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The Serotonin (5-HT) 5-HT_{2A} Receptor is a Priority Target to Reduce Impulsive Action in Substance Use Disorders: Preclinical Evidence

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The Serotonin (5-HT) 5-HT_{2A} Receptor is a Priority Target to Reduce Impulsive Action in Substance Use Disorders: Preclinical Evidence

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The Serotonin (5-HT) 5-HT$_{2A}$ Receptor is a Priority Target to Reduce Impulsive Action in Substance Use Disorders: Preclinical Evidence

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The National Institute on Drug Abuse (NIDA) Division of Therapeutics and Medical Consequences (DTMC) recently highlighted the need for pharmacological targets to treat substance use disorders (SUDs) in the *near term* as well as *entirely new* mechanistic directions for SUDs research. A hallmark of SUDs is "continued drug use despite adverse consequences," which aligns with the definition of impulsivity, a predisposition toward rapid unplanned reactions to stimuli without regard to negative consequences. Serotonin (5-HT) neurotransmission in the medial prefrontal cortex (mPFC) is an important neuromodulator of impulsive action (inability to withhold premature responses), and 5-HT actions at the G protein-coupled 5-HT$_{2A}$ receptor (5-HT$_{2A}$R) regulate impulsive action and the attentional orienting to drug cues (cue reactivity) based upon extensive pharmacological studies with investigational 5-HT$_{2A}$R antagonists/inverse agonists. We first established the potential for repurposing the recently FDA-

approved 5-HT_{2A}R antagonist/inverse agonist pimavanserin (Nuplazid®) as a therapeutic to forestall relapse vulnerability in cocaine use disorder (CUD). Pimavanserin suppressed impulsive action while baseline levels of impulsive action predicted the effectiveness of pimavanserin to suppress incubated cue reactivity in late abstinence from cocaine self-administration at doses that were ineffective in early abstinence. We then demonstrated that pimavanserin attenuated impulsive action evoked by the prescription opioid oxycodone, providing early evidence that pimavanserin may suppress impulsive opioid misuse. We explored the mPFC as a potential site of action for the 5-HT_{2A}R to modulate impulsive action using viral-mediated knockdown of 5-HT_{2A}R protein expression. However, 5-HT_{2A}R knockdown in the mPFC did not alter impulsive action or 5-HT_{2A}R ligand sensitivity, possibly due to the complex, cell type-specific architecture of the 5-HT_{2A}R in mPFC. Lastly, we established a transcriptomic landscape in the mPFC that may characterize individual differences in impulsive action, proposing novel gene targets for future impulsivity research. Our cumulative evidence suggests that the 5-HT_{2A}R is a promising target to improve the health status of SUDs patients in the *near term*, and we propose novel gene targets in the mPFC that provide *entirely new* directions for impulsivity research.

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2-OG	**2-oxoglutarate**
5-HT	**serotonin**
5-HT$_x$R	**5-HT receptor**
AAT	**aspartate aminotransferase**
AAV	**adeno-associated virus**
ACC	**anterior cingulate cortex**
ANCOVA	**analysis of covariance**
ANOVA	**analysis of variance**
ATP	**adenosine triphosphate**
ATP2B4	**ATPase plasma membrane calcium transporting 4**
BACODA	**Bay Area Council on Drugs and Alcohol**
BIS	**Barrett Impulsiveness Scale**
CACNA1C	**calcium voltage-gated channel subunit alpha1 C**
CACNA1E	**calcium voltage-gated channel subunit alpha1 E**
Ca_i^{2+}	**intracellular calcium**
CALN1	**calneuron 1**
CAR	**Center for Addiction Research**
$Ca_v2.3$	**R-type voltage-gated calcium channel**
CDC	**Centers for Disease Control**
ChIP	**chromatin immunoprecipitation**
CLSTN1	**calsyntenin 1**
CMV	**cytomegalovirus**
CPM	**counts per million**
CSRT	**choice serial reaction time**
CT	**crossing threshold**
CUD	**cocaine use disorder**
DA	**dopamine**
DEA	**Drug Enforcement Administration**
DOI	**2,5-dimethoxy-4-iodoamphetamine**
DRL	**differential reinforcement of low rate**
DSM-5	**Diagnostic and Statistical Manual, 5th edition**
DTMC	**Division of Therapeutics and Medical Consequences**
eGFP	**enhanced green fluorescent protein**
EAAT	**excitatory amino acid transporter**
EGFR	**epidermal growth factor receptor**
ERBB4	**Erb-B2 receptor tyrosine kinase 4**
FA	**forced abstinence**
FDA	**Food and Drug Administration**
FDR	**false discovery rate**
FR	**fixed ratio**
GABA	**γ-aminobutyric acid**
GABA-T	**GABA transaminase**
GAD	**glutamic acid decarboxylase**
GAT	**GABA transporter**
GDH	**glutamate dehydrogenase**
GO	**Gene Ontology**
GPCR	**G protein-coupled receptor**
GRIN2A	**glutamate ionotropic receptor NMDA type subunit 2A**
HDAC4	**histone deacetylase 4**
HI	**high impulsive**
ID_{50}	**50% of the maximum suppression**
IL	**infralimbic cortex**
i.m.	**intramuscular**
i.p.	**intraperitoneal**
IPA	**Ingenuity Pathway Analysis**
ITI	**intertrial interval**
KCNMA1	**potassium calcium-activated channel subfamily M alpha 1**
LI	**low impulsive**
LNPEP	**leucyl and cystinyl aminopeptidase**

MAP	**mitogen-associated protein**
MAT	**medication assisted treatment**
mPFC	**medial prefrontal cortex**
NAc	**nucleus accumbens**
NAcSh	**nucleus accumbens shell**
NIDA	**National Institute on Drug Abuse**
NSC	**non-silencing control**
OUD	**opioid use disorder**
PANTHER	**Protein Analysis Through Evolutionary Relationships**
PDE4D	**phosphodiesterase 4D**
PKC	**protein kinase C**
PSD95	**postsynaptic density protein of 95 kDa**
PL	**prelimbic cortex**
PTPRT	**protein tyrosine phosphatase receptor type T**
qRT-PCR	**quantitative reverse transcription polymerase chain reaction**
REST	**RE1 silencing transcription factor**
RNA-seq	**RNA-sequencing**
s.c.	**subcutaneous**
SEM	**standard error of the mean**
shRNA	**short hairpin RNA**
SLC8A1	**solute carrier family 8 member A1**
SMAD	**mothers against decapentaplegic homolog**
SSA	**succinic semialdehyde**
SUD	**substance use disorder**
TCA	**tricarboxylic acid cycle**
TMM	**trimmed mean of M-values**
VEH	**vehicle**
VGAT	**vesicular GABA transporter**
vGLUT	**vesicular glutamate transporter**
vmPFC	**ventromedial prefrontal cortex**
VTA	**ventral tegmental area**
μOR	**μ-opioid receptor**
XKR4	**X-linked Kx blood group related 4**

Chapter 1: Introduction

IMPULSIVITY: FOR BETTER OR WORSE

Impulsivity, or action without sufficient forethought, underlies the age-old question "*what was I thinking?*" Most people are familiar with this question; accelerating through a yellow-turning-red stoplight could yield an expensive traffic ticket or accident, while an unplanned chocolate bar at the grocery store checkout counter may adversely impact one's wallet and diet alike. We have even adopted figures of speech to reference our inability to withhold untimely behavior ("impulsive action") despite its negative consequences. For example, the idiom "hold your horses" is derived from Homer's *The Iliad,* where Menelaus scolded Antilochus for attempting to pass his horsecar on a flooded road, shouting "*Antilochus, thou art driving recklessly; nay, rein in thy horses! Here is the way straitened, but presently it will be wider for passing; lest haply thou work harm to us both by fouling my car*," (Lines 423 – 429) (Homer and Murray, 1965).

Impulsivity is an aspect of normal behavior, but it is marked by complex, biopsychosocial features (Moeller et al., 2001a). First, impulsivity is not a single act but rather a predisposition toward a pattern of behavior. Second, impulsivity involves actions that are rapid or unplanned, and they occur before one can consciously weigh the consequences of behavior (Moeller et al., 2001a). Third, an impulsive action occurs without regard to its consequences (Moeller et al., 2001a). Impulsivity was introduced as a question of "what was I thinking?" to emphasize the tendency to act with less forethought when this tendency may cause problems

(i.e., dysfunctional impulsivity) (Dickman, 1990). However, from an evolutionary perspective, the tendency to take spontaneous action when it is optimal (i.e., functional impulsivity) (Dickman, 1990) was likely advantageous for early humans. For example, Lewis Binford provided archaeological evidence that early humans obtained food by scavenging the consumed kills of other predators rather than hunting (Binford, 1985), suggesting a cycle of vigilant waiting and spontaneous action upon food availability. In this light, the overlap between the biopsychosocial features of impulsivity and scavenging behavior is apparent. First, scavenging would require early humans to closely follow the patterned behavior of predators in their pursuit for food. Second, early humans competed with other carnivores, so securing food at a death site would have to be rapid and reactive at the risk of losing food availability. Third, the limited, unpredictable availability of food may have engendered scavenging despite lethal consequences from predators (Binford, 1985). Thus, the tendency to take swift action to secure calorie-rich, energy-dense foods was advantageous in an environment where food sources were scarce and otherwise unpredictable, and the brain evolved to respond to these natural rewards (Volkow et al., 2012). However, in what Kelley and Berridge call a “quirk of evolutionary fate” (Kelley and Berridge, 2002), humans discovered how to artificially stimulate this reward system with psychoactive substances (McLellan, 2017). In the following section, we explore the role of impulsivity in the cycle of substance misuse and abuse that engenders substance use disorders (SUDs).

IMPULSIVITY AND SUDS

The 2017 National Survey on Drug Use and Health (SAMHSA, 2018) approximated that 19.7 million people aged 12 or older had a SUD, or a clinically significant impairment (i.e., health problems, failure to fulfill obligations) caused by recurrent substance misuse. A psychiatric-motivational theoretical framework describes SUDs as a three-stage "addiction cycle" (SAMHSA, 2016; Koob and Volkow, 2010; Koob and Volkow, 2016):

1. **Binge/Intoxication**, or the stage at which an individual consumes an intoxicating substance and experiences its rewarding or pleasurable effects
2. **Withdrawal/Negative Affect**, or the stage at which an individual experiences a negative emotional state in the absence of the substance
3. **Preoccupation/Anticipation**, or the stage at which an individual seeks substances again after a period of abstinence

Within this framework, the initial impulsive action to consume a substance is proposed to dominate the binge/intoxication stage while impaired inhibitory control and impulsivity contribute to the preoccupation/anticipation stage that precipitates relapse (Koob and Volkow, 2010). However, the diverse behaviors encompassed by the term "impulsivity" transcend a single definition. Thus, the field has converged upon two primary dimensions of impulsivity: impulsive choice and impulsive action. Impulsive choice, or the preference for small, immediate rewards over larger but delayed rewards, predominantly measures the decision-making aspect of impulsive behavior (Winstanley et al., 2006). Impulsive action, or the

inability to withhold a premature, inappropriately-timed response, represents the failure of inhibitory control mechanisms that suppress conditioned and reflexive responses (Winstanley et al., 2006). Although linked to core definitions of impulsivity, impulsive action and impulsive choice have been shown to correlate weakly or not at all, perhaps due to different neurobiological bases (Hamilton et al., 2015). Nonetheless, both types of impulsivity are proposed to sustain the SUD cycle (de Wit, 2009), and the present studies explore how *impulsive action* uniquely contributes to SUDs.

Impulsive action can be measured in a variety of laboratory tasks such as continuous performance tests, go/no-go tasks, and the family of choice serial reaction time (CSRT) tasks. In these impulsive action tasks, an inappropriate response that occurs before a signal constitutes the measure of impulsive action (Dougherty et al., 2000; Dougherty et al., 2002; Winstanley, 2011). In particular, the CSRT tasks are widely employed to study impulsive action, and reverse translation of these tasks for humans reinforces their validity as a measure of impulsive action (Voon et al., 2013; Worbe et al., 2014). In the basic rodent version of the task, called the 5-choice serial reaction time (5-CSRT) task, animals respond to a visual stimulus in one of five spatially distinct locations for delivery of a reinforcer, after which the animal must inhibit a premature response during an inter-trial interval (ITI) until presentation of the next stimulus. The inability to withhold a response during the ITI, termed a premature response, is interpreted as a measure of impulsive action. Specifically, we employ the 1-choice serial reaction time (1-CSRT) task, which reduces visuospatial-attentional demands by limiting

the stimulus to a single, spatially distinct location, thereby increasing the dynamic range of the assay to detect differences in premature responding (Anastasio et al., 2011; Anastasio et al., 2014b; Anastasio et al., 2019; Cunningham et al., 2013; Dalley et al., 2002; Fink et al., 2015; Sholler et al., 2018). By reducing the visuospatial-attentional demands of the standard 5-CSRT task, the 1-CSRT is a useful tool to measure the temporal aspects of impulsive action. For example, it is possible to quantify reaction times that occur related to center stimulus hole illumination, allowing researchers to assess the extent to which rodents utilize implicit temporal strategies versus explicit stimulus presentations to guide 1-CSRT task responding (Cope et al., 2016) (Garcia, Fink, Sholler et al., *in preparation*).

We discuss the contribution of impulsivity and, where available, impulsive action on cocaine use disorder (CUD) and opioid use disorder (OUD) due to their immediate impacts on public health. For example, the number of overdose deaths involving opioids has skyrocketed over the past two decades, rising from 8,048 in 1999 to 47,600 in 2017 (Scholl et al., 2018). A startling feature of the opioid crisis is the linked increase in cocaine-involved overdose deaths, which increased 52.4% from 2015 to 2016 (Kariisa et al., 2019). Strikingly, opioids were involved in 72.7% of cocaine-involved overdoses (Kariisa et al., 2019), underscoring the need to understand the patterned, problematic behaviors that precipitate cocaine and opioid overdoses.

The diagnosis of CUD and OUD in the United States requires that a patient meets criteria set forth in the *Diagnostic and Statistical Manual of Mental Disorders-5* (DSM-5) which defines symptoms including a persistent desire and

preoccupation to obtain cocaine or opioids, inability to control cocaine or opioid use, and continued cocaine or opioid use despite harmful consequences. In a sense, the symptomatology of CUD and OUD overlaps with the psychiatric aspects of impulsivity, including a perseverance of a response that is punished or unrewarded, inability to withhold behavior, and disregard for the negative consequences of behavior (Moeller et al., 2001a). Taken together, there is an immediate need to explore impulsivity and linked behaviors as potential risk factors for (1) the repeated misuse of cocaine and opioids that may evoke overdose, and (2) the susceptibility to relapse that hinders recovery and abstinence in CUD and OUD.

COCAINE USE DISORDER (CUD)

The trajectory from cocaine misuse to CUD is a significant public health challenge in the United States, and CUD affected nearly one million individuals aged 12 or older in 2017 (SAMHSA, 2018). Impulsivity (Evenden, 1999a) is a major risk factor for the initiation, maintenance, and relapse to cocaine use (Koob and Volkow, 2010; Rogers et al., 2010). Studies utilizing human laboratory and questionnaire measures of impulsivity reveal that cocaine-dependent subjects often present with high levels of impulsivity that predict the likelihood to drop out of CUD treatment (Moeller et al., 2001a; Moeller et al., 2004; Moeller et al., 2002; Patkar et al., 2004). Moreover, acute administration of cocaine increases impulsive action across several preclinical assays, including the go/no-go task (Paine et al., 2003; Paine and Olmstead, 2004), differential reinforcement of low rate (DRL) task

(Anastasio et al., 2011; Stoffel and Cunningham, 2008; Wang et al., 2001; Wenger and Wright, 1990; Woolverton et al., 1978), and 1- and 5-CSRT tasks (Anastasio et al., 2011; Fletcher et al., 2011; van Gaalen et al., 2006).

We demonstrated that levels of impulsive action positively correlate with the attentional bias toward cocaine-associated cues ("cue reactivity") in cocaine-dependent humans (Liu et al., 2011) two meta-analyses recently corroborated this link (Coskunpinar and Cyders, 2013; Leung et al., 2017). Further, high baseline levels of self-reported impulsivity [Barrett Impulsiveness Scale-11 (BIS-11)] (Moeller et al., 2001b; Moeller et al., 2007) or attentional bias for cocaine-associated cues (Cocaine-word Stroop task) (Carpenter et al., 2006) predicts poorer retention of cocaine-dependent participants in outpatient treatment trials. Excitingly, our laboratory demonstrated that levels of impulsive action in the 1-CSRT task positively correlate with cocaine cue reactivity in a rodent preclinical model (Anastasio et al., 2014b), enabling translational studies on these interlocked behavioral phenotypes. In this light, the self-administration paradigm is the gold standard in rodent preclinical models of CUD, where rodents are trained to respond (e.g., lever press) to obtain an intravenous infusion of cocaine. Cocaine delivery is paired with the presentation of a discrete cue complex (stimulus light, sound of the infusion pump) that acquires incentive motivational and reinforcing properties after repeated pairings. Once rats achieve stable cocaine self-administration, the extinction/reinstatement or forced abstinence models can be employed to evaluate cue reactivity, defined as lever presses reinforced by the discrete cocaine-paired cue complex. In the extinction/reinstatement model, extinction training disrupts the

association between the operant response (e.g., lever press) and cocaine delivery, and re-exposure to the cocaine-paired discrete cue complex can reinstate operant responding (Cunningham et al., 2011; Fuchs et al., 1998; Nic Dhonnchadha et al., 2009). However, the extinction/reinstatement model is limited in its translational value as abstinent humans do not typically undergo similar extinction learning.

Thus, we employ the forced abstinence model; the key difference in the forced abstinence model is an experimenter-imposed withdrawal from the self-administration chamber and retention in the home cage rather than extinction training (Anastasio et al., 2014a; Anastasio et al., 2014b; Conrad et al., 2008; Grimm et al., 2001; Koya et al., 2009; Neisewander et al., 2000). This model may be more clinically-relevant given that human drug users can experience periods of forced abstinence, including incarceration and inpatient rehabilitation. Taken together, these findings suggest that pharmacotherapeutic strategies that effectively diminish impulsive action as well as linked cue reactivity may reduce relapse during abstinence in CUD patients. We explore the link between impulsive action and cocaine cue reactivity in **Chapter 2**.

OPIOID USE DISORDER (OUD)

The escalating, problematic use of prescription or illicit opioids can progress to OUD (Jamison and Mao, 2015; Volkow et al., 2014), with ~75% of those seeking treatment for heroin addiction in 2013 self-reporting initial non-medical use of prescription opioids (Cicero et al., 2014). Human laboratory studies have shown that opioid-dependent participants exhibit high levels of impulsivity on the BIS

relative to healthy controls (Garami et al., 2017; Kirby et al., 1999; Madden et al., 1997). Moreover, treatment-seeking heroin-dependent individuals exhibit increased impulsive action assessed in the affective go/no-go task relative to healthy controls (Baldacchino et al., 2015). Moreover, daily heroin use in heroin-dependent subjects positively correlates with impulsive action assessed in the immediate and delayed memory task (Jones et al., 2016). Taken together, these studies support the interpretation that high impulsive action is observed in OUD patients.

Clinical or human laboratory investigations into the *acute* effects of opioids on impulsive behavior have received limited attention to date. One study found that the prescription opioid oxycodone did not alter impulsive action or impulsive choice in healthy volunteers, although the authors cite intersubject variability and low number of subjects (as low as $n = 7$ for measures of impulsive action) as key limitations that warrant a larger sample for future studies (Zacny and de Wit, 2009). However, the preclinical literature suggests that systemically-administered opioids increase impulsive action. For example, systemic administration of the abused opioid morphine dose-dependently increases impulsive action in the 5-CSRT task, an effect that is blocked by systemic administration of the opioid antagonist naloxone (Pattij et al., 2009). Moreover, genetic deletion of the μ-opioid receptor (μOR), a site of action for the reinforcing effects of abused opioids, decreases impulsive action (Olmstead et al., 2009). Overall, the neuropharmacology of opioid-evoked impulsive action has received limited attention to date, and we explore this further in **Chapter 3**.

TARGETING IMPULSIVE ACTION IN SUDS

In response to the opioid crisis, the National Institute on Drug Abuse (NIDA) Division of Therapeutics and Medical Consequences (DTMC) published a list of high-priority pharmacological mechanisms for rapid therapeutic development (Rasmussen et al., 2019). In particular, the NIDA DTMC emphasized pharmacological mechanisms with the "highest probability of a path to FDA approval" for the treatment of SUDs "*in the near term*," including therapeutics that are useful at different stages of the SUD cycle (Rasmussen et al., 2019). Precisely, the NIDA DTMC states that many of their high-priority mechanisms are active in more than one model of drug abuse, proposing "the intriguing possibility of their potential efficacy in treating polydrug abuse or other substance use disorders" (Rasmussen et al., 2019). In line with this call to action is the suggestion that treatments targeting impulsivity should be considered to reduce the impulsive misuse of drugs as well as the vulnerability to relapse (Moeller et al., 2001b). Thus, pharmacological mechanisms to reduce impulsive action and promote abstinence have broad applications to the treatment of SUDs, such as CUD and OUD, and may provide immediate relief for patients. The need to identify candidate pharmacological mechanisms to treat SUDs warrants a closer look into the neurocircuitry and neuropharmacology of impulsive action.

NEUROCIRCUITRY OF IMPULSIVE ACTION

The neurocircuitry of impulsive action has largely focused on the medial prefrontal cortex (mPFC) and its connections with the ventral striatum [e.g.,

nucleus accumbens, (NAc)], which confer “top-down” control over the ability to withhold an inappropriately-timed, premature response (Dalley et al., 2011). In particular, the ventral portion of the mPFC (vmPFC) and its connections with the NAc shell (NAcSh) have received considerable attention. Excitotoxic lesion of the vmPFC, but not the dorsal PFC, increases premature responses, a measure of impulsive action, in the 5-CSRT task relative to sham control (Chudasama et al., 2003). Moreover, pharmacological inactivation of the vmPFC, but not dmPFC, with the N-methyl-D-aspartate receptor antagonist 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid increases premature responses in the 5-CSRT task (Murphy et al., 2005). Interestingly, we recently demonstrated that chemogenetic stimulation of the vmPFC $\rightarrow$ NAcSh pathway decreases premature responses in the 1-CSRT task (Anastasio et al., 2019). This pathway is particularly relevant to impulsive action in CUD since both pharmacological and chemogenetic simulation of the vmPFC inhibit cocaine seeking (Augur et al., 2016; LaLumiere et al., 2012; Peters et al., 2008) while optogenetic studies have confirmed that excitation of the monosynaptic vmPFC glutamate projection to NAcSh suppresses cocaine-seeking (Pascoli et al., 2014; Stefanik et al., 2013; Van den Oever et al., 2013). Taken together, the mPFC represents an integral node in the pathophysiology of impulsive action, and studies that clarify the role of the mPFC in these behaviors could inform medications development efforts to minimize impulsive action and forestall relapse vulnerability in SUDs.

NEUROPHARMACOLOGY OF IMPULSIVE ACTION: A ROLE FOR SEROTONIN

The functional activity of serotonin (5-hydroxytryptamine, 5-HT), dopamine (DA), and norepinephrine systems governs these corticostriatal dynamics and are implicated in impulsive action (for review) (Pattij and Vanderschuren, 2008). We focus on the extensive evidence from pharmacological, lesion, and genetic manipulation studies supporting that 5-HT neurotransmission in the mPFC and linked nodes of the ventral striatum modulate impulsive action (for reviews) (Cunningham and Anastasio, 2014; Dalley et al., 2002; Eagle and Baunez, 2010; Fineberg et al., 2010; Jupp et al., 2013). Moreover, a recent study in humans demonstrated a serotonergic influence over vmPFC effective connectivity with the NAc that correlates with premature responses in humans in the 5-CSRT task (Neufang et al., 2016).

Serotonin actions are transduced by 14 subtypes of 5-HT receptors (5-HT_xR) grouped into seven families (5-HT_1R – 5-HT_7R) based on their structural and functional characteristics (for reviews) (Bockaert et al., 2006; Hoyer et al., 2002). The 5-HT_xR family consists of 13 distinct G protein-coupled receptors (GPCRs) and one ligand gated ion channel; in particular, the $G\alpha_{q/11}$-coupled 5-HT_{2A}R modulates impulsive action as suggested by extensive pharmacological studies with 5-HT_{2A}R ligands. Preferential 5-HT_{2A}R agonists [e.g., 2,5-dimethoxy-4-iodoamphetamine (DOI)] increase, while selective 5-HT_{2A}R antagonists/inverse agonists (e.g., M100907) decrease inherent- and cocaine-evoked impulsive action assessed in the 1- and 5-CSRT tasks when administered systemically (Anastasio et al., 2011; Cunningham et al., 2013; Fletcher et al., 2007; Koskinen et al., 2000b;

Sholler et al., 2018; Winstanley et al., 2004). Intriguingly, M100907 also suppresses cocaine- and cue-evoked reinstatement of cocaine seeking following extinction training from cocaine self-administration (Filip, 2005; Fletcher et al., 2002; Lacosta and Roberts, 1993; Nic Dhonnchadha et al., 2009), suggesting that the 5-HT_{2A}R may modulate the interwoven phenotypes of impulsive action and cue reactivity that engender relapse vulnerability in CUD.

The 5-HT_{2A}R is expressed in high density in the rodent mPFC (Miner et al., 2000) and is important in establishing cortical excitatory/inhibitory balance (Puig et al., 2003). The 5-HT_{2A}R in corticostriatal circuits was suggested as a key driver of impulsive action given that high 5-HT_{2A}R binding density in frontal cortical regions was observed in the selectively-bred Roman high-avoidance rat which exhibits high impulsive traits (Klein et al., 2014). In keeping with this concept, we found that high impulsive (HI) rats, separated based upon levels of premature responses in the 1-CSRT task, exhibit a higher density of synaptosomal 5-HT_{2A}R protein expression in mPFC vs. low impulsive (LI) rats (Anastasio et al., 2015; Fink et al., 2015). Moreover, HI rats exhibit increased pharmacological sensitivity to the effects of M100907 to decrease impulsive action relative to LI rats (Fink et al., 2015). These findings suggest that the 5-HT_{2A}R, possibly through actions in the mPFC (**Chapter 4**), is a promising target to reduce impulsive action and promote abstinence in SUDs.

THE 5-HT_{2A}R IS A PRIORITY TARGET TO REDUCE IMPULSIVE ACTION IN SUDS

The NIDA DTMC recognized these promising preclinical data and declared 5-HT_{2A}R antagonism/inverse agonism a pharmacological mechanism of interest for the rapid development of therapeutics in response to the opioid crisis (Rasmussen et al., 2019). However, pharmacological evaluation of a 5-HT_{2A}R antagonist/inverse agonist in humans has been impeded by the lack of clinically-available ligands that selectively target this receptor. One example of a promising 5-HT_{2A}R antagonist/inverse agonist that was tested in clinical trials but never marketed is M100907 (volinanserin; Sanofi-Aventis). M100907 exhibits > 100-fold selectivity for the 5-HT_{2A}R over the homologous 5-HT_{2B}R and 5-HT_{2C}R and other monoamine receptors (Kehne et al., 1996; Knight et al., 2004), serving as an excellent investigational compound to interrogate 5-HT_{2A}R function. The efficacy of M100907 was first investigated in combination with escitalopram to improve sleep, fatigue, and reduce cognitive impairment in treatment-resistant depression (NCT00070694). Similarly, M100907 was evaluated for the treatment of sleep initiation and maintenance disorders in a polysomnographic study (NCT00464243). In both cases, M100907 failed to demonstrate efficacy over placebo and was never marketed.

A pivotal moment for 5-HT_{2A}R research occurred in April 2016 following the approval of the selective 5-HT_{2A}R antagonist/inverse agonist pimavanserin (Nuplazid®, Acadia Pharmaceuticals) by the Food and Drug Administration (FDA). Pimavanserin was approved for the treatment of hallucinations and delusions associated with Parkinson's disease psychosis, and a clinical trial was recently initiated to evaluate the efficacy of pimavanserin to treat impulse control disorders

in Parkinson's disease (NCT03947216). Clinical trials involving M100907 and pimavanserin are intriguing considering that sleep disorders (Morgan et al., 2010; Sharkey et al., 2011) commonly co-occur with CUD and OUD, and dopamine replacement therapy in Parkinson's disease elicits side effects including increased risk-taking and impulsive behaviors (Park and Stacy, 2011). Taken together with the preclinical evidence, a compelling case can be made that pimavanserin or other 5-HT_{2A}R antagonists/inverse agonists could be efficacious in reducing impulsive action, relapse vulnerability, and improving concomitant sleep and psychiatric disorders seen in CUD and OUD. However, preclinical evidence is needed to support for these potential clinical investigations, which we generate in **Chapters 2 and 3**.

NOVEL TARGET DISCOVERY: AN ONGOING EFFORT TO REDUCE IMPULSIVITY IN SUDS

Our cumulative evidence suggests that a 5-HT_{2A}R antagonist/inverse agonist, perhaps through its actions in the mPFC, may be efficacious to suppress impulsive action and promote abstinence in SUD patients, and the clinical availability of pimavanserin provides an avenue for immediate action to treat SUDs. This aligns with the NIDA DTMC medications development priorities, as their most wanted pharmacological mechanisms are "biased towards proximal action" (Rasmussen et al., 2019). However, the NIDA DTMC also highlighted the need to uncover new mechanisms that could be useful for SUDs treatment and the creation of "entirely new directions" in SUD research (Rasmussen et al., 2019). We agree; the 5-HT_{2A}R mechanism is just one brushstroke in a larger biological

signature that typifies impulsive action. Moreover, experimental approaches to identify novel neuropharmacological targets *in vivo* have blossomed with the emergence of functional genomics. In particular, next-generation sequencing strategies have rapidly developed over the past decade, allowing researchers to profile the complete set of gene transcripts in the brain (transcriptome) and identify gene expression differences across organisms (Geschwind and Konopka, 2009; Volkow et al., 2015; Wang et al., 2009). Simultaneously, the development of publicly-available, human-curated gene enrichment tools allow researchers to map large gene lists to common biological processes, pathways, and molecular functions, illuminating an otherwise complex neurobiology (Huang da et al., 2009). The Gene Ontology (GO) Consortium (http://geneontology.org/), which is funded by the National Human Genome Research Institute, is an excellent example. Here, specific gene products are annotated based on experimental findings; for example, GO includes findings from over 150,000 published articles and encompasses over 700,000 annotations supported by the published literature. By annotating each gene, a logical structure of the otherwise complex biology can be created, and genes can be linked to common biological processes, pathways, and molecular functions.

The tandem use of RNA-sequencing and bioinformatics provides new avenues for neuropharmacological target discovery in SUDs research. In **Chapter 5**, we employ this strategy to characterize differences in the mPFC transcriptome between stably identified HI and LI rats using the 1-CSRT task. In doing so, we

suggest novel candidate gene targets in the mPFC to be pursued in future impulsivity research.

PRECLINICAL STUDIES TO ADVANCE MEDICATIONS DEVELOPMENT PRIORITIES

The studies reported herein provide preclinical evidence that the 5-HT_{2A}R is a promising target to improve the health status of SUDs patients in the *near term*, and we propose novel gene targets in the mPFC that provide *entirely new* directions for impulsivity research. The studies presented in **Chapter 2** generate preclinical evidence for repurposing pimavanserin as a therapeutic to forestall relapse vulnerability in CUD, and we hypothesized that pimavanserin would suppress impulsive action and cocaine cue reactivity following abstinence from cocaine self-administration. In **Chapter 3**, we transition from cocaine to oxycodone, considering that oxycodone products (i.e., OxyContin®, Percocet®) are among the most commonly abused prescription opioids (Comer et al., 2010a; Mendelson et al., 2008), to test the hypothesis that pimavanserin would decrease oxycodone-evoked impulsive action assessed in the 1-CSRT task. **Chapter 4** explores the causal directionality in the association between 5-HT_{2A}R density in the mPFC and impulsive action, and we hypothesized that viral-mediated knockdown of the 5-HT_{2A}R in the mPFC decreases impulsive action in the 1-CSRT task. Lastly, in **Chapter 5**, we established a transcriptomic landscape in the mPFC that may characterize individual differences in impulsive action, proposing novel gene targets for future impulsivity research.

Chapter 2: The 5-HT_{2A} Receptor Regulates Impulsivity and Cocaine Cue Reactivity in Male Sprague-Dawley Rats[1]

INTRODUCTION

CUD represents a significant public health challenge in the United States. The lack of effective pharmacotherapeutics to suppress relapse vulnerability is an unmet need in the treatment of CUD (Volkow and Skolnick, 2012). Impulsivity and cue reactivity are two key behavioral phenotypes that engender relapse vulnerability (Drummond, 2001; Koob and Volkow, 2010; Moeller et al., 2001a; O'Brien et al., 1998; Saunders et al., 2013). Impulsivity has been defined clinically as rapid, unplanned reactions to stimuli, reduced sensitivity to negative consequences, and a disregard for long-term consequences (Moeller et al., 2001a). Rapid response impulsivity (impulsive action; difficulty withholding a prepotent response) and impulsive choice (decision-making; delayed reward measures) are dimensions of impulsivity that have been associated with CUD in humans (Moeller et al., 2001a; Moeller et al., 2001b; Patkar et al., 2004) and in rodents (Anastasio et al., 2014a; Belin et al., 2008; Perry et al., 2005). Cue reactivity refers to the attentional orienting response to drug-related stimuli that predict reward (Carter and Tiffany, 1999; Field and Cox, 2008; Garavan et al., 2000; Maas et al., 1998). We demonstrated that levels of impulsive action

[1] Sholler, D.J., Stutz, S.J., Fox, R.G., Boone, E.L., Wang, Q., Rice, K.C., Moeller, F.G., Anastasio, N.C., Cunningham, K.A. (2019, January). The 5-HT_{2A} Receptor (5-HT_{2A}R) Regulates Impulsivity and Cocaine Cue Reactivity in Male Sprague-Dawley Rats. *J. Pharmacol. Exp. Ther.* 368:41–49. PMCID: PMC 30373886

positively correlated with the attentional bias toward cocaine-associated cues in cocaine-dependent humans (Liu et al., 2011) and rodents (Anastasio et al., 2014b); two meta-analyses recently corroborated this link (Coskunpinar and Cyders, 2013; Leung et al., 2017). High baseline levels of self-reported impulsivity (BIS-11) (Moeller et al., 2001b; Moeller et al., 2007) or attentional bias for cocaine-associated cues (Cocaine-word Stroop task) (Carpenter et al., 2006) predicted poorer retention of cocaine-dependent participants in outpatient treatment trials. These findings suggest that pharmacotherapeutic strategies that effectively diminish impulsive action and cocaine cue reactivity may reduce relapse during abstinence in CUD patients.

Serotonin neurotransmission regulates the limbic-corticostriatal circuitry associated with the development and maintenance of addiction as well as vulnerability to relapse (for reviews) (Cunningham and Anastasio, 2014; Koob and Volkow, 2016). Serotonin actions are transduced by 14 receptor subtypes, and the G protein-coupled 5-HT_{2A}R has been implicated in the neural mechanisms underlying impulsive action defined as premature responses in the CSRT tasks (for review) (Cunningham and Anastasio, 2014). After systemic administration, the preferential 5-HT_{2A}R agonist DOI (Titeler et al., 1988; Wischhof et al., 2011) elevated impulsive action measured in the 5-CSRT task (Blokland et al., 2005; Koskinen et al., 2000a; Koskinen et al., 2000b). The selective 5-HT_{2A}R antagonist/inverse agonist M100907 (volinanserin) administered systemically suppressed premature responding assessed in the 1 or 5-CSRT task (Anastasio et al., 2011; Anastasio et al., 2015; Cunningham et al., 2013; Fink et al., 2015;

Fletcher et al., 2011; Fletcher et al., 2007; Winstanley et al., 2004) and also suppressed cocaine- and cue-evoked reinstatement following extinction training from cocaine self-administration (Filip, 2005; Fletcher et al., 2002; Lacosta and Roberts, 1993; Nic Dhonnchadha et al., 2009). A time-sensitive increase in attentional bias toward cocaine-associated cues in rats (Grimm et al., 2001; Lu et al., 2004; Neisewander et al., 2000; Swinford-Jackson et al., 2016) has been observed during extended abstinence; this is referred to as “incubation” (for review) (Pickens et al., 2011). In rodents, escalation of cue reactivity (lever presses that are reinforced by the discrete drug-paired cue complex) occurs up to six months following termination of cocaine self-administration (Grimm et al., 2001; Lu et al., 2004; Neisewander et al., 2000; Swinford-Jackson et al., 2016). However, the relationship between levels of impulsive action and incubated cue reactivity as well as the effectiveness of a 5-HT_{2A}R antagonist/inverse agonist to suppress cocaine cue reactivity during early vs. late abstinence from cocaine self-administration is unknown.

Preclinical and early clinical evaluations of selective 5-HT_{2A}R antagonists support their potential efficacy as therapeutics in sleep disorders (Ancoli-Israel et al., 2011), psychosis (Weiner et al., 2001), psychosis in Parkinson’s disease (Meltzer et al., 2012), and other psychological disorders (Roberts, 2006). However, the investigational compound M100907 (volinanserin), which exhibits > 100-fold selectivity for the 5-HT_{2A}R over the homologous 5-HT_{2B}R and 5-HT_{2C}R and other monoamine receptors (Kehne et al., 1996; Knight et al., 2004), never achieved approval for a clinical indication. The selective 5-HT_{2A}R antagonist/inverse agonist

pimavanserin (Nuplazid®) is now clinically approved for treatment of psychosis in Parkinson's disease (Sahli and Tarazi, 2018) and exhibits > 100-fold selectivity for the 5-HT_{2A}R over 5-HT_{2B}R, 5-HT_{2C}R, and other monoamine receptors (Hacksell et al., 2014; Vanover et al., 2006). Notably, both M100907 and pimavanserin are potent 5-HT_{2A}R antagonists *in vivo*; for example, both compounds suppressed head twitch behaviors and prepulse inhibition deficits induced by DOI in rats (Fantegrossi et al., 2010; McFarland et al., 2011; Sipes and Geyer, 1995) while M100907 is well-characterized to suppress the discriminative stimulus properties of DOI and other 5-HT_{2A}R agonists (Smith et al., 2003; Winter et al., 2007).

The present preclinical study was designed to establish the potential for repurposing pimavanserin as a therapeutic to forestall relapse vulnerability in CUD. Given that previous studies support the utility of the rodent CSRT tasks for screening pharmacological interventions on impulsive action (for review) (Winstanley, 2011), we employed the 1-CSRT task to test the hypothesis that pimavanserin would suppress impulsive action and cocaine cue reactivity during abstinence from cocaine self-administration, similar to M100907. Given that baseline levels of impulsive action positively correlated with cocaine cue reactivity in humans (Liu et al., 2011) and rodents (Anastasio et al., 2014b), we evaluated whether baseline levels of impulsive action are related to the effectiveness of pimavanserin to control cocaine cue reactivity in early (Day 1) vs. late abstinence (Day 30).

METHODS

General Methods

ANIMALS. Male, Sprague–Dawley rats (*n*=160; Harlan, Houston, TX) weighed 250–275 g upon arrival and housing in the colony room. Rats were housed two/cage under a 12-h light–dark cycle with monitored and controlled temperature (21–23°C) and humidity levels (45–50%). Rats were acclimated to the colony room for seven days before handling and experimentation commenced. Rats were food restricted to ~90% free-feeding weight (confirmed by daily weights) during 1-CSRT task training and had *ad libitum* access to water except during daily operant sessions. All experiments were conducted in accordance with the National Institutes of Health *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.

DRUGS. (-)-Cocaine (National Institute on Drug Abuse Drug Supply Program, Bethesda, MD) was dissolved in 0.9% NaCl. M100907 [(R)-(2,3-dimethoxyphenyl)-[1-[2-(4-fluorophenyl)ethyl]piperidin-4-yl]methanol] (synthesized by Kenner Rice, National Institute on Drug Abuse, Bethesda, MD) was dissolved in 1% Tween-80 in 0.9% NaCl [vehicle (VEH) employed for comparison to M100907]. Pimavanserin [1-(4-fluorobenzyl)-3-(4-isobutoxybenzyl)-1-(1-methylpiperidin-4-yl)urea] (Trylead Chemical Technology Co., Ltd., Hangzhou, China) was dissolved in 0.9% NaCl brought to a pH ~6.0 using 1M HCl (VEH employed for comparison to pimavanserin). M100907 and pimavanserin were administered by the intraperitoneal (i.p.) or subcutaneous (s.c.) route, respectively.

1-Choice Serial Reaction Time (1-CSRT) task. All sessions conducted in the 1-CSRT task occurred in five-hole, nose-poke operant chambers containing a houselight, food tray, and an external pellet dispenser that delivered 45 mg dustless precision food pellets (Bio-Serv, Frenchtown, NJ) housed within ventilated, sound-attenuated cubicles (MedAssociates, St Albans, VT). The 1-CSRT task methodology was previously reported in detail (Anastasio et al., 2013; Anastasio et al., 2011; Anastasio et al., 2014b; Cunningham et al., 2013; Fink et al., 2015). To summarize, rats were initially exposed to a pre-training stage during which they were habituated to the test chamber. A nose-poke response into the illuminated center stimulus hole (i.e., a "target" response) resulted in the simultaneous illumination of the magazine light on the opposite chamber wall and the delivery of a 45 mg food pellet. Rats were progressed through a series of training stages following completion of the pre-training stage. Each stage consisted of daily 100-trial sessions to be completed in a maximum of 30 min. The stimulus duration was incrementally shortened throughout each training stage until a final stage of 0.5 sec was achieved with a limited hold of 5 sec and an ITI of 5 sec (ITI5).

A maximum of 100 target responses in a session resulted in a maximum of 100 reinforcers delivered. Incorrect "non-target" responses, premature responses, or omissions resulted in a time-out period (5 sec) that reduced the potential number of reinforcers delivered. Before progressing through each training stage, rats were required to achieve acquisition criteria: ≥ 50 reinforcers earned, > 80% accuracy (target responses/(target + non-target responses) * 100) and < 20% omissions

(omitted responses/trials completed * 100) (Anastasio et al., 2013; Anastasio et al., 2011; Anastasio et al., 2014b; Cunningham et al., 2013; Fink et al., 2015).

The number of premature responses, omissions, and reinforcers earned, percent accuracy, latency to first response, and time to finish the 1-CSRT task were recorded. Premature responses, the primary output measure to assess impulsive action, were categorized into three types: target, non-target, and total (target + non-target). The number of reinforcers earned assessed task competency and provides an additional measure of impulsive action. The percent accuracy was a general indication of attentional capacity. Percent omissions indicated motivation to perform the task, and latency to first response in the 1-CSRT task provided a secondary measure of motivation and an indication of general motor impairment.

COCAINE SELF-ADMINISTRATION AND CUE REACTIVITY. Rats (*n* = 144) were anesthetized (8.6 mg/kg of xylazine, 1.5 mg/kg of acepromazine, 43 mg/kg of ketamine in bacteriostatic saline) prior to surgical implantation of indwelling jugular catheters with back mounts; rats were allotted seven days to permit postoperative recovery (Anastasio et al., 2014a; Anastasio et al., 2014b; Cunningham et al., 2013; Cunningham et al., 2011). Rats received a 0.1 mL infusion of a bacteriostatic saline solution that contained heparin sodium (10 U/mL; American Pharmaceutical Partners, East Schaumburg, IL), streptokinase (0.67 mg/mL; Sigma Chemical, St. Louis, MO), and ticarcillin disodium (66.67 mg/mL; Research Products International, Mt. Prospect, IL) into the catheter immediately following daily cocaine self-administration sessions to ensure catheter patency during experimentation.

The cocaine self-administration assay utilized standard operant conditioning chambers (Med Associates, Inc., St. Albans, VT) housed within sound-attenuated, ventilated cubicles equipped with fans (Med Associates, Inc.). Operant chambers were fitted with two retractable response levers, a stimulus light above each of the response levers, a houselight on the wall opposite of the response levers, and an external pellet dispenser. Cocaine infusions were delivered through syringes that were loaded daily into infusion pumps (Med Associates, Inc.) located outside of the cubicles. The infusion pumps were connected to liquid swivels (Instech, Plymouth Meeting, PA) fastened to catheters via polyethylene 20 tubing encased inside a metal spring leash (Plastics One, Roanoke, VA).

Cocaine self-administration training sessions were 180-min in duration and occurred daily. Rats were trained to perform a lever press response reinforced by a cocaine infusion (0.75 mg/kg/0.1 mL infusion) (Anastasio et al., 2014a; Anastasio et al., 2014b; Cunningham et al., 2011; Swinford-Jackson et al., 2016). Schedule responses on the active lever resulted in delivery of a cocaine infusion over a 6-sec period; each infusion was simultaneously paired with the illumination of the house and stimulus lights and activation of the infusion pump (i.e., discrete cue complex paired with cocaine delivery). Inactive lever presses were recorded, but had no scheduled consequences. The stimulus light and infusion pump were inactivated following delivery of cocaine. The house light remained on to signal a timeout period (20 sec); lever presses committed during the timeout period had no scheduled consequences. Rats were trained on a fixed ratio (FR) 1 schedule of

reinforcement and progressed to a FR5 schedule after achieving seven infusions/hr with less than 10% variability for three consecutive days. Once stable cocaine self-administration was acquired, rats were subjected to forced abstinence (FA) from cocaine self-administration for one day (FA Day 1) or 30 days (FA Day 30). During the FA period, rats were returned to their home cages, weighed, and handled daily. Following the assigned FA period, rats were evaluated in a cue reactivity test session (60 min) in which presses on the previously active lever were reinforced by the discrete cue complex (stimulus light illuminated, infusion pump activated) on a FR1 schedule. Inactive lever presses were recorded, but had no scheduled consequences.

Research Design

Cohort 1: Pimavanserin and M100907 suppress impulsive action measured in the 1-CSRT task. Rats (*n* = 16) were required to meet acquisition criteria in the 1-CSRT task [≥ 50 target responses, > 80% accuracy, and < 20% omissions on the final training stage (0.5 sec stimulus duration, 5 sec limited hold, and ITI5)] for at least three consecutive days. Performance in the 1-CSRT task was assessed following systemic administration of M100907 or pimavanserin. Pretreatment with VEH (1 mL/kg, i.p.) or M100907 (0.001, 0.01, 0.1 mg/kg, i.p) occurred 30 min prior to the start of the 1-CSRT task session under an ITI5 schedule. Five rats failed to meet the acquisition criteria under an ITI5 schedule in the 1-CSRT task and were excluded from analysis of M100907 (*n* = 11 rats analyzed).

Following pharmacological evaluations with M100907, rats established stable 1-CSRT task training under an ITI5 schedule for a minimum of 30 days to permit drug washout. Pharmacological test sessions with pimavanserin commenced once rats met the 1-CSRT task acquisition criteria for at least three consecutive days. Pretreatment with VEH (1 mL/kg, s.c.) or pimavanserin (0.3, 1, 3 mg/kg, s.c.) occurred 30 min prior to the start of 1-CSRT task sessions under an ITI5 schedule. Two rats failed to maintain stable performance under an ITI5 schedule in the 1-CSRT task and were excluded from analysis of pimavanserin (*n* = 9 rats analyzed). Rats were treated with VEH the day before drug pretreatments which were separated by a minimum of three days. The order of M100907 and pimavanserin injections was randomized across rats in a within-subjects design.

COHORT 2: BASELINE LEVELS OF IMPULSIVE ACTION PREDICT THE EFFICACY OF PIMAVANSERIN TO SUPPRESS CUE REACTIVITY. Rats (*n* = 144) were trained on the 1-CSRT task. Baseline levels of impulsive action were established once rats met the acquisition criteria of ≥ 50 target responses, > 80% accuracy, and < 20% omissions on the final training stage (0.5 sec stimulus duration, 5 sec limited hold, ITI5) for three consecutive days. Three rats failed to maintain stable performance and were excluded from analysis. Following identification of baseline levels of impulsive action, 1-CSRT task sessions ceased. Rats were returned to their home cages and were freely fed for at least five days prior to surgical implantation of indwelling jugular catheters. Cocaine self-administration began following at least five days post catheterization. Following acquisition of stable cocaine self-administration, rats were assigned to either FA Day 1 or FA Day 30 and returned to their home

cages for the assigned FA duration. Fifteen rats were excluded from analysis due to technical issues in the cocaine self-administration assay (*n* = 126 rats analyzed).

Pharmacological test sessions were used to evaluate the efficacy of pimavanserin to suppress cocaine cue reactivity on FA Day 1 or FA Day 30. Pretreatment with VEH (1 mL/kg, s.c.) or pimavanserin (0.3, 1, 3, 10 mg/kg, s.c.) occurred 30 min before the cue reactivity session on FA Day 1 (*n* = 68; *n* = 12-15/group) or FA Day 30 (*n* = 58; *n* = 11-12/group) in a between-subjects design.

Statistical Analyses

A one-way repeated measures analysis of variance (ANOVA) was employed to evaluate the effects of either M100907 (VEH, 0.001, 0.01, 0.1 mg/kg) or pimavanserin (VEH, 0.3, 1, 3 mg/kg) on 1-CSRT task measures (target, non-target, or total premature responses, reinforcers earned, % omissions, % accuracy, latency to first response, time to finish the 1-CSRT task); Dunnett's procedure was employed to analyze planned comparisons (Keppel, 1973). The dose of M100907 or pimavanserin estimated to decrease total premature responses by 50% of the maximum suppression (ID_{50}) in the 1-CSRT task was quantified by a four parameter logistic nonlinear regression (Ratkowsky and Reedy, 1986; Tallarida and Murray, 1987). Student's *t*-test was employed to assess total cocaine intake throughout acquisition and maintenance of cocaine self-administration. A two-way ANOVA with the factors of FA Day (FA Day 1, FA Day 30) and medication pretreatment (VEH, 0.3, 1, 3, 10 mg/kg of pimavanserin) was employed to analyze 1-CSRT task performance to assure equal distribution

of rats across groups. A two-way ANOVA with the factors of FA Day (FA Day 1, FA Day 30) and medication pretreatment (VEH, 0.3, 1, 3, 10 mg/kg of pimavanserin) was employed to analyze previously active lever presses, inactive lever presses, and the latency to first response during the cue reactivity test session. A one-way ANOVA was used to analyze previously active lever presses at each FA Day following medication pretreatment; planned comparisons were assessed using Dunnett's procedure or Student's *t*-test, where appropriate (Keppel, 1973). A four parameter logistic nonlinear regression was used to calculate the ID_{50} of pimavanserin to suppress previously active lever presses during the cue reactivity session on FA Day 1 vs. FA Day 30 (Ratkowsky and Reedy, 1986; Tallarida and Murray, 1987). A one-way analysis of covariance (ANCOVA) was employed to evaluate the relationship between target premature responses and previously active lever presses on FA Day 1 vs. FA Day 30 following pretreatment with VEH. A one-way ANCOVA was employed to evaluate the relationship between target premature responses and previously active lever presses at each FA Day with five between-subject pretreatment conditions (VEH, 0.3, 1, 3, 10 mg/kg of pimavanserin) and the covariate (target premature responses in the 1-CSRT task).

RESULTS

M100907 and pimavanserin suppress impulsive action

We tested the hypotheses that M100907 or pimavanserin would suppress impulsive action measured in the 1-CSRT task relative to VEH. **Figure 2.1A**

displays the mean ± standard error of the mean (SEM) number of target premature responses following pretreatment with VEH or M100907 (n = 11). There was a main effect of M100907 on target premature responses [$F_{3,30}$=4.17; $p < 0.05$]; planned comparisons with Dunnett's procedure showed that 0.1 mg/kg of M100907 decreased target premature responses vs. VEH ($p < 0.05$; **Figure 2.1A**). The ID_{50} of M100907 to suppress target premature responses was 0.007 mg/kg. In addition to target premature responses, there was a main effect of M100907 on total (target+non-target) premature responses [$F_{3,30}$=4.88; $p < 0.05$], % omissions [$F_{3,30}$=8.69; $p < 0.05$], and time to finish the 1-CSRT task [$F_{3,30}$=7.21; $p < 0.05$]; planned comparisons with Dunnett's procedure showed that 0.1 mg/kg of M100907 decreased total premature responses, increased % omissions, and increased the time to finish the 1-CSRT task vs. VEH ($p < 0.05$; **Table 2.1**). There was no main effect of M100907 on non-target premature responses [$F_{3.30}$=1.55; n.s.], reinforcers earned [$F_{3,30}$=1.13; n.s.], % accuracy [$F_{3,30}$=0.422; n.s.], or latency to first response in the 1-CSRT task [$F_{3,30}$=1.04; n.s.] vs. VEH (**Table 2.1**).

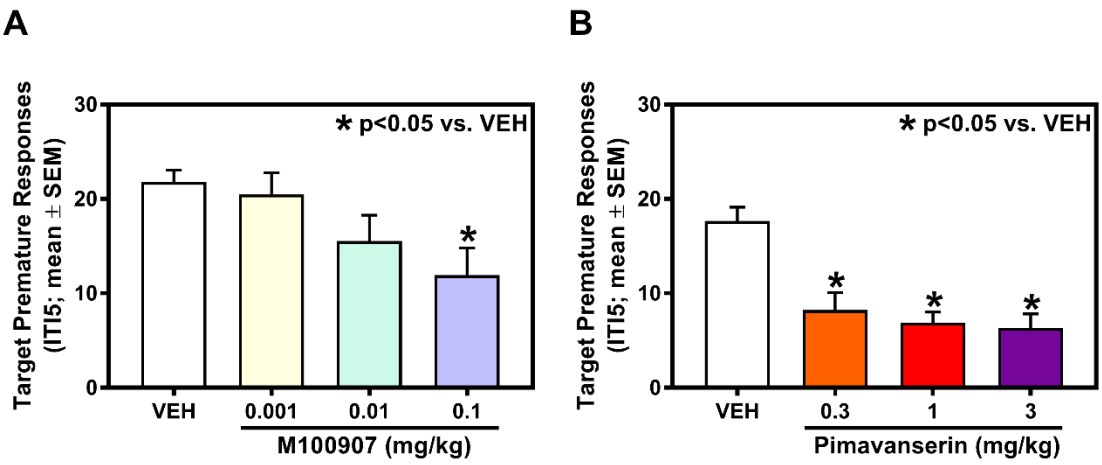

Figure 2.1: M100907 and pimavanserin suppress target premature responses in the 1-CSRT task.

The effects of M100907 (0.001, 0.01, and 0.1 mg/kg; *n* = 11) or pimavanserin (0.3, 1, 3 mg/kg; *n* = 9) were each evaluated under an ITI5 schedule in the 1-CSRT task. (A) M100907 significantly decreased target premature responses at 0.1 mg/kg (**p* < 0.05 vs. VEH). (B) Pimavanserin significantly decreased target premature responses at 0.3, 1, and 3 mg/kg (**p* < 0.05 vs. VEH).

Figure 2.1B displays the mean (± SEM) number of target premature responses following pretreatment with pimavanserin (*n* = 9). There was a main effect of pimavanserin on target premature responses [$F_{3,24}$=17.3; *p* < 0.05]; planned comparisons with Dunnett's procedure showed that 0.3, 1, 3 mg/kg of pimavanserin decreased target premature responses vs. VEH (*p* < 0.05; **Figure 2.1B**). The ID_{50} of pimavanserin to suppress target premature responses was 0.05 mg/kg. In addition to target premature responses, there was a main effect of pimavanserin on total premature responses [$F_{3,24}$=15.8; *p* < 0.05], % omissions [$F_{3,24}$=5.29; *p* < 0.05], and time to finish the 1-CSRT task [$F_{3,24}$=7.79; *p* < 0.05]; planned comparisons with Dunnett's procedure showed that 0.3, 1, and 3 mg/kg of pimavanserin decreased total premature responses while 1 and 3 mg/kg of pimavanserin increased % omissions as well as the time to finish the 1-CSRT task

vs. VEH ($p < 0.05$; **Table 2.1**). There was no main effect of pimavanserin on non-target premature responses [$F_{3,24}$=1.15; n.s.], reinforcers earned [$F_{3,24}$=1.25; n.s.], % accuracy [$F_{3,24}$=0.713; n.s.], or latency to first response in the 1-CSRT task [$F_{3,24}$=2.54; n.s.] vs. VEH (**Table 2.1**).

Pimavanserin suppresses cue reactivity on FA Day 30, but not FA Day 1

Rats (n = 126) were trained in the cocaine self-administration assay following screening on the 1-CSRT task. Rats stably acquired cocaine self-administration (i.e., FR5 schedule; seven infusions/hr for a minimum of three sessions) and displayed < 10% variation in the number of cocaine infusions earned during maintenance (**Figure 2.2A**). Rats were assigned to specific FA Day and medication pretreatment group based upon counterbalancing across target premature responses (**Table 2.2A**) and total cocaine intake across acquisition and maintenance of self-administration. The results of a two-way ANOVA indicated no main effect of FA Day, pretreatment, or FA Day X pretreatment interaction for premature responses (target, non-target, total), reinforcers earned, % accuracy, % omissions, latency to first response, and time to finish the 1-CSRT task between rats assigned to FA Day 1 or FA Day 30 and pimavanserin pretreatment groups (**Table 2.2B**), indicating that rats were appropriately counterbalanced prior to training on cocaine self-administration. Total cocaine intake across self-administration sessions was not different between rats assigned to FA Day 1 (361.8 ± 7.68 mg/kg; n = 68) or FA Day 30 (364.3 ± 7.85 mg/kg; n = 58) [t_{124}=0.234; n.s.].

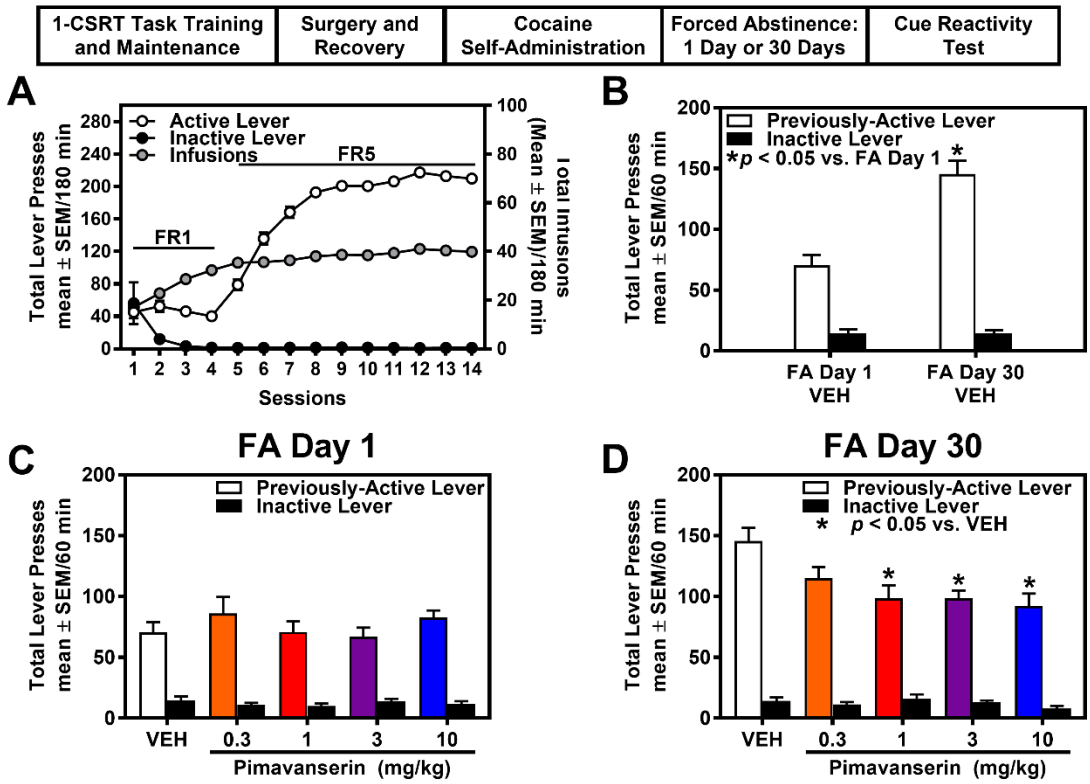

Figure 2.2: Pimavanserin suppresses cue reactivity on FA Day 30, but not FA Day 1, from cocaine self-administration.

(A) Total presses (mean ± SEM) on the active (white circles) or inactive lever (black circles; left Y-axis), and total number of cocaine infusions (mean ± SEM) obtained (gray circles; right Y-axis) are presented for the acquisition and maintenance phase of cocaine self-administration. (B) Previously active and inactive lever presses (mean ± SEM) are presented for the cue reactivity test session in VEH-treated rats on FA Day 1 and FA Day 30 from cocaine self-administration. Cue reactivity is significantly elevated on FA Day 30 vs. FA Day 1 from cocaine self-administration (*$p < 0.05$; n = 11-14/group). The effects of pimavanserin (0.3, 1, 3, 10 mg/kg) on previously active and inactive lever presses (mean ± SEM) on (C) FA Day 1 (n = 12-15/group) or (D) FA Day 30 (n = 11-12/group) from the last cocaine self-administration session are presented. Pimavanserin suppressed previously active, but not inactive, lever presses on FA Day 30 from cocaine self-administration (*$p < 0.05$ vs VEH).

We tested the hypothesis that pimavanserin would suppress cue reactivity (previously active lever presses for the discrete cue complex) during FA from cocaine self-administration. A main effect of FA Day [$F_{1,116}$=34.1; $p < 0.05$], pretreatment [$F_{4,116}$=2.75; $p < 0.05$], and a FA Day X pretreatment interaction [$F_{4,116}$=3.29; $p < 0.05$] were observed for previously active lever presses. Planned

comparisons indicated that previously active lever presses were significantly elevated in rats treated with VEH on FA Day 30 vs. FA Day 1 from cocaine self-administration [$t_{23=}$5.546; $p < 0.05$] (**Figure 2.2B**). Further, planned comparisons revealed that pimavanserin (1, 3, 10 mg/kg) did not alter previously active lever presses on FA Day 1 (**Figure 2.2C**), but did suppress previously active lever presses on FA Day 30 (**Figure 2.2D**), relative to VEH ($p < 0.05$). The ID_{50} of pimavanserin to suppress previously active lever presses was 0.22 mg/kg on FA Day 30; however, the four parameter logistic nonlinear regression was unable to model the data for FA Day 1, and an ID_{50} value was not determinable. There was no main effect of FA Day [$F_{1,116}$=0.038; n.s.], pretreatment [$F_{4,116}$=1.154; n.s.], or a FA Day X pretreatment interaction [$F_{4,116}$=1.003; n.s.] for inactive lever presses in the cue reactivity session. Further, there was no main effect of FA Day [$F_{1,116}$=0.603; n.s.], pimavanserin pretreatment [$F_{4,116}$=0.465; n.s.], or a FA Day X pretreatment interaction [$F_{4,116}$=1.227; n.s.] for latency to first response in the cue reactivity session.

Baseline levels of impulsive action predict the efficacy of pimavanserin to suppress cocaine cue reactivity on FA Day 30

We tested the hypothesis that baseline levels of impulsive action would predict previously active lever presses during the cue reactivity test on FA Day 1 or FA Day 30 in VEH-treated rats (**Figure 2.3A**). We found that the covariate target premature responses predicted the number of previously active lever presses exhibited on FA Day 1 and on FA Day 30 in VEH-treated rats [$F_{1,22}$= 5.081, $p <$

0.05] (**Figure 2.3A**). We also tested the hypothesis that baseline levels of impulsive action would predict the efficacy of pimavanserin to suppress previously active lever presses during the cue reactivity test on FA Day 1 or FA Day 30. There was no relationship between target premature responses and the number of previously active lever presses exhibited on FA Day 1 following pretreatment with VEH or pimavanserin [$F_{4,62}$= 0.405; n.s.] (**Figure 2.3B**). Target premature responses predicted the number of previously active lever presses exhibited on FA Day 30 following pretreatment with VEH or pimavanserin [$F_{4,52}$= 4.04, p = 0.05] (**Figure 2.3C**).

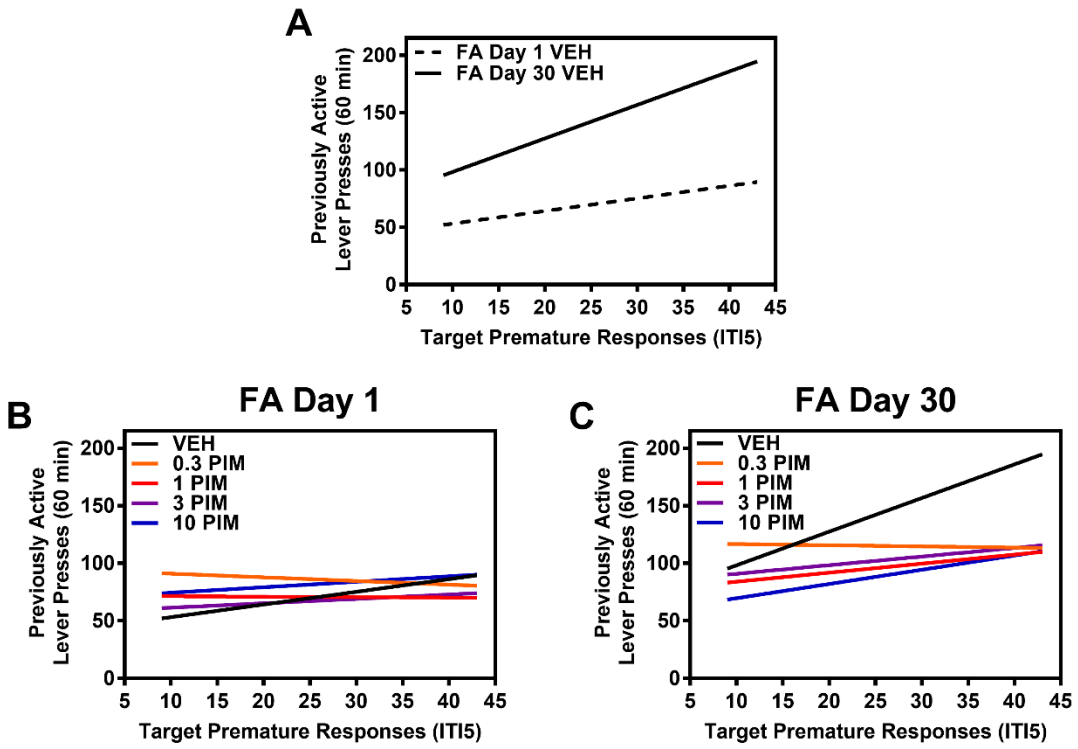

Figure 2.3: Baseline levels of impulsive action predict the efficacy of pimavanserin to suppress cocaine cue reactivity on FA Day 30 from cocaine self-administration.

Target premature responses under an ITI5 schedule in the 1-CSRT task (mean ± SEM) are presented on the X-axis, and previously active lever presses (mean ± SEM) on the cue reactivity test session are presented on the Y-axis. The relationship between target premature responses and previously active lever presses following pretreatment with VEH or pimavanserin (PIM; 0.3, 1, 3, 10 mg/kg) is represented by a linear regression line for each pretreatment condition. (A) Target premature responses predicted the number of previously active lever presses exhibited on FA Day 1 and on FA Day 30 in VEH-treated rats [$F_{1,22}$= 5.081, $p < 0.05$]. (B) There was no relationship between target premature responses and the number of previously active lever presses exhibited on FA Day 1 following pretreatment with VEH or pimavanserin [$F_{4,62}$= 0.405; n.s.]. (C) Target premature responses predicted the number of previously active lever presses exhibited on FA Day 30 following pretreatment with VEH or pimavanserin [$F_{4,52}$= 4.04, p = 0.05].

DISCUSSION

We demonstrated that pimavanserin suppressed impulsive action measured in the 1-CSRT task, similar to M100907 (Anastasio et al., 2011; Anastasio et al., 2015; Cunningham et al., 2013; Fink et al., 2015; Fletcher et al., 2011; Fletcher et al., 2007; Winstanley et al., 2004). Moreover, baseline levels of

impulsive action predicted incubated levels of cocaine cue reactivity on FA Day 30, replicating our previous observations that baseline impulsive action predicted cocaine cue reactivity on FA Day 14 (Anastasio et al., 2014b). We also found that baseline impulsive action predicted the effectiveness of pimavanserin to suppress incubated cue reactivity in late abstinence from cocaine self-administration at doses that were ineffective in early abstinence. Taken together with the extinction-reinstatement studies with M100907 (Filip, 2005; Fletcher et al., 2002; Lacosta and Roberts, 1993; Nic Dhonnchadha et al., 2009), these data suggest that the length of abstinence and associated neuroadapations that drive incubated cocaine cue reactivity are interlocked with 5-HT_{2A}R mechanisms that underlie rapid response impulsivity.

Our findings suggest that identification of baseline levels of impulsive action could be useful in guiding pharmacotherapeutic intervention in CUD. Baseline levels of impulsive action can be defined clinically through self-report questionnaire measures (e.g., BIS; Eysenck Impulsiveness Questionnaire) or behavioral laboratory tasks (e.g., Go/No Go Task; Continuous Performance Test; Stop Signal Task; CSRT tasks) (for reviews) (Hamilton et al., 2015; Moeller et al., 2001a). The integration of clinical measures of impulsive action may inform refined, personalized pharmacotherapeutic intervention for the treatment of relapse vulnerability in CUD and improve patient care for afflicted populations.

Pimavanserin is marketed as a 5-HT_{2A}R inverse agonist to treat Parkinson's disease psychosis (Meltzer et al., 2010; Sahli and Tarazi, 2018; Vanover et al., 2008). Both pimavanserin and M100907 act as 5-HT_{2A}R inverse agonists to

attenuate basal constitutive 5-HT_{2A}R signaling in cells designed with overexpression of the native 5-HT_{2A}R or transfection with a 5-HT_{2A}R mutation targeted to increase constitutive activity (Muntasir et al., 2006; Vanover et al., 2004; Vanover et al., 2006; Weiner et al., 2001). A definitive role for negative 5-HT_{2A}R efficacy in the control of behavior is suggested by a limited literature in conditioned behaviors (Romano et al., 2006; Welsh et al., 1998). For example, reduced constitutive 5-HT_{2A}R activity is proposed to account for the impairment of conditioned learning evoked by the 5-HT_{2A}R antagonist/inverse agonist MDL11,939 (Welsh et al., 1998). Presently, we are unable to definitively attribute the effects of M100907 or pimavanserin to their 5-HT_{2A}R antagonist vs. inverse agonist properties.

Pimavanserin and M100907 promote the maintenance of sleep in humans (Ancoli-Israel et al., 2011; Rosenberg et al., 2008). Notably, sleep disorders (Morgan et al., 2010) as well as psychosis (Caton et al., 2000; Peer et al., 2009) commonly co-occur with CUD, and dopamine replacement therapy in Parkinson's disease elicits side effects including increased risk-taking and impulsive behaviors (Park and Stacy, 2011). Excitingly, our present findings demonstrate that pimavanserin suppressed impulsive action as well as cocaine cue reactivity. Thus, a compelling case can be made that pimavanserin or other 5-HT_{2A}R antagonists/inverse agonists that may be ultimately available for clinical use will be efficacious in suppressing relapse vulnerability and potentially improve concomitant sleep and psychiatric disorders seen in CUD. The marketing of

pimavanserin provides the opportunity to assess the efficacy of pimavanserin to extend abstinence and improve the health status of CUD patients.

Table 2.1: 1-CSRT task descriptive statistics for M100907 and pimavanserin pretreatment (mean ± SEM)

Pretreatment	Dose	Premature Responses			Reinforcers Earned	Accuracy (%)	Omissions (%)	Latency to Start (sec)	Time to Finish (sec)
		Target	Non-target	Total					
M100907	Vehicle	21.8 ± 1.27	1.61 ± 0.265	23.4 ± 1.43	68.4 ± 1.33	97.0 ± 0.433	6.15 ± 0.817	3.38 ± 1.85	872 ± 15.9
	0.001 mg/kg	20.5 ± 2.30	1.91 ± 0.719	22.4 ± 2.32	69.5 ± 1.06	97.1 ± 0.674	6.00 ± 1.95	1.00 ± 0.185	874 ± 23.7
	0.01 mg/kg	15.5 ± 2.71	0.818 ± 0.325	16.4 ± 2.88	71.2 ± 2.60	97.5 ± 0.483	10.6 ± 2.83	2.88 ± 1.49	938 ± 40.6
	0.1 mg/kg	11.9 ± 2.87*	1.36 ± 0.338	13.3 ± 2.86*	66.8 ± 2.12	97.7 ± 0.750	18.3 ± 2.70*	2.17 ± 0.862	1022 ± 35.8*
Pimavanserin	Vehicle	17.7 ± 1.44	0.963 ± 0.286	18.6 ± 1.50	73.6 ± 1.40	97.5 ± 0.704	5.89 ± 0.957	1.44 ± 0.258	859 ± 11.5
	0.3 mg/kg	8.22 ± 1.88*	1.00 ± 0.441	9.22 ± 2.09*	79.6 ± 2.06	98.4 ± 0.631	9.89 ± 1.96	0.613 ± 0.161	898 ± 25.9
	1.0 mg/kg	6.89 ± 1.11*	0.333 ± 0.167	7.22 ± 1.16*	77.2 ± 3.36	98.3 ± 0.674	14.1 ± 3.85*	1.16 ± 0.378	948 ± 41.9*
	3.0 mg/kg	6.33 ± 1.47*	0.667 ± 0.236	7.00 ± 1.53*	74.4 ± 4.11	98.3 ± 0.657	17.1 ± 4.74*	1.51 ± 0.236	984 ± 46.0*

*** p <0.05 vs. Vehicle in each respective pretreatment group**

Table 2.2: 1-CSRT task (A) descriptive statistics (mean ± SEM) and (B) results of two-way ANOVA

(a) Descriptive Statistics									
Forced Abstinence (FA) Day	**Pimavanserin Pretreatment**	**Premature Responses**			**Reinforcers Earned**	**Accuracy (%)**	**Omissions (%)**	**Latency to Start (sec)**	**Time to Finish (sec)**
		Target	**Non-target**	**Total**					
FA Day 1	Vehicle	25.7 ± 2.33	1.43 ± 0.327	27.1 ± 2.26	62.7 ± 1.91	98.6 ± 0.377	9.29 ± 1.98	1.16 ± 0.370	981 ± 49.4
	0.3 mg/kg	25.5 ± 2.00	2.00 ± 0.467	27.5 ± 2.21	61.6 ± 2.36	98.3 ± 0.530	9.77 ± 1.86	1.02 ± 0.221	953 ± 24.4
	1.0 mg/kg	21.1 ± 2.25	1.40 ± 0.306	22.5 ± 2.19	65.2 ± 1.89	98.1 ± 0.371	11.1 ± 1.47	1.17 ± 0.347	948 ± 32.4
	3.0 mg/kg	24.4 ± 2.05	1.21 ± 0.318	25.6 ± 2.11	65.9 ± 2.64	98.5 ± 0.597	7.57 ± 1.34	1.03 ± 0.171	912 ± 22.6
	10.0 mg/kg	27.7 ± 1.76	2.00 ± 0.357	29.7 ± 1.76	61.9 ± 1.85	96.8 ± 0.895	6.58 ± 0.839	1.32 ± 0.241	948 ± 31.8
FA Day 30	Vehicle	26.2 ± 2.28	2.27 ± 0.384	28.5 ± 2.33	60.4 ± 1.91	97.8 ± 0.702	9.91 ± 1.09	1.15 ± 0.299	984 ± 42.6
	0.3 mg/kg	26.9 ± 2.33	1.67 ± 0.541	28.6 ± 2.06	63.4 ± 2.09	98.7 ± 0.568	7.17 ± 1.24	1.57 ± 0.307	935 ± 26.5
	1.0 mg/kg	28.5 ± 2.51	1.36 ± 0.432	29.9 ± 2.71	56.9 ± 2.09	97.5 ± 0.601	11.8 ± 2.42	1.37 ± 0.432	977 ± 44.2
	3.0 mg/kg	20.5 ± 2.28	1.17 ± 0.322	21.7 ± 2.51	66.5 ± 3.29	98.4 ± 0.783	10.9 ± 2.25	1.55 ± 0.469	972 ± 37.2
	10.0 mg/kg	28.3 ± 2.63	1.00 ± 0.275	29.3 ± 2.80	64.1 ± 1.96	99.2 ± 0.323	6.08 ± 1.52	14.6 ± 10.8	943 ± 42.2
(b) Between-Subjects Effects									
		Premature Responses			**Reinforcers Earned**	**Accuracy (%)**	**Omissions (%)**	**Latency to Start (sec)**	**Time to Finish (sec)**
		Target	**Non-target**	**Total**					
FA Day		$F_{1,116}$=0.716	$F_{1,116}$=0.208	$F_{1,116}$=0.563	$F_{1,116}$=0.671	$F_{1,116}$=0.509	$F_{1,116}$=0.087	$F_{1,116}$=1.96	$F_{1,116}$=0.358
Pretreatment		$F_{4,116}$=1.63	$F_{4,116}$=1.06	$F_{4,116}$=1.87	$F_{4,116}$=1.53	$F_{4,116}$=0.513	$F_{4,116}$=2.26	$F_{4,116}$=1.63	$F_{4,116}$=0.451
FA Day x Pretreatment		$F_{4,116}$=1.66	$F_{4,116}$=1.36	$F_{4,116}$=1.63	$F_{4,116}$=1.75	$F_{4,116}$=2.18	$F_{4,116}$=0.787	$F_{4,116}$=1.52	$F_{4,116}$=0.373

Chapter 3: Engagement of the 5-HT$_{2A}$ Receptor in Oxycodone-Evoked Impulsive Action

INTRODUCTION

The misuse of prescription opioids such as the selective μOR agonist oxycodone (Monory et al., 1999; Yoburn et al., 1995) is a major contributor to the drug overdose crisis in the United States (**Chapter 1**). High impulsivity (action without sufficient forethought) (Evenden, 1999a; Moeller et al., 2001a) may predispose individuals to be at higher risk for problematic use of prescription opioids (Garami et al., 2017). The μOR was hypothesized as engaged in impulsive action (motor impulsivity; inability to withhold a premature response) based on the finding that acute administration of the μOR agonist morphine increases premature responses in the 5-CSRT task (Moeller and Cunningham, 2018; Pattij et al., 2009). However, the behavioral pharmacology of opioid-evoked impulsive action has received limited attention to date.

The mechanism of action of systemically administered μOR agonists converges upon limbic-corticostriatal circuit structures (for review) (Nestler, 2005) with systemic administration of morphine increasing extracellular 5-HT levels in the frontal cortex (Tao and Auerbach, 1995). Interestingly, high impulsive action is associated with elevated 5-HT release in the mPFC (Dalley et al., 2002; Puumala and Sirviö, 1998) while systemically-administered 5-HT$_{2A}$R antagonist/inverse agonists decrease premature responses in the 1- and 5-CSRT tasks (**Chapter 2**) (Anastasio et al., 2011; Anastasio et al., 2015; Cunningham et al., 2013; Fink et

al., 2015; Fletcher et al., 2011; Fletcher et al., 2007; Sholler et al., 2018; Winstanley et al., 2004). Moreover, systemic administration of a 5-HT_{2A}R antagonist/inverse agonist reduces morphine-evoked hyperactivity (Auclair et al., 2004; Pang et al., 2016). However, the efficacy of a 5-HT_{2A}R antagonist/inverse agonist to modulate opioid-evoked impulsive action is currently unknown. We tested the hypothesis that oxycodone would dose-dependently increase impulsive action assessed in the 1-CSRT task. Further, we hypothesized that the FDA-approved 5-HT_{2A}R antagonist/inverse agonist pimavanserin (**Chapter 2**) would decrease oxycodone-evoked impulsive action assessed in the 1-CSRT task.

METHODS

General Methods

ANIMALS. Male, Sprague–Dawley rats (*n* = 24; Envigo, Haslett, MI) weighed 250–275 g upon arrival and were housed in a colony room. Rats were housed two/cage under a 12-h light–dark cycle with maintained temperature (21–23°C) and humidity (45–50%). Prior to handling, rats were first acclimated to the colony room for seven days. Rats were maintained at ~90% free-feeding weight during 1-CSRT task training and were provided with access to water *ad libitum* except during daily operant sessions. All experiments were conducted in accordance with the National Institutes of Health *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.

DRUGS. Oxycodone [(4R,4aS,7aR,12bS)-4a-hydroxy-9-methoxy-1,2,3,4,5,6,7a,13-octahydro-4,12-methanobenzofuro[3,2-e]isoquinoline-7-one] hydrochloride (Millipore Sigma, St. Louis, MO) was dissolved in vehicle (VEH; 0.9% NaCl). Pimavanserin [1-(4-fluorobenzyl)-3-(4-isobutoxybenzyl)-1-(1-methylpiperidin-4-yl)urea] (Trylead Chemical Technology Co., Ltd., Hangzhou, China) was dissolved in VEH (0.9% NaCl). Oxycodone and pimavanserin were administered by the s.c. route.

1-CHOICE SERIAL REACTION TIME (1-CSRT) TASK. 1-CSRT task sessions occurred in five-hole, nose-poke operant chambers with a houselight, food receptacle, and an external pellet dispenser that delivered 45 mg grain-based food pellets (Bio-Serv, Frenchtown, NJ) housed within ventilated, sound-attenuated cubicles (MedAssociates, St Albans, VT). A detailed 1-CSRT task methodology is published (Anastasio et al., 2013; Anastasio et al., 2011; Anastasio et al., 2014b; Cunningham et al., 2013; Fink et al., 2015; Sholler et al., 2018). In sum, rats underwent a pre-training stage that included habituation to the test chamber. A nose-poke response into the illuminated center hole (i.e., a “target” response) resulted in delivery of a food pellet that was paired with the illumination of the food receptacle light. Rats then continued through a series of training stages that lasted 100 trials or 30 min, whichever occurred first. The stimulus duration was incrementally shortened at each training stage until a final stage of 0.5 sec was achieved with a limited hold of 5 sec and an ITI5.

In each session, a maximum of 100 target responses yielded a maximum of 100 reinforcers earned. Incorrect “non-target” responses, premature responses,

or omissions resulted in a 5 sec time-out period that decreased the potential number of reinforcers earned. At each training stage, rats were required to meet acquisition criteria: ≥ 50 reinforcers earned, > 80% accuracy (target responses/(target + non-target responses) * 100) and < 20% omissions (omitted responses/trials completed * 100) (Anastasio et al., 2013; Anastasio et al., 2011; Anastasio et al., 2014b; Cunningham et al., 2013; Fink et al., 2015; Sholler et al., 2018).

The number of premature responses, omissions, and reinforcers earned, percent accuracy, latency to first response, and time to finish the 1-CSRT task were recorded. Premature responses primarily assessed impulsive action and were categorized into three types: target, non-target, and total (target + non-target) (Sholler et al., 2018). The number of reinforcers earned measured task competency and provided a secondary measure of impulsive action. Percent accuracy indicated attentional capacity. Percent omissions assessed motivation to perform the task, and the latency to first response in the 1-CSRT task gave a secondary measure of motivation and an assessment of general motor impairment. Five rats failed to maintain stable responding under the ITI5 schedule in the 1-CSRT task and were excluded from analysis (*n* = 19 rats analyzed).

Research Design

Dose-effect relationship for oxycodone in the 1-CSRT task. Rats were required to meet acquisition criteria in the 1-CSRT task [≥ 50 target responses, > 80% accuracy, and < 20% omissions on the final training stage (0.5 sec stimulus

duration, 5 sec limited hold, and ITI5)] for at least three consecutive days. Performance in the 1-CSRT task was assessed following systemic administration of oxycodone. Treatment with VEH (1 mL/kg, s.c.) or oxycodone (0.2, 0.5, 1, 2 mg/kg, s.c.) occurred 30 min prior to the start of the 1-CSRT task session under an ITI5 schedule.

Effects of pimavanserin on oxycodone-evoked impulsive action. Following pharmacological evaluations with oxycodone, rats (*n* = 19) were restablized on the ITI5 schedule. Rats were pretreated with vehicle or pimavanserin (0.3 mg/kg) (Sholler et al., 2018) immediately prior to treatment with vehicle or oxycodone (0.5 mg/kg) 30 min prior to the start of the 1-CSRT task session. Rats received VEH injections on the session preceding the test session, and tests were separated by a minimum of three days. To control for order effects, drug doses were administered in random sequence to individual rats across pharmacological test sessions. Pharmacological test sessions in the 1-CSRT were conducted using a within-subjects design.

Statistical Analyses

A repeated measures one-way ANOVA was employed to evaluate the effects of oxycodone (vehicle, 0.2, 0.5, 1, 2 mg/kg) on 1-CSRT task measures (premature responses, reinforcers earned, % omissions, % accuracy, latency to first response, time to finish the 1-CSRT task). Subsequent *a priori* comparisons were analyzed using Dunnett's procedure. A repeated-measures two-way ANOVA was employed to analyze behavioral endpoints with the factors of pretreatment

(VEH, pimavanserin) and treatment (VEH, oxycodone); subsequent *a priori* comparisons were analyzed using Bonferroni's method. Behavioral endpoints included premature responses, reinforcers earned, % omissions, % accuracy, latency to first response, and time to finish the 1-CSRT task. An experiment-wise error rate of $\alpha = 0.05$ was utilized for all analyses.

RESULTS

Oxycodone dose-dependently alters 1-CSRT task measures

We tested the hypothesis that oxycodone would increase impulsive action measured in the 1-CSRT task relative to VEH. **Figure 3.1** displays the mean (± SEM) number of target premature responses, reinforcers earned, and percent omissions following treatment with VEH or oxycodone ($n = 19$). There was a main effect of oxycodone on target premature responses [$F_{4,72}=10.23$; $p < 0.05$; **Figure 3.1A**], reinforcers earned [$F_{4,72}=39.01$; $p < 0.05$; **Figure 3.1B**], and % omissions [$F_{4,72}=63.45$; $p < 0.05$; **Figure 3.1C**]. Planned comparisons with Dunnett's procedure showed that 0.5 and 1 mg/kg of oxycodone increased target premature responses vs. VEH ($p < 0.05$; **Figure 3.1A**). Planned comparisons with Dunnett's procedure showed that 0.5, 1 and 2 mg/kg of oxycodone decreased reinforcers earned vs. VEH ($p < 0.05$; **Figure 3.1B**) while 2 mg/kg of oxycodone increased % omissions vs. VEH ($p < 0.05$; **Figure 3.1C**).

There was a main effect of oxycodone on total premature responses [$F_{4,72}=10.47$; $p < 0.05$] and time to finish the 1-CSRT task [$F_{4,72}=50.67$; $p < 0.05$] (**Table 3.1**). Planned comparisons with Dunnett's procedure showed that 0.5 and

1 mg/kg of oxycodone increased total premature responses while 2 mg/kg of oxycodone increased the time to finish the 1-CSRT task vs. VEH ($p < 0.05$; **Table 3.1**). There was a main effect of oxycodone on non-target premature responses [$F_{4,72}=3.96$; $p < 0.05$]; however, Dunnett's procedure revealed no significant differences between any dose of oxycodone tested vs. VEH (n.s., **Table 3.1**). There was no main effect of oxycodone on latency to first response in the 1-CSRT task vs. VEH [$F_{4,72}=1.93$; n.s.; **Table 3.1**]. Following administration with 2 mg/kg oxycodone, some rats ($n = 5$) failed to make any target responses; this precluded the statistical analyses of % accuracy via the repeated measures one-way ANOVA. However, the mean for percent accuracy following oxycodone (0.2 – 1 mg/kg) was ≥ 97% (**Table 3.1**), and an ordinary one-way ANOVA indicated no main effect of oxycodone on percent accuracy in the 1-CSRT task vs. VEH [$F_{4,85}=1.77$; n.s.; Table 3.1].

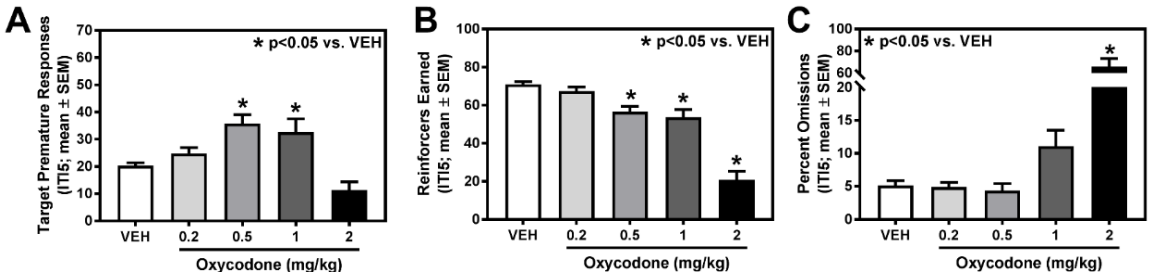

Figure 3.1: Oxycodone alters1-CSRT task measures.

The effects of oxycodone (0.2, 0.5, 1, 2 mg/kg; $n = 19$) were evaluated under an ITI5 schedule in the 1-CSRT task. (A) Target premature responses, (B) reinforcers earned, and (C) percent omissions are presented. Oxycodone significantly increased target premature responses and decreased reinforcers earned at 0.5 and 1 mg/kg (*$p < 0.05$ vs. VEH). At 2 mg/kg, oxycodone decreased reinforcers earned and increased percent omissions.

Pimavanserin decreases oxycodone-evoked impulsive action in the 1-CSRT task

We tested the hypothesis that pimavanserin (0.3 mg/kg) would decrease oxycodone-evoked (0.5 mg/kg) impulsive action in the 1-CSRT task. **Figure 3.2** displays the mean (± SEM) number of target premature responses following pretreatment with VEH + VEH, VEH + oxycodone, pimavanserin + VEH, or pimavanserin + oxycodone. There was a main effect of oxycodone treatment [$F_{1,18}$=63.12; $p < 0.05$] and pimavanserin pretreatment [$F_{1,18}$=58.55; $p < 0.05$] but no interaction [$F_{1,18}$=2.51; n.s.] observed for target premature responses (**Figure 3.2A**). Planned comparisons with Bonferroni's method revealed that 0.3 mg/kg pimavanserin decreased while 0.5 mg/kg oxycodone increased total premature responses vs. VEH-VEH ($p < 0.05$). The combination of 0.3 mg/kg pimavanserin *plus* 0.5 mg/kg oxycodone reduced total premature responses vs. treatment with 0.5 mg/kg oxycodone alone ($p < 0.05$; **Figure 3.2A**).

There was a main effect of oxycodone treatment [$F_{1,18}$=55.77; $p < 0.05$] and pimavanserin pretreatment [$F_{1,18}$=54.33; $p < 0.05$] but no interaction [$F_{1,18}$=1.38; n.s.] observed for total premature responses (**Table 3.2**). Planned comparisons with Bonferroni's method revealed that 0.3 mg/kg pimavanserin decreased while 0.5 mg/kg oxycodone increased total premature responses vs. VEH-VEH ($p < 0.05$), and the combination of 0.3 mg/kg pimavanserin *plus* 0.5 mg/kg oxycodone reduced total premature responses vs. treatment with 0.5 mg/kg oxycodone alone ($p < 0.05$; **Table 3.2**). There was a main effect of oxycodone treatment [$F_{1,18}$=41.63; $p < 0.05$] and pimavanserin pretreatment [$F_{1,18}$=5.369; $p < 0.05$] but no interaction [$F_{1,18}$=4.273; n.s.] observed for reinforcers earned (**Figure 3.2B**). Planned comparisons with Bonferroni's method revealed that 0.5 mg/kg

oxycodone decreased reinforcers earned vs. VEH-VEH (p < 0.05), and the combination of 0.3 mg/kg pimavanserin *plus* 0.5 mg/kg oxycodone increased reinforcers earned vs. treatment with 0.5 mg/kg oxycodone alone (p < 0.05; **Figure 3.2B**). There was a main effect of oxycodone treatment [$F_{1,18}$=6.194; p < 0.05] and pimavanserin pretreatment [$F_{1,18}$=12.69; p < 0.05] but no interaction [$F_{1,18}$=3.756; n.s.] observed for % omissions (**Figure 3.2C**). Planned comparisons with Bonferroni's method revealed that 0.3 mg/kg pimavanserin modestly increased % omissions vs. VEH-VEH (p < 0.05; **Figure 3.2C**).

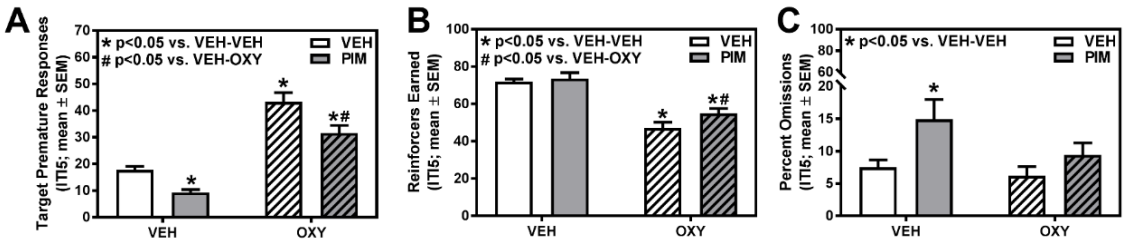

Figure 3.2: Pimavanserin decreases oxycodone-evoked impulsive action in the 1-CSRT task.

The effects of oxycodone (OXY; 0.5 mg/kg), pimavanserin (PIM; 0.3 mg/kg), or their combination were evaluated under ITI5 conditions in the 1-CSRT task (n = 19). (A) Target premature responses, (B) reinforcers earned, and (C) percent omissions are presented. Pimavanserin significant decreased target premature responses and increased percent omissions (*p < 0.05 vs. VEH-VEH). Oxycodone significantly increased target premature responses and decreased reinforcers earned (*p < 0.05 vs. VEH-VEH). The combination of oxycodone *plus* pimavanserin significantly decreased target premature responses and increased reinforcers earned relative to treatment with oxycodone alone (*p < 0.05 vs. VEH-OXY).

There was a main effect of pimavanserin pretreatment [$F_{1,18}$=6.670; p < 0.05] but no main effect of oxycodone treatment [$F_{1,18}$=4.24; n.s.] and no interaction [$F_{1,18}$=2.59; n.s.] observed for time to finish the 1-CSRT task (**Table 3.2**). There was a main effect of oxycodone treatment [$F_{1,18}$=6.20; p < 0.05] but no main effect of pimavanserin pretreatment [$F_{1,18}$=0.01; n.s.] and no interaction

[$F_{1,18}$=0.23; n.s.] observed for non-target premature responses (**Table 3.2**). Planned comparisons with Bonferroni's method identified no significant group differences for time to finish the 1-CSRT task or non-target premature responses (p < 0.05; **Table 3.2**). There was no main effect of oxycodone treatment [$F_{1,18}$=1.08; n.s.], pimavanserin pretreatment [$F_{1,18}$=0.83; n.s.] and no interaction [$F_{1,18}$=2.87; n.s.] observed for % accuracy (**Table 3.2**). There was no main effect of oxycodone treatment [$F_{1,18}$=2.05; n.s.], pimavanserin pretreatment [$F_{1,18}$=1.10; n.s.] and no interaction [$F_{1,18}$=0.14; n.s.] observed for latency to first response (**Table 3.2**).

DISCUSSION

We demonstrated that oxycodone (0.5 – 1 mg/kg) increased impulsive action measured in the 1-CSRT task, consistent with previous reports that the μOR agonist morphine increases premature responses in the 5-CSRT task (Pattij et al., 2009). The highest dose of oxycodone evaluated (2 mg/kg) dramatically increased percent omissions similar to morphine (Pattij et al., 2009), suggesting impaired behavior that impedes 1-CSRT task performance. Additionally, we found that pretreatment with the 5-HT_{2A}R antagonist/inverse agonist pimavanserin (0.3 mg/kg) suppressed premature responses evoked by the lowest effective dose of oxycodone (0.5 mg/kg). An interesting additional finding was that pimavanserin modestly increased percent omissions within 1-CSRT task criteria in the absence of oxycodone, which is consistent with decreased motivation to obtain a food reinforcer. However, there was no effect of pimavanserin pretreatment on percent

omissions in the presence of oxycodone. Intriguingly, the μOR agonist morphine increases feeding when administered systemically (Bakshi and Kelley, 1993; Hoebel, 1984; Kelley et al., 2002; Leibowitz, 1985; Levine and Billington, 1989; Levine et al., 1985; Reid, 1985) while systemic administration of non-specific 5-HT_{2A}R antagonists/inverse agonists attenuates feeding (Arolfo and McMillen, 1999; Gasque et al., 2013; Nonogaki et al., 2006). Thus, the efficacy of pimavanserin to decrease oxycodone-evoked impulsive action could be linked to decreased goal-directed behavior that mediates pursuit of food reward (Berridge and Robinson, 2003), which is a target for future studies.

The efficacy of a 5-HT_{2A}R antagonist/inverse agonist to decrease impulsive action evoked by oxycodone likely involves multiple nodes of the limbic-corticostriatal circuit, including the ventral tegmental area (VTA), mPFC, and NAc. Canonically, μOR agonists evoke their reinforcing effects through μOR receptors localized to VTA γ-aminobutyric (GABA) neurons, which disinhibit VTA DA neurons to increase DA efflux in projection targets such as the NAc and mPFC (Fields and Margolis, 2015; Johnson and North, 1992; Koob and Volkow, 2010; Koob and Volkow, 2016; Wise, 1996). Interestingly, levels of DA and its metabolite DOPAC rise in the mPFC of rats during performance of the 1-CSRT task, while highly impulsive rats have higher DA turnover rates in the mPFC (Dalley et al., 2002). Thus, oxycodone-evoked DA release in the mPFC or NAc may represent one mechanism by which oxycodone increases impulsive action.

Meanwhile, intra-mPFC infusion of the preferential 5-HT_{2A}R agonist DOI increases (Passetti et al., 2003; Winstanley et al., 2003; Wischhof et al., 2011)

while selective 5-HT_{2A}R antagonists/inverse agonists decrease impulsive action (Anastasio et al., 2011; Anastasio et al., 2015; Cunningham et al., 2013; Fink et al., 2015; Fletcher et al., 2011; Fletcher et al., 2007; Sholler et al., 2018; Winstanley et al., 2004). The 5-HT_{2A}R is present on approximately 55% of pyramidal neurons in the mPFC that project to the VTA (Vazquez-Borsetti et al., 2009), and there is a general consensus that 5-HT_{2A}R activation stimulates DA release within limbic-corticostriatal circuits based on pharmacological studies with 5-HT_{2A}R agonists and antagonists/inverse agonists (for review) (Howell and Cunningham, 2015). For example, both systemic and direct administration of DOI into the PFC increases the firing rate of VTA DA neurons and increases DA release in the VTA and PFC, effects blocked by the selective 5-HT_{2A}R antagonist/inverse agonist M100907 (Bortolozzi et al., 2005). Furthermore, systemic DOI increases glutamate release in the VTA, which is blocked by intra-PFC infusion of M100907 (Pehek et al., 2006). Together, these data suggest that a 5-HT_{2A}R antagonist/inverse agonist like pimavanserin may decrease mPFC pyramidal cell glutamate activity and decrease DA release in limbic-corticostriatal circuit structures including the mPFC and NAc (Bortolozzi et al., 2005; Pehek et al., 2006), presenting an important mechanism by which the 5-HT_{2A}R may decrease inherent and oxycodone-evoked impulsive action (Koskinen et al., 2003; Winstanley et al., 2004). Further exploration into the complex regulation of the 5-HT_{2A}R over 5-HT and DA neurotransmission in limbic-corticostriatal circuits may clarify the utility of 5-HT_{2A}R antagonist/inverse agonists to decrease opioid-evoked impulsive action.

Table 3.1: 1-CSRT task descriptive statistics for oxycodone treatment (mean ± SEM)

Treatment	Dose	Premature Responses			Reinforcers Earned	Accuracy (%)	Omissions (%)	Latency to Start (sec)	Time to Finish (sec)
		Target	Non-Target	Total					
Oxycodone	VEH	20.3 ± 1.12	1.53 ± 0.36	21.8 ± 1.19	70.9 ± 1.45	97.0 ± 0.47	5.16 ± 0.72	1.61 ± 0.18	882 ± 16.1
	0.2 mg/kg	24.7 ± 2.21	1.42 ± 0.29	26.2 ± 2.27*	67.3 ± 2.24	97.5 ± 0.72	4.89 ± 0.73	1.19 ± 0.24	882 ± 23.4
	0.5 mg/kg	35.8 ± 3.23*	2.26 ± 0.46	38.1 ± 3.40*	56.4 ± 2.97*	97.8 ± 0.48	4.37 ± 1.05	1.35 ± 0.20	860 ± 11.1
	1 mg/kg	32.6 ± 4.84*	1.68 ± 0.50	34.3 ± 5.12	53.7 ± 4.04*	98.4 ± 4.04	11.1 ± 2.39	1.36 ± 0.23	919 ± 25.8
	2 mg/kg	11.4 ± 2.93	0.42 ± 0.18	11.8 ± 3.02	20.7 ± 4.62*	N.D.	65.8 ± 7.36*	51.5 ± 36.0	1367 ± 59.3*

*** p <0.05 vs. Vehicle**

Table 3.2: 1-CSRT task (A) descriptive statistics and (B) within-subjects effects for pimavanserin pretreatment ± oxycodone treatment (mean±SEM)

(a) Descriptive Statistics									
Pretreatment	**Treatment**	**Premature Responses**			**Reinforcers Earned**	**Accuracy (%)**	**Omissions (%)**	**Latency to Start (sec)**	**Time to Finish (sec)**
		Target	**Non-Target**	**Total**					
VEH	**VEH**	17.8 ± 1.26	1.23 ± 0.20	19.1 ± 1.31	71.8 ± 1.44	97.7 ± 0.45	7.49 ± 1.15	1.33 ± 0.23	916 ± 29.3
Pimavanserin	**VEH**	9.32 ± 1.05*	1.00 ± 0.23	10.3 ± 1.16*	73.6 ± 3.11	98.4 ± 0.41	14.9 ± 3.07*	1.17 ± 0.25	994 ± 41.5
VEH	**Oxycodone**	43.3 ± 3.46*	2.58 ± 0.82	45.8 ± 3.92*	47.2 ± 2.95*	98.1 ± 0.52	6.21 ± 1.42	1.08 ± 0.23	903 ± 30.9
Pimavanserin	**Oxycodone**	31.5 ± 2.94#	2.74 ± 0.74	34.3 ± 3.31#	54.8 ± 2.70#	96.9 ± 0.94	9.42 ± 1.85#	0.81 ± 0.10	918 ± 26.5
*** p<0.05 vs. VEH-VEH; # p<0.05 vs. VEH-Oxycodone**									
(b) Within-Subjects Effects									
		Premature Responses			**Reinforcers Earned**	**Accuracy (%)**	**Omissions (%)**	**Latency to Start (sec)**	**Time to Finish (sec)**
		Target	**Non-target**	**Total**					
Pretreatment		$F_{1,18}$=58.6	$F_{1,18}$=0.01	$F_{1,18}$=54.3	$F_{1,18}$=5.37	$F_{1,18}$=0.83	$F_{1,18}$=12.7	$F_{1,18}$=1.10	$F_{1,18}$=6.70
Treatment		$F_{1,18}$=63.1	$F_{1,18}$=6.20	$F_{1,18}$=55.8	$F_{1,18}$=41.6	$F_{1,18}$=1.08	$F_{1,18}$=6.19	$F_{1,18}$=2.05	$F_{1,18}$=4.24
Pretreatment x Treatment		$F_{1,18}$=2.51	$F_{1,18}$=0.23	$F_{1,18}$=1.38	$F_{1,18}$=4.27	$F_{1,18}$=2.87	$F_{1,18}$=3.76	$F_{1,18}$=0.14	$F_{1,18}$=2.59

Chapter 4: Exploring 5-HT$_{2A}$ Receptor Involvement in the Medial Prefrontal Cortex as a Neurobiological Driver of Impulsive Action

INTRODUCTION

The Office of Disease Prevention and Health Promotion highlights substance abuse and the progression to SUDs as a nationwide health improvement priority for the "Healthy People 2020" campaign. Impulsivity is linked to SUDs including CUD and OUD (Verdejo-Garcia et al., 2008). Both cocaine- (Coffey et al., 2003; Moeller et al., 2004) and heroin-dependent users (Kirby et al., 1999; Madden et al., 1997) exhibit high levels of impulsivity on self-report questionnaire measures. Moreover, high levels of impulsivity predict treatment dropout in CUD participants (Moeller et al., 2001b; Patkar et al., 2004) while high impulsivity is evident during active opioid intake and abstinence (Jones et al., 2016; Schippers et al., 2012; Verdejo-Garcia et al., 2008; Winstanley et al., 2010). Thus, a comprehensive understanding of the neurobiological circuitry and substrates underlying impulsivity will provide new approaches to suppress impulsive misuse of cocaine and opioids and enhance our efforts to decrease relapse vulnerability and improve treatment outcomes in SUDs.

The rodent mPFC, particularly the ventral subregion, and its striatal connections modulate impulsive action as demonstrated by lesion, reversible inactivation, and genetic manipulation (Anastasio et al., 2014b; Chudasama et al., 2003; Muir et al., 1996; Narayanan et al., 2006). The 5-HT$_{2A}$R is expressed in high

density in the rodent mPFC (Miner et al., 2000) and is important in establishing cortical excitatory/inhibitory balance (Puig et al., 2003). Importantly, we found that stably-identified HI rats exhibit a higher density of synaptosomal 5-HT_{2A}R protein expression in mPFC vs. LI rats (Anastasio et al., 2015; Fink et al., 2015). Moreover, HI rats exhibit a greater number of head-twitch responses induced by the preferential 5-HT_{2A}R agonist DOI and greater pharmacological sensitivity to the effects of the selective 5-HT_{2A}R antagonist/inverse agonist M100907 to decrease impulsive action relative to LI rats (Fink et al., 2015). However, the effect of intra-mPFC infusion of 5-HT_{2A}R antagonists/inverse agonists to suppress impulsive action are mixed, with decreases in impulsive action assessed in the 5-CSRT task reported in some studies (Passetti et al., 2003; Winstanley et al., 2003), while others found no effect (Mirjana et al., 2004; Robinson et al., 2008). Thus, novel pharmacological strategies are needed to clarify the role of the 5-HT_{2A}R in the mPFC to regulate impulsive action.

Genetic manipulation of 5-HT_{2A}R in the mPFC was employed to assess the causal directionality in the association between 5-HT_{2A}R density and impulsive action (Fink et al., 2015). The present study was designed to test the hypothesis that viral-mediated knockdown of the 5-HT_{2A}R in the mPFC decreases impulsive action in the 1-CSRT task. Moreover, we hypothesized that engineered loss of the 5-HT_{2A}R in the mPFC would decrease pharmacological sensitivity to the suppressive effects of the 5-HT_{2A}R antagonist/inverse agonist pimavanserin in the 1-CSRT task. Lastly, we hypothesized that 5-HT_{2A}R knockdown in the mPFC

would decrease sensitivity to the preferential 5-HT_{2A}R agonist DOI in the head-twitch response assay.

METHODS

General Methods

ANIMALS. Male, Sprague–Dawley rats (*n* = 48; Envigo, Haslett, MI) weighed 250–275 g upon arrival and were housed in the colony room. Rats were housed two/cage under a 12-h light–dark cycle with monitored and controlled temperature (21–23°C) and humidity levels (45–50%). Rats were acclimated to the colony room for seven days before handling and experimentation commenced. Rats were food restricted to ~90% free-feeding weight (confirmed by daily weights) during 1-CSRT task training and were provided with access to water *ad libitum* except during daily operant sessions. All experiments were conducted in accordance with the National Institutes of Health *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. Investigators conducting the experiments were blinded to all treatment assignments during ligand administration and endpoint analyses.

DRUGS. Pimavanserin [1-(4-fluorobenzyl)-3-(4-isobutoxybenzyl)-1-(1-methylpiperidin-4-yl)urea] (Trylead Chemical Technology Co., Ltd., Hangzhou, China) was dissolved in VEH (0.9% NaCl, brought to pH ~6.0 using 1M NaOH). (-)-2,5-dimethoxy-4-iodoamphetamine (DOI; Sigma-Aldrich, St. Louis, MO) was dissolved in VEH (0.9% NaCl). Pimavanserin and DOI were administered by the s.c. route.

SHORT HAIRPIN RNA (SHRNA) DESIGN AND PRODUCTION. A 20-nucleotide sequence within the coding region of the *HTR2A* was identified using methods we have previously reported (Anastasio et al., 2015; Anastasio et al., 2014b; Hommel et al., 2003). Two sets of oligonucleotides (Integrated DNA Technology, Coralville, IA) for cloning were synthesized [(5-HT_{2A}R shRNA; top, 5′-TTTGAACTGCAGTGGCTTTCTGTTTTCCTTCCTGTCAGAAAACAGAAAGCCACTGCAGTTATTTTT-3′; bottom, 5′-CTAGAAAAATAACTGCAGTGGCTTTCTGTTTTCTGACAGGAAGGAAAACAGAAAGCCACTGCAGTTC-3′); non-silencing control (NSC) shRNA (top, 5′-TTTGTGGAGCCGAGTTTCTAAATTCCGCTTCCTGTCACGGAATTTAGAAACCCGGCTCCAATTTTT-3′ bottom, 5′-CTAGAAAAATTGGAGCCGGGTTTCTAAATTCCGTGACAGGAAGCGGAATTTAGAAACTCGGCTCCAC-3′)]. Oligonucleotides were designed with Sap1 and Xbal overhangs to allow ligation downstream of the mU6pro region of a modified plasmid adeno-associated virus (AAV) plasmid AAV-MCS vector, plasmid AAV-shRNA, which was designed to co-express hairpin RNAs, under the control of a mU6pro and an SV40 polyadenylation site, as well as enhanced green fluorescent protein (eGFP) controlled by an independent cytomegalovirus (CMV) promoter and hGH polyadenylation sequence (Anastasio et al., 2015; Anastasio et al., 2014b; Hommel et al., 2003). AAV serotype type 2 (AAV2) vectors were packaged using a helper-free packaging system (Life Technologies) and purified viral stocks were assayed in camptothecin-treated HT1080 cells to confirm titers of $1 \times 10^{12\text{-}13}$ transducing units/mL.

1-Choice Serial Reaction Time (1-CSRT) task. All sessions conducted in the 1-CSRT task occurred in five-hole, nose-poke operant chambers containing a houselight, food tray, and an external pellet dispenser that delivered 45 mg dustless precision food pellets (Bio-Serv, Frenchtown, NJ) housed within ventilated, sound-attenuated cubicles (MedAssociates, St Albans, VT). The 1-CSRT task methodology is previously reported in detail (Anastasio et al., 2013; Anastasio et al., 2011; Anastasio et al., 2014b; Cunningham et al., 2013; Fink et al., 2015; Sholler et al., 2018). To summarize, rats were initially exposed to a pre-training stage during which they were habituated to the test chamber. A nose-poke response into the illuminated center stimulus hole (i.e., a "target" response) resulted in the simultaneous illumination of the magazine light on the opposite chamber wall and the delivery of a 45 mg food pellet. Rats progressed through a series of training stages following completion of the pre-training stage. Each stage consisted of daily 100-trial sessions to be completed in a maximum of 30 min. The stimulus duration was incrementally shortened throughout each training stage until a final stage of 0.5 sec was achieved with a limited hold of 5 sec and ITI5.

A maximum of 100 target responses in a session resulted in a maximum of 100 reinforcers delivered. Incorrect "non-target" responses, premature responses, or omissions resulted in a time-out period (5 sec) that reduced the potential number of reinforcers delivered. Before progressing through each training stage, rats were required to achieve acquisition criteria: ≥ 50 reinforcers earned, > 80% accuracy (target responses/(target + non-target responses) * 100) and < 20% omissions (omitted responses/trials completed * 100) (Anastasio et al., 2013; Anastasio et

al., 2011; Anastasio et al., 2014b; Cunningham et al., 2013; Fink et al., 2015; Sholler et al., 2018).

The number of premature responses, omissions, and reinforcers earned, percent accuracy, latency to first response, and time to finish the 1-CSRT task were recorded. Premature responses, the primary output measure to assess impulsive action, were categorized into three types: target, non-target, and total (target + non-target) (Sholler et al., 2018). The number of reinforcers earned assessed task competency and a secondary measure of impulsive action. The percent accuracy was a general indication of attentional capacity. Percent omissions indicated motivation to perform the task, and latency to first response in the 1-CSRT task provided a secondary measure of motivation and an indication of general motor impairment.

5-HT$_{2A}$R PROTEIN ANALYSES. The mPFC was homogenized in 10X w/v extraction buffer (20 mm HEPES, 10 µL/mL protease inhibitor cocktail and 10 µL/mL phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich, St Louis, MO). The homogenate was centrifuged at 1000 g for 10 min at 4 °C to pellet the nuclear fraction. The postnuclear supernatant was supplemented with 100 mM NaCl and 0.5% NP-40 (Anastasio et al., 2013; Anastasio et al., 2010). The postnuclear supernatant was probed for 5-HT$_{2A}$R protein expression using the Wes™ automated western blotting system (ProteinSimple, San Jose, CA), which utilizes capillary electrophoresis-based immunodetection (Anastasio et al., 2015; Fink et al., 2015; Swinford-Jackson et al., 2016). 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. Postnuclear supernatant protein (1-3 µg) was 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 min. Levels of 5-HT_{2A}R were assessed using a polyclonal rabbit antibody (LS-C172270, LifeSpan Biosciences, Seattle, WA) at a concentration of 1:500 (Fink et al., 2015). Separation electrophoresis (375 V, 31 min, 25 °C) and immunodetection in the capillaries were fully automated using the following settings: separation matrix load for 200-s, stacking matrix load for 14-s, sample load for 7-s, antibody diluent for 30 min, primary antibody incubation for 60 min, secondary antibody incubation for 30 min, and chemiluminescent signal exposure for 1-s, 2-s, 4-s, 8-s, 16-s, 32-s, 64-s, 128-s, and 512-s. Data analyses were performed using the Compass Software (ProteinSimple). The Western blot analysis signal was defined as the area under the curve for the 5-HT_{2A}R peak normalized to total biotinylated protein per capillary, and representative "virtual blot" electrophoretic images for 5-HT_{2A}R were automatically generated by the Compass Software (ProteinSimple).

Research Design

Effect of 5-HT_{2A}R knockdown in the mPFC on 1-CSRT task performance. Rats (*n* = 19 analyzed) were trained to stability on the 1-CSRT task

(with < 20% variability over last three sessions); five rats were excluded from all analyses due to failure to maintain stable performance throughout behavioral evaluations. Prior to stereotaxic surgery, an ITI8 challenge session was conducted in which the ITI was 8 s for the entirety of the session (Anastasio et al., 2014b; Dalley et al., 2002). Following ITI8 challenge, rats were restabilized on ITI5 sessions (< 20% variability over three sessions) before a second ITI8 challenge. Following completion of two pre-surgery ITI8 challenges, rats were anesthetized via intramuscular (i.m.) delivery of 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) were lowered bilaterally at 11° from the midsaggital plane relative to bregma (Paxinos and Watson, 2014) to target the mPFC encompassing the ventral prelimbic (PL) and infralimbic (IL) subnuclei; the coordinates were anteroposterior +3 mm, mediolateral +1.3 mm, and dorsoventral −4.4 mm from the skull. The NSC-eGFP (‘control;' 1.0 µL) or 5-HT_{2A}R-shRNA-eGFP (‘5-HT_{2A}R knockdown,' 1.0 µL) AAV2 vectors were infused bilaterally at 0.1 µL/min over 10 min. Rats were allowed three weeks to recover and to allow for stable transgene expression. AAV infection has been well-characterized with stabilization of gene expression in rodent brain at t weeks and with stability for at least 12–18 months post infection (Daly, 2004; Leff et al., 1999).

Following intra-mPFC transgene delivery and stable viral vector expression, NSC-eGFP and 5-HT_{2A}R-shRNA-eGFP rats were restabilized on ITI5 sessions (<

20% variability over three sessions) before post-surgery performance in the 1-CSRT task was evaluated. A third ITI8 challenge was conducted, and rats were restabilized on ITI5 sessions (< 20% variability over three sessions) before a fourth ITI8 challenge.

Effect of 5-HT$_{2A}$R knockdown in the mPFC on pharmacological sensitivity to pimavanserin in the 1-CSRT task. Following ITI8 challenge sessions in the 1-CSRT task, rats were required to meet acquisition criteria in the 1-CSRT task [≥ 50 target responses, > 80% accuracy, and < 20% omissions on the final training stage (0.5 sec stimulus duration, 5 sec limited hold, and ITI5)] for at least three consecutive days. Performance under an ITI5 schedule in the 1-CSRT task was assessed following systemic administration of pimavanserin. Pretreatment with VEH (1 mL/kg, s.c.) or pimavanserin (0.01, 0.1, 1 mg/kg, s.c.) occurred 30 min prior to the start of the 1-CSRT task session under an ITI5 schedule.

Following pimavanserin challenge on an ITI5 schedule, rats were restabilized on the acquisition criteria in the 1-CSRT task [≥ 50 target responses, > 80% accuracy, and < 20% omissions on the final training stage (0.5 sec stimulus duration, 5 sec limited hold, and ITI5)] for at least three consecutive days. Performance under an ITI8 schedule in the 1-CSRT task was assessed following systemic administration of pimavanserin. Pretreatment with VEH (1 mL/kg, s.c.) or pimavanserin (0.1 mg/kg, s.c.) occurred 30 min prior to the start of the 1-CSRT task session.

Effect of 5-HT$_{2A}$R knockdown in the mPFC on response to a 5-HT$_{2A}$R agonist in the head-twitch assay. Following pharmacological challenge with pimavanserin in the 1-CSRT task, rats were kept in home cages for at least three days under maintenance of food restriction so as not to perturb 5-HT$_{2A}$R sensitivity (Serafine and France, 2014). The preferential 5-HT$_{2A}$R agonist DOI was dissolved in VEH (sterile saline). Following injection with DOI (1 mg/kg, s.c.), rats were immediately placed into transparent cages and video recorded in high-definition (HDR-XR550V; Sony, Tokyo, Japan) for 30 min (Fink et al., 2015). Head twitches were operationally defined as a rapid rotational head movements (Canal et al., 2013) and scored manually over a 15 min period beginning 10 min after injection (Fink et al., 2015).

Rats were returned to their home cages for at least five days following the last behavioral evaluation to permit drug washout. Rats were anesthetized (chloral hydrate; 400 mg/kg, i.p.) and killed, and tissue samples were taken for visualization of bilateral viral placement and immunoblot analyses of 5-HT$_{2A}$R protein knockdown. A 1-mm coronal section containing the mPFC was placed on a cold glass slide and rapid visualization of eGFP *ex vivo* was accomplished with a DFP-1 Dual Fluorescent Protein Flashlight by the investigator wearing a pair of VG2 barrier filter glasses (Nightsea, Bedford, MA) (Li and Wolf, 2011). Photomicrographs of coronal sections were taken with a DSLR camera equipped with a macro lens and yellow filter (Li and Wolf, 2011). Fluorescent regions from the mPFC were then microdissected and assayed for immunoblotting (as describe above) to assess knockdown *ex vivo*. In the NSC-eGFP group only, some rats (*n*

= 6) exhibited only unilateral targeting of the virus to the mPFC. However, a post-placement analysis revealed that rats receiving unilateral vs. bilateral placement of NSC-eGFP to the mPFC did not exhibit any differences on ITI8 performance in the 1-CSRT task, sensitivity to pimavanserin under an ITI5 or ITI8 schedule in the 1-CSRT task, the DOI-induced head twitch response (data not shown). Thus, rats exhibiting unilateral and bilateral targeting of NSC-eGFP to the mPFC were collapsed in the data analysis. All rats receiving 5-HT_{2A}R-shRNA-eGFP exhibited bilateral targeting to the mPFC.

Transcriptome profile of the mPFC following 5-HT_{2A}R knockdown. A second group of rats (*n* = 16) were anesthetized (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) were lowered bilaterally at 11° from the midsaggital plane relative to bregma (anteroposterior +3 mm; mediolateral +1.3) (Paxinos and Watson, 2014). To target the entire medial pole of the PFC [encompassing anterior cingulate cortex (ACC), PL, and IL], microsyringes were first lowered in the dorsoventral plane at -5.1 mm from bregma, and 0.7 uL of the NSC-eGFP ("control") or 5-HT_{2A}R-shRNA-eGFP ("5-HT_{2A}R knockdown") AAV2 vectors were infused bilaterally at 0.1 µL/min over 7 min. Then, microsyringes were raised 1 mm (dorsoventral: -4.1 mm from bregma), and 0.7 uL of the NSC-eGFP or 5-HT_{2A}R-shRNA-eGFP AAV2 vectors were infused bilaterally at 0.1 µL/min over 7 min. Last, microsyringes were raised an additional 1 mm (dorsoventral: -3.1 mm from

bregma), and 0.5 ul of the NSC-eGFP or 5-HT$_{2A}$R-shRNA-eGFP AAV2 vectors were infused bilaterally at 0.1 µL/min over 5 min. Rats were allowed three weeks to recover and to allow for stable transgene expression.

Following three weeks of recovery and transgene expression, rats were anesthetized (chloral hydrate; 400 mg/kg, i.p.) and killed, and tissue samples were taken for visualization of bilateral viral placement as described above. The mPFC was homogenized in 400 µL (~10X w/v) extraction buffer (20 mM HEPES, 200 mM NaCl,1 mM EDTA, 1 mM DTT, 10 µl/ml protease inhibitor cocktail, 10 µL/ml phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich, St Louis, MO), and 5 µL/ml RNaseOUT™ Recombinant Ribonuclease Inhibitor (Thermo-Fisher Scientific, Waltham, MA). Immediately following initial homogenization, samples were aliquoted in which 300 µL of brain homogenate was utilized for immunoblot analyses of 5-HT$_{2A}$R protein knockdown (as described above) while the remaining 100 µL of sample was utilized for RNA extraction. This 100 µL sample was transferred to 500 µL of TRI Reagent (Life Technologies, Grand Island, NY), and purified RNA was isolated using RNeasy Mini Kit (Qiagen, Germantown, MD). RNA concentration and quality were analyzed using Cytation 5 Cell Imaging Multi-Mode Reader (BioTek Instruments, Winooski, VT). RNA was stored at −80 °C until assayed. Isolated RNA from the mPFC of NSC-eGFP (n = 8) and 5-HT$_{2A}$R-shRNA-eGFP (n = 8) AAV2 rats was utilized for next-generation RNA sequencing.

RNA-sequencing services were provided by the University of Texas Medical Branch Next Generation Sequencing Core. RNA samples were quantified using a Qubit fluorometer (Thermo Scientific, Waltham, MA), and total RNA quality control

analysis was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). In brief, libraries for RNA sequencing were prepared with Kapa Stranded RNA-Seq Kit (Kapa Biosystems, Wilmington, MA). The workflow consisted of mRNA enrichment, cDNA generation, and end repair to generate blunt ends, A-tailing, adaptor ligation, and polymerase chain reaction (PCR) amplification. Sequencing was performed on NextSeq 550 for a single read (75 base pairs) run. The reads were aligned to the rat genome using Bowtie2 version 2.3.2 and counts were assigned to genes using HTSeq-Count. Counts were imported into R statistical programming language. The DESeq2 rlog function was employed to filter samples. K-means clustering via principal component analysis revealed $n = 1$ sample in the NSC-eGFP group that failed to cluster with the other NSC-eGFP samples; this sample was removed from analysis. Differential gene expression analysis was performed using DESeq2. Genes exhibiting a Benjamini-Hochberg adjusted $p < 0.01$ were considered significant different between NSC-eGFP and 5-HT_{2A}R-shRNA-eGFP rats.

Heat maps were generated using Java Treeview v1.1.6r4 (http://www.sourceforge.net/projects/jtreeview/files) by computing *Z*-scores from the gene counts, and *Z*-score were hierarchically clustered using Gene Cluster v3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/). RNA-sequencing data were analyzed using Ingenuity Pathway Analysis (Qiagen, Germantown, MD) core analysis to explore the biological pathways that are changed following 5-HT_{2A}R knockdown; transcripts displaying a Benjamini-Hochberg adjusted $p < 0.01$ were considered significantly different between control and 5-HT_{2A}R knockdown rats.

First, canonical pathway analysis was employed to identify biological pathways that are different between 5-HT_{2A}R knockdown and control rats using a -log(*p* -value) < 1.3 (i.e., $p < 0.05$) and absolute *z*-score > 2. Second, causal network analysis using a network bias-correct $p < 0.05$ was employed to identify which causal relationships previously reported in the literature are likely relevant for the biological mechanisms underlying our data. Third, our secondary functional gene enrichment analyses were carried out using Enrichr (updated August, 2017), a web-based gene set enrichment analysis tool (Chen et al., 2013; Kuleshov et al., 2016). Enrichr uses the Fisher exact test ($\alpha = 0.05$), the *z*-score of the deviation from the expected rank by the Fisher exact test, and a combined score that multiplies the log of the Benjamini-Hochberg unadjusted *p*-value computed with the Fisher exact test by the *z*-score (Moloney et al., 2019).

Statistical Analyses

The effects of pre- vs. post-surgery ITI8 performance in control and knockdown rats were analyzed by two-way repeated-measures ANOVA for the factors of pretreatment (control or knockdown) and ITI8 challenge (ITI8-1, ITI8-2, ITI8-3, ITI8-4). The effects of pimavanserin on 1-CSRT task performance in control and knockdown rats were analyzed by two-way repeated-measures ANOVA for the factors of treatment (control or knockdown) and pretreatment (VEH or pimavanserin); the effects of pretreatment in control and knockdown rats were assessed by one-way repeated-measures ANOVA followed by Dunnett's

procedure (for comparisons of pretreatment means vs. VEH). The DOI head-twitch response and 5-HT_{2A}R protein expression data were assessed by two-tailed Student's *t*-test. The experimenter was blinded to the group allocation (e.g., control vs. knockdown) throughout the duration of the study. These analyses were performed in GraphPad Prism (version 7; San Diego, CA) with an experiment-wise error rate of $\alpha = 0.05$. Gene transcripts displaying a Benjamini-Hochberg adjusted $p < 0.01$ were considered significantly different between control and 5-HT_{2A}R knockdown rats. Canonical pathways displaying a -log(*p* - value) < 1.3 (i.e., $p < 0.05$) and absolute Z-score >2 were considered significantly different between 5-HT_{2A}R knockdown and control rats. Causal networks displaying a network bias-correct $p < 0.05$ were considered significantly different between 5-HT_{2A}R knockdown and control rats. Functional gene enrichment using Enrichr (biological processes) was analyzed using Fisher's exact test, and FDR multiplicity correction was performed using the Benjamini-Hochberg method with an experiment-wise error rate of $\alpha = 0.05$.

RESULTS

Engineered loss of 5-HT_{2A}R in mPFC does not alter 1-CSRT task performance

Our cumulative evidence suggests that individual differences in impulsive action reflect variation in the cortical 5-HT_{2A}R system (Fink et al., 2015). We tested the hypothesis that loss of the 5-HT_{2A}R in the mPFC would decrease impulsive action assessed in the 1-CSRT task. Rats received two ITI8 challenge sessions (ITI8-1, ITI8-2) prior to and two ITI8 challenge sessions (ITI8-3, ITI8-4) following

intra-mPFC infusion of 5-HT_{2A}R-shRNA-eGFP AAV2 (“knockdown”) or NSC-eGFP (“control”). There was no main effect of AAV2 treatment [$F_{1,17}$=0.04, n.s] or ITI8 challenge session [$F_{3,51}$=0.26, n.s.] and no interaction [$F_{3,51}$=0.60, n.s.] on target premature responses (**Figure 4.1A**). There was no main effect of AAV2 treatment [$F_{1,17}$=2.26, n.s.], no main effect of ITI8 challenge session [$F_{3,51}$=0.63, n.s.], and no interaction [$F_{3,51}$=0.82, n.s.] on reinforcers earned (**Figure 4.1B**). There was no main effect of AAV2 treatment [$F_{1,17}$=0.38, n.s.] or ITI8 challenge session [$F_{3,51}$=0.94, n.s.] and no interaction [$F_{3,51}$=0.19, n.s.] on percent omissions (**Figure 4.1C**). There was no main effect of AAV2 treatment or ITI8 challenge session and no interaction on total or non-target premature responses, percent accuracy, latency to first response, or time to complete the task; outcomes of the two-way ANOVA are provided in **Table 4.1**.

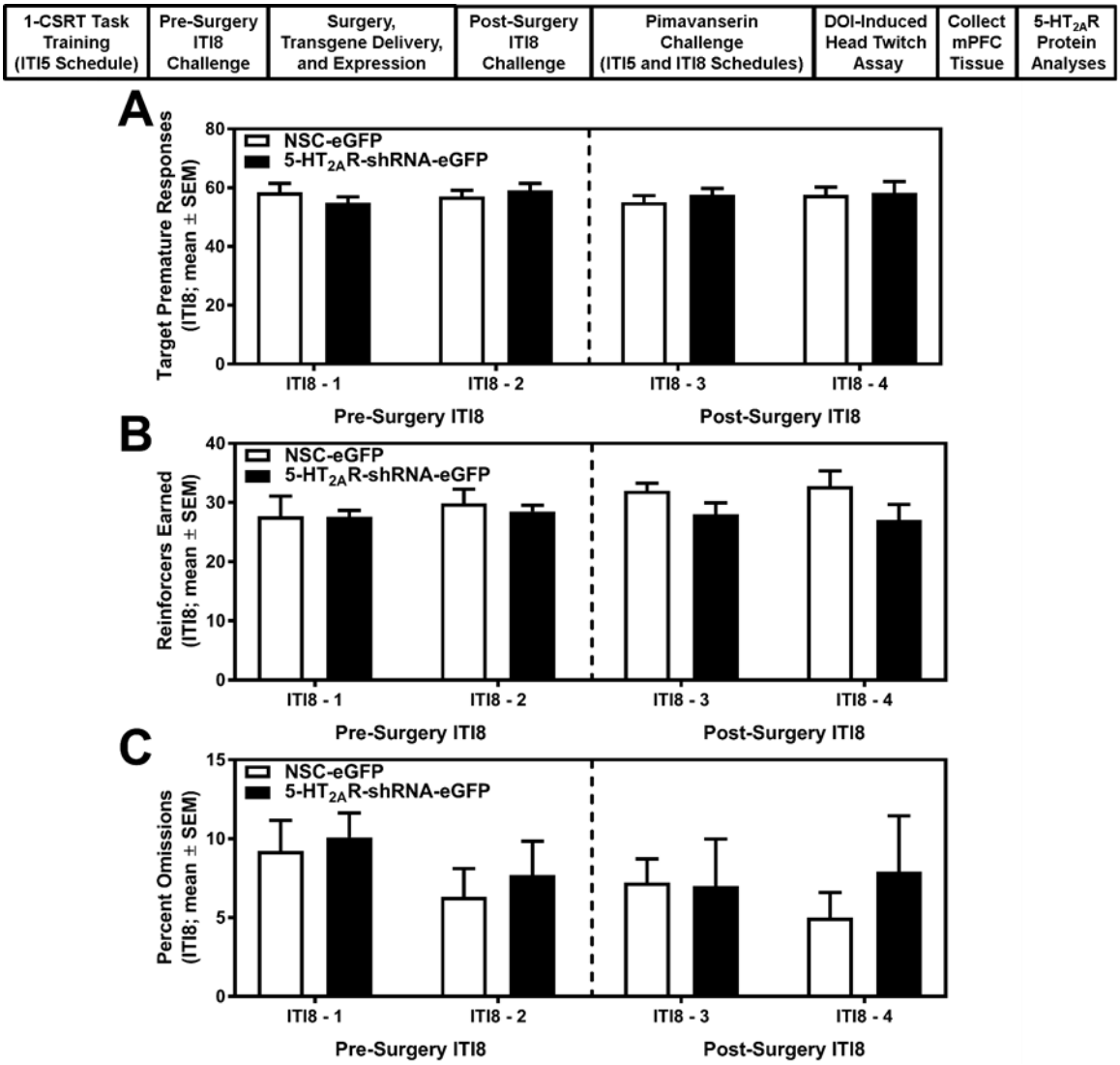

Figure 4.1: Engineered loss of the 5-HT$_{2A}$R in the mPFC does not alter 1-CSRT task performance.

Prior to intra-mPFC infusion of NSC-eGFP (*n* = 9) or 5-HT$_{2A}$R-shRNA-eGFP (*n* = 10), baseline levels of impulsive action pre-surgery were evaluated under an ITI8 schedule in the 1-CSRT task (ITI8 – 1; ITI8 – 2). Following stereotaxic surgery, rats were restabilized before receiving two additional ITI8 challenge sessions post-surgery (ITI8 – 3; ITI8 – 4) No difference in (A) target premature responses, (B) reinforcers earned, or (C) percent omissions were observed between NSC-eGFP vs. 5-HT$_{2A}$R-shRNA-eGFP rats (mean ± SEM).

Engineered loss of 5-HT$_{2A}$R in mPFC does not alter pharmacological sensitivity to pimavanserin in the 1-CSRT task

Our laboratory found that HI rats were more sensitive to the effects of the selective 5-HT_{2A}R antagonist/inverse agonist M100907 to suppress impulsive action relative to LI rats (Fink et al., 2015). We tested the hypothesis that engineered loss of the 5-HT_{2A}R in the mPFC decreases sensitivity to the 5-HT_{2A}R antagonist/inverse agonist pimavanserin on an ITI5 schedule in the 1-CSRT task relative to control rats. There was a main effect of pimavanserin pretreatment [$F_{3,51}$=25.4, $p < 0.05$], but no main effect of AAV2 treatment [$F_{1,17}$=0.35, n.s.] and no interaction [$F_{3,51}$=0.44, n.s.] on target premature responses (**Figure 4.2A**). Planned comparisons with Dunnett's revealed that 0.1 and 1 mg/kg pimavanserin decreased target premature response relative to VEH in both the control and knockdown rats ($p < 0.05$). Similarly, there was a main effect of pimavanserin pretreatment [$F_{3,51}$=26.8, $p < 0.05$] but no main effect of AAV2 treatment [$F_{1,17}$=0.76, n.s.] and no interaction [$F_{3,51}$=0.31, n.s.] on total premature responses (**Table 4.2**). Planned comparisons with Dunnett's revealed that 0.1 and 1 mg/kg pimavanserin decreased total premature response relative to VEH in both the control ($p < 0.05$) and knockdown rats ($p < 0.05$). There was a main effect of pimavanserin pretreatment [$F_{3,51}$=7.31, $p < 0.05$] but no main effect of AAV2 treatment [$F_{1,17}$=3.52, n.s.] and no interaction [$F_{3,51}$=0.33, n.s.] on reinforcers earned (**Figure 4.2B**). Planned comparisons failed to detect any differences in reinforcers earned relative to VEH in both the knockdown and control rats (n.s.). Further, there was a main effect of pimavanserin pretreatment [$F_{3,51}$=5.99, $p < 0.05$] but no main effect of AAV2 treatment [$F_{1,17}$=1.01, n.s.] and no interaction [$F_{3,51}$=0.55, n.s.] on percent omissions (**Figure 4.2C**). Planned comparisons with

Dunnett's revealed that 1 mg/kg of pimavanserin modestly increased percent omissions relative to VEH in the knockdown rats ($p < 0.05$) but not control rats (n.s.). The outcomes of the two-way ANOVA for non-target premature responses, percent accuracy, latency to first response, and time to complete the task are provided in **Table 4.2**.

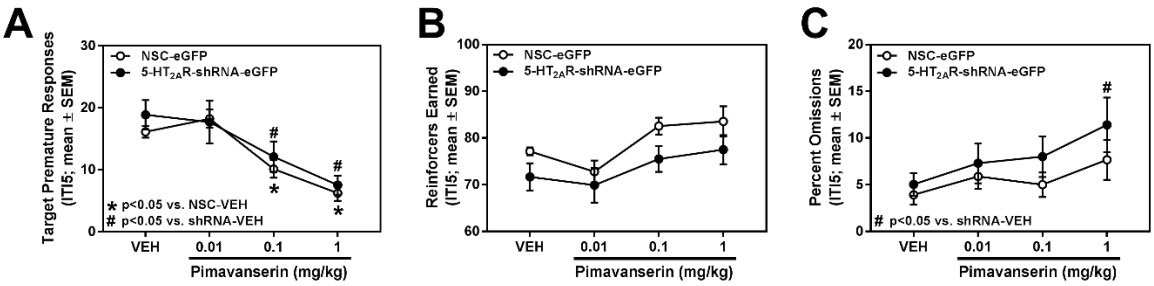

Figure 4.2: Engineered loss of the 5-HT$_{2A}$R in the mPFC does not alter pharmacological sensitivity to pimavanserin under ITI5 conditions in the 1-CSRT task.

The effects of pimavanserin (0.01, 0.1, 1 mg/kg; mean ± SEM) were evaluated under ITI5 conditions in NSC-eGFP (n = 9) and 5-HT$_{2A}$R-shRNA-eGFP rats (n = 10). (A) Pimavanserin (0.1 and 1 mg/kg) significant decreased target premature responses in both NSC-eGFP and 5-HT$_{2A}$R-shRNA-eGFP rats relative to VEH (*$p < 0.05$ vs. NSC-VEH; $^{\#}p < 0.05$ vs. shRNA-VEH). (B) Pimavanserin did not alter reinforcers earned in the NSC-eGFP or 5-HT$_{2A}$R-shRNA-eGFP rats relative to VEH. (C) Pimavanserin (1 mg/kg) decreased percent omissions relative to VEH in 5-HT$_{2A}$R-shRNA-eGFP rats but not NSC-eGFP rats.

Employment of the ITI8 challenge during pharmacological test sessions enhances the sensitivity to detect differences in premature responding (Anastasio et al., 2014b; Besson et al., 2013; Caprioli et al., 2014; Dalley et al., 2007; Economidou et al., 2012; Fink et al., 2015). We tested the hypothesis that loss of the 5-HT$_{2A}$R in the mPFC decreases sensitivity to an effective dose of pimavanserin on an ITI8 schedule in the 1-CSRT task relative to control rats. There was a main effect of pimavanserin pretreatment [$F_{1,17}$=18.5, $p < 0.05$] but no main effect of AAV2 treatment [$F_{1,17}$=0.68, n.s.] and no interaction [$F_{1,17}$=0.65, n.s.] on

target premature responses (**Figure 4.3A**). Planned comparisons with Dunnett's revealed that 0.1 mg/kg pimavanserin decreased target premature response relative to VEH in knockdown rats ($p < 0.05$) with an observed trend for the control rats ($p = 0.055$). Similarly, there was a main effect of pimavanserin pretreatment [$F_{1,17}=22.7$, $p < 0.05$] but no main effect of AAV2 treatment [$F_{1,17}=1.93$, n.s.] and no interaction [$F_{1,17}=0.20$, n.s.] on total premature responses (**Table 4.3**). Planned comparisons with Dunnett's revealed that 0.1 mg/kg of pimavanserin decreased total premature response relative to VEH in both the control ($p < 0.05$) and knockdown rats ($p < 0.05$). There was a main effect of pimavanserin pretreatment [$F_{1,17}=20.6$, $p < 0.05$] but no main effect of AAV2 treatment [$F_{1,17}=3.49$, n.s.] and no interaction [$F_{1,17}=1.24$, n.s.] on reinforcers earned (**Figure 4.3B**). Planned comparisons with Dunnett's revealed that 0.1 mg/kg of pimavanserin decreased reinforcers earned relative to VEH in both the control ($p < 0.05$) and knockdown rats ($p < 0.05$). Further, there was no main effect of pimavanserin pretreatment [$F_{1,17}=0.07$, n.s.] or AAV2 treatment [$F_{1,17}=2.82$, n.s.] and no interaction [$F_{1,17}=2.54$, n.s.] on percent omissions (**Figure 4.3C**). The outcomes of the two-way ANOVA for non-target premature responses, percent accuracy, latency to first response, and time to complete the task are provided in **Table 4.3**.

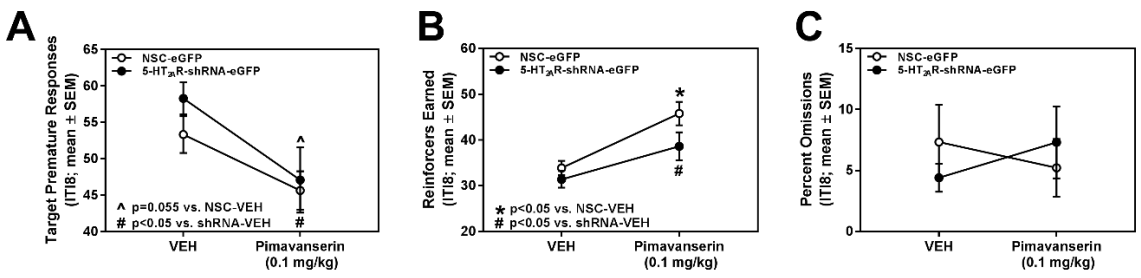

Figure 4.3: Engineered loss of the 5-HT$_{2A}$R in the mPFC does not alter pharmacological sensitivity to 0.1 mg/kg pimavanserin under ITI8 conditions in the 1-CSRT task.

The effects of pimavanserin (0.1 mg/kg; mean ± SEM) were evaluated under ITI8 conditions in NSC-eGFP (n = 9) and 5-HT$_{2A}$R-shRNA-eGFP rats (n = 10). (A) Pimavanserin significant decreased target premature responses in both NSC-eGFP and 5-HT$_{2A}$R-shRNA-eGFP rats relative to VEH ($^{\wedge}p$ = 0.055 vs. NSC-VEH; $^{\#}p$ < 0.05 vs. shRNA-VEH). (B) Pimavanserin increased reinforcers earned in both NSC-eGFP and 5-HT$_{2A}$R-shRNA-eGFP rats relative to VEH ($^{*}p$ < 0.05 vs. NSC-VEH; # p < 0.05 vs. shRNA-VEH). (C) Pimavanserin did not alter percent omissions in the NSC-eGFP or 5-HT$_{2A}$R-shRNA-eGFP rats relative to VEH.

Engineered loss of 5-HT$_{2A}$R in mPFC does not alter the DOI-induced head-twitch response

Many 5-HT$_{2A}$R agonists induce a head-twitch response in rodents that is dependent upon 5-HT$_{2A}$R activation (Canal and Morgan, 2012), possibly in the mPFC (Willins and Meltzer, 1997). Moreover, HI rats exhibited a greater head-twitch response following administration of the preferential 5-HT$_{2A}$R agonist DOI relative to LI rats (Fink et al., 2015). We hypothesized that the preferential 5-HT$_{2A}$R agonist DOI would elicit fewer head-twitch responses in knockdown rats relative to control rats. However, the number of head-twitch responses elicited following systemic administration of DOI did not differ between knockdown vs. control rats [t_{17} = 0.33, n.s., **Figure 4.4**].

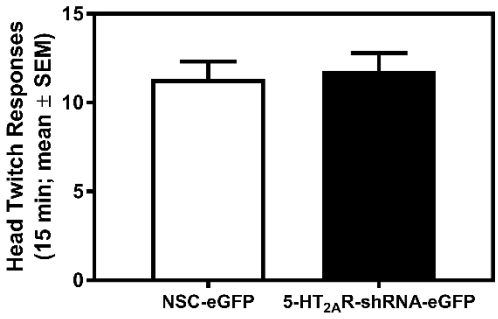

Figure 4.4: Engineered loss of the 5-HT_{2A}R in the mPFC does not alter pharmacological sensitivity to DOI in the head-twitch response assay.

Injection of DOI (1 mg/kg, s.c.) resulted in head-twitches that were quantified over a 15-min period in NSC-eGFP (n = 9) and 5-HT_{2A}R-shRNA-eGFP rats (n = 10). There was no significant differences in the number of DOI-induced head-twitch responses exhibited in NSC-eGFP vs. 5-HT_{2A}R-shRNA-eGFP rats.

Intra-mPFC infusion of 5-HT_{2A}R-shRNA-eGFP reduces 5-HT_{2A}R protein expression in the mPFC relative to NSC-eGFP rats

We then confirmed that intra-mPFC infusion of 5-HT_{2A}R-shRNA-eGFP AAV2 decreases 5-HT_{2A}R protein expression relative to control rats. *Ex vivo* analyses of microinfusion placements in individual rats illustrated that viral infection was localized to the greatest extent within the PL subregion of the mPFC, with lesser localization to the dorsal IL subregion and minimal localization in the ACC (**Figure 4.5A**). Representative photomicrographs depict coronal brain sections under regular light (**Figure 4.5B**, left) and using the fluorescent flashlight to visualize GFP (**Figure 4.5B**, right) in NSC-eGFP and 5-HT_{2A}R-shRNA-eGFP rats. *Ex vivo* analyses indicated that the 5-HT_{2A}R-shRNA-eGFP AAV2 significantly reduced 5-HT_{2A}R protein expression relative to control rats [$t_{17} = 3.02$, $p < 0.05$., **Figure 4.5C**].

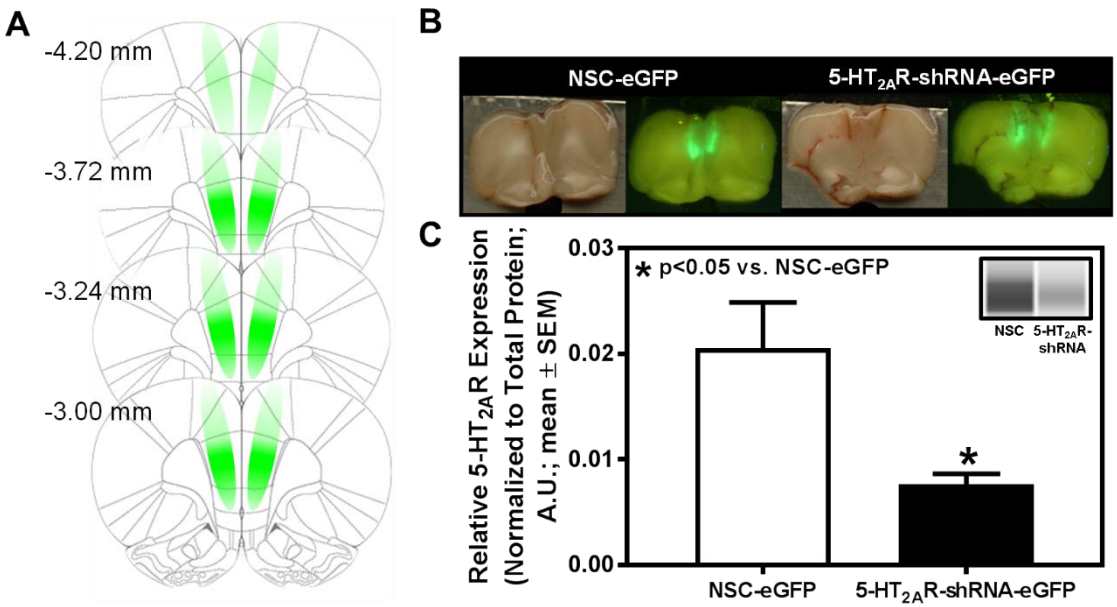

Figure 4.5: Intra-mPFC infusion of 5-HT$_{2A}$R-shRNA-eGFP reduces 5-HT$_{2A}$R protein expression in the mPFC relative to NSC-eGFP rats.

(A) The extent of viral spread (displayed as distance from bregma) in mPFC is illustrated, where the darkest shade of green indicates robust expression in all rats and lighter green indicates areas of weaker expression and/or expression in a subset of rats. (B) Representative photomicrographs depict coronal brain sections under regular light (left) and using the fluorescent flashlight to visualize GFP (right) in NSC-eGFP and 5-HT$_{2A}$R-shRNA-eGFP rats. (C) *Ex vivo* biochemical analyses indicate that rats receiving 5-HT$_{2A}$R-shRNA-eGFP display lower mPFC 5-HT$_{2A}$R protein levels relative to NSC-eGFP rats (* $p < 0.05$ vs. NSC-eGFP). The insets are representative electrophoretic bands. Arbitrary units (A.U.) of densitometry are presented.

RNA-sequencing reveals 1132 gene transcripts that are different in the mPFC of 5-HT$_{2A}$R knockdown vs. control rats

Stably-identified HI rats exhibit a higher density of synaptosomal 5-HT$_{2A}$R protein expression in mPFC vs. LI rats (Anastasio et al., 2015; Fink et al., 2015), while numerous studies demonstrate that intra-mPFC infusion of 5-HT$_{2A}$R antagonists/inverse agonists suppress impulsive action in the 5-CSRT task (Passetti et al., 2003; Winstanley et al., 2003). However, 5-HT$_{2A}$R knockdown in the mPFC did not alter impulsive action or pharmacological sensitivity to 5-HT$_{2A}$R

ligands. In a second group of behaviorally-naïve, male Sprague-Dawley rats, we hypothesized that rats receiving intra-mPFC infusion of 5-HT$_{2A}$R-shRNA-eGFP exhibit a distinct gene transcript profile (transcriptome) in the mPFC relative to NSC-eGFP rats, reflecting a potential molecular response to the consequence of 5-HT$_{2A}$R knockdown. RNA sequencing of the mPFC revealed *n* = 1132 genes of interest exhibiting a Benjamini-Hochberg adjusted $p < 0.01$; *n* = 686 gene transcripts were higher (red) while *n* = 446 were lower (green) in the mPFC of 5-HT$_{2A}$R knockdown vs. control rats (**Figure 4.6A**). The heat map of *z*-scores in **Figure 4.6B** visualizes 100 gene transcripts that exhibited the greatest absolute log-fold change values in 5-HT$_{2A}$R knockdown vs. control rats.

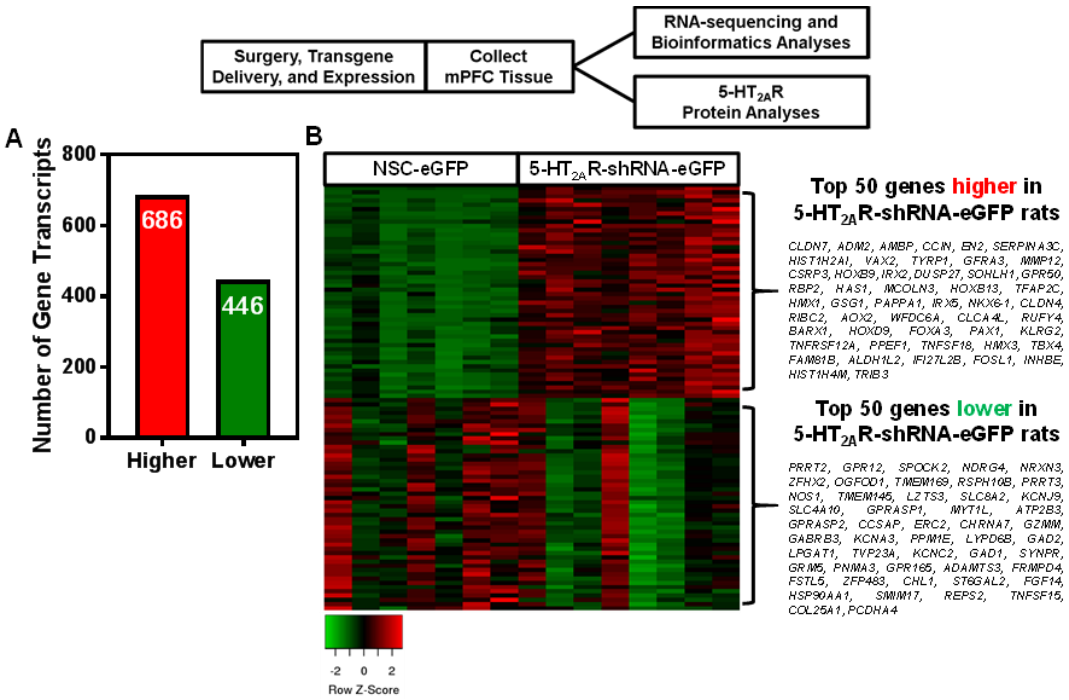

Figure 4.6: RNA-sequencing reveals *n* = 1132 gene transcripts that are higher (*n* = 686) and lower (*n* = 446) in the mPFC of 5-HT$_{2A}$R knockdown vs. control rats.

(A) The total number of gene transcripts that are higher (red) and lower (green) in the mPFC of 5-HT$_{2A}$R knockdown vs. control rats is plotted. (B) The heat map of *z*-scores visualizes gene transcripts that exhibited the greatest absolute log fold change in expression in 5-HT$_{2A}$R knockdown vs. control rats.

Canonical pathway analysis identifies 29 biological pathways that are overrepresented in the mPFC of 5-HT$_{2A}$R knockdown vs. control rats

The development of gene enrichment tools allow researchers to map large gene lists to common biological processes, pathways, and molecular functions, illuminating an otherwise complex neurobiology (Huang da et al., 2009). We employed Ingenuity Pathway Analysis (IPA) to identify canonical biological pathways that are modulated by 5-HT$_{2A}$R knockdown in the mPFC. In total, we identified 29 canonical pathways exhibiting a -log(*p*-value) > 1.3 and absolute *z*-score > 2 (**Figure 4.7**). In particular, several pathways relevant to synaptic transmission were predicted as inhibited (indicated by a negative *z*-score) following 5-HT$_{2A}$R knockdown, including synaptogenesis signaling pathway [-log (*p*) = 5.28; *z* = -4.22, pathway coverage ratio = 0.13, **Table 4.4**], synaptic long term potentiation [-log (*p*) = 3.39; *z* = -2.83, pathway coverage ratio = 0.15, **Table 4.4**], calcium signaling [-log (*p*) = 3.26; *z* = -3.44, pathway coverage ratio = 0.13, **Table 4.4**], G$_{\alpha q}$ signaling [-log (*p*) = 2.30; *z* = -2.18, pathway coverage ratio = 0.12, **Table 4.4**], and synaptic long term depression [-log (*p*) = 2.03; *z* = -2.36, pathway coverage ratio = 0.11, **Table 4.4**]; a complete list of canonical pathways is found in **Table 4.4**.

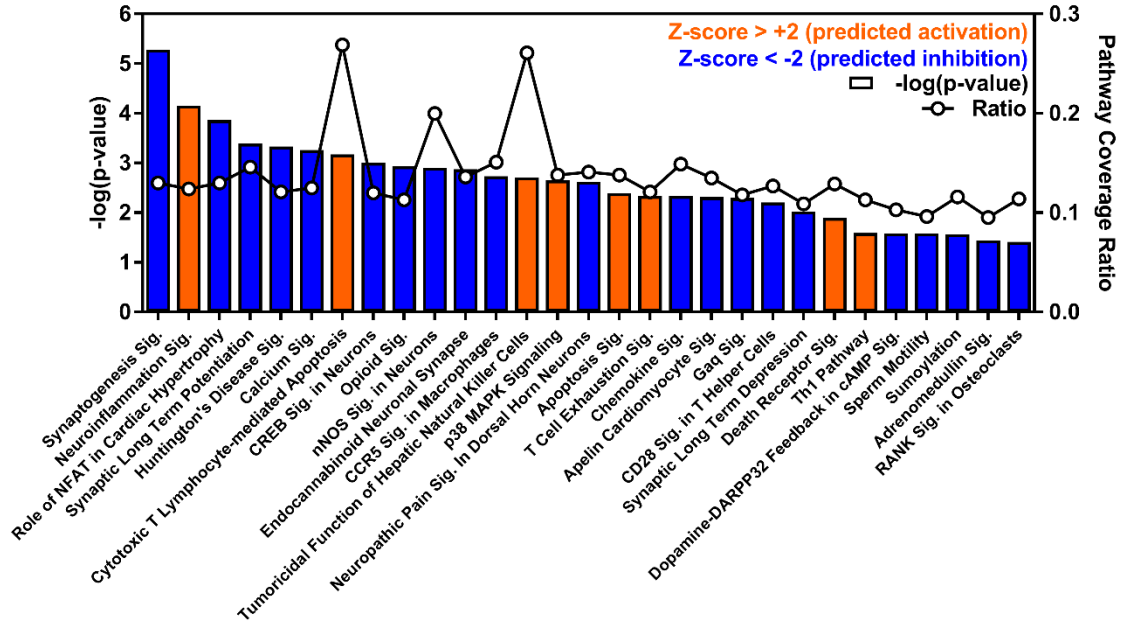

Figure 4.7: Ingenuity Pathway Analysis reveals canonical pathways that are overrepresented in the mPFC of 5-HT$_{2A}$R knockdown vs. control rats.

The left *y*-axis depicts the –log(*p*-value) and the right *y*-axis depicts the proportion of genes enriched within a biological pathway (*x*-axis). Canonical pathways exhibiting a *z*-score > 2 (predicted activation) are indicated by orange bars, and pathways exhibiting a *z*-score < - 2 (predicted inhibition) are indicated by blue bars.

HTR2A **is the master regulator of a causal network containing *n* = 21 gene transcripts in 5-HT$_{2A}$R knockdown vs. control rats**

Biological pathways related to synaptic transmission (e.g., $G_{\alpha q}$ signaling, calcium signaling, synaptic long term potentiation/depression) were overrepresented in our gene list following engineered loss of the 5-HT$_{2A}$R in the mPFC. Next, we performed a causal network analysis of our gene list, which integrates *cause-effect* relationships reported in the literature to leverage knowledge about the causal directionality in a gene list rather than correlative associations (Kramer et al., 2014). Interestingly, we found that the *HTR2A* gene is

the predicted master regulator of a causal network containing *n* = 21 downstream transcripts identified in our gene list, including *CASP3, CD24A, CD74, CDKN1A, CDKN1C, CTSD, CXCL10, FOSL1, GAD1, GAD2, GRM1, ICAM1, KCND3, MMP2, MYC, ODC1, PRKCZ, RIPK3, RSAD2, RUNX1,* and *TIMP1* (network bias-corrected $p < 0.05$; **Figure 4.9**). Strikingly, the expression pattern of these 21 transcripts was predicted following *HTR2A activation* ($z = 1.09$, $p < 0.05$) in 5-HT_{2A}R knockdown rats. The identification of a causal gene network that is predicted following *HTR2A* activation suggests a potential, compensatory molecular response to the consequence of 5-HT_{2A}R knockdown in mPFC.

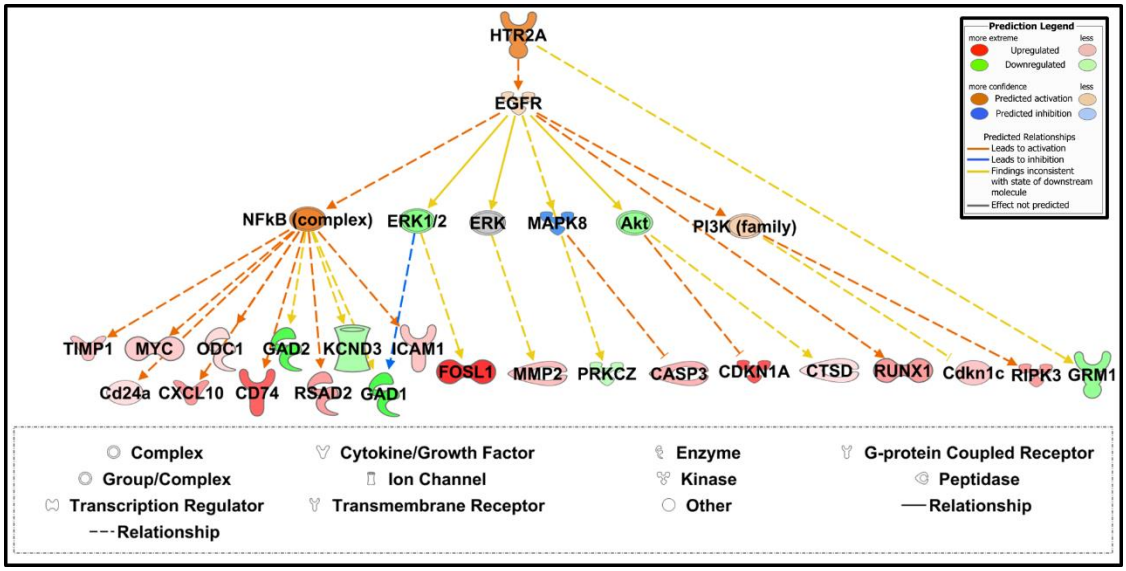

Figure 4.8: *HTR2A* is the master regulator of a causal network containing *n* = 21 gene transcripts in 5-HT_{2A}R knockdown vs. control rats.

HTR2A (top tier) is the master regulator causal network containing six intermediate regulators (middle tier) that predict the increase (red) and decrease (green) in expression of the 21 gene transcripts shown in the bottom tier. The regulators are shaded based on their predicted activation (orange) or inhibition (blue) state in which darker shades represent higher absolute *z*-scores. Pointed arrows indicate that the downstream regulator is expected to be activated if the connected upstream regulator connected is activated, and blunted arrows indicate that the downstream regulator is expected to be inhibited if the connected upstream regulator is activated.

Intra-mPFC infusion of 5-HT$_{2A}$R-shRNA-eGFP reduces 5-HT$_{2A}$R protein expression in the mPFC relative to NSC-eGFP rats

We then confirmed that intra-mPFC infusion of 5-HT$_{2A}$R-shRNA-eGFP AAV2 decreases 5-HT$_{2A}$R protein expression relative to control rats. *Ex vivo* analyses of microinfusion placements in individual rats illustrated that viral infection was localized to the entire medial pole of the mPFC (encompassing ACC, PL, and IL) (**Figure 4.9A**). Representative photomicrographs depict coronal brain sections under regular light (**Figure 4.9B**, left) and using the fluorescent flashlight to visualize GFP (**Figure 4.9B**, right) in NSC-eGFP and 5-HT$_{2A}$R-shRNA-eGFP rats. *Ex vivo* analyses indicated that the 5-HT$_{2A}$R-shRNA-eGFP AAV2 significantly reduced 5-HT$_{2A}$R protein expression relative to control rats [$t_{12} = 2.43$, $p < 0.05$., **Figure 4.9C**].

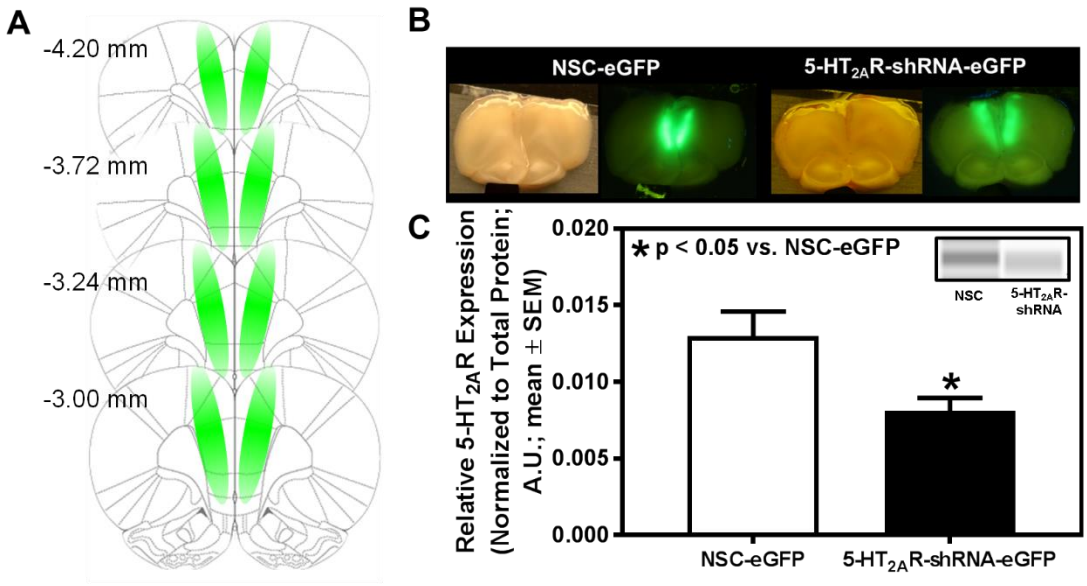

Figure 4.9: Intra-mPFC infusion of 5-HT$_{2A}$R-shRNA-eGFP reduces 5-HT$_{2A}$R protein expression in the mPFC relative to NSC-eGFP rats in a second group of behaviorally-naïve rats.

(A) The extent of viral spread (displayed as distance from bregma) in mPFC is illustrated, where the darkest shade of green indicates robust expression in all rats and lighter green indicates areas of weaker expression and/or expression in a subset of rats. (B) Representative photomicrographs depict coronal brain sections under regular light (left) and using the fluorescent flashlight to visualize GFP (right) in NSC-eGFP and 5-HT$_{2A}$R-shRNA-eGFP rats. (C) *Ex vivo* biochemical analyses indicate that rats receiving 5-HT$_{2A}$R-shRNA-eGFP display lower mPFC 5-HT$_{2A}$R protein levels relative to NSC-eGFP rats (* $p < 0.05$ vs. NSC-eGFP). The insets are representative electrophoretic bands. Arbitrary units (A.U.) of densitometry are presented.

DISCUSSION

Our finding that intra-mPFC infusion of 5-HT$_{2A}$R-shRNA-eGFP did not alter impulsive action relative to NSC-eGFP rats warrants further discussion of previous studies evaluating intra-mPFC infusion of selective 5-HT$_{2A}$R antagonists/inverse agonists (e.g., M100907) on CSRT task performance (Mirjana et al., 2004; Passetti et al., 2003; Robinson et al., 2008; Winstanley et al., 2003). Importantly, none of the studies by Winstanley et al. (0.3 µg), Robinson et al. (0.3 µg) or Carli et al. (10

– 40 µg/kg) report a change in premature responses following intra-PL or intra-IL cortex infusion of M100907 when a 0.5-s center stimulus duration was employed. However, Winstanley et al., discovered that M100907 decreases premature responses when the stimulus duration was *shortened from 0.5-s to 0.125-s*. We glean two interpretations from these previous studies. First, the present study employed a 0.5-s stimulus duration, and thus, our findings are in agreement with the available literature using M100907. Second, the mPFC 5-HT_{2A}R may modulate impulsive action under increased attentional demand (i.e., shorter stimulus duration, in the presence of a distractor stimulus). The 1-CSRT task inherently requires less visuospatial attentional demand than the 5-CSRT task because the stimulus is only presented in the center stimulus hole. We proposed in **Chapter 3** that a 5-HT_{2A}R antagonist/inverse agonist may reduce premature responses, in part, by decreasing the motivational factors that influence impulsive responding. Perhaps ligands that decrease motivation also improve the ability to attend to the impending stimulus, an idea that has been proposed but, to our knowledge, not assessed under conditions of high attentional demand (Bizarro and Stolerman, 2003). Thus, a worthwhile future direction is to evaluate the effect of mPFC 5-HT_{2A}R knockdown under increased attentional demand in the 5-CSRT task.

Studies employing intra-mPFC infusion of M100907 represent an *acute* interrogation of this region. Meanwhile, the inability of 5-HT_{2A}R knockdown to alter 1-CSRT task performance or sensitivity to 5-HT_{2A}R ligands relative to control rats suggests that intra-mPFC infusion of 5-HT_{2A}R-shRNA-eGFP over the long term (e.g., greater than three weeks in the present studies) may engender a molecular

response that profoundly negates the involvement of mPFC in impulsive action. Indeed, we identified biological pathways related to synaptic transmission (e.g., $G_{\alpha q}$ signaling, calcium signaling, synaptic long term potentiation/depression) that were predicted as inhibited following engineered loss of the 5-HT_{2A}R in the mPFC. Interestingly, the 5-HT_{2A}R preferentially couples to $G\alpha_{q/11}$ to activate a signaling cascade resulting in release of intracellular calcium (Ca_i^{2+}), activation of the mitogen-associated protein (MAP) kinase cascade, and phosphorylation of extracellular signal-regulated kinases (Casanueva et al., 2008; Chang et al., 2000; Conn and Sanders-Bush, 1986; Hoyer et al., 1989; Pazos et al., 1985; Pazos and Palacios, 1985; Werry et al., 2005). Taken together, predicted inhibition of the $G_{\alpha q}$ and calcium signaling pathways is consistent with a loss of 5-HT_{2A}R tone in the mPFC.

The predicted inhibitory biological pathways related to synaptic transmission are relevant, but not specific, to 5-HT_{2A}R function in the mPFC. A limitation of canonical pathway analyses is the mapping of genes to a defined biological pathway without the incorporation of knowledge on the cause-and-effect relationships across gene expression profiles. Stated differently, canonical pathway analysis tells you *if* a gene belongs to a pathway, but it does not explain *how* that gene interacts with other genes in the pathway in terms of directional expression changes. Thus, we employed a causal network analysis to explore “genetic compensation,” or changes in RNA levels that can functionally compensate for the loss of function of another gene (El-Brolosy and Stainier, 2017). The causal network analysis is more powerful than canonical pathway

analysis due to incorporation of the *directionality* of gene-gene relationships. The network consistency score incorporates a penalty when the directional expression of one gene is inconsistent with the direction predicted in the Ingenuity Knowledge Base (Kramer et al., 2014). Intriguingly, our unbiased, causal network analysis predicted *HTR2A* as an upstream transcriptional regulator of *n* = 21 gene transcripts whose expression was significantly different between 5-HT_{2A}R-shRNA-eGFP and NSC-eGFP rats. Excitingly, the gene expression patterns of these 21 gene transcripts were predicted following *HTR2A activation* in rats receiving 5-HT_{2A}R knockdown, which suggests that their expression may change to rescue 5-HT_{2A}R function in the face of 5-HT_{2A}R knockdown. In particular, a key intermediate node was epidermal growth factor receptor (*EGFR*), which was predicted as activated in response to *HTR2A* activation. Interestingly, the 5-HT_{2A}R transactivates EGFR, potentially through a protein kinase C (PKC)-dependent mechanism (Gooz et al., 2006), which illuminates a novel functional interaction in the mPFC that may modulate impulsive action.

Closer examination of this gene network revealed several gene transcripts related to glutamate and GABA synthesis, metabolism, transport, and metabolism, including glutamic acid decarboxylase 1 (*GAD1*) and 2 (*GAD2*), that were decreased following 5-HT_{2A}R knockdown. *GAD1* and *GAD2* encode the enzymes glutamic acid decarboxylase 67 (GAD67) and 65 (GAD65), respectively, which are rate-limiting enzymes in the synthesis of GABA from glutamate (Petroff, 2002). Thus, these data generated another question: does 5-HT_{2A}R knockdown in the mPFC engender a decrease in *GAD1* and *GAD2* as a molecular response to

decrease the synthesis of GABA from glutamate, and why? Thus, we performed a secondary analysis and manually reviewed our RNA-sequencing data ($p < 0.05$) to identify 20 gene transcripts related to GABA and glutamate synthesis, release, transport and metabolism (Benjamini-Hochberg adjusted $p < 0.05$; **Figure 4.10A**). We uploaded this list to Enrichr, a comprehensive gene set enrichment analysis suite (Kuleshov et al., 2016), to confirm the fidelity of these genes to GABAergic and glutamatergic biological processes. Indeed, we identified GABA signaling pathway, GABAergic synaptic transmission, glutamate metabolic process, dicarboxylic acid catabolic and biosynthetic processes, acidic amino acid transport, L-glutamate transport, aspartate family amino acid metabolic process, glutamatergic synaptic transmission, chemical synaptic transmission as biological processes overrepresented in 5-HT_{2A}R knockdown vs. control rats ($p < 0.05$ for all biological processes; **Figure 4.10B**).

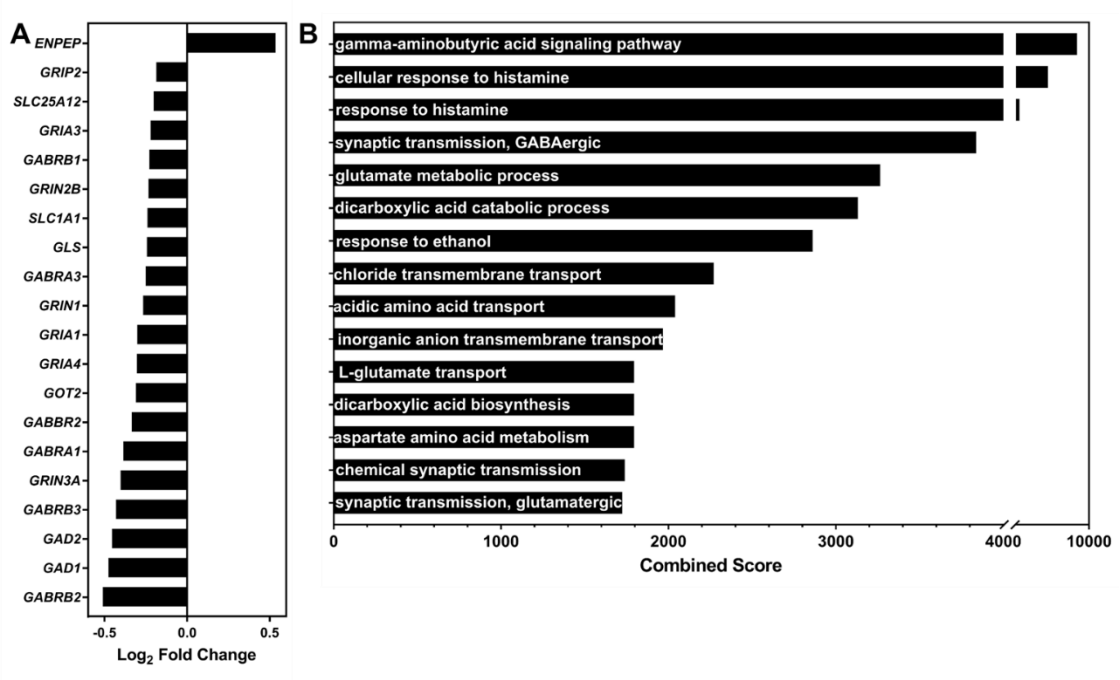

Figure 4.10: Engineered loss of the 5-HT_{2A}R in the mPFC may modulate gene transcripts related to glutamate and GABA synthesis, release, transport, and metabolism relative to control rats.

(A) The log fold change value (x-axis) for identified gene transcripts (y-axis) are displayed (black bars). Genes showing a Benjamini-Hochberg adjusted $p < 0.05$ are considered significantly different. (B) The x-axis depicts the combined score, which multiplies the log of the unadjusted *p*-value computed with the Fisher exact test by the *z*-score. A Benjamini-Hochberg adjusted $p < 0.05$ was considered significant for functional gene enrichment analyses.

These data prompt many questions related to the potential impact of 5-HT_{2A}R knockdown on GABA and glutamate synthesis, release, metabolism, and transport in neuronal and non-neuronal cells in the mPFC, particularly *if* the directionality of protein changes is empirically consistent with changes in mRNA expression shown here. We first must consider the cell types that could contribute to the excitatory/inhibitory balance of the mPFC depicted in **Figure 4.11A**. First, the mPFC is innervated by glutamate-releasing fibers originating in the thalamus (Beique et al., 2007), and stimulation of the 5-HT_{2A}R localized to presynaptic

thalamocortical terminals triggers the release of glutamate in the mPFC (Marek et al., 2001; Zhou and Hablitz, 1999). Second, about ~25% of the neuronal cell bodies in the mPFC are GABAergic local circuit interneurons while the remaining ~75% of neurons project to downstream brain regions; these are primarily glutamatergic pyramidal neurons (Santana and Artigas, 2017), although it was recently demonstrated that a small population of these projection neurons are GABAergic (Bravo-Rivera et al., 2015). The 5-HT_{2A}R localizes to a greater extent in glutamate vs. GABA neurons in the mPFC (Amargos-Bosch et al., 2004; Burnet et al., 1995; Lopez-Gimenez et al., 2001; Pompeiano et al., 1994; Santana and Artigas, 2017). Third, the mPFC contains non-neuronal cell types that express the 5-HT_{2A}R including astrocytes (Xu and Pandey, 2000), which clear glutamate from the extrasynaptic space (Sanacora et al., 2012) and produce glutamate as a product of the tricarboxylic acid (TCA) cycle (Rowley et al., 2012); the role of astrocytic 5-HT_{2A}R in the mPFC is largely unexplored, but this is a target for future studies. Thus, there is a diverse population of 5-HT_{2A}R-expressing cell types in the mPFC, and we have to consider *how* our NSC-eGFP AAV2 or 5-HT_{2A}R-shRNA-eGFP-AAV2 constructs could impact these different cells types based on the properties of the viral vectors.

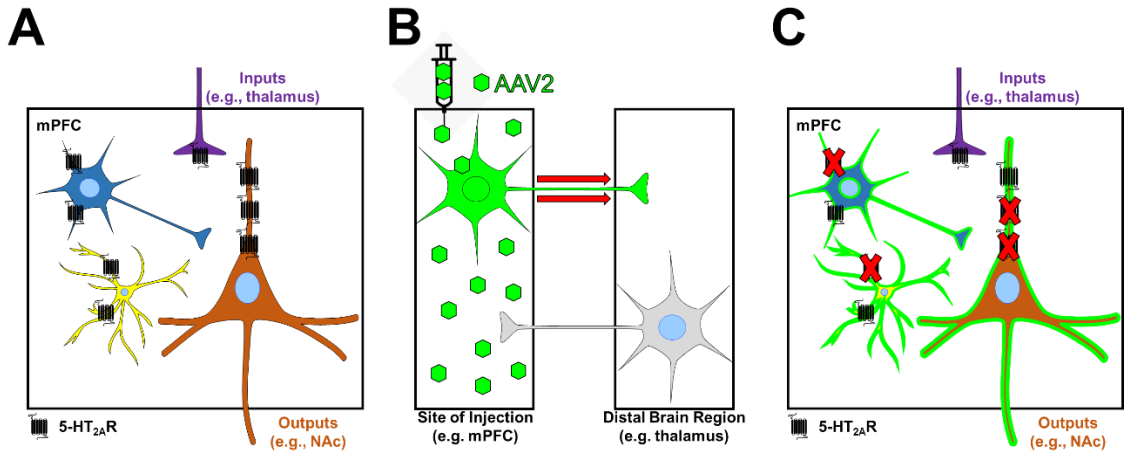

Figure 4.11: Schematic overview of (A) the cell types that modulate glutamate and GABA synthesis, release, metabolism, and transport in the mPFC, (B) AAV2 anterograde transport between two brain regions following infusion, and (C) cells hypothesized to be transduced by intra-mPFC infusion of NSC-eGFP AAV2 or 5-HT_{2A}R-shRNA-eGFP AAV2.

Based on the viral properties of AAV serotype 2, NSC-eGFP AAV2 and 5-HT_{2A}R-shRNA-eGFP AAV2 are hypothesized to be uptaken by the cell body and transported down the axon towards the presynaptic terminal of the neuron (i.e., anterograde transport). Thalamocortical fibers innervate the mPFC (purple), but because these cell bodies do not reside in the mPFC, they are not hypothesized to be transduced by NSC-eGFP AAV2 or 5-HT_{2A}R-shRNA-eGFP AAV2. The excitatory/inhibitory balance of the mPFC is largely governed by local GABA interneurons (blue) and glutamatergic pyramidal neurons (orange) that project to output regions like the NAc. The mPFC also contains resident glial cells such as astrocytes (yellow). The cell bodies of these GABAergic neurons, glutamatergic pyramidal neurons, and astrocytes reside in the mPFC. Thus, these cell bodies are hypothesized to be transduced by NSC-eGFP AAV2 or 5-HT_{2A}R-shRNA-eGFPAAV2 and are outlined in green.

The AAV serotype 2 transduces the cell body, and the virus undergoes robust axonal *anterograde* transport when injected into the rat brain (Salegio et al., 2013). Axonal anterograde transport is the transport of viral particles from the cell body down the axon towards the presynaptic terminal; we provide a graphical definition in **Figure 4.11B**, modified from (Salegio et al., 2013). In line with these properties of the virus, the AAV2 serotype was demonstrated to transduce all major cell bodies of the cortex (including neurons and astrocytes) (Aschauer et al., 2013).

Thus, the cell bodies **outlined in green in Figure 4.11C**, including glutamatergic pyramidal neurons, GABAergic interneurons, and glial cells such as astrocytes, are hypothesized to be transduced by the NSC-eGFP AAV2 or 5-HT$_{2A}$R-shRNA-eGFP AAV2. However, the thalamocortical fibers that terminate in the mPFC will *not* be transduced by the virus because AAV2 vectors are *not* hypothesized to be taken up by presynaptic terminals (i.e., the AAV2 does not undergo axonal *retrograde* transport, or the transport of viral particles from the presynaptic terminal down the axon towards the cell body). Taken together, our 5-HT$_{2A}$R-shRNA-eGFP AAV2 is hypothesized to knockdown the 5-HT$_{2A}$R on local GABAergic interneurons, projection neurons (including glutamate pyramidal neurons), and astrocytes in the mPFC, but the 5-HT$_{2A}$R will not be knocked down on the thalamocortical fibers that terminate in the mPFC (visual summary in **Figure 4.11C**).

Finally, how do we align our RNA-sequencing data, knowledge of AAV2 transduction and axonal transport, and knowledge of mPFC circuitry to infer how 5-HT$_{2A}$R knockdown could impact the excitatory/inhibitory balance of the mPFC [**Figure 4.12**, modified from (Rowley et al., 2012)]? First, we hypothesize that intra-mPFC infusion of 5-HT$_{2A}$R-shRNA-eGFP AAV2 decreases GABA synthesis and sequestration into synaptic vesicles, perhaps on local GABAergic interneurons, which may decease GABA release in the mPFC as a consequence of 5-HT$_{2A}$R knockdown (**Figure 4.12, red**). Consistent with this hypothesis, our RNA-sequencing data revealed lower expression of gene transcripts encoding glutaminase (*GLS)* and GAD (*GAD1/*2), which suggests decreased synthesis of

GABA from glutamate in 5-HT$_{2A}$R knockdown vs. control rats. Additionally, lower expression of vesicular GABA transporter (VGAT; *SLC25A12)* suggests less GABA sequestration into synaptic vesicles in 5-HT$_{2A}$R knockdown vs. control rats; synaptic vesicles store neurotransmitters that are released at the synapse. Second, we hypothesize that intra-mPFC infusion of 5-HT$_{2A}$R-shRNA-eGFP AAV2 increases glutamate release from thalamocortical terminals as a *molecular, compensatory response* to the effects of 5-HT$_{2A}$R knockdown in the mPFC (**Figure 4.12, cyan**). This is an intriguing hypothesis considering that these terminals are not hypothesized as *directly* impacted by the 5-HT$_{2A}$R-shRNA-eGFP AAV2 [because AAV2 is not hypothesized to be taken up by presynaptic terminals (Salegio et al., 2013)]. In particular, we observed lower expression of glutamate transporter 3 (EAAT3; *SLC1A1*), which suggests decreased glutamate reuptake that could indicate increased synaptic glutamate levels in 5-HT$_{2A}$R knockdown vs. control rats (Rowley et al., 2012). Additionally, we observed lower expression of aspartate aminotransferase (AAT*; GOT2*) in 5-HT$_{2A}$R knockdown vs. control rats, suggesting that less glutamate is sequestered in mitochondria for utilization in the TCA cycle in 5-HT$_{2A}$R knockdown vs. control rats, which may yield higher glutamate concentrations in the thalamocortical presynaptic terminal to be released into the synapse (Kharazia and Weinberg, 1994). Third, we propose that future studies should also explore the role of astrocytes in the excitatory/inhibitory balance of the mPFC, considering that reduced astrocyte function can decrease glutamate clearance from the extrasynaptic space and impair rates of glutamine synthesis from both glutamate and GABA (Sanacora et al., 2012). Precisely why

glutamate and GABA sequestration and release in the mPFC may be altered in the face of 5-HT$_{2A}$R knockdown is a keen area for future studies, perhaps using *in vivo* microdialysis to quantify local efflux levels of each neurotransmitter.

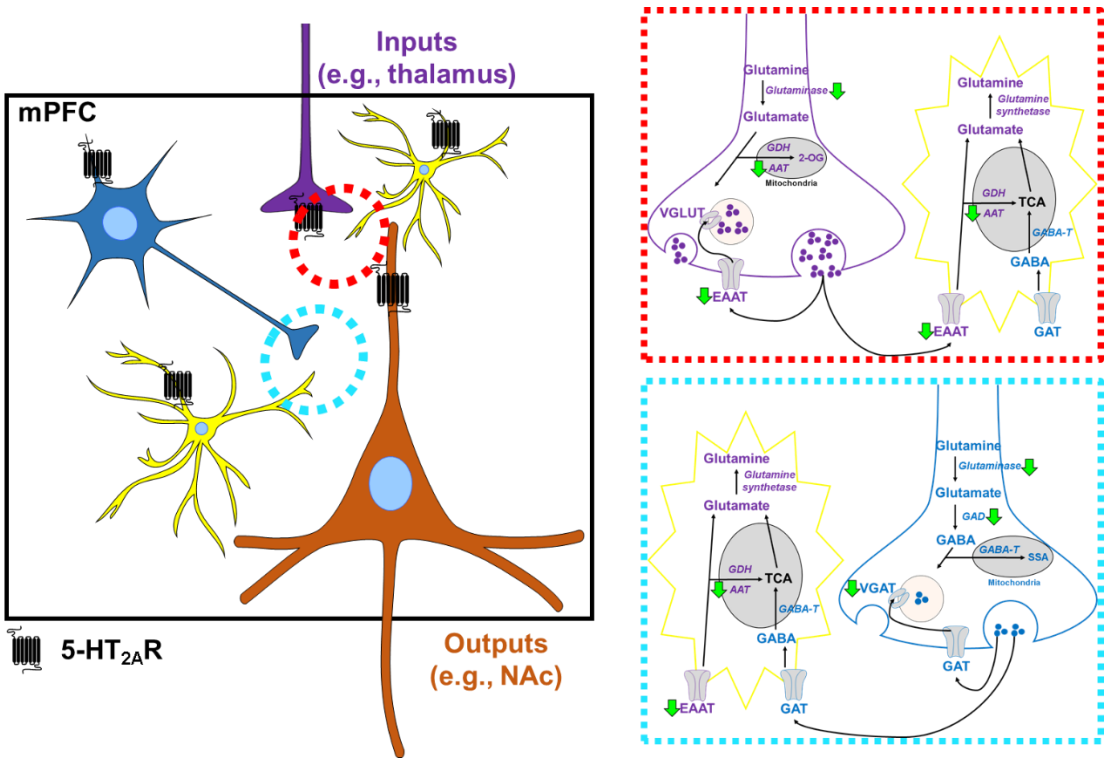

Figure 4.12: Potential sites of action for 5-HT$_{2A}$R knockdown to modulate the excitatory/inhibitory balance of the mPFC relative to control rats.

Visual summary of gene transcripts involved in the synthesis, packaging, release, transport, and metabolism of glutamate and GABA (Rowley et al., 2012). Genes decreased in the mPFC of 5-HT$_{2A}$R-shRNA-eGFP vs. NSC-eGFP rats are denoted by green arrows. 2-OG, 2-oxoglutarate; AAT, aspartate aminotransferase; EAAT, glutamate transporter; GABA-T, GABA transaminase; GAD, glutamic acid decarboxylase; GAT; GABA transporter, GDH, glutamate dehydrogenase; SSA, succinic semialdehyde; TCA, tricarboxylic acid cycle; vGAT, vesicular GAT; vGLUT; vesicular glutamate transporter.

Our cumulative evidence suggest that infusion of 5-HT$_{2A}$R-shRNA-eGFP may establish a new excitatory/inhibitory balance in the mPFC, which underscores the profound influence that this brain region confers over impulsive action. A potential next step, then, is to selectively target the 5-HT$_{2A}$R-shRNA-eGFP to

specific neuronal and non-neuronal cell types in the mPFC and elucidate the effects on impulsive action. For example, 5-HT_{2A}R knockdown in mPFC glutamate pyramidal vs. GABA neurons on impulsive action may be achieved by using gene promoters that direct shRNA expression to GABAergic [e.g., fugu-somatostatin (Nathanson et al., 2009)] or glutamatergic neurons [e.g., calcium/calmodulin-dependent protein kinase II (Scheyltjens et al., 2015)]. Moreover, 5-HT_{2A}R knockdown in thalamocortical fibers may be achieved using an AAV serotype with *retrograde* properties [e.g., AAV6 (Kasper et al., 2016)].

There are limitations in the framework of this interpretation. In particular, future studies will need to appreciate efferent regulation of the mPFC which includes 5-HT terminal input from the raphe (with or without the co-release of glutamate and/or other neuropeptides), VTA DA efferents (with or without the co-release of glutamate and/or other neuropeptides), and other brain regions that may modulate impulsive action (Dalley and Robbins, 2017; Noori et al., 2012), perhaps through future transcriptomic or proteomic studies. Additionally, a critical question moving forward is: how do the molecular consequences of mPFC 5-HT_{2A}R knockdown interact with the potential, neurobiological adaptations over repeated 1-CSRT task training? There are no observed differences in 5-HT levels in cortical dialysate sampled from rats under basal conditions vs. during 5-CSRT task performance (Dalley et al., 2002). However, the tonic firing rate of dorsal raphe 5-HT neurons – some of which terminate in the mPFC (Hoover and Vertes, 2007) – increases in the initial seconds that rats are required to wait for a delayed food reward (Miyazaki et al., 2011). Whether sensitization of 5-HT neuronal firing occurs

over repeated exposure to delay periods in the 1-CSRT task is an interesting avenue for future studies and could clarify how the molecular consequences of mPFC 5-HT_{2A}R knockdown interact with repeated 1-CSRT task training. Nonetheless, exploring how the 5-HT_{2A}R system modulates impulsive action at both micro- and macro-circuit levels are critical steps towards understanding the role of this receptor in the pathophysiology of impulsive action.

Table 4.1: 1-CSRT task (A) descriptive statistics and (B) two-way ANOVA outcomes for AAV2 treatment and ITI8 challenge (mean±SEM)

(a) Descriptive Statistics									
AAV2 Treatment	**ITI8 Challenge**	**Premature Responses**			**Reinforcers Earned**	**Accuracy (%)**	**Omissions (%)**	**Latency to Start (sec)**	**Time to Finish (sec)**
		Target	**Non-target**	**Total**					
NSC-eGFP	ITI8-1	58.4 ± 3.04	4.00 ± 0.88	62.4 ± 2.60	27.7 ± 3.41	97.8 ± 0.78	9.22 ± 1.94	1.12 ± 0.148	1139 ± 17.5
	ITI8-2	57.0 ± 2.13	5.56 ± 1.17	62.6 ± 2.69	29.9 ± 2.36	95.9 ± 1.35	6.33 ± 1.77	0.787 ± 0.16	1155 ± 16.1
	ITI8-3	55.1 ± 2.20	4.22 ± 0.88	59.3 ± 2.32	32.0 ± 1.27	95.9 ± 1.50	7.22 ± 1.50	0.784 ± 0.15	1151 ± 14.9
	ITI8-4	57.6 ± 2.67	4.11 ± 1.16	61.7 ± 2.57	32.8 ± 2.57	98.2 ± 0.75	5.00 ± 1.60	1.51 ± 0.36	1134 ± 15.3
5-HT$_{2A}$R-shRNA-eGFP	ITI8-1	54.9 ± 2.03	5.50 ± 0.60	60.4 ± 1.90	27.6 ± 1.10	93.8 ± 2.09	10.1 ± 1.53	0.66 ± 0.19	1181 ± 14.1
	ITI8-2	59.1 ± 2.40	3.80 ± 0.71	62.9 ± 2.51	28.5 ± 1.05	96.9 ± 1.10	7.70 ± 2.14	1.28 ± 0.291	1152 ± 18.3
	ITI8-3	57.6 ± 2.16	5.90 ± 1.37	63.5 ± 2.38	28.0 ± 1.94	94.6 ± 2.12	7.00 ± 2.98	1.38 ± 0.361	1171 ± 24.3
	ITI8-4	58.3 ± 3.79	5.20 ± 1.02	63.5 ± 3.58	27.1 ± 2.59	94.9 ± 1.74	7.90 ± 3.55	1.31 ± 0.563	1179 ± 26.7
(b) Two-Way ANOVA									
		Premature Responses			**Reinforcers Earned**	**Accuracy (%)**	**Omissions (%)**	**Latency to Start (sec)**	**Time to Finish (sec)**
		Target	**Non-target**	**Total**					
AAV2 Treatment		$F_{1,17}$=0.04, n.s.	$F_{1,17}$=0.63, n.s.	$F_{1,17}$=0.22, n.s.	$F_{1,17}$=2.26, n.s.	$F_{1,17}$=2.04, n.s.	$F_{1,17}$=0.38, n.s.	$F_{1,17}$=0.12, n.s.	$F_{1,17}$=2.84, n.s.
ITI8 Challenge		$F_{3,51}$=0.26, n.s.	$F_{3,51}$=0.08, n.s.	$F_{3,51}$=0.18, n.s.	$F_{3,51}$=0.63, n.s.	$F_{3,51}$=0.35, n.s.	$F_{3,51}$=0.94, n.s.	$F_{3,51}$=1.35, n.s.	$F_{3,51}$=0.08, n.s.
Interaction		$F_{3,51}$=0.60, n.s.	$F_{3,51}$=1.40, n.s.	$F_{3,51}$=0.60, n.s.	$F_{3,51}$=0.82, n.s.	$F_{3,51}$=1.26, n.s.	$F_{3,51}$=0.19, n.s.	$F_{3,51}$=1.85, n.s.	$F_{3,51}$=0.73, n.s.

Table 4.2: 1-CSRT task (A) descriptive statistics and (B) two-way ANOVA outcomes for AAV2 treatment and pimavanserin pretreatment on an ITI5 schedule (mean±SEM)

(a) Descriptive Statistics									
AAV2 Treatment	**Pimavanserin Pretreatment**	**Premature Responses**			**Reinforcers Earned**	**Accuracy (%)**	**Omissions (%)**	**Latency to Start (sec)**	**Time to Finish (sec)**
		Target	**Non-target**	**Total**					
NSC-eGFP	VEH	16.1 ± 0.96	0.96 ± 0.23	17.1 ± 0.98	77.1 ± 0.85	97.7 ± 0.77	3.93 ± 1.06	0.98 ± 0.13	843 ± 17.6
	0.01 mg/kg	18.2 ± 1.46	1.00 ± 0.33	19.2 ± 1.62	72.8 ± 2.42	97.3 ± 0.82	5.89 ± 1.34	1.38 ± 0.40	888 ± 32.1
	0.1 mg/kg	10.1 ± 1.42*	0.78 ± 0.40	10.9 ± 1.65*	82.6 ± 1.80	98.2 ± 0.62	5.00 ± 1.31	0.68 ± 0.11	859 ± 24.6
	1 mg/kg	6.22 ± 1.27*	0.33 ± 0.24	6.56 ± 1.41*	83.6 ± 3.24	97.3 ± 1.20	7.67 ± 2.15	0.93 ± 0.23	909 ± 34.1*
5-HT$_{2A}$R-shRNA-eGFP	VEH	18.9 ± 2.38	1.73 ± 0.61	20.6 ± 2.46	71.7 ± 2.90	96.2 ± 1.25	5.03 ± 1.21	1.68 ± 0.47	898 ± 29.6
	0.01 mg/kg	17.7 ± 3.43	2.10 ± 0.66	19.8 ± 3.37	69.9 ± 3.70	95.6 ± 1.45	7.30 ± 2.15	2.82 ± 0.76	931 ± 35.4
	0.1 mg/kg	12.1 ± 2.46$^{\#}$	1.20 ± 0.29	13.3 ± 2.42$^{\#}$	75.5 ± 2.74	95.8 ± 1.20	8.00 ± 2.17	3.89 ± 1.96	933 ± 31.1
	1 mg/kg	7.50 ± 1.54$^{\#}$	0.60 ± 0.27	8.10 ± 1.66$^{\#}$	77.5 ± 3.10	96.1 ± 1.03	11.4 ± 2.92$^{\#}$	1.58 ± 0.35	1010 ± 45.3$^{\#}$
(b) Two-Way ANOVA									
		Premature Responses			**Reinforcers Earned**	**Accuracy (%)**	**Omissions (%)**	**Latency to Start (sec)**	**Time to Finish (sec)**
		Target	**Non-target**	**Total**					
AAV2 Treatment		$F_{1,17}$=0.35	$F_{1,17}$=2.38	$F_{1,17}$=0.78	$F_{1,17}$=3.52	$F_{1,17}$=1.62	$F_{1,17}$=1.01	$F_{1,17}$=5.17†	$F_{1,17}$=2.83
Pimavanserin Pretreatment		$F_{3,51}$=25.4†	$F_{3,51}$=3.75†	$F_{3,51}$=26.8†	$F_{3,51}$=7.31†	$F_{3,51}$=0.32	$F_{3,51}$=5.99†	$F_{3,51}$=0.88	$F_{3,51}$=8.64†
Interaction		$F_{3,51}$=0.43	$F_{3,51}$=0.58	$F_{3,51}$=0.31	$F_{3,51}$=0.33	$F_{3,51}$=0.27	$F_{3,51}$=0.55	$F_{3,51}$=1.13	$F_{3,51}$=0.95
* $p < 0.05$ vs. NSC-eGFP VEH; # $p < 0.05$ vs. 5-HT$_{2A}$R-shRNA-eGFP VEH; † $p < 0.05$ for a significant outcome of the two-way ANOVA									

Table 4.3: 1-CSRT task (A) descriptive statistics and (B) two-way ANOVA outcomes for AAV2 treatment and pimavanserin pretreatment on an ITI8 schedule (mean±SEM)

(a) Descriptive Statistics									
AAV2 Treatment	**Pimavanserin Pretreatment**	**Premature Responses**			**Reinforcers Earned**	**Accuracy (%)**	**Omissions (%)**	**Latency to Start (sec)**	**Time to Finish (sec)**
		Target	**Non-target**	**Total**					
NSC-eGFP	VEH	53.3 ± 2.53	3.78 ± 1.09	57.1 ± 2.52	33.9 ± 1.57	95.4 ± 1.24	7.33 ± 3.05	1.87 ± 0.89	1176 ± 24.3
	0.1 mg/kg	45.7 ± 2.64*	2.33 ± 0.87	48.0 ± 2.61^	45.8 ± 2.55*	97.8 ± 1.16	5.22 ± 2.36	0.69 ± 0.25	1162 ± 22.5
5-HT$_{2A}$R-shRNA-eGFP	VEH	58.3 ± 2.19	4.70 ± 1.12	63.0 ± 1.90	31.4 ± 1.82	96.0 ± 1.04	4.40 ± 1.15	0.98 ± 0.24	1171 ± 18.9
	0.1 mg/kg	47.1 ± 4.49#	4.90 ± 1.34	52.0 ± 4.06#	38.6 ± 3.07#	94.9 ± 1.45	7.30 ± 2.94	8.44 ± 6.50	1221 ± 31.0#
(b) Two-Way ANOVA									
		Premature Responses			**Reinforcers Earned**	**Accuracy (%)**	**Omissions (%)**	**Latency to Start (sec)**	**Time to Finish (sec)**
		Target	**Non-target**	**Total**					
AAV2 Treatment		$F_{1,17}$=0.68	$F_{1,17}$=1.33	$F_{1,17}$=1.93	$F_{1,17}$=3.49	$F_{1,17}$=3.56	$F_{1,17}$=2.82	$F_{1,17}$=0.98	$F_{1,17}$=0.72
Pimavanserin Pretreatment		$F_{1,17}$=18.5†	$F_{1,17}$=1.45	$F_{1,17}$=22.7†	$F_{1,17}$=20.6†	$F_{1,17}$=0.51	$F_{1,17}$=0.07	$F_{1,17}$=0.83	$F_{1,17}$=1.90
Interaction		$F_{1,17}$=0.65	$F_{1,17}$=2.54	$F_{1,17}$=0.20	$F_{1,17}$=1.24	$F_{1,17}$=0.61	$F_{1,17}$=2.54	$F_{1,17}$=1.56	$F_{1,17}$=5.58†

* $p < 0.05$ vs. NSC-eGFP VEH; # $p < 0.05$ vs. 5-HT$_{2A}$R-shRNA-eGFP VEH; ^ $p = 0.055$ vs. NSC-eGFP VEH; † $p < 0.05$ for a significant outcome of the two-way ANOVA

Table 4.4: Ingenuity Pathway Analysis reveals canonical pathways that are overrepresented in the mPFC of 5-HT_{2A}R knockdown vs. control rats.

Ingenuity Canonical Pathways	-log(p-value)	Pathway Coverage Ratio	z-score
Synaptogenesis Signaling Pathway	5.28	0.13	-4.22
Neuroinflammation Signaling Pathway	4.15	0.12	2.41
Role of NFAT in Cardiac Hypertrophy	3.86	0.13	-2.35
Synaptic Long Term Potentiation	3.39	0.15	-2.83
Huntington's Disease Signaling	3.33	0.12	-2.24
Calcium Signaling	3.26	0.13	-3.44
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	3.17	0.27	2.00
CREB Signaling in Neurons	3.00	0.12	-2.52
Opioid Signaling Pathway	2.93	0.11	-2.20
nNOS Signaling in Neurons	2.90	0.20	-2.00
Endocannabinoid Neuronal Synapse Pathway	2.87	0.14	-3.15
CCR5 Signaling in Macrophages	2.73	0.15	-2.33
Tumoricidal Function of Hepatic Natural Killer Cells	2.71	0.26	2.00
p38 MAPK Signaling	2.65	0.14	2.50
Neuropathic Pain Signaling In Dorsal Horn Neurons	2.62	0.14	-3.21
Apoptosis Signaling	2.39	0.14	2.50
T Cell Exhaustion Signaling Pathway	2.34	0.12	2.12
Chemokine Signaling	2.34	0.15	-2.71
Apelin Cardiomyocyte Signaling Pathway	2.31	0.14	-2.31
Gαq Signaling	2.30	0.12	-2.18
CD28 Signaling in T Helper Cells	2.20	0.13	-2.12
Synaptic Long Term Depression	2.03	0.11	-2.36
Death Receptor Signaling	1.89	0.13	2.11
Th1 Pathway	1.59	0.11	3.00
Dopamine-DARPP32 Feedback in cAMP Signaling	1.58	0.10	-2.32
Sperm Motility	1.58	0.10	-2.33
Sumoylation Pathway	1.56	0.12	-2.12
Adrenomedullin signaling pathway	1.44	0.10	-2.50
RANK Signaling in Osteoclasts	1.41	0.11	-2.53

Chapter 5: Inherent Impulsive Action Associates with Specific Gene Targets in the Rat Medial Prefrontal Cortex

INTRODUCTION

Impulsivity is a biopsychosocial construct characterized by decreased sensitivity to the negative consequences of behavior, rapid, unplanned reactions to stimuli, and a disregard for long-term consequences (Moeller et al., 2001a). High impulsivity is observed across neuropsychiatric diseases, including attention deficit hyperactivity disorder, bipolar disorder, and substance use disorders (for review) (Bari and Robbins, 2013; Moeller et al., 2001a). Thus, identifying targets for neuropharmacological intervention that reduce impulsivity could reveal novel, transdiagnostic treatment strategies (Robbins et al., 2012; Volkow et al., 2015). Next-generation sequencing strategies have rapidly developed over the past decade, allowing researchers to profile the complete set of gene transcripts in the brain (transcriptome) and identify gene expression differences between organisms (Geschwind and Konopka, 2009; Volkow et al., 2015; Wang et al., 2009). Simultaneously, the development of publicly-available, human-curated gene enrichment tools allows researchers to map large gene lists to common biological processes, pathways, and molecular functions, illuminating an otherwise complex neurobiology (Huang da et al., 2009). The tandem use of RNA-sequencing and bioinformatics is providing new avenues for neuropharmacological target discovery for the treatment of disorders with an impulsive dimension (Eipper-Mains

et al., 2013; Moloney et al., 2019; Robison and Nestler, 2011; Zhang et al., 2016; Zhang et al., 2018).

Impulsivity is a multifaceted construct generally subdivided into impulsive action (motor impulsivity; inability to withhold a premature response) and impulsive choice (selection of a small, immediate reward over a larger but delayed reward) (Evenden, 1999a; Moeller et al., 2001a). Individual differences in impulsive action can be stably identified in the rat using the CSRT tasks including the 1-CSRT task (Anastasio et al., 2014b; Besson et al., 2013; Caprioli et al., 2014; Dalley et al., 2007; Economidou et al., 2012; Fink et al., 2015; Sholler et al., 2018). Limbic-corticostriatal circuit structures modulate impulsive action (for review) (Cunningham and Anastasio, 2014) while a recent transcriptome study revealed that HI rats identified in the 2-CSRT task exhibit a specific gene transcript profile in the NAc relative to LI rats (Moloney et al., 2019). The inability to withhold a premature response reflects in part the failure of “top-down” executive control by the mPFC over the NAc; this concept is supported by lesion, pharmacological, and genetic manipulation studies (Anastasio et al., 2014b; Anastasio et al., 2019; Chudasama et al., 2003; Muir et al., 1996; Narayanan et al., 2006). Catecholamine, glutamate, and 5-HT neurotransmission are key neuromodulators of the mPFC and signal through an array of receptor targets including GPCRs (e.g., 5-HT_{2A}R) that have been implicated in impulsive action (for reviews) (Cunningham and Anastasio, 2014; Pattij and Vanderschuren, 2008; Robbins, 2002). Taken together, the mPFC represents an integral node in the

pathophysiology of impulsive action as well as a hotbed for neuropharmacological target discovery for disorders with an impulsive dimension.

The present study was designed to characterize individual differences in the mPFC transcriptome of HI vs. LI male, Sprague-Dawley rats to identify novel gene targets for future impulsivity research. We hypothesized that individual differences in impulsive action are characterized by gene transcript expression differences in the mPFC. We identified HI and LI rats based upon premature responses committed in the 1-CSRT task (Anastasio et al., 2015; Anastasio et al., 2014b; Fink et al., 2015). We employed RNA-sequencing to identify a registry of gene transcripts that are differentially expressed in the mPFC of HI vs. LI rats. Further, we conducted a battery of bioinformatics analyses to extract biological insight from our gene list. The following set of experiments establishes a transcriptomic landscape in the mPFC that may characterize individual differences in impulsive action.

METHODS

General Methods

ANIMALS. Male, Sprague–Dawley rats (*n* = 96; Envigo, Haslett, MI) weighed 250–275 g upon arrival and housing in the colony. Rats were housed two per cage and maintained on a 12-h light–dark cycle with maintained temperature (21–23°C) and humidity (45–50%). Rats were acclimated to the colony for five to seven days before the beginning of handling (handling occurred for one to three days prior to the start of 1-CSRT task training). A mild food restriction (~90% free-feeding

weight) was imposed during 1-CSRT task training, and rats had *ad libitum* access to water except during daily operant sessions. Experiments were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (2011) and with the University of Texas Medical Branch Institutional Animal Care and Use Committee approval.

1-Choice Serial Reaction Time (1-CSRT) task. 1-CSRT task training occurred in five-hole, nose-poke operant chamber equipped with a houselight, food tray, and an external pellet dispenser that delivered 45 mg dustless precision food pellets (Bio-Serv, Frenchtown, NJ). Operant chambers were housed within ventilated, sound-attenuated cubicles (MedAssociates, St Albans, VT). A detailed explanation of 1-CSRT task methodology has been previously published (Anastasio et al., 2013; Anastasio et al., 2011; Anastasio et al., 2014b; Cunningham et al., 2013; Fink et al., 2015; Sholler et al., 2018). In summary, rats were first exposed to a pre-training stage to permit habituation to the test chamber. A nose-poke response into the illuminated center stimulus hole (i.e., a "target" response) resulted in the illumination of the magazine light on the opposite chamber wall as well as the delivery of a food pellet. After the pre-training stage, rats progressed through a series of training stages. Each daily session lasted 100 trials or 30 minutes, whichever occurred first. Throughout each training stage, the stimulus duration was incrementally decreased until a final stage of 0.5 sec was achieved with a limited hold of 5 sec and an ITI5.

In each session, a maximum of 100 target responses resulted in a maximum of 100 reinforcers delivered. Incorrect "non-target" responses, premature

responses, or omissions resulted in a 5-sec time-out period that decreased the possible number of reinforcers earned. Before advancing through each training stage, rats were required to meet acquisition criteria: ≥ 50 reinforcers earned, > 80% accuracy (target responses/(target + non-target responses) * 100) and < 20% omissions (omitted responses/trials completed * 100) (Anastasio et al., 2013; Anastasio et al., 2011; Anastasio et al., 2014b; Cunningham et al., 2013; Fink et al., 2015; Sholler et al., 2018). The number of premature responses, omissions, and reinforcers earned, percent accuracy, latency to first response, and time to finish the 1-CSRT task were recorded. Premature responses, the primary measure of impulsive action, were categorized into three types: target, non-target, and total (target + non-target) (Sholler et al., 2018). The number of reinforcers earned measured task competency and provided a secondary measure of impulsive action. The percent accuracy gave a general indication of attentional capacity. Percent omissions indicated motivation to perform the task, and latency to first response in the 1-CSRT task served as a secondary measure of motivation and an indication of general motor impairment.

IDENTIFICATION OF IMPULSIVE ACTION PHENOTYPE. Once rats achieved the stability criteria for the final training stage over three consecutive ITI5 sessions (with < 20% variability over the last three sessions), an ITI8 challenge session was conducted in which the ITI was 8-s for the entirety of the session; the ITI8 session enhances sensitivity to detect phenotypic differences in inherent impulsive action (Anastasio et al., 2014b; Dalley et al., 2002; Fink et al., 2015). Following the ITI8 challenge, rats were restabilized on ITI5 sessions (< 20% variability over three

sessions) before a second ITI8 challenge. Premature responses averaged over both ITI8 challenge sessions were used to identify impulsive action phenotype for each rat. HI and LI rats were defined by the upper and lower quartile of premature responses, respectively, averaged over both ITI8 challenge sessions.

Research Design

Gene transcripts in the mPFC of HI and LI rats ("RNA-seq cohort"). Following phenotype identification of rats in the RNA-seq cohort (*n* = 48), 1-CSRT task sessions were discontinued, and rats were returned to their home cages for six days. Rats were anesthetized with chloral hydrate (400 mg/kg), and brains were extracted. The mPFC (encompassing ACC, PL, and IL) (Paxinos and Watson, 2005) was microdissected immediately over ice, flash frozen in liquid nitrogen, and stored at −80 °C for subsequent RNA extraction. The mPFC was homogenized in 10X w/v extraction buffer (20 mM HEPES, 200 mM NaCl,1 mM EDTA, 1 mM DTT, 10 µL/ml protease inhibitor cocktail, 10 µl/ml phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich, St Louis, MO), and 5 µL/ml RNaseOUT™ Recombinant Ribonuclease Inhibitor (Thermo-Fisher Scientific, Waltham, MA). Immediately following initial homogenization, the sample was transferred to 500 µL of TRI Reagent (Life Technologies, Grand Island, NY), and purified RNA was isolated using RNeasy Mini Kit (Qiagen, Germantown, MD). RNA concentration and quality were analyzed using Cytation 5 Cell Imaging Multi-Mode Reader (BioTek Instruments, Winooski, VT). RNA was stored at −80 °C until assayed.

The RNA samples from a subset of HI (n = 4) and LI (n = 4) rats were utilized for RNA-sequencing of genes in the mPFC. Total RNA sequencing was performed at the University of California, Los Angeles Technology Center for Genomics and Bioinformatics. Libraries were prepared using Kapa Stranded RNA-Seq Kit (Kapa Biosystems, Wilmington, MA). During the library preparation step, samples underwent enrichment, cDNA conversion, end repair, A-tailing, multiplexing, and amplification. Libraries were sequenced as single-end, 50 base-pair reads using the Illumina Hiseq3000 instrument generating between 12 and 23 million reads per sample. Data quality control was done using CLC Genomics Workbench 12.0.2 (Qiagen Bioinformatics, Seoul, Korea) using "QC for sequencing reads" workflow.

As the first step of the analysis, reads were mapped to the University of California at Santa Cruz transcript set (updated February, 2017) (Tyner et al., 2017) using Bowtie2 version 2.1.0 (Langmead and Salzberg, 2012) and gene expression levels were estimated using RNA-Seq by Expectation Maximization (v1.2.15) (Li and Dewey, 2011). The trimmed mean of M-values was used to normalize gene expression. Differentially expressed genes were identified using Fisher's exact test in the Empirical Analysis of Digital Gene Expression Data in R program (v3) (Robinson et al., 2010). Genes showing a false discovery rate (FDR) adjusted $p < 0.1$ (calculated using Benjamini–Hochberg procedure) (Moloney et al., 2019) and log counts per million mapped reads (CPM) > 1 were considered significantly different in the mPFC of HI vs. LI rats. Functional gene enrichment analysis was carried out using Enrichr (updated May, 2017), a web-based gene set enrichment analysis tool (Chen et al., 2013; Kuleshov et al., 2016). Enrichr

uses the Fisher exact test (α = 0.05), the *z*-score of the deviation from the expected rank by the Fisher exact test, and a combined score that multiplies the log of the Benjamini-Hochberg adjusted *p*-value computed with the Fisher exact test by the *z*-score (Moloney et al., 2019).

The ConTra online portal (v3) (Kreft et al., 2017) was used to predict transcription factor binding sites in the calcium voltage-gated channel subunit alpha1 E (*CACNA1E*) gene (Kreft et al., 2017). Transcription factor DNA-binding motifs for Mothers Against Decapentaplegic Homolog (*SMAD)* (matrix profile MA0513.1) and RE1 Silencing Transcription Factor (*REST*) (matrix profile MA0138.2) were gathered from the JASPAR open-access database of curated, non-redundant transcription factor binding profiles (Khan et al., 2018). ConTra visualization analysis was employed for the region encompassing 500 base pairs upstream of the *CACNA1E* transcriptional start site (TSS; chr1:181382294, number of introns: 48, ENST00000367573) to cover the putative promoter region. A core match score cutoff > 0.95 and a similarity matrix cutoff > 0.85 was applied to minimize false positive results.

Gene target quantification with quantitative reverse transcription PCR ("qRT-PCR cohort"). Following phenotype identification of rats in the qRT-PCR cohort (*n* = 48), 1-CSRT task sessions were discontinued, and rats were returned to their home cages for eight days. Rats were anesthetized [chloral hydrate solution (400 mg/kg)], and brains were extracted. The mPFC (containing IL, PL, and ACC) (Paxinos and Watson, 2005) was microdissected immediately

over ice, flash frozen in liquid nitrogen, and stored at −80 °C for subsequent RNA extraction as described above.

A subset of HI (*n* = 4) and LI (*n* = 6) rats were utilized to quantify gene targets in the mPFC using qRT-PCR. Reverse transcription was performed using SuperScript III First Strand Synthesis System (ThermoFisher Scientific, Waltham, MA) with random hexamer primers. qRT-PCR reactions were assayed in technical triplicate on a 7500 Fast RT PCR System using TaqMan Fast Advanced Master Mix and TaqMan gene specific primer/probes [glutamate ionotropic receptor N-methyl-D-aspartate subunit 2A (*GRIN2A)*: Rn00561341_m1; Erb-B2 receptor tyrosine kinase 4 (*ERBB4)*: Rn00572447_m1; *CACNA1E*: Rn00494444_m1; calneuron 1 (*CALN1)*: Rn01481697_m1; leucyl and cystinyl aminopeptidase (*LNPEP)*: Rn01441624_m1; cyclophilin A (*PPIA*): Rn00690933_m1; Life Technologies, Carlsbad, CA]. Data were analyzed in terms of the crossing threshold (CT) for a gene target relative to the reference gene cyclophilin A (*PPIA*) [$\Delta CT = CT_{(Target)} - CT_{(PPIA)}$].The difference in ΔCT values for a gene target were then compared between HI vs. LI rats [$\Delta\Delta CT = \Delta CT_{(HI)} - \Delta CT_{(LI)}$]. The difference in gene target expression in presented as a fold change value [expression fold change = $2^{(-\Delta\Delta CT)}$].

Statistical Analyses

A two-way ANOVA with the factors of phenotype (HI, LI) and cohort (RNA-seq cohort, qRT-PCR cohort) was used to analyze 1-CSRT task measures (total, target, and non-target premature responses, reinforcers earned, % omissions, %

accuracy, latency to first response, time to finish the 1-CSRT task). Subsequent *a priori* comparisons were analyzed using Dunnett's procedure with an experiment-wise error rate of $\alpha = 0.05$. Fisher's exact test was used to analyze differential gene expression data, and FDR multiplicity correction was performed using the Benjamini-Hochberg method with an experiment-wise error rate of $\alpha = 0.1$. Functional gene enrichment using Enrichr (biological processes, pathway analysis, and *in silico* transcription factor binding analysis) was analyzed using Fisher's exact test, and FDR multiplicity correction was performed using the Benjamini-Hochberg method with an experiment-wise error rate of $\alpha = 0.05$. Student's *t*-test was employed to evaluate differences in expression fold change for each gene target between HI vs. LI rats with an experiment-wise error rate of $\alpha=0.05$.

RESULTS

HI and LI rats are identifiable using the 1-CSRT task

Two cohorts of outbred rats were stratified for phenotypic levels of impulsive action using the 1-CSRT task (**Figure 5.1, see experimental timeline**); rats successfully achieved the stability criteria for the final training stage over three consecutive ITI5 sessions (with < 20% variability over the last three sessions) before ITI8 challenge sessions were administered. **Figure 5.1A** demonstrates phenotypic stratification of rats in a representative population (RNA-seq cohort) by upper (HI, red) and lower (LI, blue) quartiles of premature responding during ITI8 challenge sessions; mid impulsive (MI, white) rats represent the middle two quartiles of premature responding (Anastasio et al., 2015; Anastasio et al., 2014b;

Cunningham and Anastasio, 2014; Fink et al., 2015). Rats from the RNA-seq cohort were utilized for RNA-sequencing (n = 4 LI, n = 4 HI) while rats from the qRT-PCR cohort were utilized for qRT-PCR analyses (n = 6 LI, n = 4 HI). Importantly, there was no main effect of cohort on any measure in the 1-CSRT task (see statistical analyses in **Table 5.1**). In both cohorts, HI rats displayed increased total (**Figure 5.1B**; $p < 0.05$) and target ($p < 0.05$, **Table 5.1**) premature responses (**Figure 5.1B**; $p < 0.05$), decreased reinforcers earned, (**Figure 5.1C**; $p < 0.05$), decreased percent omissions (**Figure 5.1D**; $p < 0.05$), and decreased time to finish the 1-CSRT task ($p < 0.05$, **Table 5.1**) than LI rats during ITI8 challenge sessions, as we have demonstrated previously (Anastasio et al., 2015; Anastasio et al., 2014b; Fink et al., 2015). There was no difference in non-target premature responses, percent accuracy, or latency to start the 1-CSRT task between HI vs. LI rats (n.s., **Table 5.1**). See **Table 5.1** for details and statistical analyses of 1-CSRT task performance.

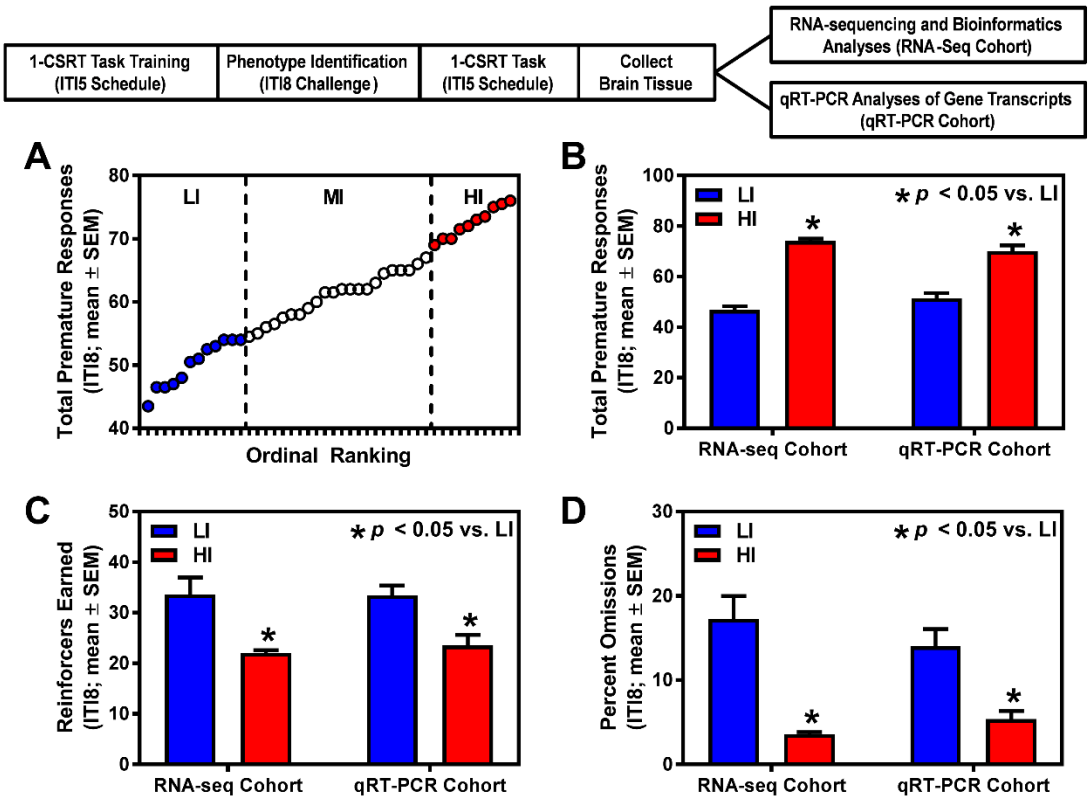

Figure 5.1: The 1-CSRT task differentiates impulsive action phenotypes.

The experimental timeline is shown above. (A) Ordinal ranking of a representative cohort (RNA-seq cohort) based on premature responses averaged over two ITI8 challenge sessions. Low impulsive (LI, blue circles) and high impulsive rats (HI, red circles) were defined by lower and upper quartiles, respectively, from mid impulsive rats (MI, white circles). In both the RNA-seq (n = 4 HI; n = 4 LI) and qRT-PCR (n = 4 HI; n = 6 LI) cohorts, HI rats (red bars) exhibited (B) increased total premature responses, (C) decreased reinforcers earned, and (D) decreased percent omissions relative to LI rats (blue bars; *p < 0.05 vs LI).

RNA sequencing reveals 18 gene transcripts of interest in high impulsive action

Our goal was to identify a registry of candidate gene targets in the mPFC that may engender impulsive action phenotypes. RNA sequencing of the mPFC revealed n = 18 genes of interest exhibiting a FDR-adjusted p < 0.1 (Moloney et al., 2019); all of the 18 genes of interest were higher in the mPFC of HI (n = 4) vs.

LI (*n* = 4) rats (**Figure 5.2**). The five gene targets exhibiting the greatest expression fold change in HI vs. LI rats were *CACNA1E (*log fold change = 0.800; $p < 0.1$), *CALN1 (*log fold change = 0.752; $p < 0.1$), *LNPEP (*log fold change = 0.748; $p < 0.1$), *ERBB4 (*log fold change = 0.740; $p < 0.1$), and *GRIN2A (*log fold change = 0.714; $p < 0.1$).

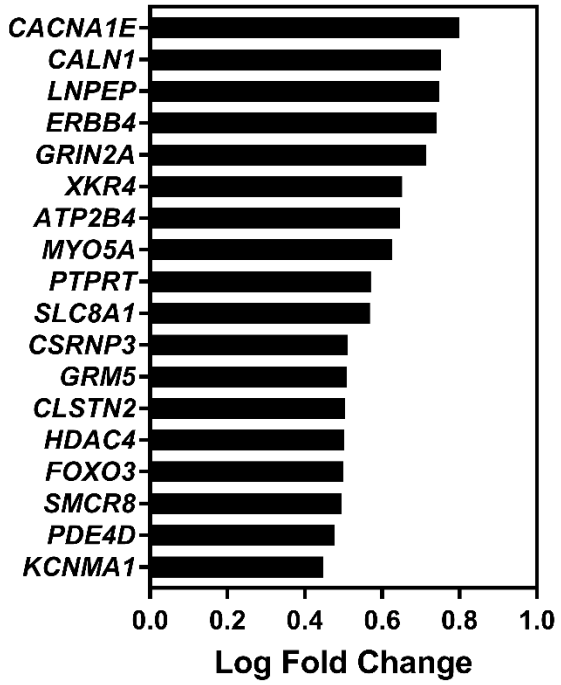

Figure 5.2: RNA-sequencing reveals *n*=18 gene transcripts that are higher in the mPFC of HI vs. LI rats.

The log fold change value (x-axis) for identified gene transcripts (y-axis) are displayed (black bars). Trimmed mean of M-values (TMM) were used to normalize gene expression in the mPFC of HI (*n* = 4) vs. LI (*n* = 4) rats. Differentially expressed genes were identified using the Empirical Analysis of Digital Gene Expression Data in R program. Genes showing a false discovery rate (FDR) adjusted $p < 0.1$ and counts per millions (CPM) > 1 were considered significantly different.

Gene enrichment identifies overrepresented biological pathways in high impulsive action

Gene set enrichment analysis extracts biological insight from RNA-sequencing datasets, providing insight into biological mechanisms that may

underlie individual differences in impulsive action (Huang da et al., 2009). We first utilized “Gene Ontology (GO): biological process” to explore biological processes which involve the targeted genes in our list. In total, 72 biological processes exhibiting a Benjamini-Hochberg adjusted $p < 0.05$ were implicated in our enrichment analysis; the ten biological processes exhibiting the greatest combined score are listed in **Table 5.2**. Functional enrichment suggested that genes related to adenylate cyclase-inhibiting G-protein coupled glutamate receptor signaling (combined score = 18.089; $p < 0.05$; **Figure 5.3**) and regulation of cell communication by electrical coupling (combined score = 21.835; $p < 0.05$; **Figure 5.3**) were overrepresented in the mPFC of HI vs. LI rats. Moreover, a specific role for metal ion homeostasis (especially, calcium homeostasis) was implicated by several biological processes, including calcium ion transport into cytosol (combined score = 27.262; $p < 0.05$; **Figure 5.3**), calcium ion import (combined score = 20.397; $p < 0.05$; **Figure 5.3**), cellular monovalent inorganic cation homeostasis (combined score = 18.822; $p < 0.05$; **Figure 5.3**), inorganic cation import across plasma membrane (combined score = 18.802; $p < 0.05$; **Figure 5.3**), and cellular metal ion homeostasis (combined score = 18.197; $p < 0.05$; **Figure 5.3**). The last three biological processes were regulation of cardiac conduction (combined score = 22.909; $p < 0.05$; **Figure 5.3**), regulation of release of sequestered calcium ion into cytosol by sarcoplasmic reticulum (combined score = 20.039; $p < 0.05$; **Figure 5.3**), and vascular smooth muscle contraction (combined score = 16.158; $p < 0.05$; **Figure 5.3**). Genes belonging to these three biological processes included solute carrier family 8 member A1 (*SLC8A1*),

ATPase plasma membrane calcium transporting 4 (*ATP2B4*), and phosphodiesterase 4D (*PDE4D*), and a secondary analysis of tissue expression profiles using GeneCards® (Weizmann Institute of Science, Rehovot, Israel) confirmed that these gene transcripts are expressed in brain as well as peripheral tissues (i.e., heart and smooth muscle), which explains why these three biological processes were overrepresented in our enrichment analysis.

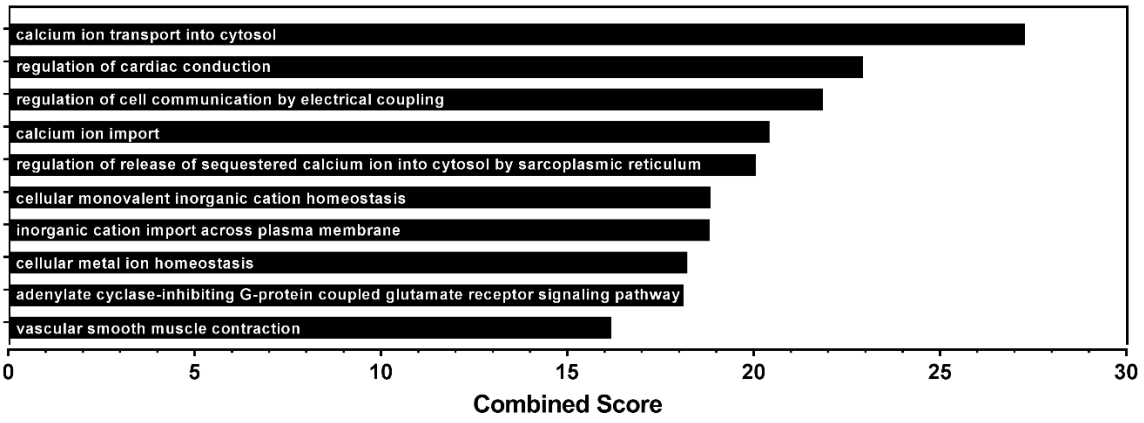

Figure 5.3: Functional gene enrichment reveals biological processes that are overrepresented in the mPFC of HI vs. LI rats.

The x-axis depicts the combined score, which multiplies the log of the *p*-value computed with the Fisher exact test by the *z*-score. A Benjamini-Hochberg adjusted $p < 0.05$ was considered significant for functional gene enrichment analyses.

Next, we employed Protein Analysis Through Evolutionary Relationships (PANTHER) Pathway Analysis tools to explore biological pathways that are overrepresented in our gene list. Four PANTHER pathways were implicated in our enrichment analysis (**Table 5.3**). Interestingly, genes in the ionotropic glutamate receptor pathway (combined score = 9.344; $p < 0.05$; **Figure 5.4**) and metabotropic glutamate receptor group 1 (combined score = 10.910; $p < 0.05$; **Figure 5.4**) and group 3 (combined score = 18.901; $p < 0.05$; **Figure 5.4**) signaling pathways were

overrepresented in the mPFC of HI vs. LI rats. Additionally, genes in the heterotrimeric G-protein G_q alpha and G_o alpha mediated signaling pathways (combined score = 7.676; $p < 0.05$; **Figure 5.4**) were overrepresented in the mPFC of HI vs. LI rats.

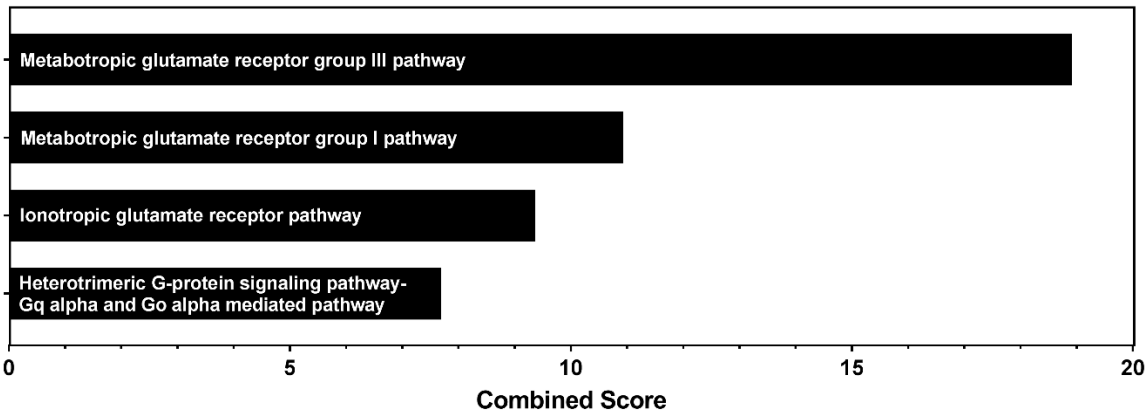

Figure 5.4: Functional gene enrichment reveals PANTHER pathways that are overrepresented in the mPFC of HI vs. LI rats.

The x-axis depicts the combined score, which multiplies the log of the *p*-value computed with the Fisher exact test by the *z*-score. A Benjamini-Hochberg adjusted $p < 0.05$ was considered significant for functional gene enrichment analyses.

Transcription factor enrichment identifies *SMAD4* and *REST* as overrepresented in high impulsive action

Gene set enrichment analysis illuminates the *downstream* biological processes and pathways that are associated with our gene transcripts of interest. It is also valuable to identify candidate *upstream* transcription factors that may regulate expression of the entire gene network of interest in high impulsive action. The availability of chromatin-immunoprecipitation (ChIP) sequencing databases [e.g., Encyclopedia of DNA Elements; ChIP-X Enrichment Analysis] permits *in silico* transcription factor enrichment analysis, or the identification of transcription

factors that are predicted to bind to a high proportion of genes in our list (Moloney et al., 2019). Transcription factor enrichment analysis revealed that *REST* (combined score = 15.599, $p < 0.05$; **Table 5.4**) was overrepresented in our gene list. *REST* was predicted to bind to protein tyrosine phosphatase receptor type T (*PTPRT*), *CACNA1E*, XK related 4 (*XKR4*), histone deacetylase 4 (*HDAC4*), *GRIN2A*, calsyntenin 1 (*CLSTN1*), and *CALN1* (**Figure 5.5**). Further, *SMAD4* (combined score = 15.293, $p < 0.05$; **Table 5.4**) was overrepresented in our gene list. *SMAD4* is predicted to bind *PTPRT*, *PDE4D*, *CACNA1E*, *SLC8A1*, and potassium calcium-activated channel subfamily M alpha 1 (*KCNMA1*) (**Figure 5.5**). Thus, the 18 genes of interest in high impulsive action are linked to the transcription factors *SMAD4* and *REST*, two potential upstream regulators of this gene network.

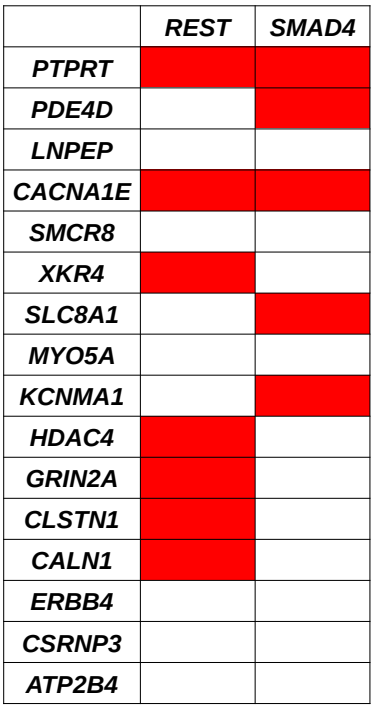

Figure 5.5: Transcription factor enrichment identifies *SMAD4* and *REST* as overrepresented in high impulsive action.

Red squares represent a transcription factor (top of the table) that is predicted to bind the corresponding gene transcript *in silico* (left of the table). A Benjamini-Hochberg adjusted $p < 0.05$ was considered significant for functional gene enrichment analyses.

In silico identification of a conserved *SMAD* binding site in the *CACNA1E* promoter region

Biological processes related to calcium homeostasis were overrepresented in our gene list while our *in silico* analysis predicted that *SMAD4* and *REST* bind to the *CACNA1E* gene. We used the ConTra web server (Kreft et al., 2017) to predict *SMAD* and *REST* binding sites on the *CACNA1E* gene 500 base pairs upstream of the transcriptional start site, or the putative promoter region. Transcripton factor DNA-binding motifs for *SMAD2/3/4* (**Figure 5.6A, left**) and *REST* (**Figure 5.6A, right**) were successfully identified in JASPAR, an open-access database of transcription factor binding profiles; JASPAR is a human-

curated database, and transcription factor binding sites are identified *in vivo* by methods such as ChIP or *in vitro* by methods based on binding of large pools of DNA fragments (e.g. protein-binding microarrays) (Khan et al., 2018). Transcription factor DNA-binding motifs for *SMAD2/3/4* and *REST* were successfully mapped *in silico* to the *CACNA1E* promoter region with a core match score cutoff > 0.95 and a similarity matrix cutoff > 0.85 (**Figure 5.6B**). In promoter region block 6 (**Figure 5.6B, dashed line**), a *SMAD* binding site was identified 215-309 base pairs upstream of the *CACNA1E* transcriptional start site that was conserved across 16 species (**Figure 5.6C, blue**). Within the same promoter region block 6, an identified *REST* binding site was conserved across three species (**Figure 5.6C, orange**).

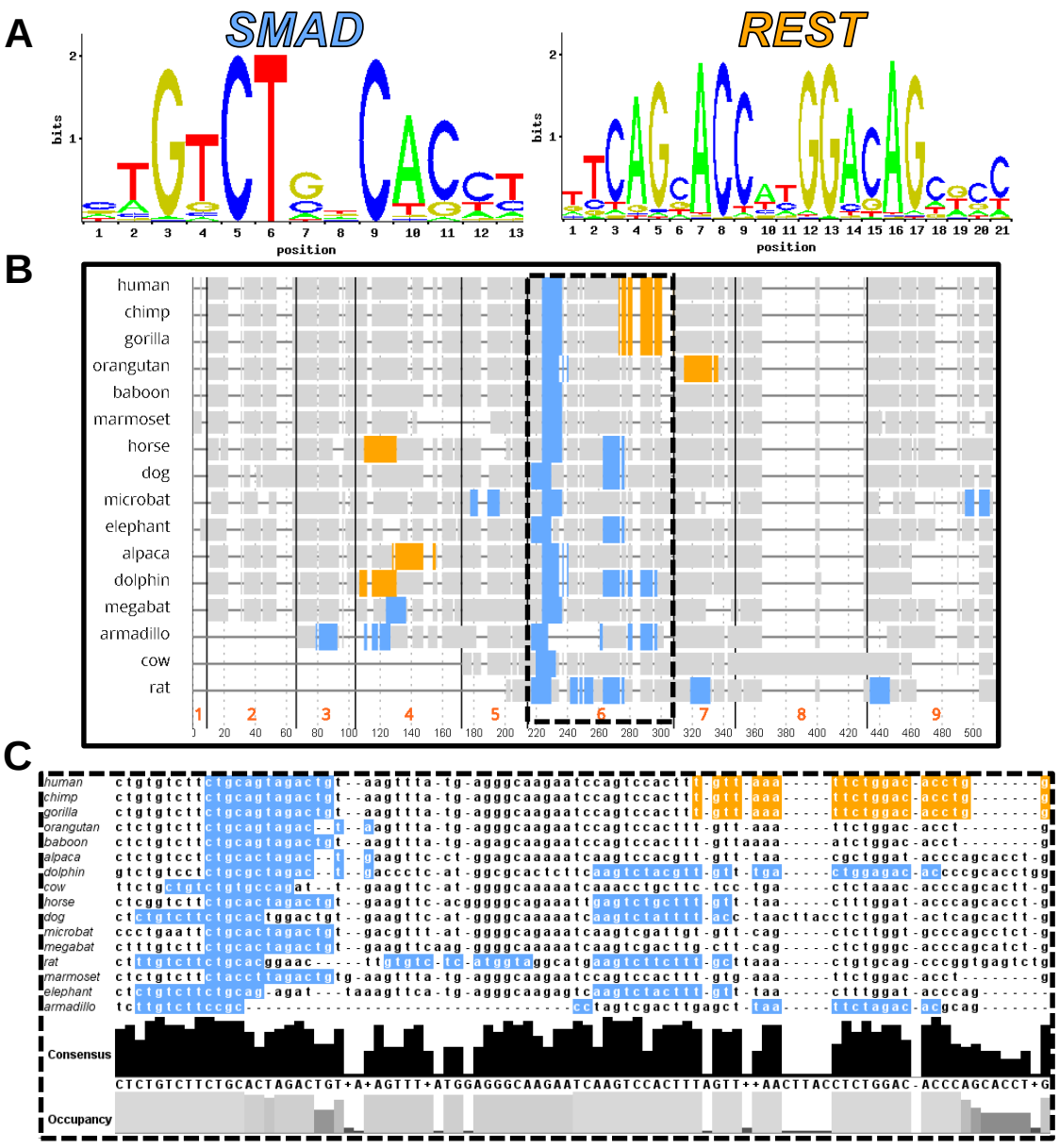

Figure 5.6: *In silico* identification of conserved *SMAD* and *REST* binding sites in the *CACNA1E* promoter region.

JASPAR sequence logos for (A) *SMAD2/3/4* (matrix profile MA0513.1) and *REST* (matrix profile MA0138.2) are shown. (B) ConTra (v3) software was used to visualize transcription factor binding 500 base pairs upstream of the *CACNA1E* transcriptional start site (TSS; chr1:181382294, number of introns: 48, ENST00000367573). (C) Visualization in block 6 of the promoter region (215-309 base pairs upstream of the *CACNA1E* TSS) a reveals *SMAD2/3/4* binding site that is conserved across 16 species (blue) and a *REST* binding sites that is conserved across 3 species (orange).

qRT-PCR quantification reveals *CACNA1E* and *LNPEP* overexpressed in HI vs. LI rats

We employed qRT-PCR as a secondary assay to quantify expression of five gene transcripts (*CACNA1E, CALN1, LNPEP, ERBB4, GRIN2A*) in the mPFC of HI vs. LI rats; these gene transcripts exhibited the greatest log fold change values in our RNA-seq experiment (see **Figure 5.2**). A two-tailed *t*-test revealed that *CACNA1E* [$t_8 = 2.793$; $p < 0.05$; **Figure 5.7A**] and *LNPEP* [$t_8 = 2.859$; $p < 0.05$; **Figure 5.7C**] mRNA expression was higher in the mPFC of HI vs. LI rats. However, no difference in mRNA expression was observed for *CALN1* [$t_8 = 1.161$; n.s.; **Figure 5.7B**], *ERBB4* [$t_8 = 0.428$; n.s.; **Figure 5.7D**], or *GRIN2A* [$t_8 = 0.115$; n.s.; **Figure 5.7E**].

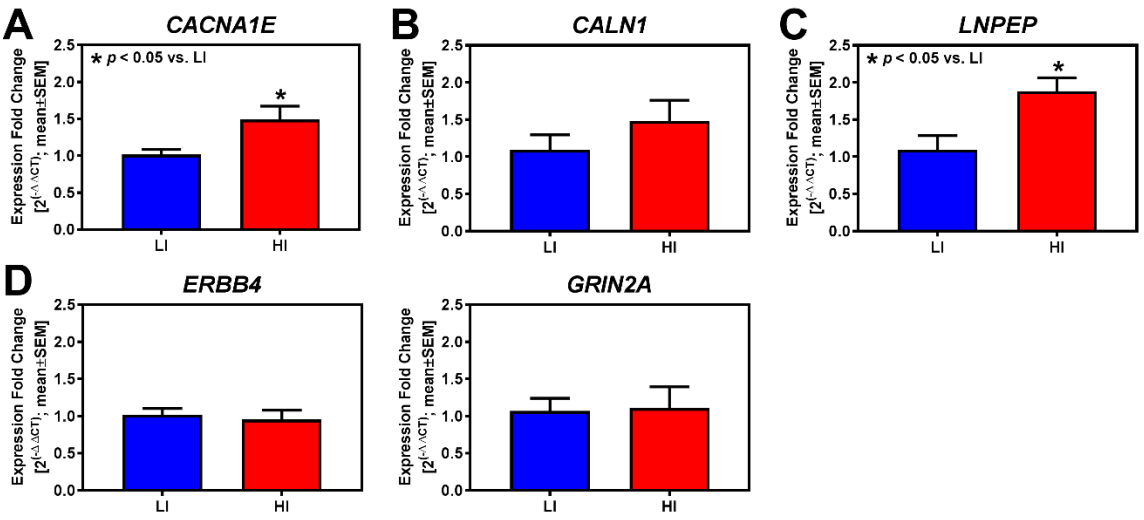

Figure 5.7: qRT-PCR quantification reveals *CACNA1E* and *LNPEP* overexpressed in HI vs. LI rats.

qRT-PCR quantification of (A) *CACNA1E*, (B) *CALN1*, (C) *LNPEP*, (D) *ERBB4*, and (E) *GRIN2A* were analyzed in terms of the crossing threshold (CT) for a gene target relative to the reference gene cyclophilin A (*PPIA*) [$\Delta CT = CT_{(Target)} - CT_{(PPIA)}$].The difference in ΔCT values for a gene target were then compared between HI vs. LI rats [$\Delta\Delta CT = \Delta CT_{(HI)} - \Delta CT_{(LI)}$]. The difference in gene target expression in presented as a fold change value in which expression fold change = $2^{(-\Delta\Delta CT)}$ (*$p < 0.05$ vs. LI, $n = 4$ HI, $n = 6$ LI).

DISCUSSION

The present study used tandem RNA-sequencing and bioinformatics to identify a registry of gene transcripts that are higher in the mPFC of HI and LI rats, proposing a transcriptomic landscape that may characterize individual differences in impulsive action. Intriguingly, all 18 gene transcripts were higher in the mPFC of HI vs. LI rats, which is interesting considering that the heterotrimeric G-protein G_q alpha and G_o alpha mediated signaling pathways and biological processes related to calcium homeostasis were overrepresented in our gene list. These data suggest that $G_{\alpha q}$ and calcium signaling pathways may be *activated* in the mPFC of HI vs. LI rats, while these same pathways were predicted as *inhibited* following 5-HT_{2A}R knockdown in the mPFC (**Chapter 4**). Taken together, perhaps differences in the $G_{\alpha q}$ and calcium signaling pathways are a shared cortical substrate of individual differences in impulsive action and variation in 5-HT_{2A}R functional status, a target for future studies.

Notably, a recent transcriptome study failed to identify gene transcripts in the PL subregion of the mPFC that associate with inherent impulsive action (Moloney et al., 2019). However, the present study utilized the entire rat mPFC (encompassing ACC, PL, and IL). Excitotoxic lesion (Chudasama et al., 2003) and chemogenetic inactivation of the ACC (Koike et al., 2016) does not alter premature responding, but does impair attentional processing on the 5-CSRT task in rats. Perhaps the ACC modulates the ability to attend to the impending stimulus, which is inherent to making an appropriately-timed response. Further, the IL modulates premature responding in CSRT tasks as demonstrated through lesion,

pharmacological, and genetic manipulation studies (Anastasio et al., 2014b; Anastasio et al., 2019; Chudasama et al., 2003). Thus, the different mPFC subregions employed for RNA-sequencing between the present study and a previous study (Moloney et al., 2019) may explain the discordance in our RNA-sequencing findings. Further, we selected five gene transcripts that exhibited the greatest log-fold change difference between HI and LI rats and evaluated these transcripts using qRT-PCR. In doing so, we confirmed that *LNPEP* and *CACNA1E* mRNA expression was higher in the mPFC of HI vs. LI rats. Our utilization of a secondary assay (qRT-PCR) strengthens our interpretation that high impulsive action may be associated with high *LNPEP* and *CACNA1E* mRNA expression in the mPFC, presenting two novel targets for future impulsivity research.

The *LNPEP* gene encodes a zinc-dependent aminopeptidase – leucyl and cystinyl aminopeptidase – that cleaves several peptide hormones including oxytocin (Elkins et al., 2017). Interestingly, spontaneously hypertensive rats, which exhibit high impulsive action (Ferguson et al., 2007; Orduna et al., 2009), express higher plasma levels of the *LNPEP* protein relative to Wistar-Kyoto rats (Prieto et al., 2018). Higher plasma levels of the *LNPEP* protein could indicate greater cleavage of oxytocin in high impulsive action, which would yield lower levels of plasma oxytocin. Consistent with this hypothesis, oxytocin levels in human serum negatively correlate with self-report impulsivity scores on the BIS-11 for both male adolescent patients with attention-deficit hyperactivity disorder as well as age-matched controls (Demirci et al., 2016). Perhaps enhanced oxytocin degradation

by the *LNPEP* protein mediates the relationship between low oxytocin levels and high impulsivity, which is an interesting avenue for future studies.

Our discovery that *CACNA1E* mRNA is higher in the mPFC of HI vs. LI rats is interesting in light of our gene enrichment analyses demonstrating that biological processes related to calcium homeostasis are overrepresented in our gene list. The *CACNA1E* gene encodes an α_1 subunit-containing R-type voltage-gated calcium channel ($Ca_V2.3$) (Berrou et al., 2002). Calcium influx into the presynaptic terminal through R-type voltage-gated calcium channels evokes synaptic neurotransmitter release, including in rodent cortex (Qian and Noebels, 2001; Wu et al., 1998), and postsynaptic GPCRs (like the 5-HT_{2A}R) mediate cellular responses to neurotransmitters and neuromodulators (Rosenbaum et al., 2009). Intriguingly, we also found that metabotropic glutamate receptor and heterotrimeric G-protein signaling pathways were overrepresented in our gene list. Several lines of evidence suggest a functional interplay between voltage-gated calcium channels and GPCR signaling (Johnson and Lovinger, 2016), and possibly through 5-HT GPCRs (Johnson and Lovinger, 2016). Little is known about the association between R-type calcium channels and 5-HT receptor activity, however, some insights can be gleaned from another family member: the L-type voltage-gated calcium channel alpha1 C (*CACNA1C*) (Catterall, 2011). For example, bath application of a 5-HT_2R agonist inhibits L-type calcium currents in layer V-VI mPFC pyramidal neurons (Day et al., 2002). Similarly, *CACNA1C* knockout selectively in dorsal raphe 5-HT neurons enhances expression of the immediate early gene *Fos* as well as response to a 5-HT_{1A} receptor antagonist, suggesting that loss of

CACNA1C disrupts 5-HT neuronal activation in the dorsal raphe, perhaps through a 5-HT_{1A} receptor-dependent mechanism (Ehlinger and Commons, 2019). It should be noted that R-type channels are presynaptically localized while the L-type channels localize to both pre- and postsynaptic neuronal terminals (Catterall, 2011), and this difference in neuronal localization should be considered as we investigate a potential functional interaction between R-type channels and 5-HT receptor activity. Nonetheless, previous studies on the association between L-type calcium channels and 5-HT receptor activity are hypothesis generating, considering that impulsive action has been extensively linked to 5-HT receptor function in the mPFC (Cunningham and Anastasio, 2014). Taken together, these studies indicate a promising future research direction to explore the functional interplay between R-type voltage-gate calcium channels and 5-HT receptor signaling as integral to inherent impulsive action.

Transcriptional regulation and mRNA synthesis are controlled by transcription factor binding to specific DNA sequences (Lambert et al., 2018). We first performed an *in silico* analysis to identify potential upstream transcriptional regulators of *CACNA1E* mRNA synthesis. We found that *SMAD4* (or “common mediator *SMAD*”) is predicted to bind the *CACNA1E* gene. *SMAD4* heterocomplexes with other *SMAD* family members to mediate activation of *SMAD*-dependent gene targets (de Caestecker et al., 2000; Ross and Hill, 2008). An intriguing future experiment, perhaps using chromatin immunoprecipitation, could test the hypothesis that *SMAD4* immuoprecipitates with *CACNA1E* to a greater extent in the mPFC of HI vs. LI rats, forging a neurobiological mechanism

by which *CACNA1E* transcriptional regulation associates with inherent impulsive action. We generated support for this mechanism by performing an *in silico* analysis to identify a potential *SMAD* binding site in the *CACNA1E* rat gene promoter; the promoter encompasses the site of transcription initiation, a process that catalyzes mRNA synthesis (Butler and Kadonaga, 2002). We predicted a *SMAD* binding site 215-309 base pairs upstream of the rat *CACNA1E* transcriptional start site *in silico*. Intriguingly, this binding site was predicted as conserved across 15 other species. Conservation of a DNA sequence does not always indicate a functional binding site (Dermitzakis and Clark, 2002; Kreft et al., 2017), but this comparative genomics approach is an effective first step to identify a potential, functional gene regulatory element (Loots et al., 2002). Thus, upstream transcriptional regulation by the *SMAD* heterocomplex is a potential mechanism by which *CACNA1E* mRNA expression is higher in the mPFC of HI vs. LI rats.

Our cumulative evidence suggest that *CACNA1E* is a promising target for impulsivity research. However, the $Ca_v2.3$ protein is highly understudied in impulsive action, and the present findings are limited to the level of the gene transcript. The application of validated antibodies, genetic inactivation (i.e., RNA interference, gene knockout) and pharmacological tools to interrogate $Ca_v2.3$ is a worthwhile starting point to illuminate the neuropharmacology of R-type voltage-gated calcium channels in impulsivity. First, the availability of $Ca_v2.3$-knockout mice provides a tool to assess global loss of the $Ca_v2.3$ on impulsive action. It should be noted that $Ca_v2.3$-knockout mice given *ad libitum* access to water and standard chow display greater body weight relative to control mice (Matsuda et al.,

2001), which could impact food-reinforced responding in the CSRT tasks. Second, we have identified a validated, commercially available antibody (ACC-006, Alomone Labs, Jerusalem, Israel) that enables future studies evaluating $Ca_V2.3$ expression in the mPFC of HI vs. LI rats. Third, pharmacological $Ca_V2.3$ blockade can be achieved using SNX-482, a selective $Ca_V2.3$ peptide antagonist that is available for commercial use (Schneider et al., 2013). Taken together, we provide a framework for evaluating $Ca_V2.3$ as a target to reduce impulsive action, providing a new mechanistic direction for impulsivity research.

Table 5.1: Two cohorts of rats exhibit stable performance in the 1-CSRT task.

LI (*n* = 4-6/cohort)								
	Premature Responses			Reinforcers Earned	Accuracy (%)	Omissions (%)	Latency to Start (sec)	Time to Finish (sec)
	Target	Non-target	Total					
RNA-seq Cohort (mean ± SEM)	46.875 ± 1.434	40.750 ± 1.362	6.125 ± 2.322	33.625 ± 3.344	93.402 ± 2.334	17.250 ± 2.742	1.078 ± 0.154	1244 ± 18.501
qRT-PCR Cohort (mean ± SEM)	51.417 ± 2.022	46.083 ± 1.399	5.333 ± 0.997	33.417 ± 1.977	96.042 ± 1.346	14.000 ± 2.070	1.471 ± 0.230	1204 ± 19.368
HI (*n* = 4/cohort)								
	Premature Responses			Reinforcers Earned	Accuracy (%)	Omissions (%)	Latency to Start (sec)	Time to Finish (sec)
	Target	Non-target	Total					
RNA-seq Cohort (mean ± SEM)	74.125 ± 0.875 *	65.583 ± 1.250 *****	8.542 ± 0.809	22.000 ± 0.612 *****	98.767 ± 0.729	3.583 ± 0.250 *	2.752 ± 1.139	1124 ± 11.216 *
qRT-PCR Cohort (mean ± SEM)	70.000 ± 2.354 *	64.375 ± 4.719 *	5.625 ± 2.392	23.500 ± 2.121 *****	95.740 ± 1.082	5.375 ± 0.966 *****	1.049 ± 0.196	1122 ± 10.437 *
Two-Way ANOVA								
	Premature Responses			Reinforcers Earned	Accuracy (%)	Omissions (%)	Latency to Start (sec)	Time to Finish (sec)
	Target	Non-target	Total					
Cohort	$F_{1, 14} = 0.012$	$F_{1, 14} = 0.708$	$F_{1, 14} = 1.228$	$F_{1, 14} = 0.082$	$F_{1, 14} = 0.016$	$F_{1, 14} = 0.141$	$F_{1, 14} = 1.464$	$F_{1, 14} = 1.491$
Phenotype	$F_{1, 14} = 143.800^{\#}$	$F_{1, 14} = 77.420^{\#}$	$F_{1, 14} = 0.655$	$F_{1, 14} = 22.870^{\#}$	$F_{1, 14} = 2.794$	$F_{1, 14} = 32.900^{\#}$	$F_{1, 14} = 1.338$	$F_{1, 14} = 34.240^{\#}$
Cohort x Phenotype	$F_{1, 14} = 5.142^{\#}$	$F_{1, 14} = 1.781$	$F_{1, 14} = 0.403$	$F_{1, 14} = 0.144$	$F_{1, 14} = 3.499$	$F_{1, 14} = 1.683$	$F_{1, 14} = 3.750$	$F_{1, 14} = 1.174$

Average performance measures of HI and LI rats during ITI8 challenge sessions
***p < 0.05 vs. LI within each cohort**
 # p < 0.05 for a significant outcome of the two-way ANOVA

Table 5.2: Functional gene enrichment reveals biological processes that are overrepresented in the mPFC of HI vs. LI rats

Term	Adjusted P-value	Z-score	Combined Score	Genes
calcium ion transport into cytosol (GO:0060402)	0.0002	-1.922	27.262	*GRIN2A;ATP2B4;SLC8A1*
regulation of cardiac conduction (GO:1903779)	0.0051	-2.197	22.909	*PDE4D;ATP2B4;SLC8A1*
regulation of cell communication by electrical coupling (GO:0010649)	0.0051	-2.244	21.835	*PDE4D;SLC8A1*
calcium ion import (GO:0070509)	0.0100	-2.521	20.397	*CACNA1E;SLC8A1*
regulation of release of sequestered calcium ion into cytosol by sarcoplasmic reticulum (GO:0010880)	0.0083	-2.365	20.039	*PDE4D;SLC8A1*
cellular monovalent inorganic cation homeostasis (GO:0030004)	0.0060	-2.077	18.822	*KCNMA1;SLC8A1*
inorganic cation import across plasma membrane (GO:0098659)	0.0088	-2.262	18.802	*ATP2B4;SLC8A1*
cellular metal ion homeostasis (GO:0006875)	0.0051	-1.943	18.197	*KCNMA1;ATP2B4;SLC8A1*
adenylate cyclase-inhibiting G-protein coupled glutamate receptor signaling pathway (GO:0007196)	0.0488	-3.568	18.089	*GRM5*
vascular smooth muscle contraction (GO:0014829)	0.0488	-3.273	16.158	*SLC8A1*

Table 5.3: Functional gene enrichment reveals PANTHER pathways that are overrepresented in the mPFC of HI vs. LI rats

PANTHER Pathway	Adjusted P-value	Z-score	Combined Score	Genes
Metabotropic glutamate receptor group III pathway	0.0002	-1.699	18.901	GRM5;GRIN2A;CACNA1E
Metabotropic glutamate receptor group I pathway	0.0015	-1.274	10.910	GRM5;GRIN2A
Ionotropic glutamate receptor pathway	0.0039	-1.296	9.344	GRIN2A;CACNA1E
Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway	0.0161	-1.392	7.676	GRM5;CACNA1E

Table 5.4: Transcription factor enrichment identifies *SMAD4* and *REST* as overrepresented in high impulsive action

Transcription Factor	Adjusted P-value	Z-score	Combined Score	Genes
REST	0.0037	-1.639	15.599	HDAC4;PTPRT;GRIN2A;CLSTN2;XKR4;CACNA1E;CALN1
SMAD4	0.0037	-1.710	15.293	PTPRT;PDE4D;KCNMA1;CACNA1E;SLC8A1

Chapter 6: Discussion

Our objectives were to elucidate the role of the 5-HT_{2A}R, particularly in mPFC, to modulate impulsive action in the context of SUDs and propose novel gene targets in the mPFC for future impulsivity research. We established the potential for repurposing pimavanserin as a therapeutic to forestall relapse vulnerability in CUD, discovering that pimavanserin suppressed impulsive action and cocaine cue reactivity following 30 days of abstinence from cocaine self-administration (**Chapter 2**). We extended these findings to the study of abused prescription opioids, providing the first indication that pimavanserin suppressed oxycodone-evoked impulsive action, which lays the foundation for future evaluations of pimavanserin as a therapeutic to promote abstinence in OUD (**Chapter 3**). Interestingly, we found that viral-mediated knockdown of the 5-HT_{2A}R in the mPFC did not alter impulsive action or sensitivity to pimavanserin in the 1-CSRT task nor DOI-induced head-twitches, but we revealed a potential compensatory gene network that may explain these findings (**Chapter 4**). Lastly, we established a transcriptomic landscape in the mPFC that may characterize individual differences in impulsive action, proposing novel gene targets such as *CACNA1E* for future impulsivity research. The following sections detail several novel contributions made by this body of work.

PIMAVANSERIN MAY REDUCE IMPULSIVE ACTION AND PROMOTE ABSTINENCE IN SUDS: PRECLINICAL EVIDENCE FOR IMMEDIATE AND FUTURE RESEARCH DIRECTIONS

There is an immediate need to introduce safe and effective medications to reduce the impulsive misuse of cocaine and opioids and promote abstinence in CUD and OUD to extend recovery (Rasmussen et al., 2019). In **Chapter 2**, we demonstrated that the 5-HT_{2A}R antagonist/inverse agonist pimavanserin suppressed impulsive action as well as cue reactivity following 30 days of forced abstinence from cocaine self-administration. This is a two-pronged approach to reduce relapse vulnerability in CUD patients, considering that high baseline levels of self-reported impulsivity (e.g., BIS-11) (Moeller et al., 2001b; Moeller et al., 2007) predict attentional bias for cocaine-associated cues (Carpenter et al., 2006) and poorer retention of cocaine-dependent participants in an outpatient treatment trial (Moeller et al., 2001b). The utility of repurposing pimavanserin to forestall relapse vulnerability in OUD is less clear, largely due to a lack of available literature. Thus, we started with a direct question: does systemically-administered pimavanserin attenuate impulsive action evoked by systemically-administered oxycodone? The data presented in **Chapter 3** demonstrated that pimavanserin suppressed oxycodone-evoked impulsive action, providing early evidence that pimavanserin could improve impulsive control deficits that underlie opioid misuse and OUD. The second prong, then, is to test the hypothesis that pimavanserin suppresses oxycodone cue reactivity following extended abstinence, considering that measures of cue reactivity predict relapse in abstinent heroin users following detoxification (Marissen et al., 2006). Excitingly, preliminary data from our laboratory suggests that pimavanserin may suppress cue reactivity following 10 days of abstinence from oxycodone self-administration (**Figure 6.1**). It should be

noted that pimavanserin significantly suppressed *cue presentations* (lever presses reinforced by presentation of the discrete cue complex) but did not suppress *total previously-active lever presses* (all lever presses during the cue-evoked drug seeking session, including perseverative responding during the timeout period). Perhaps previously-active lever presses were not different due to the shorter duration of imposed abstinence (10 vs. 30 days). Nonetheless, these data are valuable considering that ~25% of individuals completing residential treatment programs relapse within 1 to 5 years (Simpson et al., 2002). Thus, treatment with a 5-HT_{2A}R antagonist/inverse agonist during an inpatient, residential treatment program may reduce relapse vulnerability after discharge, particularly when patients return to the environment in which they previously used a drug of choice.

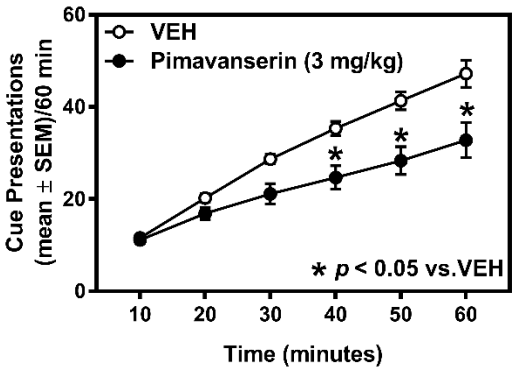

Figure 6.1: Pimavanserin may suppress cue reactivity on FA Day 10 following oxycodone self-administration.

The effects of pimavanserin (3 mg/kg) on cue presentations (mean ± SEM) on FA Day 10 (*n*=9/group) from the last oxycodone self-administration session are presented. Pimavanserin suppressed cue presentations on FA Day 10 from oxycodone self-administration (**p* < 0.05 vs VEH).

The studies presented herein, taken together with the available literature, suggest that pimavanserin is unlikely to be a cure-all for the treatment of CUD or OUD. A limitation of 5-HT_{2A}R antagonists/inverse agonists is their lack of effect on cocaine intake in rodent (Filip, 2005; Fletcher et al., 2002) or non-human primate

self-administration studies (Fantegrossi et al., 2002); the effects of 5-HT_{2A}R antagonists/inverse agonists on opioid reinforcement are unexplored. This does not discount the potential utility of pimavanserin for the treatment of CUD and OUD, as there is unlikely to be a 'silver bullet' for the treatment of SUDs (Rasmussen et al., 2019). Rather, the NIDA DTMC stated that their pharmacological mechanisms of interest are "likely to be useful at different stages of the addiction cycle," (Rasmussen et al., 2019). Indeed, our preclinical data suggest that pimavanserin could be useful to decrease impulsive action and prolong abstinence in CUD and OUD, potentially as an adjuvant pharmacotherapy.

Current FDA-approved medication assisted treatments (MAT), which target the μOR as partial or full agonists (buprenorphine, methadone) or antagonists (naltrexone), are available for OUD patients, but there are no FDA-approved medications for CUD. Acceptance of MAT in OUD has increased with long-lasting formulations of buprenorphine – often in combination with naloxone – that are prescribed on an outpatient basis (Morgan et al., 2018). Buprenorphine is commonly administered in a 4:1 ratio with naloxone (e.g., Suboxone®) to deter abuse such that, when individuals escalate their dose, naloxone directly antagonizes the positive subjective effects of buprenorphine and precipitates withdrawal symptoms (Comer et al., 2010b). Despite the efficacy of current MAT therapies for OUD, discontinuation rates are startlingly high. An analysis of 340,017 OUD patients from 2010 to 2014 found that 31% of OUD patients receiving sublingual buprenorphine in combination with naloxone discontinued treatment in 30 days or fewer, while 58% of individuals receiving sublingual

buprenorphine monotherapy discontinued treatment within this same time frame (Morgan et al., 2018). Even while patients continue on these therapies (Bickel et al., 1997; Kosten et al., 2003), recovery may be interrupted by drug misuse, with incidences of relapse prompted by precipitants including stress, exposure to drug-associated cues, or the use of an initially small amount, or priming dose, of drug (Epstein et al., 2009; Hendershot et al., 2011).

The utility of buprenorphine as a MAT for CUD is supported by the preclinical literature, as buprenorphine suppresses cocaine self-administration in rats (Carroll and Lac, 1992) and rhesus monkeys (Mello et al., 1990). The current literature evaluating buprenorphine to treat CUD patients appears insufficient at present; buprenorphine does not appear to alter cocaine use or abstinence in patients with combined cocaine and opioid use (Schottenfeld et al., 1993; Strain et al., 1994), although a recent longitudinal analysis found that buprenorphine/naloxone sublingual tablets (Suboxone®) plus extended-release injectable naltrexone (Vivitrol®) reduces cocaine urine drug screens relative to placebo in cocaine-dependent individuals (DSM-4) with past or current opioid dependence or abuse (Ling et al., 2016). We mentioned above that discontinuation rates for current MAT therapies are high, and an interesting detail is that *the additional diagnosis of CUD* is associated with a higher hazard of MAT discontinuation in OUD patients (Morgan et al., 2018). Thus, complex, interwoven problems of compliance, efficacy, and abuse liability challenge the utility of current MAT therapies for the treatment of OUD and CUD.

Strategies to increase the success of MAT maintenance such as an adjunctive medication that buffers relapse triggers may provide added coverage to extend recovery. Our preclinical data with pimavanserin suggests that combination therapy, if safe, may fill this need. First, our cumulative evidence suggests that pimavanserin suppresses oxycodone-evoked impulsive action (**Chapter 3**) and may suppress cue reactivity following 10 days of abstinence from oxycodone self-administration (**Figure 6.1**), which may overcome interruptions in OUD recovery elicited by exposure to environmental stimuli previously associated with opioid use. Second, we showed that pimavanserin suppressed cocaine cue reactivity following prolonged abstinence (**Chapter 2**), and perhaps a combination of pimavanserin plus buprenorphine or another MAT therapy would be efficacious to promote abstinence in CUD patients, which is a target for future preclinical studies. Thus, our preclinical evidence may support the utility of pimavanserin as a potential adjuvant to buprenorphine or another MAT.

A second approach is to pair pimavanserin with a novel pharmacotherapeutic of interest, which could yield a new drug combination that is "likely to be useful at different stages of the addiction cycle" (Rasmussen et al., 2019). In fact, the NIDA DTMC expressed interest in a 5-HT_{2A}R antagonist/inverse agonist combination with a 5-HT_{2C}R agonist (or positive allosteric modulator) for OUD treatment. Systemic administration of selective 5-HT_{2C}R agonists, including the FDA-approved anti-obesity medication lorcaserin (Belviq®) as well as investigational 5-HT_{2C}R agonists (e.g., Ro 60-0175, WAY163909), suppress impulsive action (Anastasio et al., 2013; Cunningham et al., 2013; Fletcher et al.,

2007; Higgins et al., 2016; Navarra et al., 2008) as well as self-administration of oxycodone (Neelakantan et al., 2017) and cocaine in preclinical models (Collins et al., 2016; Cunningham et al., 2011; Fletcher et al., 2008; Grottick et al., 2000; Manvich et al., 2012a; Manvich et al., 2012b; Neisewander and Acosta, 2007; Swinford-Jackson et al., 2016). Additionally, selective 5-HT_{2C}R agonists decrease cue reactivity assessed during abstinence from oxycodone (Neelakantan et al., 2017) or cocaine self-administration (Anastasio et al., 2014a; Cunningham et al., 2011; Fletcher et al., 2002; Grottick et al., 2000; Neisewander and Acosta, 2007; Swinford-Jackson et al., 2016). Accordingly, lorcaserin is currently under clinical evaluation for the treatment of CUD (NCT03007394, NCT03143543, NCT02393599, NCT03266939) and OUD (NCT03143543, NCT03143855) as well as cannabis use disorder (NCT03253926, NCT02932215) and nicotine use disorder (NCT02393547, NCT02906644).

Pimavanserin and lorcaserin may treat unique components of the SUD cycle, presenting the opportunity to introduce each of these pharmacotherapies at different time points in SUDs treatment. For example, we found that pimavanserin was efficacious to suppress cue-evoked drug seeking following prolonged (but not early) abstinence from cocaine self-administration (**Chapter 2**). Meanwhile, lorcaserin decreases cocaine taking (Collins et al., 2016) while another, selective 5-HT_{2C}R agonist (WAY163909) was efficacious to suppress cue-evoked drug seeking following both early and prolonged abstinence from cocaine self-administration. (Swinford-Jackson et al., 2016). Thus, lorcaserin administration may be useful to aid cessation of drug use in early abstinence while pimavanserin

might be introduced later in treatment to promote prolonged abstinence, presenting a promising strategy to reduce drug use and forestall relapse vulnerability.

The potential to mitigate adverse events while maintaining clinical efficacy is a promising aspect of using lorcaserin and pimavanserin in combination as well. For example, combined administration of a dose of the selective 5-HT_{2A}R antagonist/inverse agonist plus a 5-HT_{2C}R agonist, each ineffective alone, synergistically suppresses impulsive action as well cue- and cocaine-primed reinstatement following cocaine self-administration (Cunningham et al., 2013). Thus, the opportunity to lower the effective doses of each medication may improve tolerability and reduce the incidence of adverse events not only for pimavanserin (see below) but also lorcaserin [e.g., upper respiratory tract infection, nasopharyngitis, headache, nausea (Gustafson et al., 2013)].

A number of limitations challenge the utility of pimavanserin to extend recovery and suppress relapse in SUD patients. For example, tolerability will be an ongoing challenge; the Institute for Safe Medication Practices revealed 2236 adverse events within a 12-month post-marketing period for pimavanserin prescribed to Parkinson's disease psychosis patients; the four most commonly-reported adverse events were hallucinations (21.8%), drug ineffectiveness (14.9%), confused state (11.5%), and death (10.9%). In particular, the high incidence of mortality led the FDA to review the post-marketing reports of deaths and serious adverse events associated with the use of pimavanserin. However, the FDA did not identify any new or unexpected safety risks, considering that Parkinson's disease psychosis patients have a high mortality rate due to their older

age, advanced Parkinson's disease, and other medical conditions. Beyond these adverse events, though, is the finding that pimavanserin can elicit QT interval prolongation and arrhythmia (Tampi et al., 2019), which is particularly concerning for the treatment of CUD considering the negative consequences of acute cocaine use on cardiovascular health (e.g., electrocardiographic abnormalities, acute hypertension, arrhythmia, acute myocardial infarction) (Kim and Park, 2019), including documented QT interval prolongation (Perera et al., 1997). It is unclear how these adverse events would translate to the treatment of SUDs patients, although it is possible that the stigma associated with these adverse events alone could decrease the likelihood that pimavanserin is prescribed off-label for the treatment of SUDs.

We also recognize that, beyond the 5-HT_{2A}R antagonist/inverse agonist pimavanserin, the clinical research landscape is flooded with investigations of psychedelic 5-HT_{2A}R *partial agonists*, including 30 ongoing clinical trials evaluating psilocybin to treat cocaine-related disorders (NCT02037126), nicotine dependence (NCT01943994), and alcohol dependence (NCT02061293) among others. Superficially, this strategy appears counterintuitive to the treatment of impulsive action in SUDs, as systemically-administered 5-HT_{2A}R partial agonists (e.g., DOI) increase impulsive action assessed in the 1- and 5-CSRT tasks (Fink et al., 2015; Koskinen et al., 2000b; Winstanley et al., 2004). However, gaining a better understanding of the mechanisms governing regulation of the 5-HT_{2A}R following acute versus chronic agonist administration will lend insight into the

provocative hypothesis that agonist-mediated downregulation of the 5-HT_{2A}R may explain the potential therapeutic utility of these ligands to reduce impulsive action.

Acute administration of a 5-HT_{2A}R agonist is hypothesized to stimulate the $G\alpha_{q/11}$ effector system to activate a signaling cascade resulting in release of intracellular calcium , activation of the MAP kinase cascade, and phosphorylation of extracellular signal-regulated kinases (Casanueva et al., 2008; Chang et al., 2000; Conn and Sanders-Bush, 1986; Hoyer et al., 1989; Pazos et al., 1985; Pazos and Palacios, 1985; Werry et al., 2005). The 5-HT_{2A}R agonist-induced stimulation of the effector system is diminished over time through a process called desensitization, which is facilitated by phosphorylation of the intracellular domains of the receptor that prevents further interaction with the G protein. As a result, the 5-HT_{2A}R is endocytosed into the early endosome, where two outcomes are thought to occur: first, the agonist can dissociate from the internalized 5-HT_{2A}R, which permits 5-HT_{2A}R dephosphorylation and recycling to the plasma membrane ("resensitization") (Gray and Roth, 2001). The second mechanism, though, is thought to occur over repeated, chronic stimulation of the 5-HT_{2A}R, whereby a population of the internalized 5-HT_{2A}R is targeted to the lysosome for degradation. This phenomenon is termed downregulation, or a reduction in total specific binding sites (i.e., B_{max}) that indicates a loss of total cellular receptors (Koenig and Edwardson, 1997). The opportunity to potentiate 5-HT_{2A}R targeting to the lysosome for degradation via repeated 5-HT_{2A}R agonist administration is intriguing considering that premature responses in the 1-CSRT positively correlated with 5-HT_{2A}R density assessed by [^{3}H]-ketanserin binding in whole frontal cortex of male,

Sprague-Dawley rats (Fink et al., 2015). Thus, future studies could test the hypothesis that repeated administration of a 5-HT_{2A}R partial agonist (e.g., DOI) decreases impulsive action in the 1- or 5-CSRT tasks, perhaps via 5-HT_{2A}R downregulation in the mPFC.

In light of these 5-HT_{2A}R regulatory mechanisms, which biochemical mechanisms might mediate 5-HT_{2A}R localization to postsynaptic plasma membranes? The functional status of the 5-HT_{2A}R in the mPFC is importantly regulated by the postsynaptic density (PSD) protein of 95 kDa (PSD95), a scaffolding protein that complexes with the 5-HT_{2A}R; the 5-HT_{2A}R interaction with PSD95 enhances 5-HT_{2A}R-mediated signaling, inhibits agonist-induced 5-HT_{2A}R internalization, and promotes 5-HT_{2A}R clustering on the plasma membrane (Abbas et al., 2009; Becamel et al., 2002; Becamel et al., 2004; Xia et al., 2003). Intriguingly, our laboratory discovered that the 5-HT_{2A}R co-immunoprecipitates with PSD95 to a greater extent in the mPFC of HI vs. LI rats (Fink et al., 2015). With this in mind, selective dissociation of the 5-HT_{2A}R:PSD95 interaction may present another promising strategy to reduce impulsive action, and preclinical models to test this hypothesis might employ a commercially-available, peptidyl mimetic of the 5-HT_{2A}R C-terminus that has been shown to impede the 5-HT_{2A}R:PSD95 association *in vitro* and *in vivo* (Dupuis et al., 2017; Pichon et al., 2010; Wattiez et al., 2017; Wattiez et al., 2013).

THE 5-HT_{2A}R PROFOUNDLY INFLUENCES THE COMPLEX, CORTICAL NEUROBIOLOGY GOVERNING IMPULSIVE ACTION

Our decision to interrogate the mPFC as a site of action for the 5-HT_{2A}R to modulate impulsive action was based on several leads from the preclinical literature. For example, HI rats exhibit a higher density of synaptosomal 5-HT_{2A}R protein expression in mPFC relative to LI rats (Anastasio et al., 2015; Fink et al., 2015). Moreover, HI rats exhibit a greater number of head-twitch responses induced by the preferential 5-HT_{2A}R agonist DOI and greater pharmacological sensitivity to the effects of the selective 5-HT_{2A}R antagonist/inverse agonist M100907 to decrease impulsive action relative to LI rats. However, the effect of intra-mPFC infusion of 5-HT_{2A}R antagonist/inverse agonists to suppress impulsive action are mixed, with some studies reporting decreases in impulsive action in the 5-CSRT task (Passetti et al., 2003; Winstanley et al., 2003) while others have found no effect (Mirjana et al., 2004; Robinson et al., 2008). Thus, the studies in **Chapter 4** were designed to test the causal directionality in the association between 5-HT_{2A}R in the mPFC and impulsive action.

What mechanisms of action could account for the possible discordance between the effects of a systemically-administered 5-HT_{2A}R antagonist/inverse agonist and a shRNA-mediated 5-HT_{2A}R gene knockdown on impulsive action? First, both pimavanserin and M100907 act as 5-HT_{2A}R inverse agonists to attenuate basal constitutive 5-HT_{2A}R signaling in cells designed with overexpression of the native 5-HT_{2A}R or transfection with a 5-HT_{2A}R mutation targeted to increase constitutive activity (Muntasir et al., 2006; Vanover et al., 2004; Vanover et al., 2006; Weiner et al., 2001). Presently, we are unable to definitively attribute the effects of M100907 or pimavanserin to their 5-HT_{2A}R

antagonist vs. inverse agonist properties (see **Chapter 2, discussion**), but the degree to which 5-HT_{2A}R constitutive activity contributes to impulsive action is a target for future studies. Second, the possibility of a 5-HT_{2A}R receptor reserve, or "spare" receptors, could explain the inability of shRNA-mediated 5-HT_{2A}R gene knockdown to alter impulsive action (for review) (Kurrasch-Orbaugh et al., 2003). Receptors are said to be spare if it is possible to elicit a maximal response at a concentration of agonist that does not result in occupancy of the full complement of available receptors (Stephenson, 1956). Following 5-HT_{2A}R knockdown, perhaps the intact population of 5-HT_{2A}R in the mPFC exhibits maximal 5-HT_{2A}R responsiveness, an intriguing possibility that may explain the ineffectiveness of 5-HT_{2A}R-shRNA-eGFP on impulsive action or 5-HT_{2A}R ligand sensitivity in the 1-CSRT task and DOI-induced head-twitch response assay, an additional target for future studies. Altogether, the degree to which constitutive activity of the 5-HT_{2A}R versus a 5-HT_{2A}R receptor reserve contributes to impulsive action is an exciting area for future research.

Does the inability of 5-HT_{2A}R knockdown in the mPFC to alter 1-CSRT task performance or sensitivity to 5-HT_{2A}R ligands cast doubt on 5-HT_{2A}R antagonism/inverse agonism as a pharmacological mechanism of interest for the rapid development of therapeutics in response to the opioid crisis (Rasmussen et al., 2019)? We argue that it does not, as the molecular insights gained by these basic science results (see **Chapter 4, discussion**) are the essence of how and why FDA-approved therapeutics are repurposed for new indications, and there are several historical examples to support this. We draw from an example given by

Francis Collins, director of the National Institutes of Health, at the TEDMED 2012 conference.

The example is tamoxifen, a non-steroidal estrogen receptor antagonist that was approved by the FDA in 1977 for the treatment of metastatic breast cancer (Smith, 2014). Years later, an *in vitro* study employed the [γ-^{32}P]-adenosine triphosphate (ATP) assay, which measures kinase activity as the amount of radiolabeled phosphate from [γ-^{32}P]-ATP that is incorporated into a peptide or protein substrate, in rat brain homogenate to demonstrate that tamoxifen is a potent PKC inhibitor (O'Brian et al., 1985). Simultaneously, extensive preclinical literature suggested that lithium, which was used for over 100 years to treat acute mania and approved by the FDA in 1970 for maintenance treatment in bipolar disorder (Shorter, 2009), decreases PKC activity *in vitro* (for review) (Jope, 1999), suggesting that this mechanism of action is relevant to the treatment of bipolar disorder. Indeed, a 1993 study collected platelets from bipolar disorder subjects before and during lithium treatment; this study demonstrated that the membrane-to-cytosolic ratio of PKC activity in the [γ-^{32}P]-ATP assay is higher in manic subjects, and lithium treatment for up to two weeks reduces both cytosolic and membrane-associated PKC activity (Friedman et al., 1993). Taken together, these data culminated into the first double-blind, placebo-controlled study (NCT00026585) demonstrating that bipolar disorder subjects treated with tamoxifen show significant improvements in mania as early as five days following treatment. Even today, tamoxifen is considered a promising potential therapeutic to treat manic episodes (Palacios et al., 2019) and is recommended in some

treatment guidelines as a pharmacological option for the treatment of acute mania (Yatham et al., 2018). The tamoxifen anecdote highlights the need to continuously explore mechanism-of-action even for efficacious therapeutics. We extend this anecdote to 5-HT_{2A}R regulatory mechanisms, as the molecular consequences of mPFC 5-HT_{2A}R knockdown could inform a future indication that we have not yet considered.

Thus, our finding that systemic administration of pimavanserin decreased impulsive action and cocaine cue reactivity do not conflict with our discovery that 5-HT_{2A}R knockdown in the mPFC did not alter impulsive action or sensitivity to 5-HT_{2A}R ligands. Rather, our exploration into the molecular consequence of mPFC 5-HT_{2A}R knockdown adds to the "robust, on-going research into uncovering additional mechanisms, not yet on the list," that can be mined for their utility to reduce impulsive action in SUDs (Rasmussen et al., 2019).

CACNA1E (Ca_V2.3) IN THE MPFC IS A NOVEL TARGET FOR FUTURE IMPULSIVE ACTION RESEARCH

The NIDA DTMC emphasized the need for "*entirely new directions"* to discover novel pharmacotherapeutic mechanisms for the treatment of SUDs (Rasmussen et al., 2019). Tandem functional genomics and bioinformatics provides a platform for novel target discovery and hypothesis generation, and the provocative findings in **Chapter 5** demonstrated that *CACNA1E* mRNA expression is higher in the mPFC of HI vs. LI rats. *CACNA1E* encodes the R-type voltage-gated calcium channel Ca_V2.3, however, this protein is highly understudied while

our findings are limited to the level of the gene transcript. Two logical next steps are to (1) investigate expression of the $Ca_v2.3$ protein in the mPFC of HI vs. LI rats and (2) evaluate the efficacy of a $Ca_v2.3$ antagonist to decrease impulsive action in the 1-CSRT task, either systemically or upon microinfusion into the mPFC. First, at least one, validated, commercially-available antibody (ACC-006, Alomone Labs, Jerusalem, Israel) may guide future studies evaluating $Ca_v2.3$ expression in the mPFC of HI vs. LI rats. Second, pharmacological $Ca_v2.3$ blockade can be achieved using SNX-482, a selective $Ca_v2.3$ peptide antagonist that is available for commercial use (Schneider et al., 2013). Taken together, we provide a framework for evaluating $Ca_v2.3$ as a target to reduce impulsive action, providing an entirely new mechanistic direction for impulsivity research.

CONCLUDING REMARKS

John Evenden described impulsivity as a behavior that “adds important colour [*sic*] to everyday life,” (Evenden, 1999b). We cherish the spontaneous and unplanned; what a pleasure it is to run into an old friend on your way to the office, watch one more episode of your favorite television show even though midnight looms, or “accidentally” miss your flight to spend one more day on the beach. The tendency to act on impulse, though, can perpetuate a vicious cycle of substance misuse and abuse, exacting nearly $800 billion annually in costs related to crime, lost productivity, and health care in the United States (NIDA, 2017). The trajectory to SUDs underlies one of our greatest public health challenges, and pharmacotherapeutic strategies to treat SUDs are of immediate importance. We

provide preclinical evidence that the 5-HT_{2A}R is a promising target to reduce impulsive action and promote abstinence in CUD (**Chapter 2**) which may extend to OUD (**Chapter 3**). Our research yields mechanistic insights to the functional status of the 5-HT_{2A}R in the mPFC in impulsive action (**Chapter 4**) and offers future directions for 5-HT_{2A}R research. Finally, we reveal novel gene targets in the mPFC that are associated with inherent impulsive action (**Chapter 5**), offering entirely new directions for impulsivity research. Identifying therapeutic strategies to improve the health status of SUDs patients is much greater than the science itself. Certainly, introducing safe, effective pharmacotherapeutics to treat SUDs for what they are – chronic, relapsing diseases that directly impact roughly 20 million Americans annually (SAMHSA, 2018) – can change polarizing, societal attitudes and dissolve the stigma associated with SUDs.

Afterword

Implementation of Medication Take Back Programs Permits Safe, Anonymous Disposal of Unwanted Prescription Drugs in Galveston County, Texas

The studies in **Chapter 3** were inspired by the "first wave" of the opioid crisis, or the rise in prescription opioid overdose deaths that began in the 1990s. Still, overdose deaths involving prescription opioids were five times higher in 2017 than in 1999 while prescription opioids were involved in more than 35% of all opioid overdose deaths (CDC, 2016). Strikingly, 53.1% of individuals who misused a prescription opioid received the drug from a friend or relative (SAMHSA, 2018). The high incidence of prescription opioid diversion led us to broadly examine medication disposal practices in the United States. The Centers for Disease Control (CDC, 2016) reported that 48.9% of individuals used at least one prescription drug in the past 30 days, however, up to 40% of prescription drugs are unused (Law et al., 2015). Unwanted prescription drugs are commonly disposed of by flushing in toilets and sinks, but these routes threaten environmental contamination by passing through treatment systems and entering waterways (Lubick, 2010). Additionally, unwanted prescription drugs are vulnerable to accidental ingestion or intentional misuse (Wu and Juurlink, 2014). Thus, we identified an immediate need to design and implement community strategies to safely dispose of unwanted prescription drugs.

The UTMB Center for Addiction Research (CAR) partnered with the Bay Area Council on Drugs and Alcohol (BACODA) to implement safe, anonymous drug disposal practice in Galveston Country and the surrounding areas. First, we biannually host the Drug Enforcement Administration (DEA)-sponsored “National Prescription Drug Take Back Day,” a national initiative that provides a safe and convenient means to dispose of prescription drugs. Additionally, National Prescription Drug Take Back Day serves as an educational module to promote safe drug disposal within our community. On the 15th National Take Back Day, 949,046 lbs. of prescription drugs were collected nationally, with 82,978 lbs. coming from the state of Texas alone, to be safely disposed of via incineration.

The Secure and Responsible Drug Disposal Act of 2010 expanded DEA prescription drug take back options to include the installation of permanent collection receptacles to be housed within local police departments. Our second initiative was to partner with local police departments to install permanent prescription drug drop boxes, providing the community constant access to a safe, anonymous drug disposal option. Five police departments in Galveston County, Texas now house a prescription drop box as of October 2019: Galveston, Texas City, Dickinson, La Marque, and Santa Fe.

A third approach is to provide a medication disposal option that can be used in the home. Excitingly, BACODA received funding through the Texas Targeted Opioid Response initiative to deliver the Deterra® Personal Drug Deactivation System (Verde® Technologies) to our community. The Deterra® Personal Drug Deactivation System utilizes an activated carbon-based pouch, and it was

demonstrated to adsorb 98.72% of tested medications within eight hours (Gao et al., 2018). BACODA distributed 1,343 Deterra® Personal Drug Deactivation Systems to community members in Galveston County in 2018, providing the ability to deactivate 88,545 pills.

The CAR and BACODA have empowered the community with strategies to safely and anonymously dispose of unwanted prescription drugs including opioids. In line with the FDA guidelines, individuals should (1) identify National Prescription Drug Take Back events in their communities, available via the DEA website, (2) locate DEA-registered permanent collection sites, commonly housed within local police departments and pharmacies, and, if possible, (3) obtain at-home technologies (i.e., Deterra® Drug Deactivation System) to enable immediate, safe disposal of prescription drugs. Overall, community members and clinicians should safely dispose of expired, unwanted, or unused medicines as quickly as possible to reduce the possibility of prescription drug misuse and negative environmental impact. We hope that these evidence-based public health strategies can reduce the incidence of prescription opioid misuse and overdose that plagues the United States today.

References

Abbas, A.I., Yadav, P.N., Yao, W.D., Arbuckle, M.I., Grant, S.G., Caron, M.G., and Roth, B.L. (2009). PSD-95 is essential for hallucinogen and atypical antipsychotic drug actions at serotonin receptors. J.Neurosci. *29*, 7124-7136.

Amargos-Bosch, M., Bortolozzi, A., Puig, M.V., Serrats, J., Adell, A., Celada, P., Toth, M., Mengod, G., and Artigas, F. (2004). Co-expression and in vivo interaction of serotonin1A and serotonin2A receptors in pyramidal neurons of prefrontal cortex. Cereb. Cortex. *14*, 281-299.

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

Anastasio, N.C., Lanfranco, M.F., Bubar, M.J., Seitz, P.K., Stutz, S.J., McGinnis, A.G., Watson, C.S., and Cunningham, K.A. (2010). Serotonin 5-HT_{2C} receptor protein expression is enriched in synaptosomal and post-synaptic compartments of rat cortex. J. Neurochem. *113*, 1504-1515.

Anastasio, N.C., Liu, S., Maili, L., Swinford, S.E., Lane, S.D., Fox, R.G., Hamon, S.C., Nielsen, D.A., Cunningham, K.A., and Moeller, F.G. (2014a). Variation within the serotonin (5-HT) 5-HT_{2C} receptor system aligns with vulnerability to cocaine cue reactivity. Transl. Psychiatry *4*, e369.

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

Anastasio, N.C., Stutz, S.J., Fink, L.H., Swinford-Jackson, S.E., Sears, R.M., DiLeone, R.J., Rice, K.C., Moeller, F.G., and Cunningham, K.A. (2015). Serotonin (5-HT) 5-HT_{2A} Receptor (5-HT_{2A}R):5-HT_{2C}R imbalance in medial prefrontal cortex associates with motor impulsivity. ACS Chem. Neurosci. *6*, 1248-1258.

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

Anastasio, N.C., Stutz, S.J., Price, A.E., Davis-Reyes, B.D., Sholler, D.J., Ferguson, S.M., Neumaier, J.F., Moeller, F.G., Hommel, J.D., and Cunningham, K.A. (2019). Convergent neural connectivity in motor impulsivity and high-fat food binge-like eating in male Sprague-Dawley rats. Neuropsychopharmacology.

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

Arolfo, M.P., and McMillen, B.A. (1999). Effects of amperozide and tiospirone, atypical antipsychotic 5-HT2 drugs, on food-reinforced behavior in rats. Physiol. Behav. *68*, 93-98.

Aschauer, D.F., Kreuz, S., and Rumpel, S. (2013). Analysis of transduction efficiency, tropism and axonal transport of AAV serotypes 1, 2, 5, 6, 8 and 9 in the mouse brain. PLoS One *8*, e76310.

Auclair, A., Drouin, C., Cotecchia, S., Glowinski, J., and Tassin, J.P. (2004). 5-HT2A and alpha1b-adrenergic receptors entirely mediate dopamine release, locomotor response and behavioural sensitization to opiates and psychostimulants. Eur.J Neurosci. *20*, 3073-3084.

Augur, I.F., Wyckoff, A.R., Aston-Jones, G., Kalivas, P.W., and Peters, J. (2016). Chemogenetic activation of an extinction neural circuit reduces cue-induced reinstatement of cocaine seeking. J. Neurosci. *36*, 10174-10180.

Bakshi, V.P., and Kelley, A.E. (1993). Striatal regulation of morphine-induced hyperphagia: an anatomical mapping study. Psychopharmacology (Berl.) *111*, 207-214.

Baldacchino, A., Balfour, D.J., and Matthews, K. (2015). Impulsivity and opioid drugs: differential effects of heroin, methadone and prescribed analgesic medication. Psychol. Med. *45*, 1167-1179.

Bari, A., and Robbins, T.W. (2013). Inhibition and impulsivity: behavioral and neural basis of response control. Prog. Neurobiol. *108*, 44-79.

Becamel, C., Alonso, G., Galeotti, N., Demey, E., Jouin, P., Ullmer, C., Dumuis, A., Bockaert, J., and Marin, P. (2002). Synaptic multiprotein complexes associated with 5-HT(2C) receptors: a proteomic approach. EMBO J. *21*, 2332-2342.

Becamel, C., Gavarini, S., Chanrion, B., Alonso, G., Galeotti, N., Dumuis, A., Bockaert, J., and Marin, P. (2004). The serotonin 5-HT2A and 5-HT2C receptors interact with specific sets of PDZ proteins. J. Biol. Chem. *279*, 20257-20266.

Beique, J.C., Imad, M., Mladenovic, L., Gingrich, J.A., and Andrade, R. (2007). Mechanism of the 5-hydroxytryptamine 2A receptor-mediated facilitation of synaptic activity in prefrontal cortex. Proc.Natl.Acad.Sci.U.S.A *104*, 9870-9875.

Belin, D., Mar, A.C., Dalley, J.W., Robbins, T.W., and Everitt, B.J. (2008). High impulsivity predicts the switch to compulsive cocaine-taking. Science *320*, 1352-1355.

Berridge, K.C., and Robinson, T.E. (2003). Parsing reward. Trends Neurosci. *26*, 507-513.

Berrou, L., Klein, H., Bernatchez, G., and Parent, L. (2002). A specific tryptophan in the I-II linker is a key determinant of beta-subunit binding and modulation in Ca(V)2.3 calcium channels. Biophys. J. *83*, 1429-1442.

Besson, M., Pelloux, Y., Dilleen, R., Theobald, D.E., Lyon, A., Belin-Rauscent, A., Robbins, T.W., Dalley, J.W., Everitt, B.J., and Belin, D. (2013). Cocaine

modulation of frontostriatal expression of Zif268, D2, and 5-HT2c receptors in high and low impulsive rats. Neuropsychopharmacology *38*, 1963-1973.

Bickel, W.K., Amass, L., Higgins, S.T., Badger, G.J., and Esch, R.A. (1997). Effects of adding behavioral treatment to opioid detoxification with buprenorphine. J. Consult. Clin. Psychol. *65*, 803-810.

Binford, L.R. (1985). Human ancestors: Changing views of their behavior. Journal of Anthropological Archaeology *4*, 292-327.

Bizarro, L., and Stolerman, I.P. (2003). Attentional effects of nicotine and amphetamine in rats at different levels of motivation. Psychopharmacology (Berl.) *170*, 271-277.

Blokland, A., Sik, A., and Lieben, C. (2005). Evaluation of DOI, 8-OH-DPAT, eticlopride and amphetamine on impulsive responding in a reaction time task in rats. Behav.Pharmacol. *16*, 93-100.

Bockaert, J., Claeysen, S., Becamel, C., Dumuis, A., and Marin, P. (2006). Neuronal 5-HT metabotropic receptors: fine-tuning of their structure, signaling, and roles in synaptic modulation. Cell Tissue Res. *326*, 553-572.

Bortolozzi, A., az-Mataix, L., Scorza, M.C., Celada, P., and Artigas, F. (2005). The activation of 5-HT receptors in prefrontal cortex enhances dopaminergic activity. J Neurochem. *95*, 1597-1607.

Bravo-Rivera, C., Diehl, M.M., Roman-Ortiz, C., Rodriguez-Romaguera, J., Rosas-Vidal, L.E., Bravo-Rivera, H., Quinones-Laracuente, K., and Do-Monte, F.H. (2015). Long-range GABAergic neurons in the prefrontal cortex modulate behavior. J. Neurophysiol. *114*, 1357-1359.

Burnet, P.W., Eastwood, S.L., Lacey, K., and Harrison, P.J. (1995). The distribution of 5-HT_{1A} and 5-HT_{2A} receptor mRNA in human brain. Brain Res. *676*, 157-168.

Butler, J.E., and Kadonaga, J.T. (2002). The RNA polymerase II core promoter: a key component in the regulation of gene expression. Genes Dev. *16*, 2583-2592.

Canal, C.E., Booth, R.G., and Morgan, D. (2013). Support for 5-HT2C receptor functional selectivity in vivo utilizing structurally diverse, selective 5-HT2C receptor ligands and the 2,5-dimethoxy-4-iodoamphetamine elicited head-twitch response model. Neuropharmacology *70*, 112-121.

Canal, C.E., and Morgan, D. (2012). Head-twitch response in rodents induced by the hallucinogen 2,5-dimethoxy-4-iodoamphetamine: a comprehensive history, a re-evaluation of mechanisms, and its utility as a model. Drug Test Anal *4*, 556-576.

Caprioli, D., Sawiak, S.J., Merlo, E., Theobald, D.E., Spoelder, M., Jupp, B., Voon, V., Carpenter, T.A., Everitt, B.J., Robbins, T.W.*, et al.* (2014). Gamma aminobutyric acidergic and neuronal structural markers in the nucleus

accumbens core underlie trait-like impulsive behavior. Biol. Psychiatry *75*, 115-123.

Carpenter, K.M., Schreiber, E., Church, S., and McDowell, D. (2006). Drug Stroop performance: relationships with primary substance of use and treatment outcome in a drug-dependent outpatient sample. Addict.Behav. *31*, 174-181.

Carroll, M.E., and Lac, S.T. (1992). Effects of buprenorphine on self-administration of cocaine and a nondrug reinforcer in rats. Psychopharmacology (Berl.) *106*, 439-446.

Carter, B.L., and Tiffany, S.T. (1999). Cue-reactivity and the future of addiction research. Addiction *94*, 349-351.

Casanueva, F.F., Camina, J.P., Carreira, M.C., Pazos, Y., Varga, J.L., and Schally, A.V. (2008). Growth hormone-releasing hormone as an agonist of the ghrelin receptor GHS-R1a. Proc. Natl. Acad. Sci. U. S. A. *105*, 20452-20457.

Caton, C.L., Samet, S., and Hasin, D.S. (2000). When acute-stage psychosis and substance use co-occur: differentiating substance-induced and primary psychotic disorders. J Psychiatr.Pract. *6*, 256-266.

Catterall, W.A. (2011). Voltage-gated calcium channels. Cold Spring Harb. Perspect. Biol. *3*, a003947.

Centers for Disease Control (CDC) (2016). Wide-ranging online data for epidemiologic research (WONDER). (Atlanta, GA, Centers for Disease Control, National Center for Health Statistics).

Chang, M., Zhang, L., Tam, J.P., and Sanders-Bush, E. (2000). Dissecting G protein-coupled receptor signaling pathways with membrane-permeable blocking peptides. Endogenous 5-HT(2C) receptors in choroid plexus epithelial cells. J. Biol. Chem. *275*, 7021-7029.

Chen, E.Y., Tan, C.M., Kou, Y., Duan, Q., Wang, Z., Meirelles, G.V., Clark, N.R., and Ma'ayan, A. (2013). Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinformatics *14*, 128.

Chudasama, Y., Passetti, F., Rhodes, S.E., Lopian, D., Desai, A., and Robbins, T.W. (2003). Dissociable aspects of performance on the 5-choice serial reaction time task following lesions of the dorsal anterior cingulate, infralimbic and orbitofrontal cortex in the rat: differential effects on selectivity, impulsivity and compulsivity. Behav.Brain Res. *146*, 105-119.

Cicero, T.J., Ellis, M.S., Surratt, H.L., and Kurtz, S.P. (2014). The changing face of heroin use in the United States: a retrospective analysis of the past 50 years. JAMA Psychiatry *71*, 821-826.

Coffey, S.F., Gudleski, G.D., Saladin, M.E., and Brady, K.T. (2003). Impulsivity and rapid discounting of delayed hypothetical rewards in cocaine-dependent individuals. Exp.Clin.Psychopharmacol. *11*, 18-25.

Collins, G.T., Gerak, L.R., Javors, M.A., and France, C.P. (2016). Lorcaserin reduces the discriminative stimulus and reinforcing effects of cocaine in rhesus monkeys. J. Pharmacol. Exp. Ther. *356*, 85-95.

Comer, S.D., Sullivan, M.A., Vosburg, S.K., Kowalczyk, W.J., and Houser, J. (2010a). Abuse liability of oxycodone as a function of pain and drug use history. Drug Alcohol Depend. *109*, 130-138.

Comer, S.D., Sullivan, M.A., Vosburg, S.K., Manubay, J., Amass, L., Cooper, Z.D., Saccone, P., and Kleber, H.D. (2010b). Abuse liability of intravenous buprenorphine/naloxone and buprenorphine alone in buprenorphine-maintained intravenous heroin abusers. Addiction *105*, 709-718.

Conn, P.J., and Sanders-Bush, E. (1986). Agonist-induced phosphoinositide hydrolysis in choroid plexus. J. Neurochem. *47*, 1754-1760.

Conrad, K.L., Tseng, K.Y., Uejima, J.L., Reimers, J.M., Heng, L.J., Shaham, Y., Marinelli, M., and Wolf, M.E. (2008). Formation of accumbens GluR2-lacking AMPA receptors mediates incubation of cocaine craving. Nature *454*, 118-121.

Cope, Z.A., Halberstadt, A.L., van Enkhuizen, J., Flynn, A.D., Breier, M., Swerdlow, N.R., Geyer, M.A., and Young, J.W. (2016). Premature responses in the five-choice serial reaction time task reflect rodents' temporal strategies: evidence from no-light and pharmacological challenges. Psychopharmacology (Berl.) *233*, 3513-3525.

Coskunpinar, A., and Cyders, M.A. (2013). Impulsivity and substance-related attentional bias: a meta-analytic review. Drug Alcohol Depend. *133*, 1-14.

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

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

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

Dalley, J.W., Everitt, B.J., and Robbins, T.W. (2011). Impulsivity, compulsivity, and top-down cognitive control. Neuron *69*, 680-694.

Dalley, J.W., Fryer, T.D., Brichard, L., Robinson, E.S., Theobald, D.E., Laane, K., Pena, Y., Murphy, E.R., Shah, Y., Probst, K.*, et al.* (2007). Nucleus accumbens D2/3 receptors predict trait impulsivity and cocaine reinforcement. Science *315*, 1267-1270.

Dalley, J.W., and Robbins, T.W. (2017). Fractionating impulsivity: neuropsychiatric implications. Nat. Rev. Neurosci. *18*, 158-171.

Dalley, J.W., Theobald, D.E., Eagle, D.M., Passetti, F., and Robbins, T.W. (2002). Deficits in impulse control associated with tonically-elevated serotonergic function in rat prefrontal cortex. Neuropsychopharmacology *26*, 716-728.

Daly, T.M. (2004). Overview of adeno-associated viral vectors. Methods Mol.Biol. *246*, 157-165.

Day, M., Olson, P.A., Platzer, J., Striessnig, J., and Surmeier, D.J. (2002). Stimulation of 5-HT(2) receptors in prefrontal pyramidal neurons inhibits Ca(v)1.2 L type Ca(2+) currents via a PLCbeta/IP3/calcineurin signaling cascade. J Neurophysiol. *87*, 2490-2504.

de Caestecker, M.P., Yahata, T., Wang, D., Parks, W.T., Huang, S., Hill, C.S., Shioda, T., Roberts, A.B., and Lechleider, R.J. (2000). The Smad4 activation domain (SAD) is a proline-rich, p300-dependent transcriptional activation domain. J. Biol. Chem. *275*, 2115-2122.

de Wit, H. (2009). Impulsivity as a determinant and consequence of drug use: a review of underlying processes. Addict. Biol. *14*, 22-31.

Demirci, E., Ozmen, S., and Oztop, D.B. (2016). Relationship between impulsivity and serum oxytocin in male children and adolescents with attention-deficit and hyperactivity disorder: a preliminary study. Noro psikiyatri arsivi *53*, 291-295.

Dermitzakis, E.T., and Clark, A.G. (2002). Evolution of transcription factor binding sites in mammalian gene regulatory regions: conservation and turnover. Mol. Biol. Evol. *19*, 1114-1121.

Dickman, S.J. (1990). Functional and dysfunctional impulsivity: personality and cognitive correlates. J. Pers. Soc. Psychol. *58*, 95-102.

Dougherty, D.M., Bjork, J.M., Marsh, D.M., and Moeller, F.G. (2000). A comparison between adults with conduct disorder and normal controls on a continuous performance test: Differences in impulsive response characteristics. Psychol. Rec. *50*, 203-219.

Dougherty, D.M., Marsh, D.M., and Mathias, C.W. (2002). Immediate and delayed memory tasks: a computerized behavioral measure of memory, attention, and impulsivity. Behav. Res. Methods Instrum. Comput. *34*, 391-398.

Drummond, D.C. (2001). Theories of drug craving, ancient and modern. Addiction *96*, 33-46.

Dupuis, A., Wattiez, A.S., Pinguet, J., Richard, D., Libert, F., Chalus, M., Aissouni, Y., Sion, B., Ardid, D., Marin, P.*, et al.* (2017). Increasing spinal 5-HT2A receptor responsiveness mediates anti-allodynic effect and potentiates fluoxetine efficacy in neuropathic rats. Evidence for GABA release. Pharmacol. Res. *118*, 93-103.

Eagle, D.M., and Baunez, C. (2010). Is there an inhibitory-response-control system in the rat? Evidence from anatomical and pharmacological studies of behavioral inhibition. Neurosci.Biobehav.Rev. *34*, 50-72.

Economidou, D., Theobald, D.E., Robbins, T.W., Everitt, B.J., and Dalley, J.W. (2012). Norepinephrine and dopamine modulate impulsivity on the five-choice serial reaction time task through opponent actions in the shell and core sub-regions of the nucleus accumbens. Neuropsychopharmacology *37*, 2057-2066.

Ehlinger, D.G., and Commons, K.G. (2019). Cav1.2L-type calcium channels regulate stress coping behavior via serotonin neurons. Neuropharmacology *144*, 282-290.

Eipper-Mains, J.E., Kiraly, D.D., Duff, M.O., Horowitz, M.J., McManus, C.J., Eipper, B.A., Graveley, B.R., and Mains, R.E. (2013). Effects of cocaine and withdrawal on the mouse nucleus accumbens transcriptome. Genes Brain Behav *12*, 21-33.

El-Brolosy, M.A., and Stainier, D.Y.R. (2017). Genetic compensation: A phenomenon in search of mechanisms. PLoS genetics *13*, e1006780.

Elkins, E.A., Walti, K.A., Newberry, K.E., and Lema, S.C. (2017). Identification of an oxytocinase/vasopressinase-like leucyl-cystinyl aminopeptidase (LNPEP) in teleost fish and evidence for hypothalamic mRNA expression linked to behavioral social status. Gen. Comp. Endocrinol. *250*, 58-69.

Epstein, D.H., Willner-Reid, J., Vahabzadeh, M., Mezghanni, M., Lin, J.L., and Preston, K.L. (2009). Real-time electronic diary reports of cue exposure and mood in the hours before cocaine and heroin craving and use. Arch. Gen. Psychiatry *66*, 88-94.

Evenden, J.L. (1999a). Impulsivity: a discussion of clinical and experimental findings. J Psychopharmacol. *13*, 180-192.

Evenden, J.L. (1999b). Varieties of impulsivity. Psychopharmacology (Berl.) *146*, 348-361.

Fantegrossi, W.E., Simoneau, J., Cohen, M.S., Zimmerman, S.M., Henson, C.M., Rice, K.C., and Woods, J.H. (2010). Interaction of 5-HT2A and 5-HT2C receptors in R(-)-2,5-dimethoxy-4-iodoamphetamine-elicited head twitch behavior in mice. J. Pharmacol. Exp. Ther. *335*, 728-734.

Fantegrossi, W.E., Ullrich, T., Rice, K.C., Woods, J.H., and Winger, G. (2002). 3,4-Methylenedioxymethamphetamine (MDMA, "ecstasy") and its stereoisomers as reinforcers in rhesus monkeys: serotonergic involvement. Psychopharmacology (Berl.) *161*, 356-364.

Ferguson, S.A., Paule, M.G., Cada, A., Fogle, C.M., Gray, E.P., and Berry, K.J. (2007). Baseline behavior, but not sensitivity to stimulant drugs, differs among spontaneously hypertensive, Wistar-Kyoto, and Sprague-Dawley rat strains. Neurotoxicol. Teratol. *29*, 547-561.

Field, M., and Cox, W.M. (2008). Attentional bias in addictive behaviors: a review of its development, causes, and consequences. Drug Alcohol Depend. *97*, 1-20.

Fields, H.L., and Margolis, E.B. (2015). Understanding opioid reward. Trends Neurosci. *38*, 217-225.

Filip, M. (2005). Role of serotonin (5-HT)2 receptors in cocaine self-administration and seeking behavior in rats. Pharmacol.Rep. *57*, 35-46.

Fineberg, N.A., Potenza, M.N., Chamberlain, S.R., Berlin, H.A., Menzies, L., Bechara, A., Sahakian, B.J., Robbins, T.W., Bullmore, E.T., and Hollander, E. (2010). Probing compulsive and impulsive behaviors, from animal models to endophenotypes: a narrative review. Neuropsychopharmacology *35*, 591-604.

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

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

Fletcher, P.J., Rizos, Z., Noble, K., and Higgins, G.A. (2011). Impulsive action induced by amphetamine, cocaine and MK801 is reduced by 5-HT(2C) receptor stimulation and 5-HT(2A) receptor blockade. Neuropharmacology *61*, 468-477.

Fletcher, P.J., Rizos, Z., Sinyard, J., Tampakeras, M., and Higgins, G.A. (2008). The 5-HT(2C) receptor agonist RO 60-0175 reduces cocaine self-administration and reinstatement induced by the stressor yohimbine and contextual cues. Neuropsychopharmacology *33*, 1402-1412.

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

Friedman, E., Hoau Yan, W., Levinson, D., Connell, T.A., and Singh, H. (1993). Altered platelet protein kinase C activity in bipolar affective disorder, manic episode. Biol. Psychiatry *33*, 520-525.

Fuchs, R.A., Tran-Nguyen, L.T., Specio, S.E., Groff, R.S., and Neisewander, J.L. (1998). Predictive validity of the extinction/reinstatement model of drug craving. Psychopharmacology (Berl.) *135*, 151-160.

Gao, X., Bakshi, P., Sunkara Ganti, S., Manian, M., Korey, A., Fowler, W., and Banga, A.K. (2018). Evaluation of an activated carbon-based deactivation system for the disposal of highly abused opioid medications. Drug Dev. Ind. Pharm. *44*, 125-134.

Garami, J., Haber, P., Myers, C.E., Allen, M.T., Misiak, B., Frydecka, D., and Moustafa, A.A. (2017). Intolerance of uncertainty in opioid dependency - Relationship with trait anxiety and impulsivity. PLoS One *12*, e0181955.

Garavan, H., Pankiewicz, J., Bloom, A., Cho, J.K., Sperry, L., Ross, T.J., Salmeron, B.J., Risinger, R., Kelley, D., and Stein, E.A. (2000). Cue-induced cocaine craving: neuroanatomical specificity for drug users and drug stimuli. Am.J.Psychiatry *157*, 1789-1798.

Gasque, G., Conway, S., Huang, J., Rao, Y., and Vosshall, L.B. (2013). Small molecule drug screening in Drosophila identifies the 5HT2A receptor as a feeding modulation target. Sci. Rep. *3*, srep02120.

Geschwind, D.H., and Konopka, G. (2009). Neuroscience in the era of functional genomics and systems biology. Nature *461*, 908-915.

Gooz, M., Gooz, P., Luttrell, L.M., and Raymond, J.R. (2006). 5-HT2A receptor induces ERK phosphorylation and proliferation through ADAM-17 tumor necrosis factor-alpha-converting enzyme (TACE) activation and heparin-bound epidermal growth factor-like growth factor (HB-EGF) shedding in mesangial cells. J.Biol.Chem. *281*, 21004-21012.

Gray, J.A., and Roth, B.L. (2001). Paradoxical trafficking and regulation of 5-HT(2A) receptors by agonists and antagonists. Brain Res.Bull. *56*, 441-451.

Grimm, J.W., Hope, B.T., Wise, R.A., and Shaham, Y. (2001). Neuroadaptation. Incubation of cocaine craving after withdrawal. Nature *412*, 141-142.

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

Gustafson, A., King, C., and Rey, J.A. (2013). Lorcaserin (Belviq): A selective serotonin 5-HT_{2C} agonist In the treatment of obesity. P T *38*, 525-534.

Hacksell, U., Burstein, E.S., McFarland, K., Mills, R.G., and Williams, H. (2014). On the discovery and development of pimavanserin: a novel drug candidate for Parkinson's psychosis. Neurochem. Res. *39*, 2008-2017.

Hamilton, K.R., Littlefield, A.K., Anastasio, N.C., Cunningham, K.A., Fink, L.H., Wing, V.C., Mathias, C.W., Lane, S.D., Schutz, C.G., Swann, A.C*., et al.* (2015). Rapid-response impulsivity: definitions, measurement issues, and clinical implications. Personal Disord *6*, 168-181.

Hendershot, C.S., Witkiewitz, K., George, W.H., and Marlatt, G.A. (2011). Relapse prevention for addictive behaviors. Subst.Abuse Treat.Prev.Policy *6*, 17.

Higgins, G.A., Silenieks, L.B., Altherr, E.B., MacMillan, C., Fletcher, P.J., and Pratt, W.E. (2016). Lorcaserin and CP-809101 reduce motor impulsivity and reinstatement of food seeking behavior in male rats: Implications for understanding the anti-obesity property of 5-HT2C receptor agonists. Psychopharmacology (Berl.) *233*, 2841-2856.

Hoebel, B.G. (1984). Neurotransmitters in the control of feeding and its rewards: monoamines, opiates, and brain-gut peptides. Res. Publ. Assoc. Res. Nerv. Ment. Dis. *62*, 15-38.

Homer, and Murray, A.T. (1965). The Iliad : with an English translation (London; Cambridge (Mass.): W. Heinemann ; Harvard University Press).

Hommel, J.D., Sears, R.M., Georgescu, D., Simmons, D.L., and DiLeone, R.J. (2003). Local gene knockdown in the brain using viral-mediated RNA interference. Nat.Med. *9*, 1539-1544.

Hoover, W.B., and Vertes, R.P. (2007). Anatomical analysis of afferent projections to the medial prefrontal cortex in the rat. Brain Struct Funct *212*, 149-179.

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

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

Hoyer, D., Waeber, C., Schoeffter, P., Palacios, J.M., and Dravid, A. (1989). 5-HT1C receptor-mediated stimulation of inositol phosphate production in pig choroid plexus. A pharmacological characterization. Naunyn Schmiedebergs Arch. Pharmacol. *339*, 252-258.

Huang da, W., Sherman, B.T., and Lempicki, R.A. (2009). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. *37*, 1-13.

Jamison, R.N., and Mao, J. (2015). Opioid analgesics. Mayo Clin. Proc. *90*, 957-968.

Johnson, K.A., and Lovinger, D.M. (2016). Presynaptic G protein-coupled receptors: gatekeepers of addiction? Front. Cell. Neurosci. *10*, 264.

Johnson, S.W., and North, R.A. (1992). Opioids excite dopamine neurons by hyperpolarization of local interneurons. J.Neurosci. *12*, 483-488.

Jones, J.D., Vadhan, N.P., Luba, R.R., and Comer, S.D. (2016). The effects of heroin administration and drug cues on impulsivity. J. Clin. Exp. Neuropsychol. *38*, 709-720.

Jope, R.S. (1999). Anti-bipolar therapy: mechanism of action of lithium. Mol. Psychiatry *4*, 117-128.

Jupp, B., Caprioli, D., and Dalley, J.W. (2013). Highly impulsive rats: modelling an endophenotype to determine the neurobiological, genetic and environmental mechanisms of addiction. Dis. Model. Mech. *6*, 302-311.

Kariisa, M., Scholl, L., Wilson, N., Seth, P., and Hoots, B. (2019). Drug overdose deaths involving cocaine and psychostimulants with abuse potential - United States, 2003-2017. MMWR Morb. Mortal. Wkly. Rep. *68*, 388-395.

Kasper, J.M., McCue, D.L., Milton, A.J., Szwed, A., Sampson, C.M., Huang, M., Carlton, S., Meltzer, H.Y., Cunningham, K.A., and Hommel, J.D. (2016). Gamma-aminobutyric acidergic projections from the dorsal raphe to the nucleus accumbens are regulated by neuromedin U. Biol. Psychiatry *80*, 878-887.

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

Kelley, A.E., Bakshi, V.P., Haber, S.N., Steininger, T.L., Will, M.J., and Zhang, M. (2002). Opioid modulation of taste hedonics within the ventral striatum. Physiol Behav. *76*, 365-377.

Kelley, A.E., and Berridge, K.C. (2002). The neuroscience of natural rewards: relevance to addictive drugs. J.Neurosci. *22*, 3306-3311.

Keppel, G. (1973). Design and Analysis: A Researcher's Handbook (Englewood Cliffs, NJ: Prentice-Hall, Inc.).

Khan, A., Fornes, O., Stigliani, A., Gheorghe, M., Castro-Mondragon, J.A., van der Lee, R., Bessy, A., Cheneby, J., Kulkarni, S.R., Tan, G.*, et al.* (2018). JASPAR 2018: update of the open-access database of transcription factor binding profiles and its web framework. Nucleic Acids Res. *46*, D260-d266.

Kharazia, V.N., and Weinberg, R.J. (1994). Glutamate in thalamic fibers terminating in layer IV of primary sensory cortex. J. Neurosci. *14*, 6021-6032.

Kim, S.T., and Park, T. (2019). Acute and Chronic Effects of Cocaine on Cardiovascular Health. Int. J. Mol. Sci. *20*.

Kirby, K.N., Petry, N.M., and Bickel, W.K. (1999). Heroin addicts have higher discount rates for delayed rewards than non-drug-using controls. J. Exp. Psychol. Gen. *128*, 78-87.

Klein, A.B., Ultved, L., Adamsen, D., Santini, M.A., Tobena, A., Fernandez-Teruel, A., Flores, P., Moreno, M., Cardona, D., Knudsen, G.M*., et al.* (2014). 5-HT(2A) and mGlu2 receptor binding levels are related to differences in impulsive behavior in the Roman Low- (RLA) and High- (RHA) avoidance rat strains. Neuroscience *263*, 36-45.

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

Koenig, J.A., and Edwardson, J.M. (1997). Endocytosis and recycling of G protein-coupled receptors. Trends Pharmacol. Sci. *18*, 276-287.

Koike, H., Demars, M.P., Short, J.A., Nabel, E.M., Akbarian, S., Baxter, M.G., and Morishita, H. (2016). Chemogenetic inactivation of dorsal anterior cingulate

cortex neurons disrupts attentional behavior in mouse. Neuropsychopharmacology *41*, 1014-1023.

Koob, G.F., and Volkow, N.D. (2010). Neurocircuitry of addiction. Neuropsychopharmacology *35*, 217-238.

Koob, G.F., and Volkow, N.D. (2016). Neurobiology of addiction: a neurocircuitry analysis. Lancet Psychiatry *3*, 760-773.

Koskinen, T., Haapalinna, A., and Sirvio, J. (2003). Alpha-adrenoceptor-mediated modulation of 5-HT2 receptor agonist induced impulsive responding in a 5-choice serial reaction time task. Pharmacol Toxicol. *92*, 214-225.

Koskinen, T., Ruotsalainen, S., Puumala, T., Lappalainen, R., Koivisto, E., Mannisto, P.T., and Sirvio, J. (2000a). Activation of 5-HT2A receptors impairs response control of rats in a five-choice serial reaction time task. Neuropharmacology *39*, 471-481.

Koskinen, T., Ruotsalainen, S., and Sirvio, J. (2000b). The 5-HT(2) receptor activation enhances impulsive responding without increasing motor activity in rats. Pharmacol Biochem.Behav. *66*, 729-738.

Kosten, T., Oliveto, A., Feingold, A., Poling, J., Sevarino, K., McCance-Katz, E., Stine, S., Gonzalez, G., and Gonsai, K. (2003). Desipramine and contingency management for cocaine and opiate dependence in buprenorphine maintained patients. Drug Alcohol Depend. *70*, 315-325.

Koya, E., Uejima, J.L., Wihbey, K.A., Bossert, J.M., Hope, B.T., and Shaham, Y. (2009). Role of ventral medial prefrontal cortex in incubation of cocaine craving. Neuropharmacology *56 Suppl 1*, 177-185.

Kramer, A., Green, J., Pollard, J., Jr., and Tugendreich, S. (2014). Causal analysis approaches in Ingenuity Pathway Analysis. Bioinformatics *30*, 523-530.

Kreft, L., Soete, A., Hulpiau, P., Botzki, A., Saeys, Y., and De Bleser, P. (2017). ConTra v3: a tool to identify transcription factor binding sites across species, update 2017. Nucleic Acids Res. *45*, W490-w494.

Kuleshov, M.V., Jones, M.R., Rouillard, A.D., Fernandez, N.F., Duan, Q., Wang, Z., Koplev, S., Jenkins, S.L., Jagodnik, K.M., Lachmann, A.*, et al.* (2016). Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic Acids Res. *44*, W90-97.

Kurrasch-Orbaugh, D.M., Watts, V.J., Barker, E.L., and Nichols, D.E. (2003). Serotonin 5-hydroxytryptamine 2A receptor-coupled phospholipase C and phospholipase A2 signaling pathways have different receptor reserves. J. Pharmacol. Exp. Ther. *304*, 229-237.

Lacosta, S., and Roberts, D.C.S. (1993). MDL 72222, ketanserin, and methysergide pretreatments fail to alter breaking points on a progressive ratio schedule reinforced by intravenous cocaine. Pharmacol.Biochem.Behav. *44*, 161-165.

LaLumiere, R.T., Smith, K.C., and Kalivas, P.W. (2012). Neural circuit competition in cocaine-seeking: roles of the infralimbic cortex and nucleus accumbens shell. Eur.J.Neurosci. *35*, 614-622.

Lambert, S.A., Jolma, A., Campitelli, L.F., Das, P.K., Yin, Y., Albu, M., Chen, X., Taipale, J., Hughes, T.R., and Weirauch, M.T. (2018). The Human Transcription Factors. Cell *172*, 650-665.

Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. Nat Methods *9*, 357-359.

Law, A.V., Sakharkar, P., Zargarzadeh, A., Tai, B.W., Hess, K., Hata, M., Mireles, R., Ha, C., and Park, T.J. (2015). Taking stock of medication wastage: unused medications in US households. Res. Social Adm. Pharm. *11*, 571-578.

Leff, S.E., Spratt, S.K., Snyder, R.O., and Mandel, R.J. (1999). Long-term restoration of striatal L-aromatic amino acid decarboxylase activity using recombinant adeno-associated viral vector gene transfer in a rodent model of Parkinson's disease. Neuroscience *92*, 185-196.

Leibowitz, S.F. (1985). Brain neurotransmitters and appetite regulation. Psychopharmacol. Bull. *21*, 412-418.

Leung, D., Staiger, P.K., Hayden, M., Lum, J.A., Hall, K., Manning, V., and Verdejo-Garcia, A. (2017). Meta-analysis of the relationship between impulsivity and substance-related cognitive biases. Drug Alcohol Depend. *172*, 21-33.

Levine, A.S., and Billington, C.J. (1989). Opioids: are they regulators of feeding? Ann. N. Y. Acad. Sci. *575*, 209-219; discussion 219-220.

Levine, A.S., Morley, J.E., Gosnell, B.A., Billington, C.J., and Bartness, T.J. (1985). Opioids and consummatory behavior. Brain Res. Bull. *14*, 663-672.

Li, B., and Dewey, C.N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics *12*, 323.

Li, X., and Wolf, M.E. (2011). Visualization of virus-infected brain regions using a GFP-illuminating flashlight enables accurate and rapid dissection for biochemical analysis. J.Neurosci.Methods.

Ling, W., Hillhouse, M.P., Saxon, A.J., Mooney, L.J., Thomas, C.M., Ang, A., Matthews, A.G., Hasson, A., Annon, J., Sparenborg, S.*, et al.* (2016). Buprenorphine + naloxone plus naltrexone for the treatment of cocaine dependence: the Cocaine Use Reduction with Buprenorphine (CURB) study. Addiction *111*, 1416-1427.

Liu, S., Lane, S.D., Schmitz, J.M., Waters, A.J., Cunningham, K.A., and Moeller, F.G. (2011). Relationship between attentional bias to cocaine-related stimuli and impulsivity in cocaine-dependent subjects. Am. J. Drug Alcohol Abuse *37*, 117-122.

Loots, G.G., Ovcharenko, I., Pachter, L., Dubchak, I., and Rubin, E.M. (2002). rVista for comparative sequence-based discovery of functional transcription factor binding sites. Genome Res. *12*, 832-839.

Lopez-Gimenez, J.F., Mengod, G., Palacios, J.M., and Vilaro, M.T. (2001). Regional distribution and cellular localization of 5-HT2C receptor mRNA in monkey brain: comparison with [3H]mesulergine binding sites and choline acetyltransferase mRNA. Synapse *42*, 12-26.

Lu, L., Grimm, J.W., Dempsey, J., and Shaham, Y. (2004). Cocaine seeking over extended withdrawal periods in rats: different time courses of responding induced by cocaine cues versus cocaine priming over the first 6 months. Psychopharmacology (Berl.) *176*, 101-108.

Lubick, N. (2010). Drugs in the environment: do pharmaceutical take-back programs make a difference? Environ. Health Perspect. *118*, A210-214.

Maas, L.C., Lukas, S.E., Kaufman, M.J., Weiss, R.D., Daniels, S.L., Rogers, V.W., Kukes, T.J., and Renshaw, P.F. (1998). Functional magnetic resonance imaging of human brain activation during cue-induced cocaine craving. Am. J. Psychiatry *155*, 124-126.

Madden, G.J., Petry, N.M., Badger, G.J., and Bickel, W.K. (1997). Impulsive and self-control choices in opioid-dependent patients and non-drug-using control participants: drug and monetary rewards. Exp. Clin. Psychopharmacol. *5*, 256-262.

Manvich, D.F., Kimmel, H.L., Cooper, D.A., and Howell, L.L. (2012a). The serotonin 2C receptor antagonist SB 242084 exhibits abuse-related effects typical of stimulants in squirrel monkeys. J. Pharmacol. Exp. Ther. *342*, 761-769.

Manvich, D.F., Kimmel, H.L., and Howell, L.L. (2012b). Effects of serotonin 2C receptor agonists on the behavioral and neurochemical effects of cocaine in squirrel monkeys. J. Pharmacol. Exp. Ther. *341*, 424-434.

Marek, G.J., Wright, R.A., Gewirtz, J.C., and Schoepp, D.D. (2001). A major role for thalamocortical afferents in serotonergic hallucinogen receptor function in the rat neocortex. Neuroscience *105*, 379-392.

Marissen, M.A., Franken, I.H., Waters, A.J., Blanken, P., van den Brink, W., and Hendriks, V.M. (2006). Attentional bias predicts heroin relapse following treatment. Addiction *101*, 1306-1312.

Matsuda, Y., Saegusa, H., Zong, S., Noda, T., and Tanabe, T. (2001). Mice lacking Ca(v)2.3 (alpha1E) calcium channel exhibit hyperglycemia. Biochem. Biophys. Res. Commun. *289*, 791-795.

McFarland, K., Price, D.L., and Bonhaus, D.W. (2011). Pimavanserin, a 5-HT2A inverse agonist, reverses psychosis-like behaviors in a rodent model of Parkinson's disease. Behav.Pharmacol. *22*, 681-692.

McLellan, A.T. (2017). Substance misuse and substance use disorders: why do they matter in healthcare? Trans. Am. Clin. Climatol. Assoc. *128*, 112-130.

Mello, N.K., Mendelson, J.H., Bree, M.P., and Lukas, S.E. (1990). Buprenorphine and naltrexone effects on cocaine self-administration by rhesus monkeys. J.Pharmacol.Exp.Ther. *254*, 926-939.

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

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

Mendelson, J., Flower, K., Pletcher, M.J., and Galloway, G.P. (2008). Addiction to prescription opioids: characteristics of the emerging epidemic and treatment with buprenorphine. Exp. Clin. Psychopharmacol. *16*, 435-441.

Miner, L.H., Schroeter, S., Blakely, R.D., and Sesack, S.R. (2000). Ultrastructural localization of the serotonin transporter in superficial and deep layers of the rat prelimbic prefrontal cortex and its spatial relationship to dopamine terminals. J.Comp Neurol. *427*, 220-234.

Mirjana, C., Baviera, M., Invernizzi, R.W., and Balducci, C. (2004). The serotonin 5-HT2A receptors antagonist M100907 prevents impairment in attentional performance by NMDA receptor blockade in the rat prefrontal cortex. Neuropsychopharmacology *29*, 1637-1647.

Miyazaki, K., Miyazaki, K.W., and Doya, K. (2011). Activation of dorsal raphe serotonin neurons underlies waiting for delayed rewards. J.Neurosci. *31*, 469-479.

Moeller, F.G., Barratt, E.S., Dougherty, D.M., Schmitz, J.M., and Swann, A.C. (2001a). Psychiatric aspects of impulsivity. Am. J. Psychiatry *158*, 1783-1793.

Moeller, F.G., Barratt, E.S., Fischer, C.J., Dougherty, D.M., Reilly, E.L., Mathias, C.W., and Swann, A.C. (2004). P300 event-related potential amplitude and impulsivity in cocaine-dependent subjects. Neuropsychobiology *50*, 167-173.

Moeller, F.G., and Cunningham, K.A. (2018). Innovative therapeutic intervention for opioid use disorder. Neuropsychopharmacology *43*, 220-221.

Moeller, F.G., Dougherty, D.M., Barratt, E.S., Oderinde, V., Mathias, C.W., Harper, R.A., and Swann, A.C. (2002). Increased impulsivity in cocaine dependent subjects independent of antisocial personality disorder and aggression. Drug Alcohol Depend. *68*, 105-111.

Moeller, F.G., Dougherty, D.M., Barratt, E.S., Schmitz, J.M., Swann, A.C., and Grabowski, J. (2001b). The impact of impulsivity on cocaine use and retention in treatment. J. Subst. Abuse Treat. *21*, 193-198.

Moeller, F.G., Schmitz, J.M., Steinberg, J.L., Green, C.M., Reist, C., Lai, L.Y., Swann, A.C., and Grabowski, J. (2007). Citalopram combined with behavioral therapy reduces cocaine use: a double-blind, placebo-controlled trial. Am.J.Drug Alcohol Abuse *33*, 367-378.

Moloney, G.M., van Oeffelen, W., Ryan, F.J., van de Wouw, M., Cowan, C., Claesson, M.J., Schellekens, H., Dinan, T.G., and Cryan, J.F. (2019). Differential gene expression in the mesocorticolimbic system of innately high- and low-impulsive rats. Behav. Brain Res. *364*, 193-204.

Monory, K., Greiner, E., Sartania, N., Sallai, L., Pouille, Y., Schmidhammer, H., Hanoune, J., and Borsodi, A. (1999). Opioid binding profiles of new hydrazone, oxime, carbazone and semicarbazone derivatives of 14-alkoxymorphinans. Life Sci. *64*, 2011-2020.

Morgan, J.R., Schackman, B.R., Leff, J.A., Linas, B.P., and Walley, A.Y. (2018). Injectable naltrexone, oral naltrexone, and buprenorphine utilization and discontinuation among individuals treated for opioid use disorder in a United States commercially insured population. J. Subst. Abuse Treat. *85*, 90-96.

Morgan, P.T., Pace-Schott, E., Pittman, B., Stickgold, R., and Malison, R.T. (2010). Normalizing Effects of Modafinil on Sleep in Chronic Cocaine Users. Am. J. Psychiatry *167*, 331-340.

Muir, J.L., Everitt, B.J., and Robbins, T.W. (1996). The cerebral cortex of the rat and visual attentional function: dissociable effects of mediofrontal, cingulate, anterior dorsolateral, and parietal cortex lesions on a five-choice serial reaction time task. Cereb.Cortex *6*, 470-481.

Muntasir, H.A., Bhuiyan, M.A., Ishiguro, M., Ozaki, M., and Nagatomo, T. (2006). Inverse agonist activity of sarpogrelate, a selective 5-HT2A-receptor antagonist,

at the constitutively active human 5-HT2A receptor. J.Pharmacol.Sci. *102*, 189-195.

Murphy, E.R., Dalley, J.W., and Robbins, T.W. (2005). Local glutamate receptor antagonism in the rat prefrontal cortex disrupts response inhibition in a visuospatial attentional task. Psychopharmacology (Berl.) *179*, 99-107.

Narayanan, N.S., Horst, N.K., and Laubach, M. (2006). Reversible inactivations of rat medial prefrontal cortex impair the ability to wait for a stimulus. Neuroscience *139*, 865-876.

Nathanson, J.L., Jappelli, R., Scheeff, E.D., Manning, G., Obata, K., Brenner, S., and Callaway, E.M. (2009). Short promoters in viral vectors drive selective expression in mammalian inhibitory neurons, but do not restrict activity to specific inhibitory cell types. Front Neural Circuits. *3*, 19.

National Institute on Drug Abuse (NIDA) (2017). Trends & Statistics. Retrieved from https://www.drugabuse.gov/related-topics/trends-statistics on 2019, November 13.

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

Neelakantan, H., Holliday, E.D., Fox, R.G., Stutz, S.J., Comer, S.D., Haney, M., Anastasio, N.C., Moeller, F.G., and Cunningham, K.A. (2017). Lorcaserin

suppresses oxycodone self-administration and relapse vulnerability in rats. ACS Chem. Neurosci. *8*, 1065-1073.

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

Neisewander, J.L., Baker, D.A., Fuchs, R.A., Tran-Nguyen, L.T., Palmer, A., and Marshall, J.F. (2000). Fos protein expression and cocaine-seeking behavior in rats after exposure to a cocaine self-administration environment. J.Neurosci. *20*, 798-805.

Nestler, E.J. (2005). Is there a common molecular pathway for addiction? Nat.Neurosci. *8*, 1445-1449.

Neufang, S., Akhrif, A., Herrmann, C.G., Drepper, C., Homola, G.A., Nowak, J., Waider, J., Schmitt, A.G., Lesch, K.P., and Romanos, M. (2016). Serotonergic modulation of 'waiting impulsivity' is mediated by the impulsivity phenotype in humans. Transl. Psychiatry *6*, e940.

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

Nonogaki, K., Nozue, K., and Oka, Y. (2006). Increased hypothalamic 5-HT2A receptor gene expression and effects of pharmacologic 5-HT2A receptor

inactivation in obese Ay mice. Biochem. Biophys. Res. Commun. *351*, 1078-1082.

Noori, H.R., Spanagel, R., and Hansson, A.C. (2012). Neurocircuitry for modeling drug effects. Addict. Biol. *17*, 827-864.

O'Brian, C.A., Liskamp, R.M., Solomon, D.H., and Weinstein, I.B. (1985). Inhibition of protein kinase C by tamoxifen. Cancer Res. *45*, 2462-2465.

O'Brien, C.P., Childress, A.R., Ehrman, R., and Robbins, S.J. (1998). Conditioning factors in drug abuse: can they explain compulsion? J Psychopharmacol. *12*, 15-22.

Olmstead, M.C., Ouagazzal, A.M., and Kieffer, B.L. (2009). Mu and delta opioid receptors oppositely regulate motor impulsivity in the signaled nose poke task. PLoS One *4*, e4410.

Orduna, V., Valencia-Torres, L., and Bouzas, A. (2009). DRL performance of spontaneously hypertensive rats: dissociation of timing and inhibition of responses. Behav. Brain Res. *201*, 158-165.

Paine, T.A., Dringenberg, H.C., and Olmstead, M.C. (2003). Effects of chronic cocaine on impulsivity: relation to cortical serotonin mechanisms. Behav. Brain Res. *147*, 135-147.

Paine, T.A., and Olmstead, M.C. (2004). Cocaine disrupts both behavioural inhibition and conditional discrimination in rats. Psychopharmacology (Berl.) *175*, 443-450.

Palacios, J., Yildiz, A., Young, A.H., and Taylor, M.J. (2019). Tamoxifen for bipolar disorder: Systematic review and meta-analysis. J Psychopharmacol *33*, 177-184.

Pang, G., Wu, X., Tao, X., Mao, R., Liu, X., Zhang, Y.M., Li, G., Stackman, R.W., Jr., Dong, L., and Zhang, G. (2016). Blockade of serotonin 5-HT_{2A} receptors suppresses behavioral sensitization and naloxone-precipitated withdrawal symptoms in morphine-treated mice. Front. Pharmacol. *7*, 514.

Park, A., and Stacy, M. (2011). Dopamine-induced nonmotor symptoms of Parkinson's disease. Parkinsons.Dis. *2011*, 485063.

Pascoli, V., Terrier, J., Espallergues, J., Valjent, E., O'Connor, E.C., and Luscher, C. (2014). Contrasting forms of cocaine-evoked plasticity control components of relapse. Nature *509*, 459-464.

Passetti, F., Dalley, J.W., and Robbins, T.W. (2003). Double dissociation of serotonergic and dopaminergic mechanisms on attentional performance using a rodent five-choice reaction time task. Psychopharmacology (Berl.) *165*, 136-145.

Patkar, A.A., Murray, H.W., Mannelli, P., Gottheil, E., Weinstein, S.P., and Vergare, M.J. (2004). Pre-treatment measures of impulsivity, aggression and

sensation seeking are associated with treatment outcome for African-American cocaine-dependent patients. J.Addict.Dis. *23*, 109-122.

Pattij, T., Schetters, D., Janssen, M.C., Wiskerke, J., and Schoffelmeer, A.N. (2009). Acute effects of morphine on distinct forms of impulsive behavior in rats. Psychopharmacology (Berl.) *205*, 489-502.

Pattij, T., and Vanderschuren, L.J. (2008). The neuropharmacology of impulsive behaviour. Trends Pharmacol.Sci. *29*, 192-199.

Paxinos, G., and Watson, C. (2005). The Rat Brain in Stereotaxic Coordinates, 5th edn (Burlington, MA: Elsevier Academic Press).

Paxinos, G., and Watson, C. (2014). The rat brain in stereotaxic coordinates, Seventh edition. edn (Amsterdam ; Boston: Elsevier/AP, Academic Press is an imprint of Elsevier).

Pazos, A., Cortes, R., and Palacios, J.M. (1985). Quantitative autoradiographic mapping of serotonin receptors in rat brain. II. Serotonin-2 receptors. Brain Res. *346*, 231-249.

Pazos, A., and Palacios, J.M. (1985). Quantitative autoradiographic mapping of serotonin receptors in the rat brain. I. Serotonin-1 receptors. Brain Res. *346*, 205-230.

Peer, J., Bennett, M.E., and Bellack, A.S. (2009). Neurocognitive characteristics of individuals with schizophrenia and cocaine dependence: comparison of currently dependent and remitted groups. J Nerv.Ment.Dis. *197*, 631-634.

Pehek, E.A., Nocjar, C., Roth, B.L., Byrd, T.A., and Mabrouk, O.S. (2006). Evidence for the preferential involvement of 5-HT2A serotonin receptors in stress- and drug-induced dopamine release in the rat medial prefrontal cortex. Neuropsychopharmacology *31*, 265-277.

Perera, R., Kraebber, A., and Schwartz, M.J. (1997). Prolonged QT interval and cocaine use. J. Electrocardiol. *30*, 337-339.

Perry, J.L., Larson, E.B., German, J.P., Madden, G.J., and Carroll, M.E. (2005). Impulsivity (delay discounting) as a predictor of acquisition of IV cocaine self-administration in female rats. Psychopharmacology (Berl.) *178*, 193-201.

Peters, J., LaLumiere, R.T., and Kalivas, P.W. (2008). Infralimbic prefrontal cortex is responsible for inhibiting cocaine seeking in extinguished rats. J.Neurosci. *28*, 6046-6053.

Petroff, O.A. (2002). GABA and glutamate in the human brain. Neuroscientist *8*, 562-573.

Pichon, X., Wattiez, A.S., Becamel, C., Ehrlich, I., Bockaert, J., Eschalier, A., Marin, P., and Courteix, C. (2010). Disrupting 5-HT(2A) receptor/PDZ protein interactions reduces hyperalgesia and enhances SSRI efficacy in neuropathic pain. Mol. Ther. *18*, 1462-1470.

Pickens, C.L., Airavaara, M., Theberge, F., Fanous, S., Hope, B.T., and Shaham, Y. (2011). Neurobiology of the incubation of drug craving. Trends Neurosci. *34*, 411-420.

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

Prieto, I., Segarra, A.B., de Gasparo, M., Martinez-Canamero, M., and Ramirez-Sanchez, M. (2018). Divergent profile between hypothalamic and plasmatic aminopeptidase activities in WKY and SHR. Influence of beta-adrenergic blockade. Life Sci. *192*, 9-17.

Puig, M.V., Celada, P., Diaz-Mataix, L., and Artigas, F. (2003). In vivo modulation of the activity of pyramidal neurons in the rat medial prefrontal cortex by 5-HT2A receptors: relationship to thalamocortical afferents. Cereb. Cortex *13*, 870-882.

Puumala, T., and Sirviö, J. (1998). Changes in activities of dopamine and serotonin systems in the frontal cortex underlie poor choice accuracy and impulsivity of rats in an attention task. Neuroscience *83*, 489-499.

Qian, J., and Noebels, J.L. (2001). Presynaptic Ca2+ channels and neurotransmitter release at the terminal of a mouse cortical neuron. J. Neurosci. *21*, 3721-3728.

Rasmussen, K., White, D.A., and Acri, J.B. (2019). NIDA's medication development priorities in response to the Opioid Crisis: ten most wanted. Neuropsychopharmacology *44*, 657-659.

Ratkowsky, D.A., and Reedy, T.J. (1986). Choosing near-linear parameters in the four-parameter logistic model for radioligand and related assays. Biometrics *42*, 575-582.

Reid, L.D. (1985). Endogenous opioid peptides and regulation of drinking and feeding. Am. J. Clin. Nutr. *42*, 1099-1132.

Robbins, T.W. (2002). The 5-choice serial reaction time task: behavioural pharmacology and functional neurochemistry. Psychopharmacology (Berl.) *163*, 362-380.

Robbins, T.W., Gillan, C.M., Smith, D.G., de Wit, S., and Ersche, K.D. (2012). Neurocognitive endophenotypes of impulsivity and compulsivity: towards dimensional psychiatry. Trends Cogn. Sci. *16*, 81-91.

Roberts, C. (2006). ACP-103, a 5-HT2A receptor inverse agonist. Curr.Opin.Investig.Drugs *7*, 653-660.

Robinson, E.S., Dalley, J.W., Theobald, D.E., Glennon, J.C., Pezze, M.A., Murphy, E.R., and Robbins, T.W. (2008). Opposing roles for 5-HT2A and 5-HT2C receptors in the nucleus accumbens on inhibitory response control in the 5-choice serial reaction time task. Neuropsychopharmacology *33*, 2398-2406.

Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics *26*, 139-140.

Robison, A.J., and Nestler, E.J. (2011). Transcriptional and epigenetic mechanisms of addiction. Nat. Rev. Neurosci. *12*, 623-637.

Rogers, R.D., Moeller, F.G., Swann, A.C., and Clark, L. (2010). Recent research on impulsivity in individuals with drug use and mental health disorders: implications for alcoholism. Alcohol Clin.Exp.Res. *34*, 1319-1333.

Romano, A.G., Quinn, J.L., Liu, R., Dave, K.D., Schwab, D., Alexander, G., Aloyo, V.J., and Harvey, J.A. (2006). Effect of serotonin depletion on 5-HT2A-mediated learning in the rabbit: evidence for constitutive activity of the 5-HT2A receptor in vivo. Psychopharmacology (Berl.) *V184*, 173-181.

Rosenbaum, D.M., Rasmussen, S.G., and Kobilka, B.K. (2009). The structure and function of G-protein-coupled receptors. Nature *459*, 356-363.

Rosenberg, R., Seiden, D.J., Hull, S.G., Erman, M., Schwartz, H., Anderson, C., Prosser, W., Shanahan, W., Sanchez, M., Chuang, E.*, et al.* (2008). APD125, a selective serotonin 5-HT(2A) receptor inverse agonist, significantly improves sleep maintenance in primary insomnia. Sleep *31*, 1663-1671.

Ross, S., and Hill, C.S. (2008). How the Smads regulate transcription. Int. J. Biochem. Cell Biol. *40*, 383-408.

Rowley, N.M., Madsen, K.K., Schousboe, A., and Steve White, H. (2012). Glutamate and GABA synthesis, release, transport and metabolism as targets for seizure control. Neurochem. Int. *61*, 546-558.

Sahli, Z.T., and Tarazi, F.I. (2018). Pimavanserin: novel pharmacotherapy for Parkinson's disease psychosis. Expert Opin Drug Discov *13*, 103-110.

Salegio, E.A., Samaranch, L., Kells, A.P., Mittermeyer, G., San Sebastian, W., Zhou, S., Beyer, J., Forsayeth, J., and Bankiewicz, K.S. (2013). Axonal transport of adeno-associated viral vectors is serotype-dependent. Gene Ther. *20*, 348-352.

Sanacora, G., Treccani, G., and Popoli, M. (2012). Towards a glutamate hypothesis of depression: an emerging frontier of neuropsychopharmacology for mood disorders. Neuropharmacology *62*, 63-77.

Santana, N., and Artigas, F. (2017). Laminar and cellular distribution of monoamine receptors in rat medial prefrontal cortex. Front. Neuroanat. *11*, 87.

Saunders, B.T., Yager, L.M., and Robinson, T.E. (2013). Preclinical studies shed light on individual variation in addiction vulnerability. Neuropsychopharmacology *38*, 249-250.

Scheyltjens, I., Laramee, M.E., Van den Haute, C., Gijsbers, R., Debyser, Z., Baekelandt, V., Vreysen, S., and Arckens, L. (2015). Evaluation of the expression pattern of rAAV2/1, 2/5, 2/7, 2/8, and 2/9 serotypes with different promoters in the mouse visual cortex. J. Comp. Neurol. *523*, 2019-2042.

Schippers, M.C., Binnekade, R., Schoffelmeer, A.N., Pattij, T., and De Vries, T.J. (2012). Unidirectional relationship between heroin self-administration and impulsive decision-making in rats. Psychopharmacology (Berl.) *219*, 443-452.

Schneider, T., Dibue, M., and Hescheler, J. (2013). How "pharmacoresistant" is Cav2.3, the major component of voltage-gated R-type Ca2+ channels? Pharmaceuticals (Basel) *6*, 759-776.

Scholl, L., Seth, P., Kariisa, M., Wilson, N., and Baldwin, G. (2018). Drug and opioid-involved overdose deaths - United States, 2013-2017. MMWR Morb. Mortal. Wkly. Rep. *67*, 1419-1427.

Schottenfeld, R.S., Pakes, J., Ziedonis, D., and Kosten, T.R. (1993). Buprenorphine: Dose-related effects on cocaine and opioid use in cocaine-abusing opioid-dependent humans. Biol.Psychiatry *34*, 66-74.

Serafine, K.M., and France, C.P. (2014). Restricted access to standard or high fat chow alters sensitivity of rats to the 5-HT(2A/2C) receptor agonist 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane. Behav. Pharmacol. *25*, 44-52.

Sharkey, K.M., Kurth, M.E., Anderson, B.J., Corso, R.P., Millman, R.P., and Stein, M.D. (2011). Assessing sleep in opioid dependence: a comparison of subjective ratings, sleep diaries, and home polysomnography in methadone maintenance patients. Drug Alcohol Depend. *113*, 245-248.

Sholler, D.J., Stutz, S.J., Fox, R.G., Boone, E.L., Wang, Q., Rice, K.C., Moeller, F.G., Anastasio, N.C., and Cunningham, K.A. (2018). The 5-HT_{2A} receptor (5-

$HT_{2A}R$) regulates impulsive action and cocaine cue reactivity in male Sprague-Dawley rats. J. Pharmacol. Exp. Ther.

Shorter, E. (2009). The history of lithium therapy. Bipolar disorders *11 Suppl 2*, 4-9.

Simpson, D.D., Joe, G.W., and Broome, K.M. (2002). A national 5-year follow-up of treatment outcomes for cocaine dependence. Arch.Gen.Psychiatry *59*, 538-544.

Sipes, T.E., and Geyer, M.A. (1995). DOI disruption of prepulse inhibition of startle in the rat is mediated by 5-HT_{2A} and not by 5-HT_{2C} receptors. Behav.Pharmacol. *6*, 839-842.

Smith, G.L. (2014). The Long and Short of Tamoxifen Therapy: A Review of the ATLAS Trial. Journal of the advanced practitioner in oncology *5*, 57-60.

Smith, R.L., Barrett, R.J., and Sanders-Bush, E. (2003). Discriminative stimulus properties of 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane [(+/-)DOI] in C57BL/6J mice. Psychopharmacology (Berl.) *166*, 61-68.

Stefanik, M.T., Moussawi, K., Kupchik, Y.M., Smith, K.C., Miller, R.L., Huff, M.L., Deisseroth, K., Kalivas, P.W., and LaLumiere, R.T. (2013). Optogenetic inhibition of cocaine seeking in rats. Addict.Biol. *18*, 50-53.

Stephenson, R.P. (1956). A modification of receptor theory. Br. J. Pharmacol. Chemother. *11*, 379-393.

Stoffel, E.C., and Cunningham, K.A. (2008). The relationship between the locomotor response to a novel environment and behavioral disinhibition in rats. Drug Alcohol Depend. *92*, 69-78.

Strain, E.C., Stitzer, M.L., Liebson, I.A., and Bigelow, G.E. (1994). Buprenorphine versus methadone in the treatment of opioid-dependent cocaine users. Psychopharmacology (Berl.) *116*, 401-406.

Substance Abuse and Mental Health Services Administration (SAMHSA) (2016). Reports of the Surgeon General. In Facing Addiction in America: The Surgeon General's Report on Alcohol, Drugs, and Health (Washington (DC): US Department of Health and Human Services).

Substance Abuse and Mental Health Services Administration (SAMHSA) (2018). Key substance use and mental health indicators in the United States: results from the 2017 National Survey on Drug Use and Health. (Rockville, MD, Center for Behavioral Health Statistics and Quality, Substance Abuse and Mental Health Services Administration).

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

Tallarida, R.J., and Murray, R.B. (1987). Manual of Pharmacologic Calculations with Computer Programs, 2nd edn (New York: Springer-Verlag).

Tampi, R.R., Tampi, D.J., Young, J.J., Balachandran, S., Hoq, R.A., and Manikkara, G. (2019). Evidence for using pimavanserin for the treatment of Parkinson's disease psychosis. World journal of psychiatry *9*, 47-54.

Tao, R., and Auerbach, S.B. (1995). Involvement of the dorsal raphe but not median raphe nucleus in morphine-induced increases in serotonin release in the rat forebrain. Neuroscience *68*, 553-561.

Titeler, M., Lyon, R.A., and Glennon, R.A. (1988). Radioligand binding evidence implicates the brain 5-HT2 receptor as a site of action for LSD and phenylisopropylamine hallucinogens. Psychopharmacology (Berl.) *94*, 213-216.

Tyner, C., Barber, G.P., Casper, J., Clawson, H., Diekhans, M., Eisenhart, C., Fischer, C.M., Gibson, D., Gonzalez, J.N., Guruvadoo, L.*, et al.* (2017). The UCSC Genome Browser database: 2017 update. Nucleic Acids Res. *45*, D626-d634.

Van den Oever, M.C., Rotaru, D.C., Heinsbroek, J.A., Gouwenberg, Y., Deisseroth, K., Stuber, G.D., Mansvelder, H.D., and Smit, A.B. (2013). Ventromedial prefrontal cortex pyramidal cells have a temporal dynamic role in recall and extinction of cocaine-associated memory. J. Neurosci. *33*, 18225-18233.

van Gaalen, M.M., Brueggeman, R.J., Bronius, P.F.C., Schoffelmeer, A.N.M., and Vanderschuren, L.J.M.J. (2006). Behavioral disinhibition requires dopamine receptor activation. Psychopharmacology (Berl.) *187*, 73-85.

Vanover, K.E., Betz, A.J., Weber, S.M., Bibbiani, F., Kielaite, A., Weiner, D.M., Davis, R.E., Chase, T.N., and Salamone, J.D. (2008). A 5-HT2A receptor inverse agonist, ACP-103, reduces tremor in a rat model and levodopa-induced dyskinesias in a monkey model. Pharmacol.Biochem.Behav. *90*, 540-544.

Vanover, K.E., Harvey, S.C., Son, T., Bradley, S.R., Kold, H., Makhay, M., Veinbergs, I., Spalding, T.A., Weiner, D.M., Andersson, C.M.*, et al.* (2004). Pharmacological characterization of AC-90179 [2-(4-Methoxyphenyl)-N-(4-methyl-benzyl)-N-(1-methyl-piperidin-4-yl)-acetamide hydrochloride]: A selective serotonin 2A receptor inverse agonist. J. Pharmacol. Exp. Ther. *310*, 943-951.

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

Vazquez-Borsetti, P., Cortes, R., and Artigas, F. (2009). Pyramidal neurons in rat prefrontal cortex projecting to ventral tegmental area and dorsal raphe nucleus express 5-HT2A receptors. Cereb. Cortex *19*, 1678-1686.

Verdejo-Garcia, A., Lawrence, A.J., and Clark, L. (2008). Impulsivity as a vulnerability marker for substance-use disorders: review of findings from high-risk research, problem gamblers and genetic association studies. Neurosci. Biobehav. Rev. *32*, 777-810.

Volkow, N.D., Frieden, T.R., Hyde, P.S., and Cha, S.S. (2014). Medication-assisted therapies--tackling the opioid-overdose epidemic. N. Engl. J. Med. *370*, 2063-2066.

Volkow, N.D., Koob, G., and Baler, R. (2015). Biomarkers in substance use disorders. ACS Chem. Neurosci. *6*, 522-525.

Volkow, N.D., and Skolnick, P. (2012). New medications for substance use disorders: challenges and opportunities. Neuropsychopharmacology *37*, 290-292.

Volkow, N.D., Wang, G.J., Fowler, J.S., Tomasi, D., and Baler, R. (2012). Food and drug reward: overlapping circuits in human obesity and addiction. Curr. Top. Behav. Neurosci. *11*, 1-24.

Voon, V., Irvine, M.A., Derbyshire, K., Worbe, Y., Lange, I., Abbott, S., Morein-Zamir, S., Dudley, R., Caprioli, D., Harrison, N.A.*, et al.* (2013). Measuring "waiting" impulsivity in substance addictions and binge eating disorder in a novel analogue of rodent serial reaction time task. Biol.Psychiatry.

Wang, Q., Simpao, A., Sun, L., Falk, J.L., and Lau, C.E. (2001). Contribution of the active metabolite, norcocaine, to cocaine's effects after intravenous and oral administration in rats: pharmacodynamics. Psychopharmacology (Berl.) *153*, 341-352.

Wang, Z., Gerstein, M., and Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet *10*, 57-63.

Wattiez, A.S., Dupuis, A., Privat, A.M., Chalus, M., Chapuy, E., Aissouni, Y., Eschalier, A., and Courteix, C. (2017). Disruption of 5-HT2A-PDZ protein interaction differently affects the analgesic efficacy of SSRI, SNRI and TCA in the treatment of traumatic neuropathic pain in rats. Neuropharmacology *125*, 308-318.

Wattiez, A.S., Pichon, X., Dupuis, A., Hernandez, A., Privat, A.M., Aissouni, Y., Chalus, M., Pelissier, T., Eschalier, A., Marin, P.*, et al.* (2013). Disruption of 5-HT2A receptor-PDZ protein interactions alleviates mechanical hypersensitivity in carrageenan-induced inflammation in rats. PLoS One *8*, e74661.

Weiner, D.M., Burstein, E.S., Nash, N., Croston, G.E., Currier, E.A., Vanover, K.E., Harvey, S.C., Donohue, E., Hansen, H.C., Andersson, C.M.*, et al.* (2001). 5-hydroxytryptamine2A receptor inverse agonists as antipsychotics. J. Pharmacol. Exp. Ther. *299*, 268-276.

Welsh, S.E., Romano, A.G., and Harvey, J.A. (1998). Effects of serotonin 5-HT(2A/2C) antagonists on associative learning in the rabbit. Psychopharmacology (Berl.) *137*, 157-163.

Wenger, G.R., and Wright, D.W. (1990). Behavioral effects of cocaine and its interaction with d-amphetamine and morphine in rats. Pharmacol.Biochem.Behav. *35*, 595-600.

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

Willins, D.L., and Meltzer, H.Y. (1997). Direct injection of 5-HT2A receptor agonists into the medial prefrontal cortex produces a head-twitch response in rats. J. Pharmacol. Exp. Ther. *282*, 699-706.

Winstanley, C.A. (2011). The utility of rat models of impulsivity in developing pharmacotherapies for impulse control disorders. Br.J.Pharmacol. *164*, 1301-1321.

Winstanley, C.A., Chudasama, Y., Dalley, J.W., Theobald, D.E., Glennon, J.C., and Robbins, T.W. (2003). Intra-prefrontal 8-OH-DPAT and M100907 improve visuospatial attention and decrease impulsivity on the five-choice serial reaction time task in rats. Psychopharmacology (Berl.) *167*, 304-314.

Winstanley, C.A., Eagle, D.M., and Robbins, T.W. (2006). Behavioral models of impulsivity in relation to ADHD: Translation between clinical and preclinical studies. Clin. Psychol. Rev. *26*, 379-395.

Winstanley, C.A., Olausson, P., Taylor, J.R., and Jentsch, J.D. (2010). Insight into the relationship between impulsivity and substance abuse from studies using animal models. Alcohol Clin.Exp.Res. *34*, 1306-1318.

Winstanley, C.A., Theobald, D.E., Dalley, J.W., Glennon, J.C., and Robbins, T.W. (2004). 5-HT2A and 5-HT2C receptor antagonists have opposing effects on

a measure of impulsivity: interactions with global 5-HT depletion. Psychopharmacology (Berl.) *176*, 376-385.

Winter, J.C., Rice, K.C., Amorosi, D.J., and Rabin, R.A. (2007). Psilocybin-induced stimulus control in the rat. Pharmacol. Biochem. Behav. *87*, 472-480.

Wischhof, L., Hollensteiner, K.J., and Koch, M. (2011). Impulsive behaviour in rats induced by intracortical DOI infusions is antagonized by co-administration of an mGlu2/3 receptor agonist. Behav. Pharmacol. *22*, 805-813.

Wise, R.A. (1996). Neurobiology of addiction. Curr.Opin.Neurobiol. *6*, 243-251.

Woolverton, W.L., Kandel, D., and Schuster, C.R. (1978). Effects of repeated administration of cocaine on schedule-controlled behavior of rats. Pharmacol. Biochem. Behav. *9*, 327-337.

Worbe, Y., Savulich, G., Voon, V., Fernandez-Egea, E., and Robbins, T.W. (2014). Serotonin depletion induces 'waiting impulsivity' on the human four-choice serial reaction time task: cross-species translational significance. Neuropsychopharmacology *39*, 1519-1526.

Wu, L.G., Borst, J.G., and Sakmann, B. (1998). R-type Ca2+ currents evoke transmitter release at a rat central synapse. Proc. Natl. Acad. Sci. U. S. A. *95*, 4720-4725.

Wu, P.E., and Juurlink, D.N. (2014). Unused prescription drugs should not be treated like leftovers. CMAJ *186*, 815-816.

Xia, Z., Gray, J.A., Compton-Toth, B.A., and Roth, B.L. (2003). A direct interaction of PSD-95 with 5-HT2A serotonin receptors regulates receptor trafficking and signal transduction. J Biol Chem. *278*, 21901-21908.

Xu, T., and Pandey, S.C. (2000). Cellular localization of serotonin(2A) (5HT(2A)) receptors in the rat brain. Brain Res.Bull. *51*, 499-505.

Yatham, L.N., Kennedy, S.H., Parikh, S.V., Schaffer, A., Bond, D.J., Frey, B.N., Sharma, V., Goldstein, B.I., Rej, S., Beaulieu, S.*, et al.* (2018). Canadian Network for Mood and Anxiety Treatments (CANMAT) and International Society for Bipolar Disorders (ISBD) 2018 guidelines for the management of patients with bipolar disorder. Bipolar disorders *20*, 97-170.

Yoburn, B.C., Shah, S., Chan, K., Duttaroy, A., and Davis, T. (1995). Supersensitivity to opioid analgesics following chronic opioid antagonist treatment: relationship to receptor selectivity. Pharmacol. Biochem. Behav. *51*, 535-539.

Zacny, J.P., and de Wit, H. (2009). The prescription opioid, oxycodone, does not alter behavioral measures of impulsivity in healthy volunteers. Pharmacol. Biochem. Behav. *94*, 108-113.

Zhang, Y., Kong, F., Crofton, E.J., Dragosljvich, S.N., Sinha, M., Li, D., Fan, X., Koshy, S., Hommel, J.D., Spratt, H.M.*, et al.* (2016). Transcriptomics of Environmental Enrichment Reveals a Role for Retinoic Acid Signaling in Addiction. Front. Mol. Neurosci. *9*, 119.

Zhang, Y., Liang, Y., Randesi, M., Yuferov, V., Zhao, C., and Kreek, M.J. (2018). Chronic oxycodone self-administration altered reward-related genes in the ventral and dorsal striatum of C57BL/6J mice: an RNA-seq analysis. Neuroscience.

Zhou, F.M., and Hablitz, J.J. (1999). Activation of serotonin receptors modulates synaptic transmission in rat cerebral cortex. J.Neurophysiol. *82*, 2989-2999.

Vita

Dennis Jeremy Sholler was born on October 28th, 1992 in Somers Points, New Jersey. He graduated from Egg Harbor Township High School in Egg Harbor Township, New Jersey in 2011 and subsequently matriculated to Rutgers University in New Brunswick, New Jersey. Mr. Sholler is a proud, first-generation college graduate, and he was recruited to the Ronald E. McNair Post-baccalaureate Achievement Program in 2013, a program designed to prepare undergraduate students for doctoral studies through involvement in research. Throughout this time, he worked in the laboratory of Mark O. West, Ph.D. in the Department of Psychology studying brain activity and affective behavior in rats during cocaine self-administration. Mr. Sholler earned his B.A. in Cell Biology & Neuroscience in 2015 and subsequently pursued his interest in scientific research via the Pharmacology and Toxicology Graduate Program at the University of Texas Medical Branch in Galveston, Texas. He joined the laboratory of Kathryn A. Cunningham, Ph.D. in April 2016 and was awarded a predoctoral position on the National Institute on Drug Abuse T32 training grant secured by the UTMB Center for Addiction Research. He has presented his research at local, national, and international meetings in addition to co-authoring several publications in academic journals. Mr. Sholler proudly serves his community as a member of the Board of Directors for the Bay Area Council on Drugs and Alcohol (BACODA), a nonprofit organization whose mission is to help individuals, families and communities stop drug and alcohol abuse.

Awards:

1. E. Fitzgerald Sporar Endowment Award, Center for Addiction Research, University of Texas Medical Branch (06/2019)
2. Kenneth M. Johnson Pharmacology & Toxicology Graduate Program Endowment Award, University of Texas Medical Branch (06/2019)
3. Best Oral Presentation, Pharmacology & Toxicology Annual Symposium, University of Texas Medical Branch (06/2019)
4. Best Poster Award, 2019 Public Health Symposium, University of Texas Medical Branch (03/2019)
5. Arthur V. Simmang Scholarship Fund, Graduate School of Biomedical Sciences, University of Texas Medical Branch (12/2018)
6. Trainee Professional Development Award, Society for Neuroscience (09/2018)
7. Community Outreach Award, Center for Addiction Research, University of Texas Medical Branch (05/2018)
8. Sharon Nelson, PhD, and Odd Steinsland, PhD Endowed Scholarship for Outstanding Research in Pharmacology and Toxicology, Graduate School of Biomedical Sciences, University of Texas Medical Branch (11/2017)
9. CAR Endowment Travel Award, Center for Addiction Research, University of Texas Medical Branch (06/2017)
10. Best Poster Award, Pharmacology & Toxicology Annual Symposium, University of Texas Medical Branch (06/2017)
11. NIDA Director's Travel Award, 79th Annual Meeting of the College on Problems of Drug Dependence (03/2017)
12. William C. Levin, MD Presidential Scholarship, Graduate School of Biomedical Sciences, University of Texas Medical Branch (06/2016)
13. Chauncey Leake Memorial Fellowship Award, Department of Pharmacology & Toxicology, University of Texas Medical Branch (06/2015)
14. Frances Adoue Lynch Addiction Research Fellowship, Center for Addiction Research, University of Texas Medical Branch (06/2015)

Peer-Reviewed Publications:

1. **Sholler, Dennis J.**, Stutz, Sonja J., Fox, Robert G., Boone, Edward L., Wang, Qin, Rice, Kenner C., Moeller, Frederick G., Anastasio, Noelle C., and Cunningham, Kathryn A. (2019, January). The 5-HT_{2A} Receptor (5-HT_{2A}R) Regulates Impulsivity and Cocaine Cue Reactivity in Male Sprague-Dawley Rats. *J. Pharmacol. Exp. Ther. 368:41–49.* PMCID: PMC 30373886

2. Price, Amanda E.*, **Sholler, Dennis J.***, Stutz, Sonja J., Anastasio, Noelle C., and Cunningham, Kathryn A. (2019, January). Endogenous Serotonin 5-HT_{2A} and 5-HT_{2C} Receptors Associate in the Medial Prefrontal Cortex. *ACS Chem. Neurosci. [Epub ahead of print].* PMCID: PMC 30645940
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