R complex expression in the ACC, PL, and IL. Red puncta represent the PLA signal while DAPI-staining is visualized in blue. Significant differences in PLA puncta between brain regions were observed (**Figure 5.5B**). Composite data are represented as mean +/- SEM for biological replicates (n=3). *p<0.05

DISCUSSION

We assessed the expression patterns of the 5-HT_{2A}R and 5-HT_{2C}R in the ACC, PL, and IL regions of rat brain and demonstrated region- and layer-specific patterns of expression for both receptors that are consistent with previous reports (136, 145-149).

The highest levels of 5-HT_{2A}R expression were identified in layer V of the ACC and PL while the highest levels of 5-HT_{2C}R expression were also identified in the deeper layers of the dorsal mPFC. We further identified a dorsal-ventral gradient of expression of the 5-HT_{2A}R:5-HT_{2C}R complex in the mPFC. These results are consistent with a previously published report that identified co-localization of 5-HT_{2A}R and 5-HT_{2C}R protein in the deep layers of the PL (136), in addition to another study which identified co-expression of 5-HT_{2A}R and 5-HT_{2C}R mRNA within cells of the mPFC (150).The PLA requires that two target proteins are within 40 nm of each other to produce a quantifiable signal; this resolution cannot be ensured using traditional immunohistochemical co-localization or co-immunoprecipitation analyses. Thus, our report is the first to demonstrate the presence of a spatially-close interaction between the two receptors in *ex vivo* tissue analyses of the mPFC.

The mPFC is composed of three regions that each contribute to addictive behaviors. Of note, there are profound differences in function that occur moving in both a dorsal-ventral and rostral-caudal direction [e.g., see (151) and (152)]; thus, we have done our best to discuss our findings in light of the most anatomically comparable studies we could identify. In general, the ACC is a key regulator of motor initiation and attention, while the PL is highly implicated in executive functions such as decision making and working memory, and the IL mediates autonomic and visceral activity in addition to fear extinction (17). Each of these regions plays a complex role in regulating addictive behaviors such as cue reactivity and impulsivity that cannot be explained by the contributions of one subregion alone. For example, previous studies have demonstrated that lesions to the entire mPFC (i.e., an area encompassing the ACC, PL, and IL) result in higher levels of impulsivity than lesions affecting only the ventral regions of the mPFC (i.e., an area encompassing only the PL and IL) (151). However, another study demonstrated that lesioning the ACC alone does not alter baseline impulsivity, but lesioning the IL alone is sufficient to increase impulsivity (153). Even with these seemingly discrepant results, a

model deeming the dorsal regions of the mPFC as the primary mediators of the “Go” system and the ventral regions of the mPFC as the primary mediators of the “Stop” system has prevailed in the addiction literature (for review, see (154). This model highlights the contrasting roles of the PL to promote and the IL to inhibit drug-seeking behavior (154). The present results consistently indicated higher levels of 5-HT_{2A}R, 5-HT_{2C}R, and 5-HT_{2A}R:5-HT_{2C}R complex expression in the more dorsal regions of the mPFC. Thus, the discussion will primarily center around how these receptors may be acting within the “Go” pathway to influence addiction-related behaviors, although a contribution of these receptors within the “Stop” pathway should not be discounted.

Deeper cortical layers, including layers V and VI, consist primarily of glutamate efferent neurons that project to subcortical regions in addition to GABA interneurons that regulate the firing of these glutamate efferent neurons (155). Glutamate neurons originating in the PL densely innervate the nucleus accumbens core, a region that is known to promote drug-related behaviors upon activation, and is therefore one reason the PL is thought to be a regulator of the “Go” pathway (for review, see (154). Multiple studies have demonstrated that 5-HT_{2A}R expression in the mPFC is predominantly localized to the apical dendrites of pyramidal neurons with an additional smaller population of receptors located on GABA interneurons (156-160). The expression of the 5-HT_{2C}R in this region of the mPFC has been identified predominantly on GABA interneurons which synapse on pyramidal output neurons in the deeper layers of the cortex with an additional smaller population of receptors located on pyramidal neurons (136, 145). Thus, it is possible that regional activation of the 5-HT_{2A}R results in net activation of glutamatergic neurons that project to subcortical regions. Conversely, regional activation of the 5-HT_{2C}R may result primarily in activation of GABA interneurons, which inhibits glutamate release in subcortical reward-mediating regions. This scenario provides one hypothesis to explain how regional antagonism at the 5-HT_{2A}R and agonism at the 5-HT_{2C}R may suppress cue reactivity and impulsivity (136).

The cell-type specificity of neurons expressing the 5-HT_{2A}R:5-HT_{2C}R complex is less studied. Identification of 5-HT_{2A}R and 5-HT_{2C}R mRNA from the same cells suggests that complex formation probably occurs primarily in GABA neurons (150). Nocjar et al also posited in their recent study that 5-HT_{2A}R and 5-HT_{2C}R co-localization occurs primarily on GABA neurons due to the predominance of 5-HT_{2C}R expression seen in this cell type. They further suggest that together the receptors may co-regulate signaling of GABA firing that provides inhibitory control over efferent pyramidal projection neurons in this region (136). However, it cannot be excluded that 5-HT_{2A}R:5-HT_{2C}R complex expression may also occur on pyramidal neurons within the deep layers of the mPFC (136, 145), which may directly regulate glutamate signaling. In fact, previous studies have isolated mRNA from 5-HT_{2A}R and 5-HT_{2C}R in the same pyramidal neurons in the mPFC (161, 162). We propose that future studies assess the cell-type specificity of neurons expressing the 5-HT_{2A}R:5-HT_{2C}R complex in the dorsal mPFC. Furthermore, we observed 5-HT_{2A}R:5-HT_{2C}R complex expression in both superficial and deep layers of the mPFC. Thus, in addition to regulating cortical-subcortical pathways via expression on neurons in the deeper layers, activation of the 5-HT_{2A}R:5-HT_{2C}R complex may also regulate intracortical signaling via expression on neurons in the superficial layers of the mPFC (155).

A significant proportion of glutamate efferent neurons originating in the mPFC targets areas that are highly implicated in drug- and food-reward related behaviors, such as the ventral striatum, dorsal striatum, and lateral hypothalamus (11, 163-165). The target of the efferent neurons is responsible for differentially regulating behavior. For example, activation of the nucleus accumbens core via neurons originating in the PL increases cue reactivity while activation of the nucleus accumbens shell via neurons originating in the IL decreases cue reactivity (for review, (166). While these pathways are spatially separated, this is not always the case. For instance, neurons in the PL can also project to the basal nucleus of the amygdala which upon activation cause increased expression of conditioned fear (for review, (166). Together, these findings suggest that activation of neurons from

the PL can modulate behaviors in not just the anticipation/preoccupation stage of the addictive cycle, but also in other disorders such as posttraumatic stress disorder (166). Ultimately, serotonergic signaling on glutamate or GABA neurons in the mPFC can directly or indirectly alter each of these behaviors via top-down regulation, respectively. Because activation of specific neuronal pathways results in divergent behaviors, future studies should utilize anatomical neuronal tracing studies to determine the target of neurons in the dorsal mPFC that express the 5-HT_{2A}R:5-HT_{2C}R complex to better understand the role of the complex in neuronal pathway regulation.

The functional significance of a 5-HT_{2A}R:5-HT_{2C}R complex is only in its infancy of exploration. Previous studies have suggested cross-talk between the receptors that causes upregulation or increased function at the reciprocal receptor when the first receptor is downregulated or functionally blocked (37, 38). In addition, other studies have demonstrated additive effects when ineffective doses of specific 5-HT_{2A}R and 5-HT_{2C}R ligands are combined (35, 36). Further, recent evidence has demonstrated that lower levels of the 5-HT_{2A}R:5-HT_{2C}R protein complex in the mPFC, as assessed by co-immunoprecipitation, is associated with higher levels of impulsivity (38). A recently published report has suggested that when the two receptors form a 5-HT_{2A}R:5-HT_{2C}R heteromer, signaling is mediated via the 5-HT_{2C}R (39). Together, these findings suggest the 5-HT_{2A}R:5-HT_{2C}R complex may function differently compared to when the individual receptors act independently. Interestingly, studies have demonstrated that microinfusion of a 5-HT_{2C}R agonist into either the PL or IL can suppress cue-induced cocaine seeking (167) while viral-mediated genetic knockdown of the 5-HT_{2C}R in the mPFC increases impulsivity (52). Thus, we hypothesize that an intervention that promotes 5-HT_{2A}R:5-HT_{2C}R complex expression in the mPFC (e.g., administration of heterotopic bivalent ligands that increase the affinity between the two receptors) might be effective in suppressing addiction-related behaviors by shifting signaling from the 5-HT_{2A}R to the 5-HT_{2C}R. Future studies should assess if increased association or disruption of the 5-

HT_{2A}R:5-HT_{2C}R complex can alter functional cross-talk occurring between the two receptors and determine the specific neuronal population that contributes to behavior.

Cumulative data suggest an important functional role of a 5-HT_{2A}R:5-HT_{2C}R complex, especially in the regulation of addiction-related behaviors. We propose the following model based upon our study in light of previous literature. Serotonin-mediated activation of the 5-HT_{2A}R:5-HT_{2C}R complex occurring on GABA neurons in the deeper layers of the dorsal mPFC would result in inhibition of glutamate neurons that project to subcortical regions. Inhibition of these pathways, such as the PL-nucleus accumbens core pathway, would suppress both cue reactivity and impulsivity, therefore decreasing expression of the preoccupation/anticipation stage of the addictive cycle. This would then in turn prevent future binge/intoxication activity. Thus, activation or promotion of expression of the 5-HT_{2A}R:5-HT_{2C}R complex could act as a protective factor in the treatment of addictive disorders.

The present study has identified the presence of a dorsal-ventral gradient of 5-HT_{2A}R:5-HT_{2C}R complex expression within the mPFC. Future studies should further investigate the cell-type specificity of neurons expressing the complex in addition to identifying the pathways that the 5-HT_{2A}R:5-HT_{2C}R complex may specifically regulate. Previous work indicating interactions between the 5-HT_{2A}R and 5-HT_{2C}R confers protection against addiction-related behaviors suggests the 5-HT_{2A}R:5-HT_{2C}R complex may be a viable therapeutic target in the treatment of addictive disorders.

Chapter 6: Conclusions

The present body of work illustrates four strategies to identify therapeutic opportunities in the treatment of addictive disorders. The following sections demonstrate the opportunities that lie within each strategy – neuronal modulation, behavior-guided therapy, drug repurposing, and combined therapeutic approaches. Each strategy is discussed in depth below, with special emphasis on translation to treatment of BED and other addictive disorders.

NEURONAL MODULATION

Chapter 2 demonstrated that $insula_{ant}$ activation suppressed both HFF binge intake and cue reactivity, suggesting modulation of $insula_{ant}$ activity may be an effective therapy in BED. **Chapter 5** identified expression of the 5-HT_{2A}R:5-HT_{2C}R complex in the dorsal mPFC and discussed how this interaction may prevent binge/intoxication by suppressing activation of the "Go" pathway and thus cue reactivity and impulsivity. The following sections address techniques which could be used in humans to alter neuronal function as well as the current state of select approaches in addictive disorders. The final section considers implementation of the present findings into clinical medicine.

Electrical Stimulation

Multiple techniques, including electroconvulsive therapy (ECT), transcranial direct current stimulation (tDCS), cranial electrostimulation (CES), and deep brain stimulation (DBS), can be used to directly alter neuronal activity via electrical stimulation. ECT employs high voltage electric stimuli which induces a seizure while tDCS uses low voltage and weak current to alter resting membrane potential (168, 169). CES uses alternating current applied at a low intensity to two electrodes near the ears to alter activity of the hypothalamus, limbic system, and reticular activating system (170), while DBS requires

surgical implantation of bipolar electrodes and pulse generators into specific brain regions (168). Each technique targets precise brain regions and utilizes specific electrical frequencies and intensities to modulate neuronal function which can alter long term potentiation and depression and ultimately affect behavior. Previous studies have demonstrated that active tDCS targeting the PFC suppresses food craving, especially for sweet foods and carbohydrates, to a greater extent than sham tDCS (171, 172). This has laid the foundation for a clinical trial employing tDCS directed to the PFC in the treatment of BED (NCT02382497). In addition, tDCS targeting the PFC reduces alcohol craving (173) in addition to altering neuronal activity upon exposure to crack-related images in patients with cocaine use disorder (174). Previous studies also support the use of DBS in the treatment of addictive disorders. For example, DBS of the nucleus accumbens shell reduces binge eating in mice (175) and DBS of the lateral hypothalamic area can induce weight loss under specific conditions in patients with obesity (176).

Stimulation of specific nerves outside the brain can also be used to alter behavior. Non-invasive (e.g., transcutaneous) or invasive (e.g., intra-abdominal) vagal nerve stimulation suppresses appetite and results in weight loss (177-180). To our knowledge, there are no studies that have assessed the viability of vagal nerve stimulation in the treatment of BED; however, this technique should be explored since vagal stimulation may restore dysfunctional satiety signals (181) observed in BED (97).

Magnetic Stimulation

An alternative to electrical stimulation of neurons is TMS. TMS focuses high intensity electromagnetic pulses through a coil outside the skull, which generates an electric current that results in depolarization of superficial cortical neurons (182). Traditional TMS can only penetrate far enough to reach superficial layers, but deep TMS can modulate deeper cortical and even sub-cortical brain regions. This is accomplished through use of a specialized coil (i.e., H-coil) that concentrates magnetic energy to deeper

areas while minimizing electrical field changes in superficial layers. Repetitive TMS (rTMS) induces long-term increases or decreases in cortical functioning depending on if high frequency or low frequency repeated stimulation is utilized, respectively. Meanwhile, theta-burst stimulation (TBS) inhibits or excites neurons through continuous or intermittent bursts of three high frequency pulses, respectively. Previous studies have indicated rTMS directed at the PFC inhibits the development of food craving (183) and decreases valuation of food (184) which in part led to an ongoing clinical trial testing the effectiveness of rTMS to the PFC in BED (185). TMS is also a promising therapeutic strategy in the treatment of SUD (for review, (186); rTMS delivered to the insula and PFC with concurrent exposure to smoking cues resulted in a 6-month abstinence rate of 33% in patients with refractory nicotine use disorder (187).

Chemogenetics

Chemogenetics is a technique in which macromolecules are engineered to interact with specific exogenous small molecules and include platforms such as receptors activated solely by synthetic ligands (RASSLs), neoceptors, and DREADDs such as the one used in **Chapter 2** (188). These techniques are currently reserved for mechanistic research in animal models, but there is potential for therapeutic use in humans. Opponents may argue that the designer drugs are not specific enough to prevent adverse effects brought on by processes such as back-metabolism of CNO to clozapine (189, 190), but new systems are actively being developed that utilize truly biologically inert ligands (191). An interesting concept to consider is the possibility that fully inert ligands are not necessary. For example, perhaps a prodrug that only exhibits actions at the engineered receptor could be used to bolster the effects that the metabolized drug can elicit on its own, essentially resulting in a two-drugs-in-one scenario.

Beyond the challenges faced by the chemogenetic system itself are also those related to implementation. One proposed method for implementing a chemogenetic

system in humans is to use the same techniques used in animal studies – virally-mediated gene transduction technology. With the advent of techniques that ensure cell-type specificity, this approach could result in very limited expression of the chemogenetic system to only specific targeted areas and cell types. Alternatively, cell-type specificity could be achieved via induced pluripotent stem cells stably expressing DREADDs (188). A final approach to deliver chemogenetic systems to the brain is to use nanotechnology. Although a long path towards use in human populations remains, this technology has the potential to allow for greater cell-type specificity than virally-mediated gene transduction. Excitingly, techniques such as intracranial viral-mediated gene delivery are already being used to treat human diseases such as glioblastoma (192).

Chemogenetic approaches have been used to alter multiple addictive behaviors in animal models (for reviews, (193, 194). For example, data were presented in **Chapter 2** showing DREADD-mediated activation of the insula suppresses HFF binge intake and cue reactivity. Others have demonstrated inhibition of specific pathways can suppress drug-seeking behaviors (195). Thus, this approach is well-justified for use in humans once safe and effective technology has been developed.

Behavioral Intervention

Another way to alter specific brain region activity is through behavioral interventions. Mindfulness training, defined as attention to one's ongoing experiences with a nonjudgmental attitude (196), is thought to be mediated through interoceptive processes (197). Thus, it is not surprising that mindfulness training can alter insula activity (197-199). In fact, a recent study demonstrated that mindful watching of drug-cue videos resulted in greater insula activation compared to passive viewing (200). Thus, mindfulness training may be effective in increasing insula activation and thus preventing cue reactivity that may precipitate binge eating episodes.

Craving behavioral intervention, originally developed to reduce craving for gambling, has also been shown to increase insula activation and decrease severity of symptoms and cue-induced craving in individuals with internet gaming disorder (201). This therapy aims to help individuals with internet gaming disorder to recognize how craving promotes binge activity, reduce binge activity by decreasing cue-induced craving through amelioration of cue salience, enhance self-monitoring and behavioral control, and use coping skills instead of engaging in binge behavior (202). Other behavioral interventions such as cognitive behavioral therapy, cue-exposure based extinction training, and motivational interviewing alter neuronal activation patterns associated with dysregulated eating, addictive disorders, and other neuropsychiatric diseases (203-207). These behavioral approaches to modulate neuronal activity are particularly enticing since these techniques are non-invasive and can be achieved without specialized equipment such as what is necessary in electrical or magnetic stimulation.

Translating Findings to Therapeutics

Chapter 2 demonstrated that insula activation suppressed HFF binge intake and cue reactivity. One way to immediately translate these findings into the treatment of BED is to direct high-frequency rTMS to the insula utilizing an H-coil. Since previous studies have demonstrated that rTMS exhibits greater effectiveness when administered during cue-exposure (187), rTMS would ideally occur during exposure to food cues. Alternatively, a behavioral intervention such as mindfulness training or craving behavioral intervention could be used to increase insula activity and thus suppress cue reactivity in BED.

Chapter 5 discussed possible mechanisms by which expression of the 5-HT_{2A}R:5-HT_{2C}R complex may mediate addiction-related behaviors such as cue reactivity and impulsivity. Future studies are needed to confirm the proposed mechanism. However, once confirmed, neuronal modulation techniques could be utilized to mimic activation of the 5-HT_{2A}R:5-HT_{2C}R complex. Ideally, chemogenetic platforms under the guidance of

cell-type specific delivery techniques using nanotechnology could be used to induce expression of engineered receptors in neurons that typically express the 5-HT_{2A}R:5-HT_{2C}R complex. The receptors could then be activated via administration of a specific ligand which would regulate the neuronal activity responsible for modulating addiction-related behaviors. Admittedly, translation of this technique for the treatment of addictive disorders has a long path to successful implementation because these approaches are currently not fully developed for use in human populations.

BEHAVIOR-GUIDED THERAPY

Chapter 2 of this dissertation discussed the utility in suppressing cue reactivity in the treatment of BED. **Chapter 5** built on this further by suggesting a potential mechanism by which cue reactivity and impulsivity may be modulated to treat addictive disorders. **Chapter 3** discussed the potential for people suffering from co-morbid obesity and BED to exhibit a greater response to lorcaserin therapy compared to patients with obesity without BED. **Chapter 4** then identified specific behavioral profiles that may be more responsive to pimavanserin, lorcaserin, or pimavanserin plus lorcaserin therapy in the treatment of BED. The following sections discuss using behavior-guided therapy in two ways – as a therapeutic target and as a predictive biomarker. The final section considers implementation of the present findings into clinical medicine.

Behavior as a Therapeutic Target

There are numerous behaviors that contribute to addictive behaviors. These include cue reactivity, impulsivity, compulsivity, anxiety, and sensation-seeking, amongst others (11, 14, 208). In **Chapter 2** we demonstrated a positive association between HFF binge intake and cue reactivity and suggested cue reactivity should be directly targeted to suppress the binge/intoxication stage of addictive disorders. A recent study demonstrating the highly efficacious weight-loss drug *D*-fenfluramine may suppress food intake in part

through suppression of cue-induced food seeking (59). Further varenicline, an approved smoking cessation therapy, suppresses cue-induced drug seeking in addition to smoking (57). Excitingly, both *N*-acetylcysteine (56) and TMS (58) can successfully attenuate cue-induced food craving, and thus should be explored as possible therapeutic options in the treatment of BED.

Impulsivity has also been linked to addictive disorders including BED and SUD (for reviews, (14, 68). For example, impulsivity, as scored using the Barrett Impulsivity Scale-11 (BIS-11), is correlated with larger test meal intake in individuals with BED (209). Further, impulsive action is elevated in women with BED compared to women without BED (210) and predicts early engagement of binge eating in adolescents (211). Preclinical studies have also established a relationship between impulsivity and binge eating behavior. Impulsive choice, as measured on a delay discounting task, predicted binge eating in an intermittent access paradigm in Wistar rats (212, 213). Thus, evidence suggests an interlocked relationship between impulsivity and binge eating. The relationship between impulsivity and SUD has also been extensively characterized (for reviews, (11, 14, 214, 215). Together, these findings support suppression of impulsivity as a therapeutic target in the treatment of addictive disorders.

Behavior as a Predictive Biomarker

The identification of accurate predictive biomarkers is paramount to patient treatment. Without useful biomarkers, physicians must often make their best guess as to what therapeutic approach is best suited for an individual patient. This can increase morbidity as a patient may not respond to treatment until after multiple therapeutic trials. As discussed above, cue reactivity and impulsivity are linked to addictive disorders. Studies have previously demonstrated that neural activation patterns during tests assessing cue reactivity and impulsivity can predict relapse (for review, (216). However, few studies have assessed if these neural activation patterns can be used to predict

specific treatment response. Other studies have demonstrated that genetic screening can predict relapse potential and response to specific pharmacotherapies (for review, (217) or identify people more susceptible to relapse by predicting behaviors such as cue reactivity (83). Unfortunately, both imaging and genetic studies are expensive and often inaccessible. Therefore, a cheaper, readily-available method for predicting therapeutic response is necessary. Herein, we propose this could be achieved by using behavioral information. Behaviors such as cue reactivity and impulsivity can be easily measured by employing questionnaires (e.g., visual analogue scale for craving, BIS-11, monetary choice questionnaire) and behavioral task measures (e.g., Stroop task, Go/No-Go task, continuous performance task, stop signal task, anti-saccade task, Richards task, experiential discounting task) (20, 21). There is evidence to support this approach may work in predicting effective treatments in addictive disorders. For example, treatment of BED with lisdexamfetamine suppresses both binge eating and BIS-11 scores, particularly on the motor and nonplanning impulsivity subscales (218). To determine if impulsivity levels are a predictive biomarker for response to lisdexamfetamine, it would be beneficial to conduct a correlational analysis where a subject's binge eating activity after lisdexamfetamine treatment (e.g., percent suppression of binge episodes) is analyzed in relation to that individual's baseline impulsivity scores. A negative correlation between these two measures would suggest that higher baseline levels of impulsivity are predictive of a more robust response to lisdexamfetamine. To our knowledge, this analysis has not been conducted in humans, but future studies should explore this possibility.

Unpublished data from our laboratory also demonstrates the importance of using behavior as a predictive biomarker. We previously published a study demonstrating combined ineffective doses of the 5-HT_{2A}R antagonist M100907 plus 5-HT_{2C}R agonist WAY163909 suppresses motor impulsivity as assessed by premature responses on the 1-choice serial reaction time task (35). We tested these same doses of M100907 and WAY163909 in our binge eating model in rats that had previously been phenotyped as

high or low impulsive based on premature responses on the 1-choice serial reaction time task. Initial analyses indicated no main effect of treatment on binge intake ($F_{3,93}$=1.618, p=0.1904) for the overall group (**Figure 6.1A**). However, we discovered that while there was no main effect of treatment on low impulsive rats ($F_{3,42}$=0.3417, p=0.7953; **Figure 6.1B**), there was a main effect of treatment in high impulsive rats ($F_{3,45}$=5.323, p=0.0032; **Figure 6.1C**). Dunnett's multiple comparisons test indicated no suppression of binge intake after M100907 (p=0.2305) or WAY163909 (p=0.1850) administration alone, but a significant suppression of binge intake after administration of combined M100907 plus WAY163909 (p=0.0007). These findings suggest that behavioral analyses could be used as a predictive biomarker in the treatment of addictive disorders.

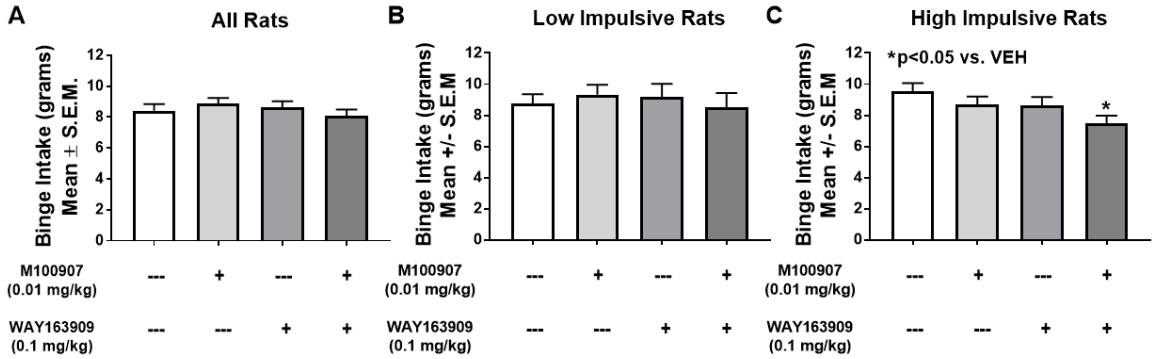

Figure 6.1: Impulsivity predicts response to M100907 plus WAY163909 on binge intake.

There was no effect of M100907 or WAY163909 alone or in combination in the full group analysis (**Figure 6.1A**) or in low impulsive rats only (**Figure 6.1B**). M100907 plus WAY163909 significantly suppressed binge intake in high impulsive rats (**Figure 6.1C**). Composite data are represented as mean +/- SEM. *p<0.05 vs. VEH (Dunnett's multiple comparisons test).

Translating Findings to Therapeutics

The findings in **Chapter 2** suggest that suppression of cue reactivity may suppress binge eating. Thus, therapeutic approaches that are known to suppress cue reactivity such as *N*-acetylcysteine may offer new possibilities in the treatment of BED. In fact, preclinical studies have demonstrated *N*-acetylcysteine can also decrease binge eating in rodent models (219). Thus, future studies should assess the potential of *N*-acetylcysteine in the treatment of BED. Alternatively, rTMS targeting the dorsolateral PFC suppresses cue-induced food cravings in addition to binge eating episodes in patients with bulimia (58). Thus, this approach may also be of benefit to patients with BED.

Chapter 5 proposes a role for the 5-HT_{2A}R:5-HT_{2C}R complex in the regulation of addiction-related behaviors. Previous studies have demonstrated that lower levels of the 5-HT_{2A}R:5-HT_{2C}R complex as identified using co-immunoprecipitation studies are associated with higher levels of impulsivity. Interestingly, rats phenotyped as high impulsive are more sensitive to administration of a 5-HT_{2A}R antagonist (127), thus suggesting that different populations of people with addictive disorders may be more responsive to specific treatments. We hypothesize that individuals with higher levels of impulsivity may be more amenable to therapies that increase 5-HT_{2A}R:5-HT_{2C}R complex formation. Future studies should assess if heterotopic bivalent ligands can increase 5-HT_{2A}R:5-HT_{2C}R complex expression and simultaneously suppress impulsivity.

Chapters 3 and 4 discuss specific behavioral profiles that may be utilized as predictive biomarkers in the treatment of obesity and BED. For example, we hypothesize that people with co-morbid obesity and BED may respond better to treatment with lorcaserin than people with obesity but without BED (**Chapter 3**). We suspect this because 5-HT_{2C}R agonists can suppress both the homeostatic intake of food as well as the hedonic intake of food (91, 92, 94). We further hypothesize that specific behavioral profiles of BED may be more amenable to treatment with pimavanserin, lorcaserin, or pimavanserin plus

lorcaserin (**Chapter 4**). For example, since low doses of pimavanserin do not cause weight change but can suppress binge intake, this may be the preferred course of treatment for an individual with a healthy BMI who has BED. Further, a person with co-morbid obesity and BED who engages in occasional binge eating sessions may respond well to lorcaserin alone since this drug is an approved weight loss agent that can also suppress binge intake. Finally, a person with both obesity and BED who engages in binge eating sessions nearly every day may respond best to combined treatment with pimavanserin plus lorcaserin since this combination suppresses binge episode occurrence, binge intake, and weight gain associated with HFF exposure.

DRUG REPURPOSING

Chapter 4 of this dissertation assessed the viability of the 5-HT_{2A}R antagonist pimavanserin and 5-HT_{2C}R agonist lorcaserin to be repurposed for the treatment of BED. The following sections highlight two methods for identifying candidates for drug repurposing. The final section considers implementation of the present findings into clinical medicine.

Shared Drug Targets

Pharmaceutical agents can be repurposed from their original indication to treat other disorders using two main concepts, shared drug targets and shared drug actions. The concept of shared drug targets refers to the possibility that a drug target can be associated with diseases other than its original indication (28). Perhaps the most well-known example of this strategy is with the drug sildenafil (Viagra®). Sildenafil was originally developed as a vasodilator that works via inhibition of phosphodiesterase type 5 to treat angina. However, this mechanism of action also results in increased blood flow to the corpus cavernosum of the penis. Since identification of this “adverse effect,” it has become a blockbuster drug for treatment of erectile dysfunction. Further studies indicate

sildenafil may be of benefit for people suffering from pulmonary hypertension and Raynaud's phenomenon (220). This same concept also led to the approval of naltrexone for opioid use disorder, alcohol use disorder, and obesity (when used in combination with bupropion) (1, 9).

Shared Drug Actions

The concept of shared drug actions refers to the ability of a drug to act on multiple targets (28). This process essentially leverages the occurrence of an off-target effect of a drug to treat a secondary disease. An example of this approach is demonstrated with tamoxifen, an estrogen receptor blocker that was originally approved for treatment of breast cancer (221). Recently it has been shown that tamoxifen also acts to inhibit protein kinase C and has proven effective in the treatment of bipolar disorder (222). This similar concept has been applied to the treatment of unintentional weight loss in the elderly. The antipsychotic olanzapine and antidepressant mirtazapine both antagonize 5-HT_2R and 5-HT_3R, which are thought to increase appetite and cause weight gain (223). Thus, clinicians often prescribe these drugs to cancer patients exhibiting cachexia.

Bupropion is an example of a drug that has been successfully repurposed for the treatment of addictive disorders. Bupropion inhibits reuptake of dopamine and noradrenaline and was originally approved for treatment of depression (224). However, its ability to attenuate the stimulant effects of nicotine through antagonism at the nicotinic acetylcholine receptors and through inhibition of nicotine-induced vesicular release of dopamine also made this drug a viable therapeutic option for use as a smoking cessation aide (225, 226). Clinical trials indicated that bupropion could induce remission in about 20% of smokers (227, 228), which ultimately led to approval of the drug for smoking cessation as a secondary indication. Recently, bupropion has also been approved as a weight loss therapy when given in combination with naltrexone. Bupropion is thought to exhibit effectiveness in the treatment of obesity by stimulating pro-opiomelanocortin

neurons to release α-melanocyte stimulating hormone, which decreases appetite and increases energy expenditure (229). Thus, bupropion has demonstrated efficacy in the treatment of major depressive disorder, nicotine use disorder, and obesity through multiple drug actions.

Translating Findings to Therapeutics

The present work has demonstrated the potential for the clinically-available 5-HT_{2A}R antagonist/inverse agonist pimavanserin and the 5-HT_{2C}R agonist lorcaserin to be repurposed for use in BED. The road to repurposing lorcaserin is potentially much easier because of the high co-morbidity rates between BED and obesity. A first step towards the approval of lorcaserin in BED is to assess the effectiveness of lorcaserin in patients attempting to lose weight with and without co-morbid BED. Clinical trials have demonstrated that a small subset of patients taking the drug exhibit hyper-responsiveness to lorcaserin. Because lorcaserin can modulate both food intake and reward-mediated behavior, it is possible that this drug would display increased efficacy in people who exhibit dysfunction in both behaviors and thus, should be tested in patients with BED. The utility of lorcaserin in other addictive disorders including cocaine use disorder (NCT03007394, NCT03143543, NCT02393599, NCT03266939), cannabis use disorder (NCT03253926, NCT02932215), opioid use disorder (NCT03143543, NCT03143855), and nicotine use disorder (NCT02393547, NCT02906644) is also currently being assessed.

Repurposing pimavanserin for the treatment of BED may prove more difficult than repurposing lorcaserin since it is not currently approved for a feeding or addictive disorder. However, there is evidence to support translating the findings of **Chapter 4** into clinical populations. For example, decreased weight was a common treatment-emergent adverse event observed in patients with Parkinson's disease psychosis who received pimavanserin in an open-label, long term study (12.4% of subjects exhibited this side effect). However, weight change from baseline was not statistically significant between subjects in the

pimavanserin and placebo groups (230). Of note, very few (9.1%) subjects with Parkinson's disease psychosis treated with pimavanserin in an open-label long term study experienced clinically significant weight gain (>7% of body weight). This effect was primarily driven by subjects with a low BMI (<19 kg/m^2); 16.7% of patients with a low BMI exhibited clinically significant weight gain while only 9.4% of subjects with a mid-range BMI (19-32 kg/m^2) and 2.0% of subjects with a high BMI (>32 kg/m^2) experienced the same event. Conversely, clinically significant weight loss (>7% of body weight) was seen in 31.4% of all subjects taking pimavanserin; 25.0%, 30.4%, and 42.0% of subjects with a low, mid-range, or high BMI exhibited clinically significant weight loss, respectively. Further, clinically significant weight loss was greater after pimavanserin treatment compared to placebo administration (230). This was an interesting finding since many atypical anti-psychotics induce weight gain via blockade of the 5-HT$_{2C}$R (34). Thus, there is evidence to suggest that pimavanserin alters eating patterns in human populations, and thus future studies should more directly assess the ability of pimavanserin to suppress binge eating.

COMBINED THERAPEUTIC APPROACHES

Chapter 4 demonstrated the potential for combined effective doses of pimavanserin plus lorcaserin to achieve greater suppression of measures of binge eating than either drug alone. The following sections describe multiple strategies to effectively combine therapeutic approaches. The final section considers implementation of the present findings into clinical medicine.

Combined Pharmacotherapies

One approach to achieve better treatment outcomes in addictive disorders is to target multiple receptors simultaneously using selective pharmaceutical agents. Different methods can be used to either decrease adverse effects or increase efficacy. The first

approach is to administer two drugs at inactive or subthreshold doses to decrease adverse events. This approach relies on an additive or synergistic interaction between the drugs to cause an effect. For example, the recommended maintenance dose for the weight loss drug Qsymia® contains 7.5 mg phentermine and 46 mg extended-release topiramate and is taken once daily. To achieve weight loss using the individual components as monotherapies, phentermine must be used at a dose of 15-30 mg daily (10) while topiramate requires 96-200 mg daily (231). Negative side effects due to phentermine or topiramate monotherapy include adverse cardiovascular and neuropsychiatric/neurocognitive sequelae, respectively. However, combination therapy results in much lower rates of adverse events, presumably through synergistic effects that allow for lower and better tolerated dosing with the drugs (9). Thus, subthreshold combination dosing of two drugs can result in a safer, but still efficacious, treatment approach.

The second combination therapy approach is to administer fully effective doses of drugs to increase efficacy. This approach is especially appropriate when effect sizes of the two individual drugs are small. An example that partially uses this method is the weight loss drug Contrave®, a combination of naltrexone and bupropion. Naltrexone alone does not produce reliable weight loss at doses ranging from 50-300 mg daily (232-234), but bupropion results in weight loss at doses of 200-400 mg daily (235-237). Although no effective dose of naltrexone alone has been identified, when used in combination with an active dose of bupropion (360 mg), significant weight loss is achieved (120). Further, naltrexone plus bupropion is more effective than bupropion alone in promoting weight loss (120).

Combined Pharmacotherapy Plus Neuronal Modulation

An interesting approach to treating addictive disorders is to combine an effective pharmaceutical agent with an effective brain modulation approach (238). Previous studies

have demonstrated potential effectiveness for this approach. For example, patients undergoing opioid detoxification with buprenorphine plus naloxone treatment were less likely to use drugs two weeks post-discharge if they received active transcutaneous electric acupoint stimulation rather than sham treatment (239). Further, low frequency DBS plus administration of a dopamine receptor antagonist, but neither treatment alone, normalized cocaine-induced neuronal changes in a mouse model (240). Thus, addictive disorders, in addition to other neuropsychiatric disorders including major depression, posttraumatic stress disorder, epilepsy, stroke, and Alzheimer's disease, may all be amenable to combined pharmacotherapy plus neuronal modulation techniques (238).

Combined Pharmacotherapy Plus Behavioral Therapy

Although this approach is not a novel one, it must still be mentioned because of the success this approach has had in the treatment of addictive disorders. For example, fluoxetine plus cognitive behavioral therapy was more effective in achieving remission for patients with BED than fluoxetine alone (241). In two additional studies, topiramate plus cognitive behavioral therapy was more effective than cognitive behavioral therapy alone at attaining binge remission (242) and orlistat plus cognitive behavioral therapy was more effective than cognitive behavioral therapy alone in suppressing binge eating behavior and weight loss (243). These studies support the utility of combining pharmacotherapy with behavioral therapy in the treatment of addictive disorders.

Translating Findings to Therapeutics

Chapter 4 demonstrated that pimavanserin alone and lorcaserin alone could suppress binge intake, but not binge episode occurrence or weight gain associated with HFF exposure. However, when active doses of pimavanserin and lorcaserin were combined, all three measures of binge eating were suppressed. Thus, combined administration of pimavanserin and lorcaserin may prove more effective than either drug

alone in treating patients with BED. Although neither drug is currently approved for use in BED (see previous discussion), future studies should assess if the combination of pimavanserin plus lorcaserin is safe and effective in the treatment of BED.

This dissertation has provided rationale for several other combined therapeutic approaches in addition to combined pharmacotherapies. For example, the finding that $insula_{ant}$ activation suppresses both binge intake and cue reactivity suggests that combined use of TMS targeting the insula plus pimavanserin and/or lorcaserin may also be a useful approach in the treatment of BED. Alternatively, a pharmacological agent and a neuromodulation technique that both suppress cue reactivity (e.g., N-acetylcysteine plus TMS targeting the insula) may result in complete suppression of cue reactivity and thus prevent the binge/intoxication stage of BED. Finally, a heterobivalent ligand consisting of a 5-HT_{2A}R antagonist plus a 5-HT_{2C}R agonist may be used to prevent binge eating in combination with a behavioral approach that increases activity of the insula (e.g., mindfulness training).

TRANSLATING APPROACHES BETWEEN ADDICTIVE DISORDERS

Overall, the behavioral changes seen within BED are very similar to other SUDs. However, it is important to remember that the specific neural mechanisms responsible for driving certain behaviors in one disorder are not necessarily the same mediators in another disorder. An example of this is illustrated with **Chapter 2**. We demonstrated that $insula_{ant}$ activation suppresses HFF cue reactivity. However, when this same technique was applied to test the effect of $insula_{ant}$ activation on cocaine cue reactivity, we saw no change in lever pressing (**Figure 6.2**). While these findings suggest different neural mechanisms responsible for HFF and cocaine cue reactivity, neural modulation techniques targeting the insula may still be useful in the treatment of cocaine use disorder. Preclinical studies have demonstrated that *inactivation* of the insula via administration of GABA agonists suppresses cue-induced reinstatement of cocaine seeking (60). Thus,

perhaps using low frequency rTMS to induce long-term depression of the insula (as opposed to high frequency rTMS which induces long-term potentiation) may be a viable therapeutic approach in the treatment of cocaine use disorder.

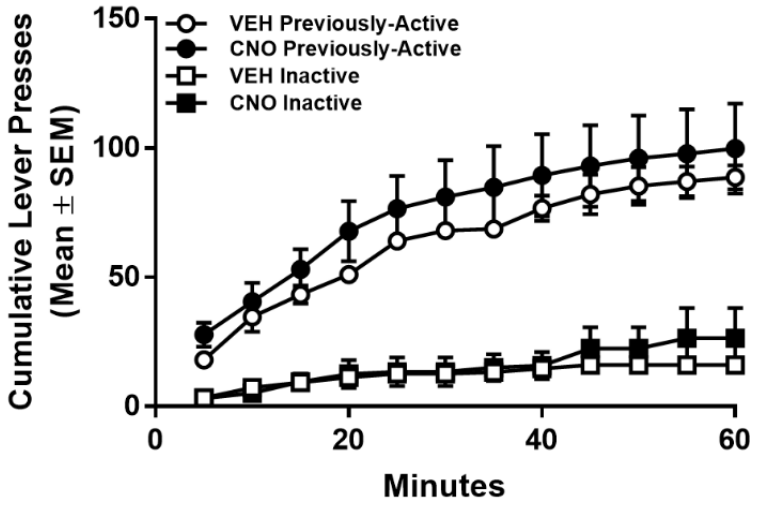

Figure 6.2. Activation of the insula$_{ant}$ does not alter cocaine cue reactivity.

Cocaine cue reactivity was assessed in rats exhibiting bilateral expression of hM3D in the insula$_{ant}$ after 1 mL/kg VEH or 2 mg/kg CNO administration. A two-way mixed model ANOVA demonstrated a main effect of time ($F_{11,66}$=44.81; p<0.0001) but not of treatment ($F_{1,6}$=0.453; p=0.5260) or a time x treatment interaction ($F_{11,66}$=0.1761; p=0.9984) on previously-active lever presses. Composite data are represented as mean +/- SEM.

CONCLUSIONS

Addictive disorders are complex diseases that are associated with substantial disruptions in neural function. While each addictive disorder contains unique features specific to the diagnosis, overlapping similarities are seen at levels spanning from neuron to behavior. This dissertation explored four strategies to identify therapeutic opportunities in the treatment of addictive disorders. We demonstrated that direct neuronal modulation can suppress cue reactivity that drives the binge/intoxication stage as well as the

binge/intoxication stage itself. We further discussed how behavior-guided therapy may be beneficial in the proper development and selection of treatment approaches. This dissertation then identified clinically-available drugs that are well-suited for repurposing in the treatment of addictive disorders. Finally, we demonstrated that combined therapeutic approaches may result in more favorable outcomes than one approach alone. Overall, patients should benefit from these strategies to identify therapeutic opportunities in the treatment of addictive disorders.

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Vita

Amanda Elizabeth Price was born on July 30, 1990 to Roxanne and Horace Price, Jr. in Thibodaux, Louisiana. She was raised in Houma, Louisiana where she met her husband, Benjamin Porche. Together they have two children – Owen David (born December 27, 2016) and Olivia Elizabeth (born August 22, 2018).

Ms. Price graduated from H. L. Bourgeois High School in 2008 and subsequently enrolled in Louisiana State University in Baton Rouge, Louisiana. She was awarded the LSU Alumni Association Top 100 Scholarship which afforded her the opportunity to participate in the Chancellor's Future Leaders in Research program. Through this program, she worked in the biochemistry laboratory of Grover L. Waldrop, Ph.D. for four years where she studied the enzyme kinetics of the bacterial enzyme acetyl-coA carboxylase. Ms. Price also completed two summer internships through the Howard Hughes Medical Institute Summer Research Program at LSU. She graduated in 2012 after earning Bachelor of Science degrees in biochemistry and microbiology with minors in chemistry and psychology. She also earned the LSU University Medal for graduating with a 4.0 GPA in addition to the LSU Distinguished Communicator Award.

Ms. Price subsequently enrolled in the M.D.-Ph.D. Combined Degree Program at the University of Texas Medical Branch in Galveston, Texas. Upon completion of the first two years of medical school, she joined the Neuroscience Graduate Program where she pursued her doctoral work in the laboratory of Kathryn A. Cunningham, Ph.D. In 2014, Ms. Price was awarded a predoctoral position on the National Institute on Drug Abuse T32 training grant secured by the UTMB Center for Addiction Research. She was later awarded an F30 training grant from the National Institute on Drug Abuse under the mentorship of Dr. Cunningham and co-mentor, Noelle C. Anastasio, Ph.D. She has presented her research at local, national, and international meetings in addition to co-authoring several publications in academic journals. Ms. Price is passionate about

education and has demonstrated this through her commitment to improving the UTMB M.D.-Ph.D. combined degree program where she has served as an officer for the student organization and as the student representative on the director search committee. Ms. Price has worked extensively as a tutor in addition to mentoring fellow graduate students in her laboratory which resulted in her receiving the Drs. Giovanni and Maria Grazia Micci Award for Mentoring Excellence.

Awards

1. Drs. Giovanni & Maria Grazia Micci Award for Mentoring Excellence (UTMB, 2017)
2. Francis Adoue Lynch Addiction Research Fund Travel Award (UTMB, 2017)
3. 1st Place Oral Presentation Neuroscience & Cell Biology Retreat (UTMB, 2016)
4. Ann & John Hamilton Endowed Scholarship (UTMB, 2016 & 2015)
5. Herin Addiction Pioneer Travel Award (UTMB, 2016)
6. Blocker Scholar Fellowship in Biomedical Research (UTMB, 2014)
7. Savage Award (UTMB, 2012)
8. University Medalist (LSU, 2012)
9. Distinguished Communicator Award (LSU, 2012)
10. Brandon J. Latiolais Memorial Scholarship (LSU, 2012)
11. Howard Hughes Medical Institute Summer Research Program (LSU, 2011 & 2010)
12. Tiger Athletic Foundation Scholarship (LSU, 2010)
13. Chancellor's Future Leaders in Research (LSU, 2008-2012)
14. Alumni Association Top 100 Scholarship (LSU, 2008-2012)

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2. **Price, A.E.**,* Sholler, D.J.,* Stutz, S.J., Anastasio, N.C., and Cunningham, K.A. (2019). Endogenous serotonin 5-HT_{2A} and 5-HT_{2C} receptors associate in the medial prefrontal cortex. *ACS Chemical Neuroscience*. 10 (7): 3241-3248. PMID: 30645940. PMCID: PMC6640104. *These authors contributed equally.

3. **Price, A.E.**, Stutz, S.J., Hommel, J.D., Anastasio, N.C., and Cunningham, K.A. (2019). Anterior insula activity regulates the associated behaviors of high fat food binge intake and cue reactivity in male rats. *Appetite*. 133: 231-239. PMID: 30447231. PMCID: PMC6594399.

4. **Price, A.E.**, Brehm, V.D., Hommel, J.D., Anastasio, N.C., and Cunningham, K.A. (2018). Pimavanserin and lorcaserin attenuate measures of binge eating in male Sprague-Dawley rats. *Frontiers in Pharmacology*. 9: 1424. PMID: 30581386. PMCID: PMC6293203.

5. **Price, A.E.**,* Anastasio, N.C.,* Stutz, S.J., Hommel, J.D., and Cunningham, K.A. (2018). Serotonin 5-HT_{2C} receptor activation suppresses binge intake and the reinforcing and motivational properties of high-fat food. *Frontiers in Pharmacology*. 9: 821. PMID: 30100875. PMCID: PMC6072841. *These authors contributed equally.

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7. Broussard, T.C., Kobe, M.J., Pakhomova, S., Neau, D.B., **Price, A.E.**, Champion, T.S., and Waldrop, G.W. (2013). The three-dimensional structure of the biotin carboxylase-biotin carboxyl carrier protein complex of *E. coli* acetyl-CoA carboxylase. *Structure* 21 (4): 650-657. PMID: 23499019. PMCID: PMC3965354.

8. Broussard, T.C., **Price, A.E.,** Laborde, S.M., and Waldrop, G.L. (2013) Complex formation and regulation of Escherichia coli acetyl-CoA carboxylase. *Biochemistry* 52 (19): 3346-3357. PMID: 23594205.

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This dissertation was typed by Amanda Price.