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Role of the Serotonin (5-HT) 5-HT_{2C} Receptor (5-HT_{2C}R) in Cocaine Cue Reactivity

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by

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Dedication

To my family, by blood and by choice, for your unwavering support.

To everyone that makes science so much fun for me.

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Cocaine use disorder is a chronic brain disorder characterized by high relapse rates and poor treatment outcomes; one variable known to engender relapse is exposure to environmental and discrete cues previously associated with drug-taking (cue reactivity). Human drug users experience an increase in craving elicited by drug-paired cues over time, and in rodent models, a time-dependent increase in cue reactivity ('incubation") is observed during forced abstinence from drug self-administration. Neuroplasticity in the mesocorticolimbic neurocircuitry mediates the incubation of cue reactivity, and a greater understanding of the mechanisms underlying incubation phenomena is needed to improve treatment outcomes and extend abstinence. Serotonin (5-HT) neurotransmitter systems play an important role in the behavioral effects of cocaine particularly through the 5-HT_{2C} receptor (5-HT_{2C}R), however, the involvement of this system in incubation of cue reactivity during abstinence from cocaine self-administration has not been investigated.

The 5-HT_{2C}R is expressed throughout reward neurocircuitry, but exploration of the region-dependent role of 5-HT_{2C}R function to modulate cocaine-related behaviors, including cue reactivity, has been limited. The present studies aimed to explore the 5-HT_{2C}R localized to nodes of the mesocorticolimbic pathway as a potential neuroregulator of cue reactivity assessed during forced abstinence from cocaine self-administration. Prolonged vs. early forced abstinence from cocaine self-administration was associated with elevated cue reactivity, a lower potency of the selective 5-HT_{2C}R agonist WAY163909 to suppress cue reactivity, and an altered subcellular distribution profile of the 5-HT_{2C}R in the medial prefrontal cortex. Levels of cue reactivity and 5-HT_{2C}R protein expression levels within specific nodes (medial prefrontal cortex, ventral tegmental area) of the mesocorticoaccumbens pathway were inversely correlated. A definitive role for the 5-HT_{2C}R in the VTA as a driver of cocaine-related behaviors could not be determined in the present study due to technical limitations in virally-mediated gene transfer experiments. Collectively, these studies illuminate the 5-HT_{2C}R as a potential contributor to the incubation of cue reactivity associated with abstinence from cocaine self-administration. These data shed new light on the involvement of pathway-specific regulation of the 5-HT_{2C}R in a key phenotype associated with relapse suggest new pharmacotherapeutic strategies to curb cue reactivity and prevent relapse to cocaine.

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5-HT	serotonin
$5\text{-HT}_{2C}\text{R}$	serotonin 2C receptor
6-OHDA	6-hydroxydopamine
AAV	adeno-associated virus
ACC	anterior cingulate cortex
ANOVA	analysis of variance
BDNF	brain derived neurotrophic factor
CNS	central nervous system
dHipp	dorsal hippocampus
$\text{ERK}_{1/2}$	extracellular signal-regulated kinase 1/2
FA	forced abstinence
FR	fixed ratio
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
i.p.	intraperitoneal
mPFC	medial prefrontal cortex
NAc	nucleus accumbens
NSC	non-silencing control
OFC	orbitofrontal cortex
$\text{pERK}_{1/2}$	phosphorylated extracellular signal-regulated kinase 1/2
PR	progressive ratio

shRNA	short hairpin RNA
SNP	single nucleotide polymorphism
VTA	ventral tegmental area

Chapter 1: Introduction

Cocaine use disorder is one of the great challenges on the public health agenda of the United States, with an enormous cost in human tragedy as well as in public health and safety; there are presently no FDA-approved medications to treat cocaine use disorder. A central contributor to the chronic and cyclic pattern of dependence and relapse is exposure to environmental and discrete stimuli previously associated with the drug experience (cue reactivity) which can precipitate relapse (Carter and Tiffany, 1999; O'Brien et al., 1998). Cue reactivity is the attentional orienting response to drug-associated stimuli that predict reward, and exposure to cocaine-associated cues includes physiological responses (e.g., elevated heart rate), subjective reactions (e.g., craving), appetitive approach behaviors (e.g., cocaine seeking) as well as neural circuit activation in humans (Carter and Tiffany, 1999; Field and Cox, 2008; Garavan et al., 2000; Maas et al., 1998). An improved understanding of the mechanisms underlying cue reactivity may suggest novel pharmacotherapeutic targets for cocaine use disorder.

Drug cue reactivity has traditionally been assessed in human subjects by presenting drug-associated cues and measuring neural activation, physiological response, and/or self-reported drug craving (reviewed in Carter and Tiffany, 1999; Jasinska et al., 2014; Modesto-Lowe and Kranzler, 1999); cocaine cues can be presented visually or audio-visually through pictures or films (Childress et al., 1999; Garavan et al., 2000; Goudriaan et al., 2013; Maas et al., 1998), in the form of an autobiographical script (Bonson et al., 2002; Kilts et al., 2001), or even using virtual reality (Hone-Blanchet et al., 2014). While the multiplicity of measures employed to assess cue reactivity

contributes to difficulties in reproducibility of findings (Modesto-Lowe and Kranzler, 1999), cue reactivity correlates positively with measurements of craving (Bell et al., 2014; Childress et al., 1999; Garavan et al., 2000; Goudriaan et al., 2013; Kilts et al., 2001; Maas et al., 1998) and is one of the phenotypic targets for pharmacotherapeutics development for cocaine use disorder. Interestingly, craving elicited by exposure to drug-associated cues increases over time in abstinent human drug abusers (Bedi et al., 2010; Li et al., 2014; Wang et al., 2013), an observation which supports the importance of ameliorating cue reactivity as a treatment target to sustain recovery and abstinence.

Experimental measures of cue reactivity may offer better reproducibility than self-report measures and are well-suited for use in pharmacological studies. One task widely employed to study cue reactivity in humans is the cocaine-word Stroop task. In this behavioral measure of cue reactivity, subjects are asked to indicate the color of cocaine-related words (e.g., crack) and neutral words (i.e., couch). Attentional bias, defined as the difference in response latency for cocaine-related words relative to neutral words, is higher in cocaine-dependent subjects relative to controls (Anastasio et al., 2014a; Hester et al., 2006; Liu et al., 2013b; Liu et al., 2012; Liu et al., 2011). Cue reactivity measured in the cocaine-word Stroop task correlates with subjective measures of craving (Copersino et al., 2004; Field et al., 2009) and predicts treatment outcomes (Carpenter et al., 2006). Advantages of this task include the possibility for inclusion of simultaneous neuroimaging and self-report measures in the study as well as the capability to assess cue reactivity without relying upon self-reports of craving (Field and Cox, 2008; Marhe et al., 2013). Additionally, translational studies can capitalize on the similarities between the

cocaine-word Stroop task and preclinical models of cue reactivity (Anastasio et al., 2014a).

The self-administration paradigm is the gold standard in rodent preclinical models of addiction, including cocaine use disorder; self-administration of saline or palatable reinforcers (i.e., sucrose) is often used as a control. In this task, rodents are trained to respond (e.g., lever press or nose poke) on a specific schedule of reinforcement to obtain a reinforcer (e.g., intravenous cocaine infusion, sucrose pellet delivery into a food hopper); delivery of the reinforcer is associated with discrete cues (stimulus light, sound of the infusion pump or pellet dispenser) which acquire incentive motivational and reinforcing properties through repeated pairings. After achieving and maintaining stable self-administration, the extinction/reinstatement or forced abstinence models can be employed to evaluate cue reactivity [operationally defined as appetitive approach behavior (lever presses reinforced by the discrete cocaine-paired cue complex)]. In the extinction/reinstatement model, extinction training is employed to disrupt the association between the operant response (e.g., lever press) and drug delivery; re-exposure to cocaine-paired environmental and/or discrete cues (e.g., stimulus lights, pump sounds) or non-contingent injection of cocaine results in reinstatement of operant responding (for example, Cunningham et al., 2011; Fuchs et al., 1998; Nic Dhonnchadha et al., 2009) with extinction regarded as a new learning process (Bouton, 2002). The extinction/reinstatement model is limited in its translatability to clinical populations because abstinent humans do not typically undergo similar extinction learning or the associated dynamic neuroplastic changes that occur during extinction training (Marchant et al., 2013). Forced abstinence refers to an imposed withdrawal from the self-

administration environment and retention in the home environment (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 under certain circumstances (e.g., incarceration, inpatient rehabilitation). Cue reactivity following extinction/reinstatement (Di Ciano and Everitt, 2002; Reichel et al., 2011) or forced abstinence escalates over the first months after cessation of self-administration of abused drugs, including cocaine (Grimm et al., 2001; Neisewander et al., 2000), and from non-drug rewards like sucrose (Grimm et al., 2006; Grimm et al., 2002); escalation of cue reactivity has been termed "incubation" in rodents (for review, Pickens et al., 2011). While the outcome measures of cocaine-seeking in these models are behaviorally similar, the neurobiological underpinnings and recruited brain circuits in the extinction/reinstatement and forced abstinence models are unique (Marchant et al., 2013; Neisewander et al., 2000; Self et al., 2004).

Cue reactivity in cocaine-dependent human and rodent subjects is associated with activation of overlapping mesocorticolimbic neurocircuits, which is thought to serve as a key driver of addictive behavior (for reviews, Jasinska et al., 2014; Kalivas and Volkow, 2005; Nestler, 2005; Pickens et al., 2011; Volkow et al., 2004). Human neuroimaging studies have identified that exposure to cocaine cues increases activation of frontal cortices [dorsolateral prefrontal cortex (PFC), anterior cingulate cortex (ACC), orbitofrontal cortex (OFC), insula (Bonson et al., 2002; Childress et al., 1999; Garavan et al., 2000; Kilts et al., 2001; Maas et al., 1998)] subcortical regions [caudate, thalamus (Garavan et al., 2000)], ventral striatum [nucleus accumbens (NAc) (Bell et al., 2014;

Kilts et al., 2001)], limbic areas [amygdala, rhinal cortex (Bonson et al., 2002; Childress et al., 1999; Kilts et al., 2001)] and the ventral tegmental area (VTA) (Goudriaan et al., 2013). The medial PFC (mPFC), NAc, amygdala and VTA, in particular, are also implicated in rodent models of cocaine cue reactivity (for reviews, Kalivas and Volkow, 2005; Koob and Volkow, 2010)). Further, incubation of cue reactivity following abstinence from cocaine self-administration has been linked to time-dependent neuronal plasticity in the mPFC (Koya et al., 2009; Ma et al., 2014; Whitfield et al., 2011), NAc (Conrad et al., 2008; Lu et al., 2003; Ma et al., 2014; Terrier et al., 2015), VTA (Lu et al., 2003; Lu et al., 2009), and amygdala (Lu et al., 2005; Lu et al., 2007), which establishes the mesocorticolimbic circuit as a central pathway which mediates cue reactivity.

Regions of the brain implicated in cocaine cue reactivity are innervated by dopamine neurons from the VTA (Fallon, 1981; Fallon and Moore, 1978) and serotonin (5-HT) neurons from the raphe nuclei (Di Matteo et al., 2008; Halliday and Tork, 1989; Herve et al., 1987), which logically follows given that the principal mechanism of action of cocaine in the brain is blockade of monoamine transporters which inhibits reuptake and increases the local concentration of 5-HT, dopamine, and norepinephrine (Ritz et al., 1990). The dopamine system has been studied extensively, but no clinically effective pharmacotherapies targeting the dopamine system have been discovered (Nutt et al., 2015). In contrast, 5-HT is thought to modulate dopamine neurotransmission, particularly through its action at the 5-HT_2 receptor (5-HT_2R) family (for review, Bubar and Cunningham, 2008; Howell and Cunningham, 2015). Serotonin exerts its effect through 14 receptor subtypes which are divided into seven families by structural and functional features (Bockaert et al., 2006; Hoyer et al., 2002). The 5-HT_2R family consists of the 5-

$HT_{2A}R$, 5-$HT_{2B}R$, and 5-$HT_{2C}R$, of which the 5-$HT_{2A}R$ and 5-$HT_{2C}R$ are prominently expressed in the brain and, despite similar signaling events, exert oppositional control over behavior ((Bubar and Cunningham, 2008; Cunningham and Anastasio, 2014). Activation of the 5-$HT_{2C}R$ increased firing of VTA γ-aminobutyric acid (GABA) neurons (Di Giovanni et al., 2001), decreased firing of VTA dopamine neurons (Di Giovanni et al., 2000; Di Matteo et al., 2000; Gobert et al., 2000), and decreased dopamine release in projection regions (e.g., NAc, mPFC) (Di Giovanni et al., 2000; Di Matteo et al., 2000; Gobert et al., 2000). The 5-$HT_{2C}R$ appears to be poised to regulate cocaine cue reactivity and merits further exploration.

The 5-$HT_{2C}R$ mRNA and protein are abundantly expressed throughout the central nervous system (CNS) with the highest levels of 5-$HT_{2C}R$ found in the choroid plexus, the region responsible for the production of cerebrospinal fluid (Abramowski and Staufenbiel, 1995; Backstrom et al., 1995; Clemett et al., 2000; Julius et al., 1988; Molineaux et al., 1989; Pompeiano et al., 1994). Peripheral tissues (e.g., heart, lung, liver, and kidney) have been shown to lack the 5-$HT_{2C}R$ (Anastasio et al., 2010; Julius et al., 1988), although recent evidence suggests some specialized peripheral cell types (i.e., islet cells of the pancreas, adipocytes) may express the 5-$HT_{2C}R$ (Cunningham laboratory, unpublished observations). Within the CNS, expression of the 5-$HT_{2C}R$ overlaps with regions known to be involved in the behavioral response to cocaine, including frontal cortices [e.g., PFC, OFC, MC (Abramowski et al., 1995; Anastasio et al., 2010; Clemett et al., 2000; Liu et al., 2007; Pompeiano et al., 1994)], striatum [e.g., NAc (Abramowski et al., 1995; Clemett et al., 2000; Pompeiano et al., 1994)], limbic regions [e.g., hippocampus, amygdala (Abramowski et al., 1995; Clemett et al., 2000;

Molineaux et al., 1989; Pompeiano et al., 1994)], and VTA (Bubar and Cunningham, 2007; Bubar et al., 2011; Molineaux et al., 1989; Pompeiano et al., 1994). Examination of the neuronal subtypes which express the 5-HT_{2C}R within a given region highlight a complex role for the 5-HT_{2C}R to modulate neuronal firing and output. In the PFC, the 5-HT_{2C}R is primarily localized to GABA interneurons (Liu et al., 2007; Pasqualetti et al., 1999; Vysokanov et al., 1998), although 5-HT_{2C}R expression has also been observed in cortical pyramidal neurons (Carr et al., 2002; Clemett et al., 2000; Liu et al., 2007). In the VTA, 5-HT_{2C}R protein is expressed in both GABA and dopamine neurons (Bubar and Cunningham, 2007; Bubar et al., 2011), despite earlier studies which only detected 5-HT_{2C}R mRNA in GABA neurons (Eberle-Wang et al., 1997). The 5-HT_{2C}R protein is expressed postsynaptically (Anastasio et al., 2010; Clemett et al., 2000; Liu et al., 2007), and neuronal firing and output from specific brain regions may be modulated directly through 5-HT_{2C}R activity on projection neurons or indirectly through 5-HT_{2C}R signaling on interneurons.

The 5-HT_{2C}R couples to $G\alpha_{q/11}$ to activate phospholipase Cβ which generates intracellular second messengers inositol-1,4,5-trisphosphate and diacylglycerol, leading to increased calcium release from intracellular stores (Hannon and Hoyer, 2008; Millan et al., 2008). Alternate signaling pathways include activation of phospholipase D (McGrew et al., 2002; Moya et al., 2007) or phospholipase A_2 to generate arachidonic acid through an unidentified pertussis toxin-sensitive G protein (Felder et al., 1990). In addition to G protein-dependent signaling pathways, the 5-HT_{2C}R activates downstream signaling through a β-$arrestin_2$-depedent, G protein-independent manner (Abbas and Roth, 2008; Labasque et al., 2008; Werry et al., 2006). Activation of the 5-HT_{2C}R triggers

independent signaling cascades, but these can also intersect on effectors like extracellular signal-regulated kinase 1/2 ($ERK_{1/2}$) (Raymond et al., 2001; Werry et al., 2005; Werry et al., 2008). Phosphorylation of $ERK_{1/2}$ ($pERK_{1/2}$) is an important integrator of upstream signaling events for the 5-HT_{2C}R and can be mediated by agonist-dependent coupling of the 5-HT_2R to G proteins (Labasque et al., 2010; Werry et al., 2005; Werry et al., 2008) or to other protein transducers (e.g., β-arrestins; Labasque et al., 2008; Lefkowitz and Shenoy, 2005; Schmid et al., 2008); however, activation of $ERK_{1/2}$ through G protein-dependent vs. β-arrestin-dependent pathways may be characterized by different temporal dynamics, subcellular localization and functional consequences (reviewed in Kholodenko et al., 2010). The ultimate consequence of 5-HT_{2C}R activation is governed by divergent or convergent signaling through these varied cascades.

The functional capacity of the 5-HT_{2C}R is dictated by an abundance of regulatory mechanisms, including interactions with varied protein partners. The 5-HT_{2C}R interacts in a functionally-oppositional manner with the scaffolding proteins postsynaptic density protein 95 (PSD-95) and the membrane-associated guanylate kinase p55 subfamily member 3 (MPP3) to promote and inhibit, respectively, receptor desensitization and internalization (Gavarini et al., 2006).The protein phosphatase and tensin homolog (PTEN) interacts with the third intracellular loop of the 5-HT_{2C}R to dephosphorylate the receptor (Anastasio et al., 2013; Ji et al., 2006); disruption of this protein:protein interaction with the small peptide 3L4F enhanced signaling through the 5-HT_{2C}R and potentiated the effect of a 5-HT_{2C}R agonist on behavioral measures (Anastasio et al., 2013; Ji et al., 2006). Protein:protein interactions with G protein-coupled receptors (GPCRs) may also govern 5-HT_{2C}R function; homodimeric conformation of the 5-HT_{2C}R

is thought to be required for activation (Mancia et al., 2008), and we have recently shown that the 5-HT_{2C}R and the 5-HT_{2A}R form a protein complex and exist in a functional balance such that lower 5-HT_{2C}R expression is associated with higher 5-HT_{2A}R expression in the mPFC (Anastasio et al., 2015).

The 5-HT_{2C}R is also governed by post-transcriptional and even genetic mechanisms, including alternative splicing, RNA editing, and single nucleotide polymorphisms. Alterative splicing of the 5-HT_{2C}R RNA generates a truncated form of the receptor protein which is retained in the endoplasmic reticulum and not secreted to the plasma membrane (Martin et al., 2013); additionally, the truncated 5-HT_{2C}R protein can dimerize with the full-length 5-HT_{2C}R in the endoplasmic reticulum to regulate membrane 5-HT_{2C}R expression (Martin et al., 2013). The 5-HT_{2C}R is the only G protein-coupled receptor known to undergo RNA editing (Burns et al., 1997), which negatively regulates alternative splicing of the 5-HT_{2C}R (Martin et al., 2013). The 5-HT_{2C}R RNA can be edited at five nucleotide sites which code for three amino acids in the second intracellular loop, resulting in as many as 32 RNA isoforms and 24 protein isoforms (Burns et al., 1997). Relative to the unedited INI isoform, RNA editing of the 5-HT_{2C}R is associated with less constitutive activity (Marion et al., 2004; Niswender et al., 1999; Price et al., 2001), higher membrane expression (Marion et al., 2004), and lower agonist-independent and β-arrestin-mediated internalization (Marion et al., 2004), which may suggest that edited isoforms may be more capable of responding to a 5-HT_{2C}R agonist. Genetic variants (i.e., single nucleotide polymorphisms, SNP) of the 5-HT_{2C}R have been observed in the human *HTR2C* gene. A SNP in the 5-HT_{2C}R gene (*HTR2C*) converts a cysteine to a serine at amino acid 23 in the N-terminus of the 5-HT_{2C}R (Cys23Ser;

rs6318) and is thought exert an inhibitory effect on 5-HT_{2C}R signaling (Lappalainen et al., 1990; Okada et al., 2004; Piva et al., 2011; Walstab et al., 2011). Human subjects expressing the Cys23Ser variant exhibited dampened sensitivity to 5-HT_{2C}R ligands (Brasch-Andersen et al., 2011; Kuhn et al., 2004; Quested et al., 1999), and cocaine-dependent subjects with the SNP demonstrated higher cue reactivity (Anastasio et al., 2014a). Individually or in combination, these means by which aspects of the 5-HT_{2C}R are regulated may broadly alter the 5-HT_{2C}R subcellular distribution, ligand binding, and coupling to signaling cascades to impact 5-HT_{2C}R function.

Genetic and pharmacological studies in rodents have established the 5-HT_{2C}R as a critical mediator of the behavioral effects evoked by cocaine (for reviews, Bubar and Cunningham, 2008; Cunningham and Anastasio, 2014). Transgenic mice which lack the 5-HT_{2C}R exhibited elevated motor activity, cocaine-evoked hyperactivity, and responding for cocaine on a progressive ratio schedule of reinforcement relative to wild-type mice (Rocha et al., 2002); however, 5-HT_{2C}R knockout mice also displayed lower anxiety-like behavior (Heisler et al., 2007) that could influence the interpretation of exploratory behavior in motor assays. Features of the 5-HT_{2C}R mutant mouse model, including enhanced neuronal excitability that causes seizures (Heisler and Tecott, 1999), limit the utility of a global 5-HT_{2C}R knockout approach. Systemic administration of ligands which activate or block the 5-HT_{2C}R produce effects on cocaine-related behaviors which are largely consistent with genetic manipulation. Cocaine-evoked hyperactivity was decreased by pretreatment with 5-HT_{2C}R agonists (Cunningham et al., 2013; Filip et al., 2004; Grottick et al., 2000; Pockros et al., 2012), whereas administration of a 5-HT_{2C}R antagonist (Filip et al., 2004; Fletcher et al., 2002) or inverse agonist (McCreary and

Cunningham, 1999) potentiated the hyperlocomotive effect of cocaine. The bidirectional modulation of 5-HT_{2C}R agonists (Callahan and Cunningham, 1995; Frankel and Cunningham, 2004) and antagonists (Filip et al., 2006) to decrease and increase, respectively, the discriminative stimulus effects of cocaine in the drug discrimination assay has also been observed. The reinforcing (Cunningham et al., 2011; Grottick et al., 2000) and/or motivational properties (Fletcher et al., 2008; Grottick et al., 2000) of cocaine in the rat self-administration paradigm were suppressed by administration of 5-HT_{2C}R agonists; 5-HT_{2C}R antagonist treatment only increased the reinforcing and/or motivational properties of self-administered cocaine at very low unit doses of cocaine (Fletcher et al., 2002). Activation of the 5-HT_{2C}R suppresses cue- and cocaine-primed reinstatement of cocaine-seeking behavior following extinction from cocaine self-administration (Burbassi and Cervo, 2008; Cunningham et al., 2013; Cunningham et al., 2011; Fletcher et al., 2002; Fletcher et al., 2008; Grottick et al., 2000; Higgins et al., 2015; Neisewander and Acosta, 2007). Recently, we demonstrated that pretreatment with a selective 5-HT_{2C}R agonist attenuates cue reactivity at an early period of forced abstinence from cocaine self-administration (Anastasio et al., 2014a). Global activation of the 5-HT_{2C}R has been shown to curb the behavioral effects of cocaine, and the contribution of individual brain regions to mediate these effects has been the subject of recent investigation.

The mPFC, NAc, VTA, and amygdala have been (incompletely) explored as sites of action for the 5-HT_{2C}R to modulate specific cocaine-related behaviors, especially through the use of intracranial pharmacology; notably, directed ligand delivery to several candidate regions which express the 5-HT_{2C}R and contribute to the behavioral effects of

cocaine (e.g., OFC, dHipp) has not been investigated. Cocaine-evoked hyperactivity is decreased by intra-mPFC (Filip and Cunningham, 2003) or intra-VTA (Fletcher et al., 2004) microinfusion of preferential 5-HT_{2C}R agonists and increased by intra-mPFC administration of a 5-HT_{2C}R antagonist (Filip and Cunningham, 2003). In contrast, the behavioral effect of intra-NAc shell delivery of 5-HT_{2C}R ligands on cocaine-evoked hyperactivity opposes systemic administration such that a 5-HT_{2C}R agonist potentiates (Filip and Cunningham, 2002) and a 5-HT_{2C}R antagonist suppresses (McMahon et al., 2001) the hyperlocomotive properties of cocaine. Intra-mPFC microinfusion of a 5-HT_{2C}R agonist decreased and a 5-HT_{2C}R antagonists increased the discriminative stimulus effects of cocaine (Filip and Cunningham, 2003); however, a contradictory role for 5-HT_{2C}R manipulation in the NAc shell on drug discrimination is also observed such that 5-HT_{2C}R agonist administration enhances and 5-HT_{2C}R antagonist administration reduces the discriminative stimulus properties of cocaine (Filip and Cunningham, 2002). Cocaine self-administration was acutely decreased by 5-HT_{2C}R agonist administration into the VTA (Fletcher et al., 2004), but not the mPFC (Pentkowski et al., 2010); differences in the training dose of cocaine (0.25 mg/kg/inf vs. 0.75 mg/kg/inf) could explain this disparity. Alternately, activation of the 5-HT_{2C}R across regions may differentially control specific cocaine-related behaviors, consistent with the role of individual brain regions to mediate aspects of the behavioral response to cocaine. For example, microinfusion of a 5-HT_{2C}R agonist into the mPFC or the VTA may similarly suppress certain cocaine-evoked behaviors (locomotor activity, drug discrimination), but in contrast, perhaps cocaine-taking is selectively modulated by activation of the 5-HT_{2C}R in the VTA but not the mPFC.

Following extinction from cocaine self-administration, intra-mPFC delivery of a 5-HT_{2C}R attenuated both cocaine- and cue-primed reinstatement of cocaine-seeking behavior (Pentkowski et al., 2010); microinfusion of a 5-HT_{2C}R agonist into the central, but not basolateral, amygdala suppressed reinstatement of cocaine-seeking behavior elicited by a cocaine priming injection but not by previously cocaine-paired cues (Pockros-Burgess et al., 2014). Surprisingly, although the behavioral impact of activation or blockade of the 5-HT_{2C}R in the NAc shell (Filip and Cunningham, 2002) diametrically opposes that observed in the mPFC (Filip and Cunningham, 2003) or with systemic administration of 5-HT_{2C}R ligands (Callahan and Cunningham, 1995; Cunningham et al., 2013; Filip et al., 2004; Frankel and Cunningham, 2004; Grottick et al., 2000; Pockros et al., 2012), a similar relationship is observed between cue reactivity following a brief period of forced abstinence from cocaine self-administration and 5-HT_{2C}R levels in the mPFC (Anastasio et al., 2014a; Anastasio et al., 2014b) and NAc shell (Cunningham laboratory, unpublished observations). Phenotypic levels of cue reactivity correlated with diminished sensitivity to the selective 5-HT_{2C}R agonist WAY163909 (Anastasio et al., 2014a) and lower synaptosomal 5-HT_{2C}R expression in the mPFC (Anastasio et al., 2014a) and NAc (Cunningham laboratory, unpublished observations). Further, cue reactivity was elevated following virally-mediated gene knockdown of the 5-HT_{2C}R selectively in the mPFC (Anastasio et al., 2014b) or NAc shell (Cunningham laboratory, unpublished observations). With the possible exception of the mPFC, the role for 5-HT_{2C}R activity within a given region to modulate the behaviors outlined above has not been systematically evaluated. Of note, the involvement of the 5-HT_{2C}R in the incubation of cocaine cue reactivity has not been interrogated.

The goal of the studies detailed herein was to explore the 5-HT_{2C}R localized to nodes of the mesocorticolimbic pathway as a potential neuroregulator of cue reactivity in the translationally-relevant cocaine self-administration and forced abstinence model. The sensitivity of the selective 5-HT_{2C}R agonist WAY163909 to suppress cocaine cue reactivity was evaluated at early vs. prolonged forced abstinence. The subcellular distribution profile of the 5-HT_{2C}R in the mPFC as a potential contributor to the incubation of cocaine cue reactivity was also examined. The association between cocaine cue reactivity and 5-HT_{2C}R protein expression levels was analyzed across various brain regions in the mesocorticolimbic neurocircuitry to identify a putative pathway-specific effect. A virally-mediated knockdown strategy was used to assess the impact of lower 5-HT_{2C}R expression selectively in the VTA on cocaine-related behaviors, including cue reactivity. Together, these studies provide insight into the regulation of cocaine cue reactivity by the 5-HT_{2C}R localized specifically within the mesocorticoaccumbens pathway.

Chapter 2: Incubation of Cocaine Cue Reactivity Associates with Neuroadaptations in the Cortical Serotonin (5-HT) 5-HT_{2C} Receptor (5-HT_{2C}R) System[1]

Introduction

Cocaine use disorder is characterized by cycles of use, abstinence and relapse (Volkow et al., 2010). Exposure to the environmental contexts and stimuli previously associated with the drug experience (cue reactivity) can precipitate relapse (Carter and Tiffany, 1999; O'Brien et al., 1998). Cue reactivity can be defined as the attentional orienting response to drug-associated stimuli that predict reward, and exposure to cocaine-associated cues includes physiological responses (e.g., elevated heart rate), subjective reactions (e.g., craving), appetitive approach behaviors (e.g., cocaine seeking) as well as neural circuit activation in humans (Carter and Tiffany, 1999; Field and Cox, 2008; Garavan et al., 2000; Maas et al., 1998). Abstinent drug abusers are reported to exhibit a time-dependent increase in craving elicited by drug-associated cues (Bedi et al., 2010; Wang et al., 2013). In rodents, cue reactivity (lever presses reinforced by the discrete drug-paired cue complex) escalates over the first months following cessation of self-administration of cocaine (Grimm et al., 2001; Neisewander et al., 2000) and other abused drugs (Abdolahi et al., 2010; Li et al., 2015) as well as non-drug rewards such as sucrose (Grimm et al., 2006; Grimm et al., 2002); escalation of cue reactivity during abstinence has been termed "incubation" (for review, Pickens et al., 2011). Plasticity of

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neuronal signaling within the mPFC (Koya et al., 2009; LaLumiere et al., 2012; Whitfield et al., 2011), in concert with other nodes within the limbic-corticostriatal circuit (Conrad et al., 2008; Lu et al., 2003; Lu et al., 2005; Ma et al., 2014; Terrier et al., 2015), plays a fundamental role in the generation of cue reactivity and incubation phenomena.

The incentive-motivational effects of cocaine and cocaine-associated cues are regulated by 5-HT systems within the mPFC, particularly through the 5-HT_{2C}R (Filip and Cunningham, 2003; Pentkowski et al., 2010) which is enriched in this region (Liu et al., 2007; Lopez-Gimenez et al., 2001; Nocjar et al., 2015). Neisewander and colleagues demonstrated that localized stimulation of the 5-HT_{2C}R in the prelimbic or infralimbic mPFC suppressed cue- and cocaine-primed reinstatement following extinction from cocaine self-administration (Pentkowski et al., 2010), an outcome identical to the effects of a selective 5-HT_{2C}R agonist administered systemically (Burbassi and Cervo, 2008; Cunningham et al., 2013; Cunningham et al., 2011; Fletcher et al., 2008; Higgins et al., 2015; Neisewander and Acosta, 2007). Interestingly, abstinent cocaine users exhibited lower sensitivity to the effects of a 5-HT_{2C}R agonist (Buydens-Branchey et al., 1997; Lee and Meltzer, 1994; Patkar et al., 2006) and we recently reported that higher cue reactivity was observed in cocaine-dependent subjects carrying a single nucleotide polymorphism in the *HTR2C* gene (Anastasio et al., 2014a) which is predicted to diminish 5-HT_{2C}R signal transduction (Lappalainen et al., 1995; Okada et al., 2004; Piva et al., 2011; Walstab et al., 2011). Likewise, we identified that high cocaine cue reactivity correlated with the lowest levels of 5-HT_{2C}R protein expression in the mPFC (Anastasio et al., 2014a) and a blunted sensitivity to the suppressive effects of the selective 5-HT_{2C}R agonist WAY163909 (Anastasio et al., 2014a). Together with our observation that

knockdown of the 5-HT_{2C}R in the mPFC resulted in vulnerability to the expression of cocaine cue reactivity in rats (Anastasio et al., 2014b), these data suggest that the functional status of the cortical 5-HT_{2C}R system may be a mechanistic driver in the generation of cue reactivity.

The involvement of the 5-HT_{2C}R system in the incubation of cue reactivity has not been investigated. The present study tested the hypothesis that incubation of cue reactivity during abstinence from cocaine self-administration is accompanied by lower potency and/or efficacy of the selective 5-HT_{2C}R agonist WAY163909 to suppress cue reactivity. Given that the pharmacological responsivity of the 5-HT_{2C}R is regulated by the localization of the receptor to cellular microdomains, such as membrane *vs.* cytoplasmic compartments (Herrick-Davis et al., 2015; Zacharias et al., 2002), we tested the hypothesis that incubation of cocaine cue reactivity (but not sucrose cue reactivity) is accompanied by a shift in the subcellular localization profile of the mPFC 5-HT_{2C}R protein. Self-administration of sucrose is a behaviorally consistent paradigm in which acquisition and lever press behavior for a natural reinforcer reasonably match those seen in cocaine self-administration (Choi et al., 2011; Edwards et al., 2011). Furthermore, incubation of sucrose cue reactivity is well-described (Grimm et al., 2006; Grimm et al., 2002), however 5-HT_{2C}R agonists do not suppress sucrose cue reactivity (Burbassi and Cervo, 2008; Cunningham et al., 2011). The outcomes of the following experiments offer the first indication that a shift in the responsivity of the 5-HT_{2C}R system, driven in part by the altered subcellular localization of the receptor, may contribute to incubation of cocaine cue reactivity.

Methods

Animals

Male Sprague-Dawley rats (n=213), Harlan, Inc., Houston, TX) weighing 250-325 g at the start of experiments were used. Rats were acclimated for seven days to a colony room maintained at a constant temperature (21-23°C) and humidity (45-50%) on a 12 hour light-dark cycle (lights on 0600-1800 h). Rats were housed two/cage and handled daily throughout the study. Food and water were available *ad libitum*. All experiments were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (2011) and with approval from the University of Texas Medical Branch Institutional Animal Care and Use Committee.

Drugs

(−)-Cocaine (National Institute on Drug Abuse, Research Triangle Park, NC) was dissolved in 0.9% NaCl. WAY163909 [(7b-R,10a-R)-1,2,3,4,8,9,10,10a-octahydro-7bH-cyclopenta[b][1,4] diazepino [6,7,1hi]indole] was a gift from Pfizer, Inc. (New York, NY) and was dissolved in 0.9% NaCl (vehicle).

Apparatus

Both cocaine and sucrose self-administration studies employed standard operant conditioning chambers (Med-Associates, Inc., St. Albans, VT, USA) housed in ventilated, sound-attenuating cubicles with fans (Med-Associates, Inc.). Each chamber was outfitted with two retractable response levers, a stimulus light above each response lever, a houselight opposite the levers, and a magazine-type pellet dispenser. The cocaine

infusions were delivered via syringes attached to infusion pumps (Med Associates, Inc.) located outside the cubicles. The infusion pumps were connected to liquid swivels (Instech, Plymouth Meeting, PA, USA) that were fastened to the catheters via polyethylene 20 tubing encased inside a metal spring leash (Plastics One, Roanoke, VA). Sucrose pellets (45 mg; Bio-Serv, Frenchtown, NJ, USA) were delivered into a pellet receptacle located between the two levers.

Cocaine self-administration and cue reactivity analyses

Rats were anesthetized (8.6 mg/kg of xylazine, 1.5 mg/kg of acepromazine, 43 mg/kg of ketamine in bacteriostatic saline) and implanted with intravenous catheters with back mounts and allowed to recover for 5-7 days (Anastasio et al., 2014a; Anastasio et al., 2014b; Cunningham et al., 2013; Cunningham et al., 2011). Catheter patency was maintained by daily flushes with a solution of 0.1 mL of bacteriostatic saline containing heparin sodium (10 U/mL; American Pharmaceutical Partners, East Schaumburg, IL), streptokinase (0.67 mg/mL; Sigma Chemical), and ticarcillin disodium (66.67 mg/mL; Research Products International, Mt. Prospect, IL) immediately following daily cocaine self-administration sessions.

Cocaine self-administration training consisted of 14 daily 180-min sessions during which rats were trained to lever press for cocaine infusions (0.75 mg/kg/0.1 mL infusion) (Anastasio et al., 2014a; Anastasio et al., 2014b; Cunningham et al., 2013; Cunningham et al., 2011). Schedule completions on the active lever resulted in delivery of a cocaine infusion over a 6-sec period paired simultaneously with illumination of the house and stimulus lights and activation of the infusion pump (discrete cue complex

paired with cocaine delivery); inactive lever presses produced no scheduled consequences. Following reinforcer delivery, the stimulus light as well as the infusion pump were inactivated; the house light remained on for an additional 20 sec to indicate a timeout period during which lever presses had no scheduled consequences. Rats were trained on a fixed ratio (FR) 1 schedule of reinforcement and progressed to an FR5 schedule after achieving seven infusions/hr with less than 10% variability for three consecutive days. Upon achieving stability on the FR5 schedule (less than 10% variability for a minimum of three consecutive days), rats were pseudorandomly assigned to either FA Day 1 or FA Day 30, and returned to their home cages for the appropriate FA period.

At the designated FA period, rats were assessed in a 60-min cue reactivity test session ("cue test" rats) in which presses on the previously-active lever were reinforced by the discrete cue complex (stimulus light illuminated, infusion pump activated) on an FR1 schedule; presses on the inactive lever were recorded but produced no scheduled consequences. "Cue test" rats were killed immediately upon removal from the cue reactivity session on FA Day 1 or FA Day 30. "No test" rats were returned to their home cage following the last self-administration session and killed immediately upon removal from their home cages on FA Day 1 or FA Day 30 without re-exposure to the operant chambers to control for the behavioral experience during the cue reactivity session in *ex vivo* neurochemical studies (Anastasio et al., 2014a).

Two cohorts of rats were trained to self-administer cocaine and assessed for cue reactivity. In the first cohort (n=149), pharmacological analyses were employed to test the hypothesis that incubation of cue reactivity during abstinence from cocaine self-

administration is accompanied by lower potency and/or efficacy of the selective 5-HT_{2C}R agonist WAY163909 to suppress cue reactivity. After meeting the criterion for stable cocaine self-administration, rats received an injection of vehicle (0.9% NaCl, 1 mL/kg; i.p.) or WAY163909 (0.05 mg/kg, 0.2 mg/kg, or 1.0 mg/kg; i.p.) 15 min prior to the cue reactivity session on FA Day 1 or FA Day 30. The doses were chosen from our previous studies with WAY163909 (Anastasio et al., 2014a; Anastasio et al., 2014b; Cunningham et al., 2013; Cunningham et al., 2011). The second cohort of rats (n=20) was trained to self-administer cocaine as above and treated with vehicle 15 min prior to the cue reactivity session on FA Day 1 or FA Day 30. The vehicle-treated rats from both cohorts were employed in *ex vivo* analyses (see below).

Sucrose self-administration and cue reactivity analyses

Sucrose self-administration training consisted of 14 daily 180-min sessions during which freely-fed rats (n=32) were trained to lever press for 45 mg sucrose pellets (Bio-Serv, Frenchtown, NJ) (Cunningham et al., 2011). Experimental parameters were identical to those employed in cocaine self-administration and cue reactivity analyses except that sucrose was substituted as the reinforcer. Rats from this cohort were employed in *ex vivo* analyses (see below).

5-HT_{2C}R protein analysis

The 5-HT_{2C}R protein expression profiles were assessed using Western blot analyses of tissue harvested from FA Day 1 or FA Day 30 "cue test" and "no test" cocaine- or sucrose-trained rats (Anastasio et al., 2014a) or naïve rats. Naïve rats were

included as an additional control to interrogate levels of 5-HT_{2C}R protein expression and subcellular distribution independent of experimental manipulation. Rats were anesthetized (400 mg/kg chloral hydrate solution), decapitated, and brains were cut in 2 mm coronal sections, rapidly microdissected with a scalpel on a cool tray (4°C) (Heffner et al., 1980), frozen in liquid nitrogen and stored at -80°C. Tissue encompassing the mPFC (cingulate cortex 1, prelimbic cortex and infralimbic cortex) was extracted at 3.00 mm from bregma (Paxinos and Watson, 1998). Protein fractionation techniques were employed to assess the synaptosomal expression profile (Anastasio et al., 2010; Liu et al., 2007) or the membrane *vs.* cytoplasmic protein expression profile of the 5-HT_{2C}R (Anastasio et al., 2013; Anastasio et al., 2014b).

The crude synaptosomal protein fraction is enriched for pre- and postsynaptic proteins [i.e., presynaptic terminals, postsynaptic membranes, postsynaptic density, synaptic protein complexes (Breukel et al., 1997)] and was prepared as described previously (Anastasio et al., 2010; Liu et al., 2007). Individual mPFC tissues were homogenized in 10 times w/v ice cold Krebs buffer (125 mM NaCl, 1.2 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 22 mM Na_2CO_3, 1 mM NaH_2PO_4, 10 mM glucose) containing 0.32 M sucrose plus protease inhibitor cocktail and phosphatase inhibitor 2 and 3 cocktails (10 μL/mL; Sigma-Aldrich, St. Louis, MO). The homogenate was centrifuged at 1000 g for 10 min at 4°C to pellet the nuclear fraction. The supernatant was collected and centrifuged at 16,000 g for 20 min at 4°C to pellet the crude synaptosome. The pellet was re-suspended in Krebs buffer with 1% dodecyl maltoside or 0.5% NP40.

Crude plasma membrane and cytoplasmic protein fractions were prepared via differential centrifugation as previously described (Anastasio et al., 2013; Anastasio et al., 2014b). The crude membrane fraction captures membrane-associated proteins localized to the plasma membrane and membranous organelles (e.g., mitochondria) (Rockstroh et al., 2011; Suski et al., 2014). The crude cytoplasmic fraction contains a number of intracellular organelles (except the nucleus) as well as the cytosol, but not a selective marker of the plasma membrane (i.e., cadherin) (Rockstroh et al., 2011; Suski et al., 2014). The mPFC was homogenized in 10 times w/v extraction buffer (10 mM HEPES, 1 mM EDTA, 2 mM EGTA, 1 mM DTT) plus protease inhibitor cocktail and phosphatase inhibitor 2 and 3 cocktails (10 μL/mL). The homogenate was centrifuged at 1000 g for 10 min at 4°C to pellet the nuclear fraction. The supernatant was collected and centrifuged at 20,000 g for 30 min at 4°C to pellet the membrane-bound enriched protein fraction. The cytoplasmic fraction was collected and reserved. The membrane-enriched pellet was washed once and resuspended in buffer [(20 mM HEPES, 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, protease inhibitor cocktail and phosphatase inhibitor 2 and 3 cocktails (10 μL/mL)] plus 0.5% NP40.

Equal amounts of crude synaptosomal protein (30 μg) were reduced with Laemmli sample buffer and heated for 20 min at 70°C then separated by SDS-PAGE using 10% Bis-Tris gels (Invitrogen, San Diego, CA) for 2-3 hrs at 110V. Proteins were transferred to a PVDF membrane (BioRad, Hercules, CA) via a wet-transfer electroblotting apparatus (BioRad) overnight at 60-70V (Anastasio et al., 2010). Membranes were blocked with Odyssey blocking buffer [LI-COR® Biosciences; 1:1 in Tris Buffered Saline (TBS), pH 7.4] followed by incubation with primary antibody

[mouse monoclonal 5-HT_{2C}R (D-12, sc-17797, Santa Cruz; 1:100), mouse monoclonal pan-cadherin (ab6528, Abcam; 1:5000)] (Anastasio et al., 2010; Anastasio et al., 2015; Fink, 2015). Membranes were rinsed in TBS + 0.1% Tween-20 (TBS-T), incubated with secondary antibody [infrared-labeled goat anti-mouse IRDye™800CW (926-32210) or IRDye™680RD (926-68070; LI-COR® Biosciences, Lincoln, NE, 1:10000)], then rinsed in TBS-T. PVDF membranes were imaged using the Odyssey® Infrared Imaging System (LI-COR® Biosciences, Lincoln, NE). The integrated intensity of immunoreactive bands normalized to the housekeeping protein cadherin were analyzed with the Odyssey® software.

The subcellular localization (synaptosomal; membrane *vs.* cytoplasmic) profile of the mPFC 5-HT_{2C}R protein was assessed via the Wes™ automated Western blotting system (ProteinSimple, San Jose, CA) which utilizes capillary electrophoresis-based immunodetection for higher resolution, sensitivity, and reproducibility (even at low sample concentrations) relative to traditional immunoblotting techniques (Anastasio et al., 2015; Fink, 2015; Liu et al., 2013a). 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, and matrix removal buffer, secondary antibodies, antibody diluent, and capillaries) were obtained from the manufacturer (ProteinSimple) and used according to the manufacturer's recommendations with minor modifications (Anastasio et al., 2015; Fink, 2015). The mouse monoclonal 5-HT_{2C}R (D-12, sc-17797, Santa Cruz; 1:50), mouse monoclonal pan-cadherin (ab6528, Abcam; 1:10000), and mouse monoclonal GAPDH antibody (6C5, Advanced Immunochemicals; 1:50000) were diluted with ProteinSimple antibody

diluent. Equal amounts of protein (3 µg) were combined with 0.1X sample buffer and 5X master mix (200 mM DTT, 5X sample buffer, 5X fluorescent standards), gently mixed, and then denatured at 95^{o}C for 5 min. The denatured samples, biotinylated ladder, antibody diluent, primary antibodies, HRP-conjugated secondary antibodies, chemiluminescent substrate, and wash buffer were dispensed to designated wells in a pre-filled microplate (ProteinSimple). Separation electrophoresis (375 V, 31 min, 25^{o}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 5-s, 15-s, 30-s, 60-s, 120-s, 240-s, and 480-s. Data analyses were performed using the Compass Software (ProteinSimple). The area under the curve of the 5-HT$_{2C}$R peak was normalized to the area under the curve of the cadherin peak (synaptosomal and membrane fractions) or area under the curve of the GAPDH peak (cytoplasmic fraction). Representative "virtual blot" electrophoretic images were automatically generated by the Compass Software (ProteinSimple).

5-HT$_{2C}$R mRNA analysis

We employed RT-PCR analyses to determine 5-HT$_{2C}$R mRNA levels on FA Day 30 *vs.* FA Day 1 from cocaine self-administration. Immediately following the cue reactivity test session, rats were sacrificed and the mPFC was harvested as described above. Samples of mPFC were homogenized in Trizol Reagent® and RNA was isolated using the Trizol Reagent Protocol (Life Technologies, Grand Island, NY) (Anastasio et

al., 2014b). Reverse transcription was performed on 250 ng RNA using SuperScript III Reverse Transcriptase (Life Technologies) with random hexamer primers. RT-PCR reactions were assayed in triplicate on a 7500 Fast RT PCR System using TaqMan Fast Advanced Master Mix and TaqMan® gene specific primer/probes [*Htr2c*: Rn00562748_m1 (spans boundary of exons 4 and 5); Cyclophilin A (*Ppia*): Rn00690933_m1; Life Technologies]. Data are presented in terms of Crossing Threshold (Ct), where ΔCt=Ct(*Htr2c*)–Ct(Cyclophilin).

Statistical analyses

Student's t-test was used to analyze total intake during acquisition and maintenance of cocaine or sucrose self-administration. A two-way ANOVA for the factors of FA Day (FA Day 1, FA Day 30) and WAY163909 treatment (vehicle, 0.05, 0.2 and 1.0 mg/kg) was used to analyze previously-active and inactive lever presses and latency to the first response during the cue reactivity test session; planned comparisons were subsequently made with a Dunnett's test (Keppel, 1973). The four parameter logistic nonlinear regression (Sigma Plot, Version 12.3, Systat Software, Inc., Chicago, IL) was used to estimate the dose of WAY163909 estimated to decrease cue reactivity by 50% of the maximum suppression by WAY163909 (ID_{50}) on FA Day 1 *vs.* FA Day 30 from cocaine self-administration (Ratkowsky and Reedy, 1986; Tallarida and Murray, 1987). A one-way ANOVA was used to analyze previously-active and inactive lever presses and latency to the first response on FA Day 1 *vs.* FA Day 30 following sucrose or cocaine (second cohort) self-administration. A two-way ANOVA for the factors of FA (FA Day 1, FA Day 30) and group ("cue test", "no test") was used to analyze

synaptosomal 5-HT_{2C}R protein expression; planned comparisons were made with a Tukey's test (Keppel, 1973). Student's t-test was used to analyze 5-HT_{2C}R mRNA levels on FA Day 1 *vs.* FA Day 30. A one-way ANOVA (FA Day 1, FA Day 30, naïve) was used to analyze synaptosomal, membrane, cytoplasmic or membrane:cytoplasmic 5-HT_{2C}R protein expression; planned comparisons were made with a Tukey test (Keppel, 1973). A Pearson's correlation was used to analyze the relationship between previously-active lever presses on the cue reactivity test session and the membrane:cytoplasmic ratio of 5-HT_{2C}R protein expression (Keppel, 1973). The experiment-wise error rate for all analyses was set at α=0.05.

Results

Incubation aligns with lower potency of a selective 5-HT_{2C}R agonist to suppress cocaine cue reactivity

Rats readily acquired cocaine self-administration to stability (i.e., seven infusions/hr on an FR5 schedule for at least three sessions) and displayed <10% variation in the number of infusions earned during the maintenance sessions (**Fig. 2.1a**). There was no difference in total cocaine intake across the self-administration phase between rats assigned to FA Day 1 (368.6 ± 8.9 mg/kg) or FA Day 30 (362.7 ± 7.2 mg/kg; $t_{1,147}$=0.26; n.s.).

We tested the hypothesis that rats assessed for cue reactivity (previously-active lever presses; mean ± SEM) on FA Day 1 and FA Day 30 from cocaine self-administration would display lower potency and/or efficacy of the selective 5-HT_{2C}R agonist WAY163909 to suppress cue reactivity (Anastasio et al., 2014a). Rats were injected with vehicle (0.9% NaCl) or WAY163909 (0.05 mg/kg, 0.2 mg/kg, or 1.0 mg/kg; i.p.) 15 min prior to the cue reactivity session on FA Day 1 or FA Day 30. A main effect of FA Day ($F_{1,132}$=74.28; p<0.05), WAY163909 treatment ($F_{3,132}$=21.81; p<0.05), and an FA Day x WAY163909 treatment interaction ($F_{3,132}$=4.38; p<0.05) were observed for previously-active lever presses for the discrete cue complex. Planned comparisons indicated that cue reactivity was significantly elevated in the vehicle-treated rats on FA Day 30 *vs.* FA Day 1 from cocaine self-administration (**Fig. 2.1b**), consistent with previous observations that cue reactivity incubates during forced abstinence from cocaine-taking (Grimm et al., 2001; Neisewander et al., 2000). Cue reactivity in vehicle-treated rats is replotted (**Fig. 2.1c**) for FA Day 1 (dotted line) and FA Day 30 (dashed

line). Planned comparisons for FA Day 1 indicated that rats treated with the intermediate (0.2 mg/kg) or high dose (1.0 mg/kg) of WAY163909 exhibited lower previously-active lever presses *vs.* rats treated with vehicle ($p<0.05$); the low dose (0.05 mg/kg) of WAY163909 had no effect *vs.* rats treated with vehicle (**Fig. 2.1c**; n.s.). Planned comparisons for FA Day 30 indicated that only the high dose (1.0 mg/kg) of WAY163909 significantly attenuated previously-active lever presses *vs.* rats treated with vehicle ($p<0.05$); the intermediate (0.2 mg/kg) and low (0.05 mg/kg) doses of WAY163909 had no effect *vs.* rats treated with vehicle (**Fig. 2.1c**). The dose of WAY163909 estimated to decrease previously-active lever presses by 50% of the maximum suppression by WAY163909 (ID_{50}) was 0.12 mg/kg on FA Day 1 and 0.39 mg/kg on FA Day 30 (~3-fold rightward shift). There was no difference in the maximum efficacy of 1 mg/kg of WAY163909 (previously active lever presses expressed as percent suppression *vs.* vehicle) on FA Day 1 (32.8 ± 8.8%) *vs.* FA Day 30 (48.0 ± 4.1%; $t_{1,34}=2.24$; n.s.). Thus, incubation was associated with lower potency of WAY163909, but no change in efficacy, to suppress cue reactivity on FA Day 30 *vs.* FA Day 1.

Inactive lever presses in vehicle-treated rats are replotted (**Fig. 2.1d**) for FA Day 1 (dotted line) and FA Day 30 (dashed line). For inactive lever presses, no main effect of FA Day ($F_{1,132}=0.94$; n.s.), a main effect of WAY163909 treatment ($F_{3,132}=3.66$; $p<0.05$), and no FA Day x WAY163909 treatment interaction ($F_{3,132}=0.22$; n.s.) were observed during the cue reactivity test session. Planned comparisons indicated that WAY163909 did not significantly alter inactive lever presses on either FA Day 1 or FA Day 30 at any dose tested (**Fig. 2.1d**). No main effect of FA Day ($F_{1,132}=1.85$; n.s.), a main effect of WAY163909 treatment ($F_{3,132}=5.57$; $p<0.05$), and no FA Day x WAY163909 treatment

interaction ($F_{3,132}$=0.04; n.s.) were observed for latency to the first press on the previously-active lever during the cue reactivity test session. On FA Day 1, planned comparisons indicated that the latency to first press (mean ± SEM) following WAY163909 treatment at 0.05 mg/kg (27.4 ± 4.6 sec; n.s.), 0.2 mg/kg (39.7 ± 6.6 sec; n.s.) and 1.0 mg/kg (57.7 ± 10.6 sec; n.s) did not differ from vehicle (32.4 ± 10.9). On FA Day 30, the latency to first press following WAY163909 treatment at 0.05 mg/kg (18.9 ± 5.5 sec; n.s.) or 0.2 mg/kg (34.0. ± 10.6 sec; n.s.) did not differ from vehicle (21.0 ± 4.9 sec). The latency to first press following 1.0 mg/kg (50.8 ± 11.3; p<0.05) was significantly higher relative to vehicle (21.0 ± 4.9 sec). Thus, WAY163909 suppressed cue reactivity with a limited impact on additional measures of behavioral performance.

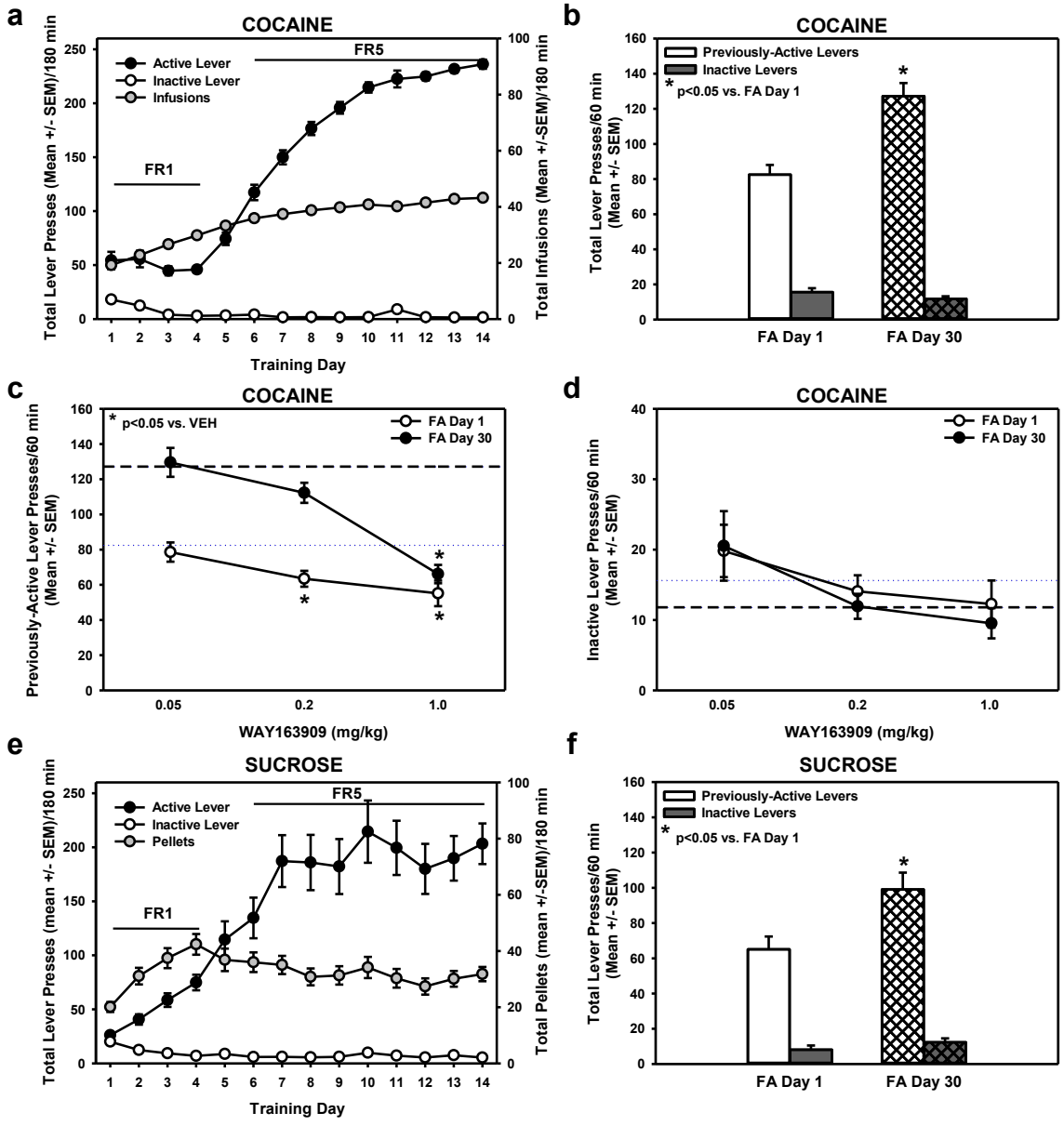

Figure 2.1: Cue reactivity incubates during forced abstinence from cocaine and sucrose self-administration, and incubation aligns with lower potency of a selective 5-HT$_{2C}$R agonist to blunt cocaine cue reactivity.

(a) Mean responses (± SEM) on the active (black circles) or inactive lever (white circles), and total number of cocaine infusions earned (± SEM; gray circles) are presented for the acquisition and maintenance phase of cocaine self-administration. (b) Mean (± SEM) previously-active lever presses and inactive lever presses are presented for the cue reactivity test session in vehicle-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=19-20/group). (c) Mean (± SEM) previously-active lever presses are presented for the cue reactivity test session on FA Day

1 and FA Day 30 from cocaine self-administration. On FA Day 1, WAY163909 at 0.2 mg/kg (n=16; $p<0.05$) and 1.0 mg/kg (n=19; $p<0.05$), but not 0.05 mg/kg (n=16; n.s.), suppressed cue reactivity *vs.* vehicle (dotted line). On FA Day 30, WAY163909 at 1.0 mg/kg (n=17; $p<0.05$), but not 0.05 mg/kg (n=17; n.s.) or 0.2 mg/kg; n=16; n.s.), suppressed cue reactivity *vs.* vehicle (dashed line). (d) Mean (± SEM) inactive lever presses are presented for the cue reactivity test session on FA Day 1 and FA Day 30 from cocaine self-administration. Treatment with WAY163909 did not alter inactive lever presses on either FA Day 1 (n.s.) or FA Day 30 (n.s.). (e) Mean responses (± SEM) on the active (black circles) or inactive lever (white circles), and total number of sucrose pellets earned (± SEM; gray circles) are presented for the acquisition and maintenance phase of sucrose self-administration. (f) Mean (± SEM) previously-active lever presses and inactive lever presses are presented for the cue reactivity test session on FA Day 1 and FA Day 30 from sucrose self-administration. Cue reactivity is significantly elevated on FA Day 30 *vs.* FA Day 1 from sucrose self-administration (*$p<0.05$; n=8-10/group).

Cue reactivity incubates during forced abstinence from sucrose self-administration

Rats readily acquired sucrose self-administration to stability and displayed <10% variation in the number of pellets earned during the maintenance sessions (**Fig. 2.1e**). There was no difference in total sucrose intake across the self-administration phase between rats assigned to FA Day 1 (19.9 ± 2.1 g) or FA Day 30 (21.0 ± 2.2 g; $t_{1,30}$=0.13; n.s.).

A main effect of FA Day ($F_{1,16}$=7.40; $p<0.05$) was observed for previously active lever presses (mean ± SEM) on the cue reactivity test session. Previously-active lever presses (mean ± SEM) were significantly elevated on FA Day 30 *vs.* FA Day 1 from sucrose self-administration (**Fig. 2.1f**). No main effect of FA Day was observed for inactive lever presses ($F_{1,16}$=1.58, n.s.; **Fig. 2.1f**). No main effect of FA Day was observed for the latency to the first press ($F_{1,16}$=0.88; n.s). The latency was 20.8 ± 8.4 sec on FA Day 1 and 13 ± 3.1 sec on FA Day 30 from sucrose self-administration. Thus, cue reactivity incubates during forced abstinence from sucrose-taking, as previously described (Grimm et al., 2006; Grimm et al., 2002).

Incubation of cocaine, but not sucrose, cue reactivity associates with lower synaptosomal 5-HT_{2C}R protein expression in the mPFC

We tested the hypothesis that incubation of cocaine cue reactivity would associate with 5-HT_{2C}R protein expression in the mPFC synaptosomal fraction. Vehicle-treated rats were sacrificed on FA Day 1 or FA Day 30 from cocaine (**Fig. 2.1a,b**) or sucrose self-administration (**Fig. 2.1e,f**) immediately following the cue reactivity test session ("cue test") or upon removal from their home cages without re-exposure to the operant chambers ("no test") (Anastasio et al., 2014a). A portion of the "no test" protein expression data from FA Day 1 from cocaine self-administration rats was previously reported (Anastasio et al., 2014a) and has been reanalyzed within the present experiment for comparison. A diagram of the mPFC region dissected for analyses is shown in **Fig. 2.2a**.

Figure 2.2 illustrates the qualitative (**inset**) and quantitative analyses (**bars**) of mPFC 5-HT_{2C}R synaptosomal protein expression at FA Day 1 and FA Day 30 from cocaine (**Fig. 2.2b**) or sucrose self-administration for both "cue test" and "no test" rats (**Fig. 2.2c**). For cocaine cue reactivity, a main effect of FA Day ($F_{1,21}$=13.19; p<0.05), but no main effect of cue group ($F_{1,21}$=3.75; n.s.) or FA Day x cue group interaction ($F_{1,21}$=0.93; n.s.) on mPFC 5-HT_{2C}R protein expression was observed. Planned comparisons indicated that synaptosomal 5-HT_{2C}R protein expression in the mPFC was lower on FA Day 30 *vs.* FA Day 1 regardless of cue group ("cue test" or "no test"; **Fig. 2.2b**). We performed RT-PCR to assess 5-HT_{2C}R mRNA levels in the mPFC from rats sacrificed immediately following the cue reactivity test session on FA Day 1 or FA Day

30 from cocaine self-administration. Expression of 5-HT_{2C}R mRNA (mean ± SEM.) was identical between FA Day 1 (4.82 ± 0.13 arbitrary units, A.U.) and FA Day 30 (4.80 ± 0.30 A.U.; $t_{1,4}$=0.00; n.s.). For sucrose cue reactivity, there was no main effect of FA Day ($F_{1,27}$=0.21; n.s.) or cue group ($F_{1,27}$=0.02; n.s.) and no FA Day x cue group interaction ($F_{1,27}$=0.70; n.s.). Synaptosomal 5-HT_{2C}R protein expression in the mPFC was identical on FA Day 30 vs. FA Day 1 under both "cue test" and "no test" conditions (**Fig. 2.2c**). Together, these data demonstrate that lower 5-HT_{2C}R expression in the mPFC is specific to incubation of cocaine cue reactivity and that the profile of 5-HT_{2C}R protein expression is potentially regulated by post-transcriptional mechanisms (e.g., trafficking and/or recycling processes).

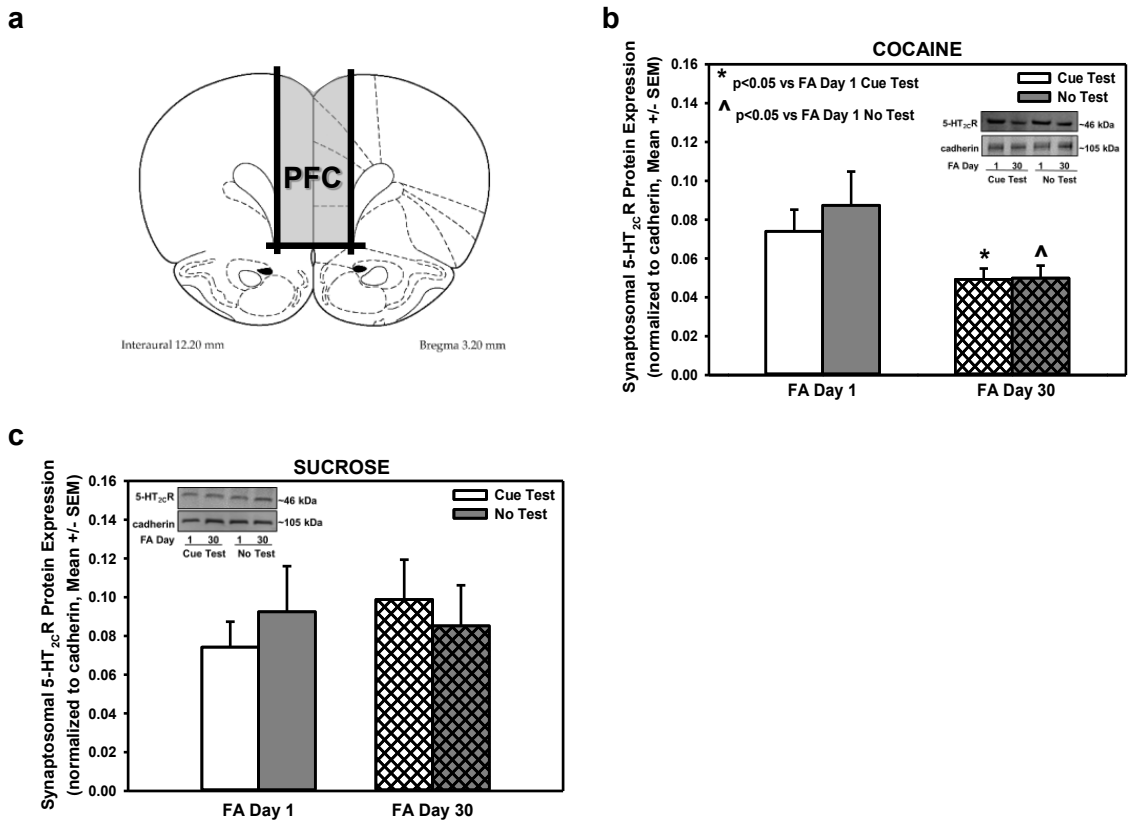

Figure 2.2: Incubation of cocaine, but not sucrose, cue reactivity associates with lower synaptosomal 5-HT_{2C}R protein expression in the mPFC.

(a) The diagram demonstrates the region of mPFC dissected for *ex vivo* analyses (Paxinos and Watson, 1998). (b) Synaptosomal 5-HT_{2C}R protein expression (normalized to cadherin) in the mPFC is lower in rats assessed in the cue reactivity test session ("cue test";*p<0.05; n=8/group) and "no test" rats sacrificed upon removal from their home cages at the expected time of that test session without re-exposure to the operant chambers (^p<0.05; n=4-5/group) on FA Day 30 *vs.* FA Day 1 from cocaine self-administration; a representative immunoblot is located in inset. There was no difference between "cue test" and "no test" groups on the same FA Day. (c) Synaptosomal mPFC 5-HT_{2C}R protein expression (normalized to cadherin) did not differ on FA Day 1 *vs.* FA Day 30 from sucrose self-administration in "cue test" rats assessed in the cue reactivity test session (n=7-9/group) or in "no test" rats sacrificed upon removal from their home cages at the expected time of that test session without re-exposure to the operant chambers (n.s.; n=7/group); a representative immunoblot is located in inset.

Incubation of cocaine cue reactivity associates with altered subcellular distribution of 5-HT_{2C}R protein in mPFC

We tested the hypothesis that incubation of cocaine cue reactivity is associated with differential subcellular distribution of 5-HT_{2C}R protein expression in the mPFC as an indicator of post-transcriptional mechanisms including receptor trafficking and/or recycling processes. Rats from the second cohort readily acquired cocaine self-administration to stability (i.e., seven infusions/hr on an FR 5 schedule for at least three sessions) and displayed <10% variation in the number of infusions earned during the maintenance sessions (data not shown). There was no difference in total cocaine intake across the self-administration phase between rats assigned to FA Day 1 (366.7 ± 18.8 mg/kg) or FA Day 30 (385.6 ± 30.0 mg/kg; $t_{1,18}$=0.29; n.s.). Cue reactivity was significantly higher on FA Day 30 *vs.* FA Day 1 (**Fig. 2.3a**; $F_{1,18}$=25.38; p<0.05) as shown for the first cohort (**Fig. 2.1b**).

Analyses of synaptosomal (**Fig. 2.3b**), membrane (**Fig. 2.3c**), and cytoplasmic (**Fig. 2.3d**) mPFC 5-HT_{2C}R protein expression in these rats are presented. A cohort of naïve rats were included as an additional neurochemical control. The horizontal bars represent the mean (solid line) ± SEM (dotted lines and shading) of 5-HT_{2C}R protein expression in the mPFC of naïve rats. A main effect of group (naïve, FA Day 1, FA Day 30) was detected for synaptosomal 5-HT_{2C}R protein expression in the mPFC ($F_{2,13}$=5.33; p<0.05). Planned comparisons indicated that synaptosomal 5-HT_{2C}R protein expression (**Fig. 2.3b, inset**) was lower on FA Day 30 *vs.* FA Day 1 (**Fig. 2.3b**; p<0.05), replicating observations presented in **Fig. 2b**. Synaptosomal 5-HT_{2C}R protein expression on FA Day 1 and FA Day 30 did not differ from naïve (**Fig. 2.3b;** n.s.). A main effect of group was

detected for membrane 5-HT_{2C}R protein expression ($F_{2,14}$=6.98; p<0.05). Membrane expression of 5-HT_{2C}R (**Fig. 2.3c, inset**) was lower on FA day 30 *vs.* FA Day 1 (**Fig. 2.3c**; p<0.05) and *vs.* naïve (**Fig. 2.3c**; p<0.05); membrane 5-HT_{2C}R protein expression on FA Day 1 did not differ from naïve (**Fig. 2.3c**; n.s.). A main effect of group was detected for cytoplasmic 5-HT_{2C}R protein expression ($F_{2,14}$=4.04; p<0.05). Cytoplasmic expression of 5-HT_{2C}R (**Fig. 2.3d, inset**) did not differ between FA Day 1 and FA Day 30 or naïve (**Fig. 2.3d**; n.s.). A main effect of group was detected for the ratio of membrane to cytoplasmic 5-HT_{2C}R protein expression ($F_{2,14}$=13.27, p<0.05). The ratio of membrane to cytoplasmic 5-HT_{2C}R expression was lower on FA Day 30 *vs.* FA Day 1 (**Fig. 2.3e**; p<0.05), but did not differ from naïve (**Fig. 2.3e**; n.s.). There was an inverse correlation between previously-active lever presses during the cue reactivity test session and the membrane:cytoplasmic ratio of 5-HT_{2C}R protein expression in individual rats (**Fig. 2.3f**; r=-0.8922; p<0.05). Together, these data suggest that incubation of cocaine cue reactivity is manifested by adaptations in trafficking and/or recycling processes that dictate the subcellular localization of the 5-HT_{2C}R protein.

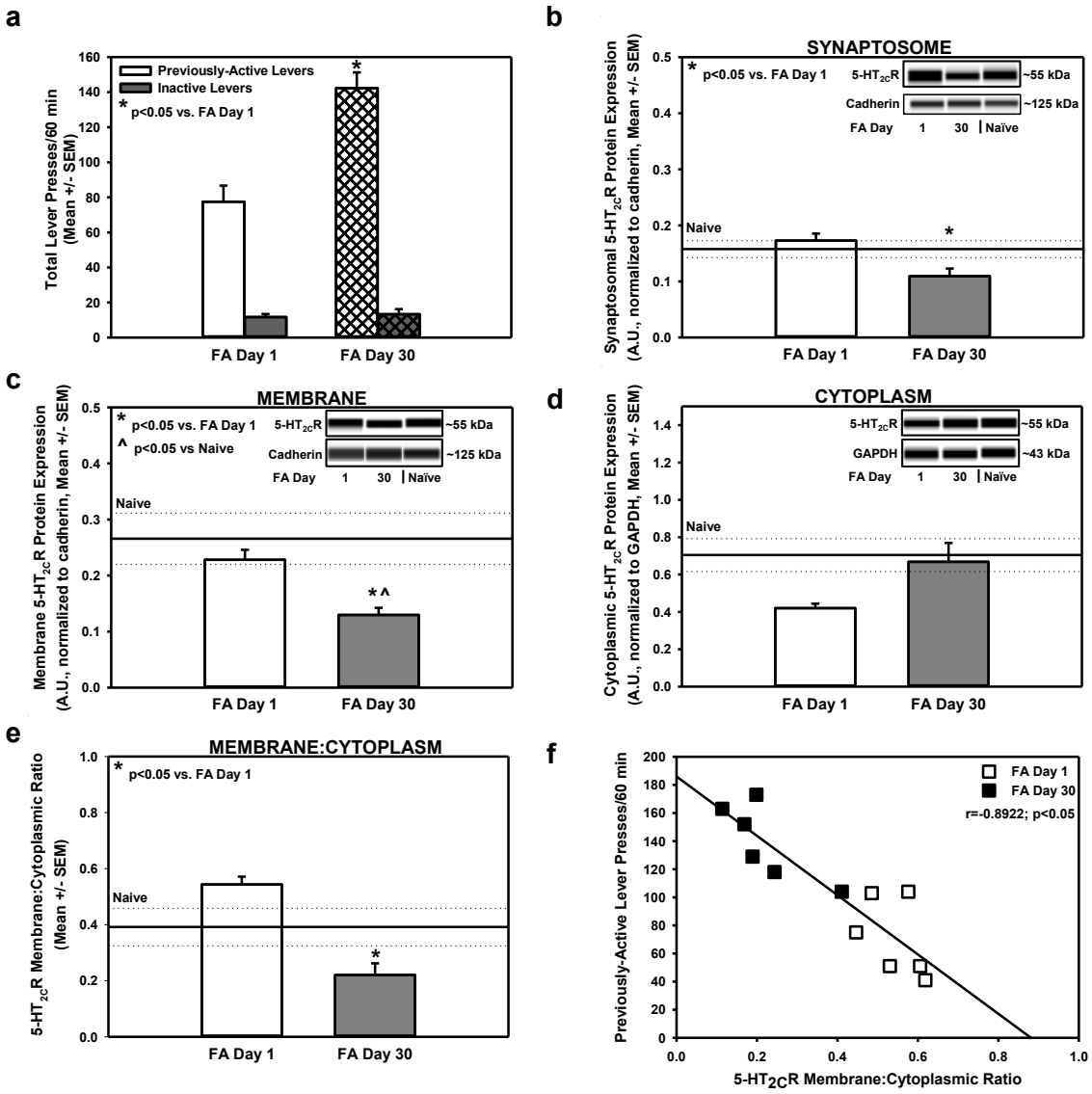

Figure 2.3: Incubation of cocaine cue reactivity associates with altered subcellular distribution of 5-HT$_{2C}$R protein in the mPFC.

(a) Mean (± SEM) previously-active and inactive lever presses are presented for the cue reactivity test session on FA Day 1 and FA Day 30 from cocaine self-administration in the second cohort. Cue reactivity is significantly elevated in vehicle-treated rats on FA Day 30 *vs.* FA Day 1 (*p<0.05; n=10/group). (b-e) The horizontal bars represent the mean (solid line) ± SEM (dotted lines and shading) 5-HT$_{2C}$R protein expression in the mPFC of naïve rats. (b) Synaptosomal 5-HT$_{2C}$R protein expression (normalized to cadherin) in the mPFC is lower on FA Day 30 *vs.* FA Day 1 from cocaine self-administration (*p<0.05; n=4-6/group); representative electrophoretic bands are located in inset. Synaptosomal 5-HT$_{2C}$R protein expression on FA Day 1 and FA Day 30 did not differ from naïve (n.s.). (c) Membrane expression of 5-HT$_{2C}$R (normalized to cadherin) is lower on FA Day 30 *vs.* FA Day 1 (*p<0.05) and lower on FA Day 30 *vs.* naïve

(^$p<0.05$; n=5-6/group); representative electrophoretic bands are located in inset. Membrane 5-HT_{2C}R protein expression on FA Day 1 did not differ from naïve (n.s.) (d) Cytoplasmic expression of 5-HT_{2C}R (normalized to GAPDH) did not differ between FA Day 1 and FA Day 30 or naïve (n.s.); representative electrophoretic bands are located in inset. (e) The ratio of membrane to cytoplasmic 5-HT_{2C}R expression in the mPFC is lower on FA Day 30 relative to FA Day 1 (*$p<0.05$), but did not differ from naïve (n.s.). (f) There was an inverse correlation between active lever presses in the cue reactivity test session and the membrane:cytoplasmic ratio of 5-HT_{2C}R protein expression for individual rats ($r=-0.8922$; $p<0.05$).

Discussion

The present studies demonstrate that the incubation of cue reactivity during prolonged abstinence from cocaine self-administration is associated with lower potency of the selective 5-HT_{2C}R agonist WAY163909 to suppress cue reactivity, with no evident change in agonist efficacy. Biochemical analyses established that incubation associates with lower synaptosomal expression of 5-HT_{2C}R protein in the mPFC, a key site that mediates the incubation phenomena (Koya et al., 2009; Ma et al., 2014; Whitfield et al., 2011). Further, a greater proportion of the expressed 5-HT_{2C}R protein was sequestered in the cytoplasmic (vs. membrane) compartment of the mPFC at prolonged *vs.* early forced abstinence and there was an inverse correlation of the membrane to cytoplasmic 5-HT_{2C}R ratio in the mPFC with levels of cocaine cue reactivity. Collectively, these outcomes are the first indication that a shift in the responsivity of the 5-HT_{2C}R system, driven in part by the altered subcellular localization of the receptor, may contribute to incubation of cocaine cue reactivity.

The findings presented here uphold the interpretation that the functional status of the 5-HT_{2C}R system is a factor in establishing the incubation phenomena. An analysis of the dose-effect curve for the high affinity (K_i=10.5 nM) and efficacy (90% *vs.* 5-HT) 5-HT_{2C}R agonist WAY163909 (Dunlop et al., 2005) substantiated a ~3-fold rightward shift in its potency to suppress cue reactivity in prolonged (ID_{50} = 0.39 mg/kg) relative to early forced abstinence (ID_{50} = 0.12 mg/kg) from cocaine self-administration. In general, a full agonist need only occupy a fraction of available receptors to activate a maximal response (Kenakin, 2002; Strange, 2008); this concept is supported here by the fact that the maximal efficacy of WAY163909 (1 mg/kg) is retained in prolonged forced abstinence.

The subcellular localization of the 5-HT_{2C}R is a key feature of receptor readiness to signal and respond to agonists (Kenakin, 2002; Strange, 2008). In the present study, the ratio of membrane to cytoplasmic 5-HT_{2C}R expression in the mPFC was significantly lower at prolonged *vs.* early abstinence, in the absence of differences in 5-HT_{2C}R mRNA levels, suggesting that post-transcriptional mechanisms govern the neuroadaptations in the 5-HT_{2C}R system that contribute to elevated cocaine cue reactivity.

Neuroimaging studies in humans (Garavan et al., 2000; Maas et al., 1998) and pharmacological and behavioral studies in rodents (Koya et al., 2009; LaLumiere et al., 2012; Ma et al., 2014; Pentkowski et al., 2010; Whitfield et al., 2011) have implicated the mPFC as a key neuronal locus that controls the persistence of cocaine-associated cues to generate susceptibility to relapse, including the 5-HT_{2C}R (Anastasio et al., 2014a; Anastasio et al., 2014b). The observations described herein support the hypothesis that disruption of a population of synaptically-localized 5-HT_{2C}R (Anastasio et al., 2010) and a reduced 5-HT_{2C}R tone in mPFC is an integral mechanism in the expression of cue reactivity during abstinence from cocaine self-administration (for review, Cunningham and Anastasio, 2014). The 5-HT_{2C}R interfaces in a functionally-coordinated manner with synaptic proteins to promote or inhibit receptor readiness and subsequent signaling capacity (Gavarini et al., 2006), most likely a reactive process to the dynamic microenvironment. A shift in receptor expression from the membrane fraction to the cytoplasmic fraction indicates that post-transcriptional processes which govern 5-HT_{2C}R trafficking and subcellular localization (e.g., RNA editing, alternative splicing) (Marion et al., 2004; Martin et al., 2013) may also be subject to regulation during periods of abstinence from cocaine. It is intriguing to propose that the neurochemical adaptations,

including fluctuations in 5-HT neurotransmission promoted by cocaine-taking and periods of withdrawal (Parsons and Justice, 1993; Parsons et al., 1995), may be responsible for shuttling 5-HT_{2C}R protein between subcellular compartments. Future analyses are required to determine the importance of other factors likely to contribute to the 5-HT_{2C}R functional capacity and responsivity to agonist *in vivo*, including receptor density, affinity, reserve and/or coupling efficiency (Kenakin, 2002; Sanders-Bush and Breeding, 1990). Our primary objective in the present studies was to uncover the late-emerging changes in 5-HT_{2C}R expression that may be involved in incubation, but shifts in 5-HT_{2C}R expression may also occur during cocaine self-administration and/or in the first 24 hours following the cessation of cocaine-taking. Interestingly, the profile of mPFC 5-HT_{2C}R membrane expression in naïve rats mirrored that observed at early forced abstinence from cocaine self-administration. Without a comprehensive understanding of 5-HT levels and 5-HT_{2C}R function during abstinence from cocaine (Parsons and Justice, 1993; Parsons et al., 1995), concise conclusions about the functional status of the 5-HT_{2C}R system relative to basal conditions are difficult to formulate. Nonetheless, while similar between naïve and early abstinence, 5-HT_{2C}R membrane expression in prolonged abstinence was lower than naïve controls and early abstinence, findings that support the interpretation that the subcellular distribution of 5-HT_{2C}R expression is dysregulated at prolonged abstinence from cocaine-taking. A greater appreciation of the subcellular distribution of the 5-HT_{2C}R and the specific mechanisms which regulate its postsynaptic functionality will advance the development of therapeutics designed to harmonize 5-HT_{2C}R signaling in the mPFC to minimize heightened cue reactivity.

The present studies implicate the involvement of synaptically expressed 5-HT_{2C}R in the mPFC as an additional molecular substrate of the incubation phenomenon. However, the manner in which diverse molecular elements, ranging from neurotrophic factors (Whitfield et al., 2011) to intracellular signaling molecules (Koya et al., 2009) to receptor localization (present study), interface and interact to control incubation remains to be elucidated. Serotonergic terminals predominantly synapse on PFC interneurons (Smiley and Goldman-Rakic, 1996) and the 5-HT_{2C}R transcript and protein are localized to PFC GABA interneurons (Liu et al., 2007; Vysokanov et al., 1998), although cortical pyramidal neurons also express the 5-HT_{2C}R (Carr et al., 2002; Clemett et al., 2000; Liu et al., 2007). Given that the 5-HT_{2C}R in the mPFC localized to parvalbumin-positive interneurons (Liu et al., 2007), which innervate and inhibit efferent signaling of pyramidal neurons (Gabbott et al., 1997; Markram et al., 2004), the 5-HT_{2C}R-mediated influence on GABA interneurons would be expected to provide inhibitory control over output of cortical pyramidal neurons under basal conditions. Thus, low 5-HT_{2C}R tone in mPFC following prolonged abstinence from cocaine self-administration may predict less inhibitory control over output to key structures (e.g., nucleus accumbens; NAc) (Conrad et al., 2008) to modulate cocaine-seeking behavior in protracted abstinence. The tightly regulated balance of mPFC projections to the NAc (Ma et al., 2014) and dynamic remodeling events within the NAc AMPA receptor system (Conrad et al., 2008) cement neuroadaptive processes within the corticoaccumbens circuit as central to the development and expression of incubation following cocaine self-administration.

We found that dysregulation of the mPFC 5-HT_{2C}R system does not appear to be involved in incubation following forced abstinence from sucrose self-administration

which is consistent with prior reports that have identified disparate mechanisms involved in incubation following cocaine *vs*. sucrose self-administration in the mPFC (Koya et al., 2009) and NAc (Counotte et al., 2014; Lu et al., 2003). In contrast, convergent mechanisms for incubation following cocaine and sucrose self-administration have been described within limbic areas of the brain, such as the amygdala (Lu et al., 2007; Uejima et al., 2007), which may implicate different neurocircuitry in incubation following abstinence from drug *vs.* non-drug reinforcers. Of note, 5-HT_{2C}R populations within the NAc (Filip and Cunningham, 2002; Navailles et al., 2008) and the ventral tegmental area (Bubar and Cunningham, 2007; Bubar et al., 2011; Navailles et al., 2008) also control corticostriatal circuitry (Pozzi et al., 2002), but have yet to be fully investigated for their role in incubation following either cocaine or sucrose self-administration. Collectively, neuroadaptations in the 5-HT_{2C}R and other neurobiological systems within the corticoaccumbens circuit could aggregately prime the neural network for an augmented response to reward-related cues in prolonged abstinence from cocaine self-administration.

An improved understanding of the mechanisms which mediate the incubation phenomenon could provide insight into treatment modalities that may be differentially effective to curb cue reactivity and prevent relapse at early *vs*. prolonged periods of abstinence from cocaine. Importantly, these data suggest that pharmacotherapy with the FDA-approved selective 5-HT_{2C}R agonist lorcaserin may be generally efficacious, but more potent and particularly useful early after cessation of cocaine use to mitigate relapse primed by exposure to drug-associated cues and perhaps bolster relapse prevention during abstinence (Cunningham and Anastasio, 2014; Harvey-Lewis et al., 2016).

Chapter 3: Pathway-Specific Neuroadaptations in 5-HT_{2C}R Expression Associate with Incubation of Cocaine Cue Reactivity

Introduction

One of the major contributions to relapse to drugs of abuse is cue reactivity (Carter and Tiffany, 1999; O'Brien et al., 1998). Cue reactivity is the attentional orienting response to drug-associated stimuli that predict reward, and exposure to cocaine-associated cues includes physiological responses (e.g., elevated heart rate), subjective reactions (e.g., craving), appetitive approach behaviors (e.g., cocaine seeking) as well as neural circuit activation in humans (Carter and Tiffany, 1999; Field and Cox, 2008; Garavan et al., 2000; Maas et al., 1998). Neuroimaging studies in humans have begun to identify neural loci involved in cocaine cue reactivity. Activation of frontal cortices [dorsolateral PFC, ACC (Garavan et al., 2000; Maas et al., 1998)] and subcortical regions [caudate, thalamus (Garavan et al., 2000)] was increased in cocaine users exposed to cocaine cues as measured by fMRI. Cocaine-dependent individuals demonstrated increased activation of the VTA in response to cocaine-paired cues (Goudriaan et al., 2013). Exposure to cocaine-associated cues triggered activation of the ventral striatum (e.g., NAc) and correlated with measures of craving in abstinent cocaine-dependent participants (Bell et al., 2014). PET imaging revealed that cocaine cues evoked craving and activated frontal (lateral OFC, dorsolateral PFC) and limbic (amygdala, rhinal cortex) regions in cocaine-dependent individuals (Bonson et al., 2002). These regions closely correspond with those identified by behavioral and pharmacological experiments in rodent cocaine self-administration and cue reactivity models (Kalivas and Volkow, 2005; Pickens et al., 2011). Preclinical and clinical efforts have identified several

neurobiological mechanisms which modulate cocaine cue reactivity, but the regional specificity of these contributions remains to be fully elucidated, particularly with respect to some neurotransmitter systems (e.g., 5-HT).

Many of the regions involved in the response to cocaine-associated cues are innervated by 5-HT neurons projecting from the raphe nuclei (Di Matteo et al., 2008; Halliday and Tork, 1989; Herve et al., 1987). Serotonin acts through 14 receptor subtypes (Bockaert et al., 2006; Hoyer et al., 2002), of which the 5-HT_{2C}R has been strongly implicated in cocaine-related behaviors including cue reactivity (see (Cunningham and Anastasio, 2014). Systemic administration of 5-HT_{2C}R agonists has been shown to suppress cue- and cocaine-primed reinstatement following extinction from cocaine self-administration (Burbassi and Cervo, 2008; Cunningham et al., 2013; Cunningham et al., 2011; Fletcher et al., 2008; Higgins et al., 2015; Neisewander and Acosta, 2007). The 5-HT_{2C}R protein is found widely throughout the rat central nervous system and is densely expressed in frontal cortices [e.g., PFC (Abramowski et al., 1995; Anastasio et al., 2010; Clemett et al., 2000; Liu et al., 2007)], striatum [e.g., NAc (Abramowski et al., 1995; Clemett et al., 2000)], limbic regions [e.g., dorsal hippocampus (dHipp), amygdala (Abramowski et al., 1995; Clemett et al., 2000)], and VTA (Bubar and Cunningham, 2007; Bubar et al., 2011).

Intracranial pharmacological and biochemical studies have begun to explore the capacity of 5-HT_{2C}R activation within individual regions to modulate cocaine-related behaviors, including cue reactivity. The mPFC is the best-characterized in this regard; localized stimulation of the 5-HT_{2C}R in the mPFC suppressed cue- and cocaine-primed reinstatement following extinction from cocaine self-administration (Pentkowski et al.,

2010). Rats phenotypically identified as expressing the highest levels of cocaine cue reactivity in early abstinence also expressed the lowest levels of 5-HT_{2C}R protein in the mPFC (Anastasio et al., 2014a) and a blunted sensitivity to the suppressive effects of the selective 5-HT_{2C}R agonist WAY163909 on cocaine cue reactivity (Anastasio et al., 2014a). In Chapter 2, we demonstrated that incubation of cocaine cue reactivity is accompanied by lower potency of the selective 5-HT_{2C}R agonist WAY163909 to suppress cue reactivity and a shift in the subcellular localization profile of the mPFC 5-HT_{2C}R protein. Additionally, virally-mediated knockdown of the 5-HT_{2C}R in the mPFC increased cocaine cue reactivity in rats (Anastasio et al., 2014b). The 5-HT_{2C}R localized to the NAc also modulates cocaine cue reactivity; higher cocaine cue reactivity correlated with lower 5-HT_{2C}R protein expression in the NAc, and selective knockdown of the 5-HT_{2C}R in the NAc shell elevated cocaine cue reactivity in abstinence from cocaine self-administration (Cunningham laboratory, unpublished observations). The effect of 5-HT_{2C}R activation within the VTA on cocaine cue reactivity has not yet been explored, but localized administration of the 5-HT_{2C}R agonist Ro60-0175 attenuates cocaine-evoked hyperactivity and the reinforcing properties of cocaine (Fletcher et al., 2004). The 5-HT_{2C}R in the VTA is poised to regulate dopamine output to other regions (e.g., NAc) (Navailles et al., 2008) and thus may also govern cocaine cue reactivity (Ikemoto and Panksepp, 1999). The impact of 5-HT_{2C}R activation in the OFC has not been determined; however, there was a trend of systemic pretreatment with the 5-HT_{2C}R agonist Ro60-0175 to prevent the cocaine-evoked increase in mRNA expression of the immediately early gene *zif268* in the OFC (Burton et al., 2013), which provides indirect evidence that 5-HT_{2C}R expression within this region may regulate the behavioral and/or biochemical

effects of cocaine. The 5-HT_{2C}R is expressed in the dHipp but its role in this region is entirely unexplored. The dHipp is known to mediate cocaine-seeking behavior elicited by contextual cues (Fuchs et al., 2007), and it is conceivable that the 5HT_{2C}R may contribute to this effect. Overall, the neuroanatomically distinct effect of 5-HT_{2C}R activation on cocaine-related behaviors, including cocaine cue reactivity, is underappreciated.

The cocaine self-administration and forced abstinence model was employed to interrogate the association between cue reactivity levels and 5-HT_{2C}R protein expression in distinct brain regions. In this model, rats exhibit a time-dependent increase in cue reactivity (lever presses reinforced by the discrete drug-paired cue complex; "incubation") exceeding two months in abstinence from self-administration of cocaine (Grimm et al., 2001; Neisewander et al., 2000) or sucrose (Grimm et al., 2006; Grimm et al., 2002). Incubation of cocaine cue reactivity is associated with molecular neuroadaptations in neurotransmitter receptors [e.g., AMPA receptor (Conrad et al., 2008)], neurotrophic factors [e.g., BDNF (Whitfield et al., 2011)] and signal transducers [e.g., $ERK_{1/2}$ (Koya et al., 2009; Lu et al., 2005)] within nodes of the limbic-corticostriatal circuit including the mPFC (Koya et al., 2009; Ma et al., 2014; Whitfield et al., 2011), NAc (Conrad et al., 2008; Lu et al., 2003; Ma et al., 2014; Terrier et al., 2015), VTA (Lu et al., 2003; Lu et al., 2009), and amygdala (Lu et al., 2005; Lu et al., 2007). In particular, phosphorylation of $ERK_{1/2}$ ($pERK_{1/2}$) has surfaced as a molecular indicator of regions involved in the incubation of cue reactivity (Koya et al., 2009; Lu et al., 2005; Lu et al., 2006; Lu et al., 2007; Whitfield et al., 2011). The present study surveyed 5-HT_{2C}R protein expression following early vs. prolonged forced abstinence from cocaine self-administration within neurocircuitry known to contribute to cocaine cue reactivity. We

tested the hypothesis that the incubation of cocaine cue reactivity would associate with altered 5-HT_{2C}R expression selectively within specific areas of the brain. Expression of 5-HT_{2C}R was sampled across regions where the 5-HT_{2C}R has been strongly implicated (PFC) or predicted (VTA) to control cue reactivity as well as regions involved in cue reactivity but where 5-HT_{2C}R activation as a potential modulator of cue reactivity is unexplored (OFC, dHipp). In the VTA, which expressed lower 5-HT_{2C}R protein at prolonged abstinence from cocaine self-administration, we further examined the association between 5-HT_{2C}R protein levels and sucrose cue reactivity (control) as well as expression of $pERK_{1/2}$ protein given its role as a downstream effector of 5-HT_{2C}R signaling (Raymond et al., 2001; Werry et al., 2005; Werry et al., 2008) and an indicator of a neural locus of cue reactivity (Koya et al., 2009; Lu et al., 2005; Lu et al., 2006; Lu et al., 2007; Whitfield et al., 2011). These studies suggest alterations in 5-HT_{2C}R protein expression within the mesocorticoaccumbens pathway may be a novel modulator of the incubation of cocaine cue reactivity.

METHODS

Animals

Male Sprague-Dawley rats (n=71), Harlan, Inc., Houston, TX) weighing 250-325 g at the start of experiments were used. Rats were acclimated for seven days to a colony room maintained at a constant temperature (21-23°C) and humidity (45-50%) on a 12 hour light-dark cycle (lights on 0600-1800 h). Rats were housed two/cage and handled daily throughout the study. Food and water were available *ad libitum*. All experiments were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (2011) and with approval from the University of Texas Medical Branch Institutional Animal Care and Use Committee.

Drugs

(−)-Cocaine (National Institute on Drug Abuse, Research Triangle Park, NC) was dissolved in 0.9% NaCl.

Self-Administration Apparatus

Both cocaine and sucrose self-administration studies employed standard operant conditioning chambers (Med-Associates, Inc., St. Albans, VT, USA) housed in ventilated, sound-attenuating cubicles with fans (Med-Associates, Inc.). Each chamber was outfitted with two retractable response levers, a stimulus light above each response lever, a houselight opposite the levers, and a magazine-type pellet dispenser. The cocaine infusions were delivered via syringes attached to infusion pumps (Med Associates, Inc.) located outside the cubicles. The infusion pumps were connected to liquid swivels

(Instech, Plymouth Meeting, PA, USA) that were fastened to the catheters via polyethylene 20 tubing encased inside a metal spring leash (Plastics One, Roanoke, VA). Sucrose pellets (45 mg; Bio-Serv, Frenchtown, NJ, USA) were delivered into a pellet receptacle located between the two levers.

Cocaine self-administration and cue reactivity analysis

Implantations of intravenous catheters with back mounts were performed under anesthesia with a cocktail containing 8.6 mg/kg of xylazine, 1.5 mg/kg of acepromazine, and 43 mg/kg of ketamine in bacteriostatic saline as described previously (Anastasio et al., 2014a; Anastasio et al., 2014b; Cunningham et al., 2013; Cunningham et al., 2011; Nic Dhonnchadha et al., 2009). Catheter patency was maintained by daily flushes with a solution of 0.1 mL of bacteriostatic saline containing heparin sodium (10 U/mL; American Pharmaceutical Partners, East Schaumburg, IL), streptokinase (0.67 mg/mL; Sigma Chemical), and ticarcillin disodium (66.67 mg/mL; Research Products International, Mt. Prospect, IL) immediately following daily cocaine self-administration sessions. Rats recovered for at least five days prior to cocaine self-administration training.

Cocaine self-administration training consisted of 14 daily 180-min sessions during which rats (n=39) were trained to lever press for cocaine infusions (0.75 mg/kg/0.1 mL infusion) (Anastasio et al., 2014a; Anastasio et al., 2014b; Cunningham et al., 2013; Cunningham et al., 2011). Schedule completions on the active lever resulted in delivery of a cocaine infusion over a 6-sec period paired simultaneously with illumination of the house and stimulus lights and activation of the infusion pump (discrete cue

complex paired with cocaine delivery); inactive lever presses produced no scheduled consequences. Following reinforcer delivery, the stimulus light and the infusion pump were inactivated; the house light remained on for an additional 20 sec to indicate a timeout period during which lever presses had no scheduled consequences. Rats were trained on a fixed ratio (FR) 1 schedule of reinforcement and progressed to an FR5 schedule after achieving seven infusions/hr with less than 10% variability for three consecutive days. Upon achieving stability on the FR5 schedule (less than 10% variability for a minimum of three consecutive days), rats were pseudorandomly assigned to either FA Day 1 or FA Day 30, and returned to their home cages for the appropriate FA period.

At the designated FA period, rats were assessed in a 60-min cue reactivity test session ("cue test" rats) in which presses on the previously-active lever were reinforced by the discrete cue complex (stimulus light illuminated, infusion pump activated) on an FR1 schedule; presses on the inactive lever were recorded but produced no scheduled consequences. Rats were killed immediately upon removal from the cue reactivity session on FA Day 1 or FA Day 30 for *ex vivo* biochemical analyses.

Sucrose self-administration and cue reactivity analyses

Sucrose self-administration training consisted of 14 daily 180-min sessions during which freely-fed rats (n=32) were trained to lever press for 45 mg sucrose pellets (Bio-Serv, Frenchtown, NJ) (Cunningham et al., 2011). Experimental parameters were identical to those employed in cocaine self-administration and cue reactivity analyses except that sucrose was substituted as the reinforcer. Rats were killed immediately upon

removal from the cue reactivity session on FA Day 1 or FA Day 30 for *ex vivo* biochemical analyses.

Protein Analyses

The 5-HT_{2C}R protein expression levels were assessed using Western blot analyses on tissue harvested from FA Day 1 or FA Day 30 rats. Rats were anesthetized (400 mg/kg chloral hydrate solution), decapitated, and brains were cut in 2 mm coronal sections, rapidly microdissected with a scalpel on a cool tray (4°C) (Heffner et al., 1980), frozen in liquid nitrogen and stored at -80°C.

A crude synaptosomal protein fraction of the mPFC enriched for pre- and postsynaptic proteins [i.e., presynaptic terminals, postsynaptic membranes, postsynaptic density, synaptic protein complexes (Breukel et al., 1997)] was prepared as described previously (Anastasio et al., 2010; Liu et al., 2007). The mPFC tissues were homogenized in 10 times w/v ice cold Krebs buffer (125 mM NaCl, 1.2 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 22 mM Na_2CO_3, 1 mM NaH_2PO_4, 10 mM glucose) containing 0.32 M sucrose plus protease inhibitor cocktail and phosphatase inhibitor 2 and 3 cocktails (10 μL/mL; Sigma-Aldrich, St. Louis, MO). The homogenate was centrifuged at 1000 g for 10 min at 4°C to pellet the nuclear fraction. The supernatant was collected and centrifuged at 16,000 g for 20 min at 4°C to pellet the crude synaptosome. The pellet was re-suspended in Krebs buffer with 1% dodecyl maltoside.

Crude plasma membrane, cytoplasmic, and nuclear protein fractions of the OFC, dHipp, and VTA were prepared (Anastasio et al., 2013; Anastasio et al., 2014b). The crude membrane fraction captures membrane-associated proteins throughout the plasma

membrane and membranous organelles (e.g., mitochondria). The crude cytoplasmic fraction encompasses a number of intracellular organelles (except the nucleus) as well as the cytosol. Tissue was homogenized in 10 times w/v extraction buffer (10 mM HEPES, 1 mM EDTA, 2 mM EGTA, 1 mM DTT) plus protease inhibitor cocktail and phosphatase inhibitor 2 and 3 cocktails (10 μL/mL). The homogenate was centrifuged at 1000 g for 10 min at 4°C to pellet the nuclear fraction. The supernatant was collected and centrifuged at 20,000 g for 30 min at 4°C to pellet the membrane-bound enriched protein fraction. The cytoplasmic fraction was collected and reserved. The nuclear and membrane-enriched pellets were washed once and resuspended in buffer [(20 mM HEPES, 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, protease inhibitor cocktail and phosphatase inhibitor 2 and 3 cocktails (10 μL/mL)] plus 1% dodecyl maltoside.

Equal amounts of protein were reduced with Laemmli sample buffer and heated for 20 min at 70°C then separated by SDS-PAGE using 10% Bis-Tris gels (Invitrogen, San Diego, CA) for 2-3 hrs at 110V. Proteins were transferred to a PVDF membrane (BioRad, Hercules, CA) via a wet-transfer electroblotting apparatus (BioRad) overnight at 60-70V (Anastasio et al., 2010). Membranes were blocked with Odyssey blocking buffer [LI-COR® Biosciences; 1:1 in Tris Buffered Saline (TBS), pH 7.4] followed by incubation with primary antibody [mouse monoclonal 5-HT_{2C}R (D-12, sc-17797, Santa Cruz; 1:100), mouse monoclonal pan-cadherin (ab6528, Abcam; 1:5000); rabbit polyclonal $ERK_{1/2}$ (9102, Cell Signaling; 1:500), mouse monoclonal $pERK_{1/2}$ (9106, Cell Signaling; 1:250)] (Anastasio et al., 2010; Anastasio et al., 2015; Fink, 2015). Membranes were rinsed in TBS + 0.1% Tween-20 (TBS-T), incubated with secondary

antibody [infrared-labeled goat anti-mouse IRDye™800CW (926-32210) or IRDye™680RD (926-68070; LI-COR® Biosciences, Lincoln, NE, 1:10000)], then rinsed in TBS-T. PVDF membranes were imaged using the Odyssey® Infrared Imaging System (LI-COR® Biosciences, Lincoln, NE). The integrated intensity of 5-HT_{2C}R immunoreactive bands normalized to the housekeeping protein cadherin and $pERK_{1/2}$ immunoreactive bands normalized to total $ERK_{1/2}$ were analyzed with the Odyssey® software.

Statistical analyses

Student's t-test was used to analyze total intake during acquisition and maintenance of cocaine or sucrose self-administration. A one-way ANOVA was used to analyze previously-active and inactive lever presses and latency to the first response on FA Day 1 *vs.* FA Day 30 following cocaine or sucrose self-administration. Student's t-test was used to analyze synaptosomal or membrane 5-HT_{2C}R protein expression and nuclear and cytoplasmic $pERK_{1/2}/ERK_{1/2}$ protein expression. The experiment-wise error rate for all analyses was set at α=0.05.

Results

Cue reactivity incubates during forced abstinence from cocaine self-administration

Rats readily acquired cocaine self-administration (0.75 mg/kg/0.1 mL infusion) to stability (i.e., seven infusions/hr on an FR5 schedule for at least three sessions) and displayed <10% variation in the number of infusions earned during the maintenance sessions. There was no difference in total cocaine intake across the self-administration phase between rats assigned to FA Day 1 (365.3 ± 19.9 mg/kg) or FA Day 30 (374.4 ± 9.7 mg/kg; $t_{1,37}$=0.18; n.s.). Cue reactivity (measured as previously-active lever presses reinforced by the discrete cue complex) was significantly elevated on FA Day 30 *vs.* FA Day 1 from cocaine self-administration (**Fig. 3.1a**; $F_{1,37}$=24.33; p<0.05), consistent with previous observations that cue reactivity incubates during forced abstinence from cocaine self-administration (Grimm et al., 2001; Neisewander et al., 2000). Inactive lever presses did not differ on FA Day 1 or FA Day 30 (**Fig. 3.1a**; $F_{1,37}$=2.18; n.s.). The latency to first press (mean ± SEM) did not differ on FA Day 1 (32.4 ± 10.9) vs. FA Day 30 (21.0 ± 4.9; $F_{1,37}$=1.18; n.s.). These data are reanalyzed and represented from Chapter 2.

Cue reactivity incubates during forced abstinence from sucrose self-administration

Rats readily acquired sucrose self-administration (45 mg pellet) to stability and displayed <10% variation in the number of pellets earned during the maintenance sessions. There was no difference in total sucrose intake across the self-administration phase between rats assigned to FA Day 1 (19.9 ± 2.1 g) or FA Day 30 (21.0 ± 2.2 g; $t_{1,30}$=0.13; n.s.). A main effect of FA Day ($F_{1,16}$=7.40; p<0.05) was observed for previously active lever presses (mean ± SEM) on the cue reactivity test session.

Previously-active lever presses (mean ± SEM) were significantly elevated on FA Day 30 *vs.* FA Day 1 from sucrose self-administration (**Fig. 3.1b**), consistent with previous observations that cue reactivity incubates during forced abstinence from sucrose self-administration (Grimm et al., 2006; Grimm et al., 2002). No main effect of FA Day was observed for inactive lever presses (**Fig. 3.1b;** $F_{1,16}$=1.58; n.s.). No main effect of FA Day was observed for the latency to the first press ($F_{1,16}$=0.88; n.s). The latency to first press (mean ± SEM) was 20.8 ± 8.4 sec on FA Day 1 and 13 ± 3.1 sec on FA Day 30 from sucrose self-administration. These data are represented from Chapter 2.

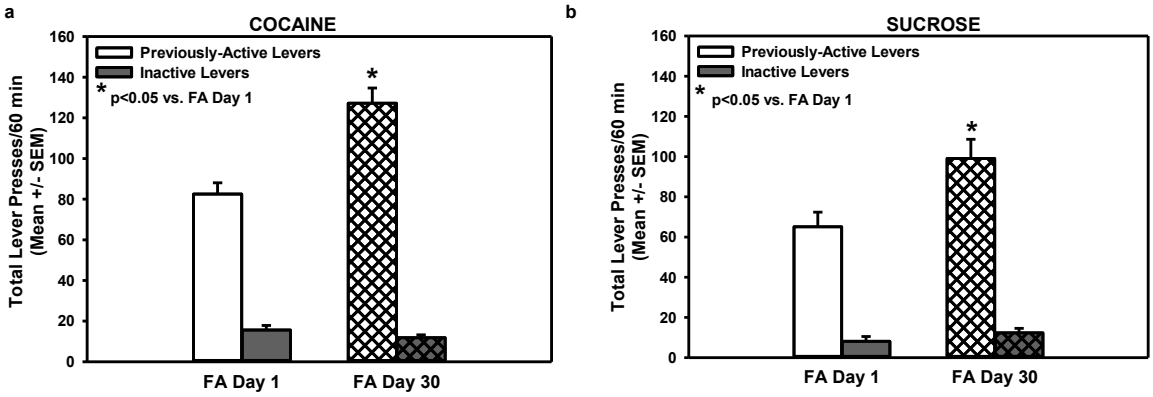

Figure 3.1: Cue reactivity incubates during forced abstinence from cocaine and sucrose self-administration.

(a) Mean (± SEM) previously-active lever presses and inactive lever presses are presented for the cue reactivity test session in vehicle-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=19-20/group). (b) Mean (± SEM) previously-active lever presses and inactive lever presses are presented for the cue reactivity test session on FA Day 1 and FA Day 30 from sucrose self-administration. Cue reactivity is significantly elevated on FA Day 30 *vs.* FA Day 1 from sucrose self-administration (**p*<0.05; n=8-10/group). These data are replotted from Chapter 2.

Incubation of cocaine cue reactivity associates with lower membrane 5-HT_{2C}R protein expression in the mPFC and VTA, but not the OFC or dHipp

We tested the hypothesis that incubation of cocaine cue reactivity is associated with differential 5-HT_{2C}R protein expression in various regions of the brain, including the mPFC, OFC, dHipp, and VTA. Analyses of membrane 5-HT_{2C}R protein expression in the mPFC (**Fig. 3.2**), OFC (**Fig. 3.3**), dHipp (**Fig. 3.4**), and VTA (**Fig. 3.5**) are presented. **Fig. 3.2a** shows a diagram of the mPFC region dissected for analyses (Paxinos and Watson, 1998). Synaptosomal 5-HT_{2C}R protein expression in the mPFC (**Fig. 3.2b, inset**) was significantly lower at FA Day 30 *vs.* FA Day 1 from cocaine self-administration (**Fig. 3.2b**; $t_{1,14}$=5.76; p<0.05); these data are represented from Chapter 2. **Fig. 3.3a** shows a diagram of the OFC region dissected for analyses (Paxinos and Watson, 1998). Membrane 5-HT_{2C}R protein expression in the OFC (**Fig. 3.3b, inset**) did not differ between FA Day 1 and FA Day 30 from cocaine self-administration (**Fig. 3.3b**; $t_{1,6}$=1.44; n.s.). **Fig. 3.4a** shows a diagram of the dHipp region dissected for analyses (Paxinos and Watson, 1998). Membrane 5-HT_{2C}R protein expression in the dHipp (**Fig. 3.4b, inset**) did not differ between FA Day 1 and FA Day 30 from cocaine self-administration (**Fig. 3.4b**; $t_{1,8}$=0.65; n.s.). **Fig. 3.5a** shows a diagram of the VTA region dissected for analyses (Paxinos and Watson, 1998). Membrane 5-HT_{2C}R protein expression in the VTA (**Fig. 3.5b, inset**) was significantly lower at FA Day 30 *vs.* FA Day 1 from cocaine self-administration (**Fig. 3.5b**; $t_{1,4}$=20.42; p<0.05). These data suggest lower 5-HT_{2C}R expression selectively within the mPFC and VTA may contribute to the incubation of cocaine cue reactivity.

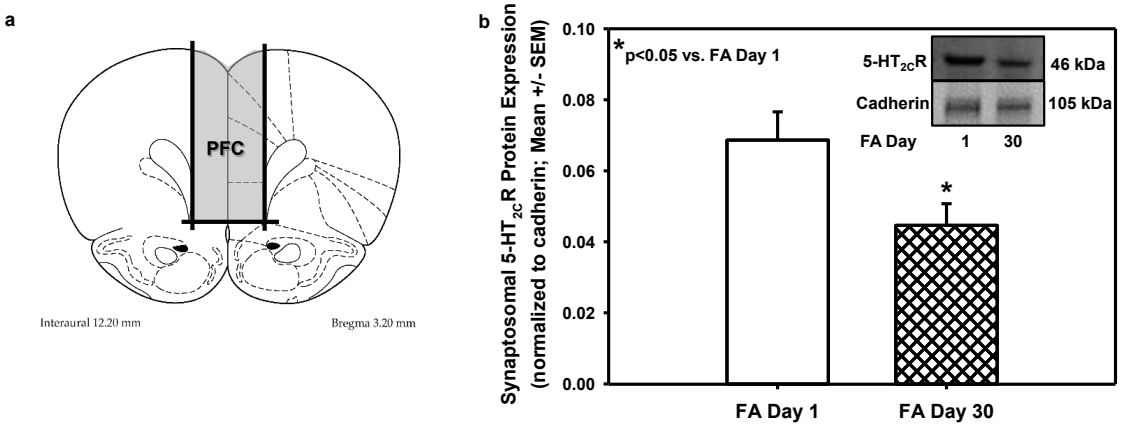

Figure 3.2: Incubation of cocaine cue reactivity associates with lower membrane 5-HT_{2C}R protein expression in the mPFC.

(a) The diagram demonstrates the region of mPFC dissected for biochemical analyses (Paxinos and Watson, 1998). (b) Synaptosomal mPFC 5-HT_{2C}R protein expression was significantly lower on FA Day 30 *vs.* FA Day 1 from cocaine self-administration *(*p*<0.05*;* n=8/group); a representative immunoblot is located in inset. These data are replotted from Chapter 2.

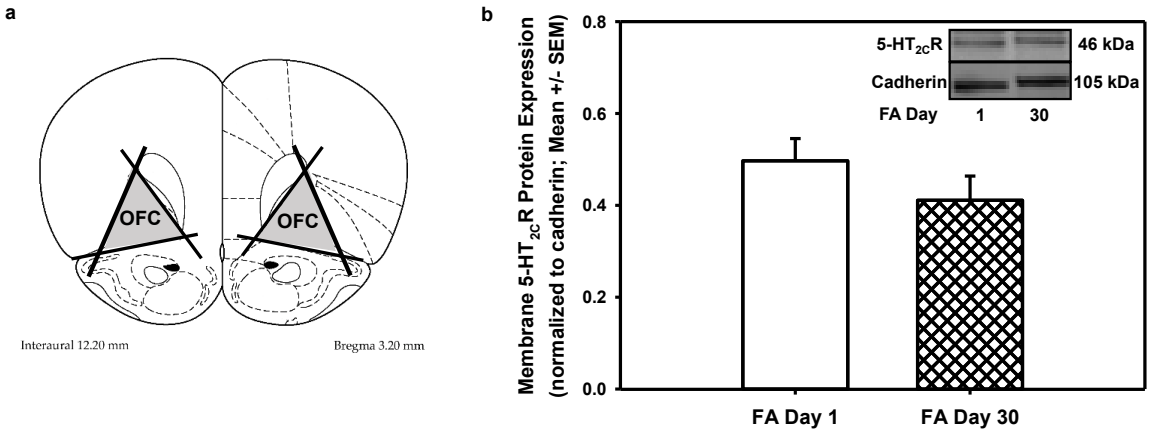

Figure 3.3: Incubation of cocaine cue reactivity did not associate with membrane 5-HT_{2C}R protein expression in the OFC.

(a) The diagram demonstrates the region of OFC dissected for biochemical analyses (Paxinos and Watson, 1998). (b) Membrane OFC 5-HT_{2C}R protein expression did not differ on FA Day 1 *vs.* FA Day 30 from cocaine self-administration (n.s.; n=4/group); a representative immunoblot is located in inset.

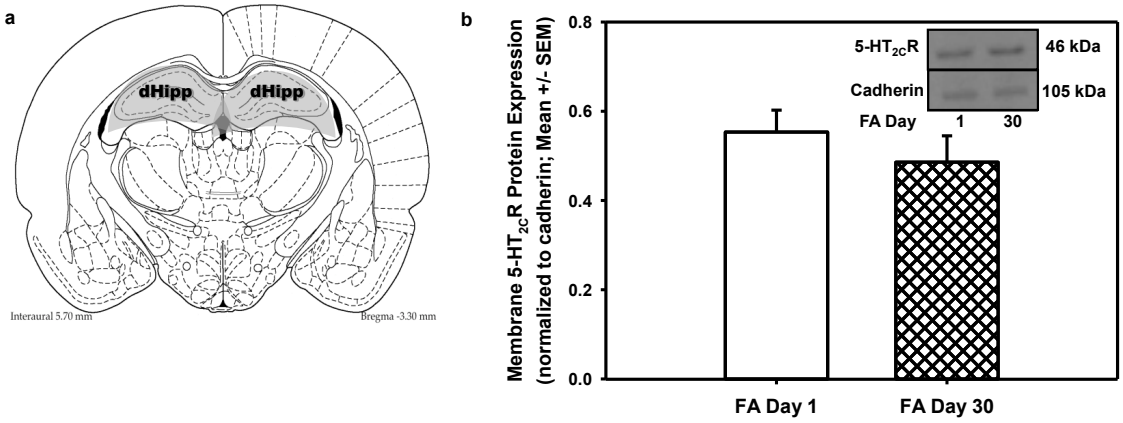

Figure 3.4: Incubation of cocaine cue reactivity did not associate with membrane 5-HT_{2C}R protein expression in the dHipp.

(a) The diagram demonstrates the region of dHipp dissected for biochemical analyses (Paxinos and Watson, 1998). (b) Membrane dHipp 5-HT_{2C}R protein expression did not differ on FA Day 1 *vs.* FA Day 30 from cocaine self-administration (n.s.; n=4-6/group); a representative immunoblot is located in inset.

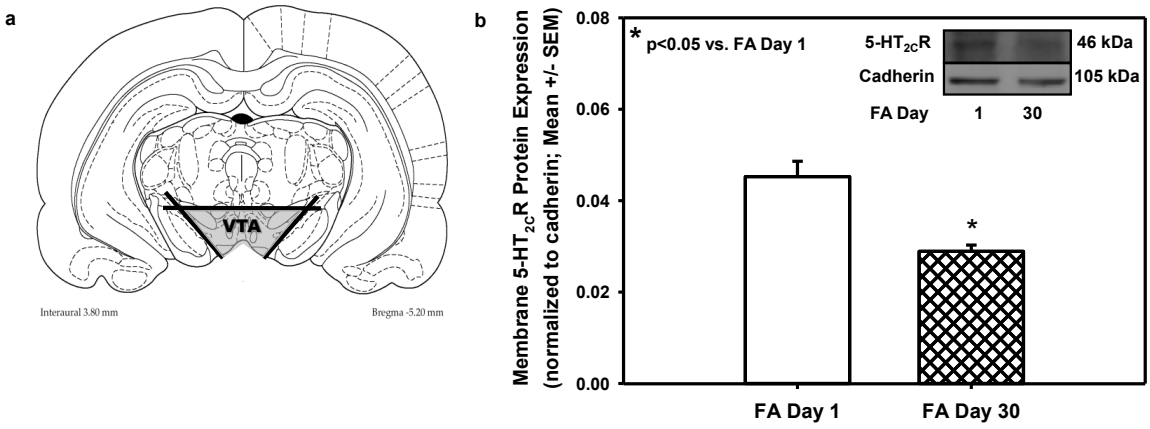

Figure 3.5: Incubation of cocaine cue reactivity associates with lower membrane 5-HT_{2C}R protein expression in the VTA.

(a) The diagram demonstrates the region of VTA dissected for biochemical analyses (Paxinos and Watson, 1998). (b) Membrane VTA 5-HT_{2C}R protein expression was significantly lower on FA Day 30 *vs.* FA Day 1 from cocaine self-administration *(*p*<0.05*;* n=3/group); a representative immunoblot is located in inset.

Incubation of cocaine cue reactivity associates with altered subcellular localization of phosphorylated extracellular signal-regulated kinase ($pERK_{1/2}$) in the VTA

Activation of ERK through undefined pathways in the mPFC (Koya et al., 2009) and amygdala (Lu et al., 2005) is critical for the incubation of cocaine cue reactivity. Impaired neuronal signal transduction in the VTA through the $ERK_{1/2}$ pathway may contribute to the incubation of cocaine cue reactivity; however, lower membrane 5-HT_{2C}R protein expression as mediator of this effect is unknown. Here, we tested the hypothesis that that incubation of cocaine cue reactivity is associated with differential subcellular localization of $pERK_{1/2}$ concomitant with lower 5-HT_{2C}R expression. Nuclear $pERK_{1/2}$ protein expression in the VTA (**Fig. 3.6, right**) was significantly higher at FA Day 30 vs. FA Day 1 from cocaine self-administration (**Fig. 3.6**; $t_{1,8}$=6.20; p<0.05). Cytoplasmic $pERK_{1/2}$ protein expression in the VTA (**Fig. 3.6, right**) did not differ between FA Day 1 and FA Day 30 from cocaine self-administration (**Fig. 3.6**; $t_{1,8}$=0.23; n.s.). Together, these data support the VTA as a locus for cocaine cue reactivity and suggest lower VTA 5-HT_{2C}R protein expression concomitant with altered $pERK_{1/2}$ subcellular localization may be a neurobiological driver of the incubation phenomenon.

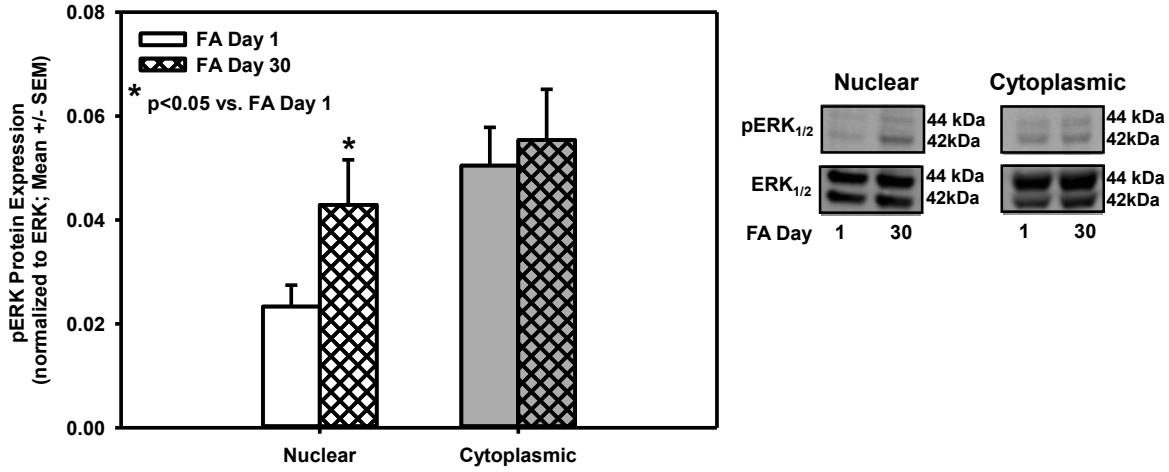

Figure 3.6: Incubation of cocaine cue reactivity associates with higher pERK$_{1/2}$ expression in the nuclear, but not cytoplasmic compartment, in the VTA.

Nuclear pERK$_{1/2}$ expression in the VTA is significantly lower on FA Day 30 *vs.* FA Day 1 from cocaine self-administration (*p<0.05*;* n=5-6/group). Cytoplasmic pERK$_{1/2}$ expression in the VTA did not differ between FA Day 1 and FA Day 30 from cocaine self-administration (n.s.; n=5-6/group). Representative immunoblots are located to the right.

Incubation of sucrose cue reactivity does not associate with membrane 5-HT$_{2C}$R protein expression in the VTA

We tested the hypothesis that the incubation of cue reactivity in rats previously trained to self-administer sucrose would be independent of the 5-HT$_{2C}$R protein expression profile in the VTA given that 5-HT$_{2C}$R signaling does not appear to be an important neurochemical mediator of cue-evoked reinstatement of sucrose-seeking (Burbassi and Cervo, 2008; Cunningham et al., 2013; Cunningham et al., 2011; Fletcher et al., 2008; Neisewander and Acosta, 2007). We previously demonstrated the incubation of sucrose cue reactivity did not associate with synaptosomal 5-HT$_{2C}$R expression in the

mPFC (Chapter 2). Membrane 5-HT_{2C}R protein expression in the VTA (**Fig. 3.7, right**) did not differ between FA Day 1 and FA Day 30 from sucrose self-administration (**Fig. 3.7**; $t_{1,8}$=0.26; n.s.). These data suggest that differential membrane 5-HT_{2C}R expression in the VTA on FA Day 30 *vs*. FA Day 1 is specific to incubation from cocaine, but not sucrose, cue reactivity.

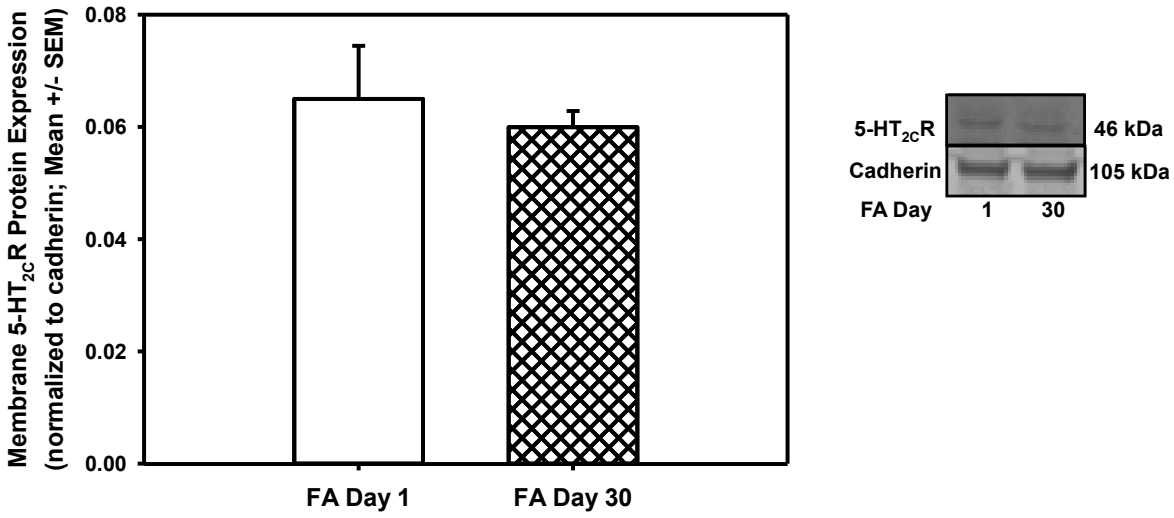

Figure 3.7: Incubation of sucrose cue reactivity did not associate with membrane 5-HT_{2C}R protein expression in the VTA.

Membrane VTA 5-HT_{2C}R protein expression did not differ on FA Day 1 *vs.* FA Day 30 from sucrose self-administration (n.s.; n=5/group); a representative immunoblot is located to the right.

Discussion

The present studies demonstrate that incubation of cocaine cue reactivity associates with lower membrane 5-HT_{2C}R expression in the mPFC and VTA. This difference in 5-HT_{2C}R expression appears to be specific to certain neural loci given that other brain regions (OFC, dHipp) did not demonstrate any association between 5-HT_{2C}R protein expression and the incubation of cocaine cue reactivity. Activation of $ERK_{1/2}$ was higher in the nuclear, but not cytoplasmic, compartment of the VTA in prolonged abstinence from cocaine self-administration, further implicating the VTA as a critical region which mediates the incubation phenomenon. These observations highlight the putative role of altered 5-HT_{2C}R protein expression in the mesocorticoaccumbens neurocircuitry in the incubation of cocaine cue reactivity.

The observations presented herein suggest that 5-HT_{2C}R expression within the mPFC and VTA may contribute to the incubation of cocaine cue reactivity. Importantly, lower 5-HT_{2C}R expression in the VTA was selectively observed in the incubation of cocaine, but not sucrose, cue reactivity, which recapitulates our results from the mPFC (Chapter 2) and corroborates prior reports which have identified distinct mechanisms for cocaine vs. sucrose cue reactivity (Counotte et al., 2014; Koya et al., 2009; Lu et al., 2003). In rats phenotypically evaluated for cue reactivity in early abstinence, we found that the highest levels of cocaine cue reactivity were associated with the lowest levels of 5-HT_{2C}R protein expression in the mPFC (Anastasio et al., 2014a) and NAc (Cunningham laboratory, unpublished observations). Additionally, cocaine cue reactivity was increased in rats with virally-mediated knockdown of the 5-HT_{2C}R in the mPFC (Anastasio et al., 2014b) or NAc shell (Cunningham laboratory, unpublished

observations). Together, these data implicate the 5-HT_{2C}R localized selectively within mesocorticoaccumbens circuit in the regulation of cocaine cue reactivity. We found no difference in membrane 5-HT_{2C}R expression in the OFC or dHipp at prolonged vs. early forced abstinence from cocaine self-administration, which further supports the conclusion that altered 5-HT_{2C}R protein expression occurs in specific neurocircuitry rather than ubiquitously throughout the CNS. Our survey was not intended to be exhaustive, and it is certainly possible that the incubation of cue reactivity may associate with altered 5-HT_{2C}R expression within other regions (e.g., amygdala) or subregions (e.g., ventral Hipp) implicated in cocaine-related behaviors; in fact, current efforts in the laboratory have shown that 5-HT_{2C}R expression in the ventral Hipp modulates cocaine-related behaviors (Cunningham laboratory, unpublished observation). An appreciation of how altered 5-HT_{2C}R protein expression in the PFC, NAc, and VTA integrate with other neurotransmitter systems (e.g., glutamate, dopamine) and affect function and signaling at the mesocorticoaccumbens pathway level is necessary.

The present study is the first to identify a potential role for the 5-HT_{2C}R in the VTA in the incubation of cocaine cue reactivity, which expands upon the cocaine-related behaviors known to be regulated by the 5-HT_{2C}R in this region. Intra-VTA microinfusion of the 5-HT_{2C}R agonist Ro 60-0175 has been shown to dose-dependently suppress cocaine-evoked hyperactivity as well as the reinforcing properties of cocaine (0.25 mg/kg/inf) under fixed and progressive ratio schedules of reinforcement in cocaine self-administration (Fletcher et al., 2004). The mesocorticolimbic dopamine pathway arises in the VTA and is well-established to mediate the rewarding properties of cocaine (Nestler, 2005; Ritz et al., 1987; Volkow et al., 2004). Intra-VTA administration of Ro60-0175

attenuates cocaine-evoked dopamine efflux measured in the NAc (Navailles et al., 2008), which is likely the mechanism underlying the suppression of cocaine-related behaviors by intra-VTA 5-HT_{2C}R activation (Fletcher et al., 2004). Under basal conditions, systemic administration of 5-HT_{2C}R agonists increased the firing of non-dopaminergic (presumably GABAergic) neurons in the VTA (Di Giovanni et al., 2001), decreased the firing of dopamine neurons in the VTA (Di Giovanni et al., 2000; Di Matteo et al., 2000; Gobert et al., 2000), and decreased dopamine release in the NAc (Di Giovanni et al., 2000; Di Matteo et al., 2000; Gobert et al., 2000) and the mPFC (Gobert et al., 2000). The 5-HT_{2C}R protein is expressed on both dopaminergic and GABAergic neurons within the VTA (Bubar and Cunningham, 2007; Bubar et al., 2011); however, the overall inhibitory impact of the 5-HT_{2C}R over VTA function is thought to occur via 5-HT_{2C}R-mediated depolarization of GABA interneurons that synapse onto dopaminergic neurons (Di Giovanni et al., 2001; Di Matteo et al., 2000; Theile et al., 2009). Loss of the 5-HT_{2C}R particularly on GABA neurons in the VTA during prolonged abstinence from cocaine self-administration may disinhibit dopamine output to regions, including the NAc and mPFC, to increase cue reactivity. Assessment of the functional capacity of the 5-HT_{2C}R (e.g., agonist-mediated signaling, RNA editing, alternative splicing) localized to GABAergic vs. dopaminergic neurons of the VTA may further resolve the impact of altered 5-HT_{2C}R expression on cocaine cue reactivity.

There is escalating interest in understanding how impaired signal transduction contributes to the long-term adaptations associated with cocaine exposure (Pickens et al., 2011), however, few studies have focused on understanding the signaling imbalances that drive the incubation of cue reactivity (Koya et al., 2009; Lu et al., 2004; Lu et al., 2005;

Whitfield et al., 2011) and none that have investigated 5-HT_{2C}R signaling in this light. Incubation of cocaine cue reactivity was associated with increased $pERK_{1/2}$ expression in the ventral mPFC (Koya et al., 2009) and central amygdala (Lu et al., 2005). Our present observation that $pERK_{1/2}$ expression is higher in the nuclear, but not cytoplasmic, compartment of the VTA during prolonged abstinence from cocaine self-administration complements these previous findings. The current study is the first to our knowledge to interrogate the subcellular distribution of $pERK_{1/2}$ expression in the incubation of cocaine cue reactivity. Phosphorylation of $ERK_{1/2}$ ($pERK_{1/2}$) is an important integrator of upstream signaling events for the 5-HT_{2C}R and can be mediated by agonist-dependent coupling of the 5-HT_2R to G proteins (Labasque et al., 2010; Werry et al., 2005; Werry et al., 2008) or to other protein transducers (e.g., β-arrestins; Labasque et al., 2008; Lefkowitz and Shenoy, 2005; Schmid et al., 2008). Activation of $ERK_{1/2}$ through G protein-dependent vs. β-arrestin-dependent pathways (i.e., "biased signaling" or "functional selectivity") may be characterized by different temporal dynamics, subcellular localization and functional consequences (reviewed in Eishingdrelo and Kongsamut, 2013; Kholodenko et al., 2010). The G protein-dependent pathway is characterized by rapid, transient phosphorylation of $ERK_{1/2}$ in the cytoplasmic and nuclear compartments (reviewed in Eishingdrelo and Kongsamut, 2013; Kholodenko et al., 2010) where it can promote gene transcription (Mattson et al., 2005; Miller and Marshall, 2005; Radwanska et al., 2005; Valjent et al., 2000); the β-arrestin-dependent pathway is characterized by slower, prolonged phosphorylation of $ERK_{1/2}$ and retention to the cytoplasm (reviewed in Eishingdrelo and Kongsamut, 2013; Kholodenko et al., 2010). Activation of $ERK_{1/2}$ can occur through a convergence of multiple receptor

systems (Valjent et al., 2005) and is influenced by a variety of cocaine-associated paradigms (for review, Lu et al., 2006); we cannot determine whether altered $pERK_{1/2}$ expression is a direct consequence of lower 5-HT_{2C}R protein expression using currently available *ex vivo* protein biochemistry methods. Still, it is intriguing to consider that lower membrane 5-HT_{2C}R protein expression may shift the net cellular balance of $pERK_{1/2}$ expression toward increased nuclear $pERK_{1/2}$, possibly via decreased β-arrestin-dependent vs. G protein-dependent $ERK_{1/2}$ activation, which could then promote gene transcription [e.g., via transcription factors like CREB or Elk1 (Mattson et al., 2005; Valjent et al., 2000) and induction of immediate early genes like *zif268* or *cFos* (Miller and Marshall, 2005; Radwanska et al., 2005)] to enhance the incubation of cocaine cue reactivity. Nonetheless, these findings assert altered $pERK_{1/2}$ expression in the VTA localized to distinct subcellular compartments is a novel contributor to the incubation of cocaine cue reactivity and suggest exciting new avenues (i.e., biased signaling using agonists which signal selectively through the G protein-dependent or β-arrestin-dependent pathway) for investigation.

Overall, these studies have identified that lower 5-HT_{2C}R protein expression in the VTA associates with incubation of cocaine cue reactivity. Future studies should explore lower 5-HT_{2C}R expression in the VTA as a neurobiological driver of underlying the incubation phenomenon. Ultimately, understanding the nuanced roles of the 5-HT_{2C}R and other receptors within specific neurocircuitry may provide insight into important relationships (e.g., serotonin-dopamine interactions) that could shed light on new treatment approaches (i.e., combination therapies) to suppress cue reactivity and promote abstinence.

Chapter 4: Exploration of 5-HT_{2C}R Expression in the Ventral Tegmental Area (VTA) as a Neurobiological Driver of the Behavioral Effects of Cocaine

Introduction

The mesocorticolimbic pathway, widely regarded as a common pathway for addiction to various drugs of abuse, contains dopaminergic projections which originate in the VTA and terminate in regions like the NAc and mPFC (for reviews, Kalivas and Volkow, 2005; Nestler, 2005; Volkow et al., 2004). Dopamine release from the VTA has been well-established to mediate cocaine-related behaviors in rats, including motor activity (Kelly and Iversen, 1976), cocaine-taking (Roberts and Koob, 1982) and cocaine-seeking behaviors (Di Ciano and Everitt, 2004; McFarland and Kalivas, 2001; See et al., 2007). Lesion of VTA dopamine neurons by injection of the neurotoxin 6-hydroxydopamine (6-OHDA) blocked motor hyperactivity elicited by acute experimenter-delivered cocaine (Kelly and Iversen, 1976), demonstrating that dopamine release from the VTA is required for the hyperlocomotive effect of cocaine. In rats previously trained to self-administer cocaine (0.75 mg/kg/inf) to stability, intra-VTA 6-OHDA lesions also reduced, and in some cases abolished, subsequent cocaine-taking (Roberts and Koob, 1982). Pharmacological inactivation of the VTA by microinfusion of the GABA agonists baclofen and muscimol inhibited the reinstatement of cocaine-seeking evoked by an acute priming injection of cocaine (McFarland and Kalivas, 2001), attenuated responding for cocaine-associated conditioned cues under a second-order schedule of reinforcement (Di Ciano and Everitt, 2004), and suppressed lever-pressing

upon reintroduction to the cocaine-paired context following 14 days of forced abstinence from cocaine self-administration (See et al., 2007). Dopaminergic output from the VTA and related behavioral effects of cocaine are controlled by a multitude of factors, including interaction with the serotonergic system.

Serotonin acts through 14 receptor subtypes (Bockaert et al., 2006; Hoyer et al., 2002), of which the 5-HT_{2C}R has emerged as a neurobiological regulator of various behaviors related to cocaine administration in rodent models (reviewed in Bubar and Cunningham, 2008; Cunningham and Anastasio, 2014). Activation of the 5-HT_{2C}R by systemic agonist administration consistently decreased the hyperlocomotive effects of acute cocaine (Cunningham et al., 2013; Filip et al., 2004; Grottick et al., 2000; Pockros et al., 2012), whereas blockade of the 5-HT_{2C}R by administration of an antagonist (Filip et al., 2004; Fletcher et al., 2002) or an inverse agonist (McCreary and Cunningham, 1999) increased hyperactivity evoked by cocaine. In rats trained to self-administer cocaine, 5-HT_{2C}R agonists suppressed cocaine-taking maintained on fixed ratio (Cunningham et al., 2011; Grottick et al., 2000) or progressive ratio (Fletcher et al., 2008; Grottick et al., 2000) schedules of reinforcement; the selective 5-HT_{2C}R antagonist SB242084 increased progressive ratio responding for cocaine infusions only at low unit doses (i.e., 0.0625 or 0.125 mg/kg/inf) (Fletcher et al., 2002). Systemic administration of 5-HT_{2C}R agonists has been shown to attenuate cue- and cocaine-primed reinstatement of cocaine-seeking behavior following extinction from cocaine self-administration (Burbassi and Cervo, 2008; Cunningham et al., 2013; Cunningham et al., 2011; Fletcher et al., 2002; Fletcher et al., 2008; Grottick et al., 2000; Higgins et al., 2015; Neisewander and Acosta, 2007). Recently, we demonstrated that the selective 5-HT_{2C}R agonist

WAY163909 also suppressed cue reactivity in forced abstinence from cocaine self-administration (Anastasio et al., 2014a), Chapter 2). In 5-HT_{2C}R knockout mice, basal motor activity, cocaine-evoked hyperactivity, and progressive ratio responding for cocaine self-administration were increased relative to wild-type mice (Rocha et al., 2002), which aligns with the outcomes of studies that employed 5-HT_{2C}R ligands. Overall, these findings support that 5-HT_{2C}R activity exerts inhibitory control over cocaine-related behaviors, but delineating the contribution of the 5-HT_{2C}R in specific regions of the brain, like the VTA, improves our understanding of the interactions between neurotransmitter systems.

The 5-HT_{2C}R protein is localized to dopaminergic and GABAergic neurons in the VTA (Bubar and Cunningham, 2007; Bubar et al., 2011); however, the overall neurochemical and behavioral effects of 5-HT_{2C}R activation or inhibition within the VTA are consistent with expression on GABAergic interneurons. Elegant electrophysiology and microdialysis experiments have characterized the effect of 5-HT_{2C}R activity in the VTA on neuronal firing and dopamine release. Systemic administration of a 5-HT_{2C}R agonist increased the basal firing rate of non-dopaminergic (presumably GABAergic) neurons in the VTA, and this effect is blocked by pretreatment with the selective 5-HT_{2C}R antagonist SB242084 (Di Giovanni et al., 2001), indicating that that 5-HT_{2C}R activity mediates the changes in neuronal firing. Systemic administration of 5-HT_{2C}R agonists also decreased the firing rate of dopaminergic neurons in the VTA (Di Giovanni et al., 2000; Di Matteo et al., 2000; Gobert et al., 2000) which corresponded with decreased dopamine release in the NAc (Di Giovanni et al., 2000; Di Matteo et al., 2000; Gobert et al., 2000) and frontal cortex (Gobert et al., 2000); pretreatment with SB242084

blocked each of these effects (Di Giovanni et al., 2000; Di Matteo et al., 2000). Interestingly, the 5-HT_{2C}R inverse agonist SB206553 increased dopamine release in the NAc and frontal cortex and increased dopamine neuron firing, especially burst (i.e, phasic) firing, in the VTA (Gobert et al., 2000). Intra-VTA administration of the preferential 5-HT_{2C}R agonist Ro60-0175 attenuated dopamine outflow measured in the NAc following an acute experimenter-delivered injection of cocaine (Navailles et al., 2008); accordingly, cocaine-related behaviors also appear to be regulated by activation of the 5-HT_{2C}R expressed in the VTA. Pharmacological activation or inhibition of the 5-HT_{2C}R in the VTA does not alter basal motor activity (Fletcher et al., 2004; McMahon et al., 2001), but cocaine-evoked hyperactivity is attenuated by intra-VTA microinfusion of the preferential 5-HT_{2C}R agonist Ro60-0175 (Fletcher et al., 2004) and unaltered by the 5-HT_{2C}R antagonist RS102221 (McMahon et al., 2001). Intra-VTA delivery of Ro60-0175 attenuated self-administration of cocaine (0.25 mg/kg/inf) under fixed and progressive ratios of reinforcement (Fletcher et al., 2004), which suggests the reinforcing and/or motivational properties of cocaine may be governed by 5-HT_{2C}R expression in the VTA. The impact of pharmacological manipulation of the 5-HT_{2C}R in the VTA on measures of cocaine-seeking behavior, including cue reactivity, has not been explored. In Chapter 3, we found that higher cue reactivity in prolonged abstinence from cocaine self-administration was associated with lower membrane 5-HT_{2C}R expression in the VTA; however, altered 5-HT_{2C}R expression in the VTA as a neuromolecular driver of elevated cocaine cue reactivity has not been investigated.

The overall inhibitory impact of the 5-HT_{2C}R over VTA function is thought to occur via 5-HT_{2C}R-mediated depolarization of GABA interneurons that synapse onto

dopaminergic neurons (Di Giovanni et al., 2001; Di Matteo et al., 2000; Theile et al., 2009). Thus, loss of the 5-HT_{2C}R in the VTA during prolonged abstinence from cocaine self-administration may disinhibit dopamine output to increase cocaine-related behaviors. In the present study, we employed a virally-mediated knockdown strategy (Anastasio et al., 2015; Anastasio et al., 2014b) to interrogate the causal role of 5-HT_{2C}R expression selectively in the VTA in cocaine-related behaviors, including cocaine-evoked hyperactivity, cocaine self-administration and cocaine cue reactivity. We tested the hypothesis that knockdown of the 5-HT_{2C}R in the VTA would confer an increased sensitivity to the hyperlocomotive, reinforcing, and motivational properties of cocaine as well as cocaine cue reactivity. Conflicting evidence exists regarding whether aspects of cocaine-taking and –seeking behaviors are influenced by anxiety (Bush and Vaccarino, 2007; Davis et al., 2008; Deroche-Gamonet et al., 2004; Homberg et al., 2002) Cunningham laboratory, unpublished observations). Anxiety-like behavior is decreased in 5-HT_{2C}R knockout mice (Heisler et al., 2007), and pharmacological experiments show that certain 5-HT_{2C}R agonists (i.e., CP809101, MK212) can increase anxiety-like behavior (de Mello Cruz et al., 2005; Pockros-Burgess et al., 2014) while 5-HT_{2C}R antagonists (i.e., SB242084) can act as anxiolytics under high anxiety conditions (Christianson et al., 2010; Craige et al., 2015). A role for the 5-HT_{2C}R in the VTA to mediate anxiety-like behavior has not been determined; nonetheless, we also assessed anxiety-like behavior following intra-VTA 5-HT_{2C}R knockdown to verify that differences in cocaine-related behaviors are not due to differences in anxiety.

Methods

Animals

Male Sprague-Dawley rats (n=38), Harlan, Inc., Houston, TX) weighing 250-325 g at the start of experiments were used. Rats were acclimated for seven days to a colony room maintained at a constant temperature (21-23°C) and humidity (45-50%) on a 12 hour light-dark cycle (lights on 0600-1800 h). Rats were housed two/cage and handled daily throughout the study. Food and water were available *ad libitum*. All experiments were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (2011) and with approval from the University of Texas Medical Branch Institutional Animal Care and Use Committee.

Drugs

(−)-Cocaine (National Institute on Drug Abuse, Research Triangle Park, NC) was dissolved in 0.9% NaCl.

shRNA design and production

A 24-nucleotide sequence within the coding region of the *Htr2c* was identified using methods we have previously reported (Hommel et al., 2003). Two sets of oligonucleotides (Integrated DNA Technology, Coralville, IA) for cloning were synthesized (*Htr2c* shRNA (top, 5′-TTGAATCCAGACGGGGCACAAATATCCTTCCTGTCAGATATTTGTGCCCCGTC TGGATTATTTTT-3′ bottom, 5′-CTAGAAAAATAATCCAGACGGGGCACAAATATCTGACAGGAAGGATATTTGT

GCCCCGTCTGGATTC-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 pAAV-MCS vector, pAAV-shRNA, which was designed to coexpress hairpin RNAs, under the control of a mU6pro and an SV40 polyadenylation site, as well as eGFP controlled by an independent CMV promoter and hGH polyadenylation sequence (Hommel et al., 2003). Adeno-associated viral (AAV) serotype type 2 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 1-2 × 10^{11} transducing units/ml.

Viral-mediated gene transfer

Intracranial viral vector delivery was performed under anesthesia with a cocktail containing 8.6 mg/kg of xylazine, 1.5 mg/kg of acepromazine, and 43 mg/kg of ketamine in bacteriostatic saline as described previously (Anastasio et al., 2015; Anastasio et al., 2014b). Rats were 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 6° from the midsaggital plane relative to bregma (Paxinos and Watson, 1998) to target the VTA; the coordinates were anteroposterior -5.3 mm, mediolateral +1.6 mm, and dorsoventral −8.2 mm from the skull. The NSC shRNA-eGFP AAV (0.3 µl/side) or 5-HT_{2C}R shRNA-eGFP AAV (0.3 µl/side) vectors were infused

bilaterally at 0.1 μl/min over 3 min. Rats were allowed 3 weeks for recovery and stable transgene expression. Transgene expression in rodent brain is stable at 3 weeks and maintained for at least 12–18 months following AAV infection (Daly, 2004; Leff et al., 1999).

Elevated plus maze

The elevated plus maze is a well-established task which measures anxiety-like behavior in rodents by capitalizing upon their inherent aversion of open spaces (i.e., open arms of the maze) and natural tendency to explore novel environments (Pellow et al., 1985). The plus-shaped apparatus consists of four arms made of black Plexiglass (50 cm long X 10 cm wide) elevated 72 cm above the floor in a dimly lit room. Two opposite arms are open (0.5 cm high edges) while the other two opposite arms are closed on three sides (40 cm high walls) (Med-Associates, Georgia, VT). Photobeam sensors are located at the entrance of each arm; beam breaks recorded explorations and entries onto each arm as well as time spent in each compartment. Rats were acclimated to the room for 1 hour prior to testing. Rats were placed in the center junction of the platform facing an open arm and allowed to freely explore the maze for 5 min during which behavior was monitored and automatically recorded.

Locomotor activity analyses

Locomotor activity was monitored and quantified under low light using a modified open field activity system (San Diego Instruments, San Diego, CA, USA). Clear Plexiglass chambers (40 × 40 × 40 cm) were surrounded by a 4 × 4 photobeam matrix

positioned 4 cm from chamber floor. Horizontal ambulation was measured by consecutive beam breaks and was further divided into central ambulation (central 16 × 16 cm) and peripheral ambulation (surrounding perimeter). Vertical activity (rearing) was also recorded by a break in the row of 16 photobeams positioned 16 cm from the activity monitor floor.

Rats were habituated to the activity chambers for 60 min; locomotor activity was recorded. Twenty four hours later, rats were evaluated for cocaine-evoked hyperactivity using a cumulative dosing regimen in a single locomotor activity session. Rats were habituated to the locomotor chambers for 30 min prior to receiving a saline injection (1 mL/kg, i.p.) and returned to the locomotor chamber for 30 min to control for the injection procedure. Rats were then treated with cocaine (10 mg/kg, i.p.) and returned the locomotor chamber for 30 min prior to a second injection of cocaine (10 mg/kg, i.p.) and another 30 min in the locomotor chamber.

Cocaine self-administration, progressive ratio, and cue reactivity analyses

Implantations of intravenous catheters with back mounts were performed as above approximately four weeks after viral-mediated gene transfer. Self-administration training employed standard operant conditioning chambers housed in ventilated sound-attenuating cubicles with fans (Med-Associates, Inc., St. Albans, VT). Each chamber was equipped with a pellet receptacle flanked by two retractable response levers, a stimulus light above each response lever, and a house light opposite the levers. Cocaine infusions were delivered by a syringe attached to an infusion pump (Med-Associates, Inc.) located outside the cubicle. The infusion pumps were connected to liquid swivels (Instech,

Plymouth Meeting, PA) that were fastened to catheters via polyethylene tubing encased inside a metal spring leash (Plastics One, Roanoke, VA).

Cocaine self-administration training consisted of 14 daily 180-min sessions during which intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats (n=14) and NSC shRNA-eGFP AAV rats (n=14) were trained to lever press for cocaine infusions (0.75 mg/kg/0.1mL inf) (Anastasio et al., 2014a; Anastasio et al., 2014b; Cunningham et al., 2013; Cunningham et al., 2011). Schedule completions on the active lever resulted in delivery of a cocaine infusion over a 6-sec period paired simultaneously with illumination of the house and stimulus lights and activation of the infusion pump (discrete cue complex paired with cocaine delivery); inactive lever presses produced no scheduled consequences. Following reinforcer delivery, the stimulus light as well as the infusion pump were inactivated; the house light remained on for an additional 20 sec to indicate a timeout period during which lever presses had no scheduled consequences. Rats were trained on a fixed ratio (FR) 1 schedule of reinforcement and progressed to an FR5 schedule after achieving seven infusions/hr with less than 10% variability for three consecutive days; all rats progressed to an FR5 by day nine of cocaine self-administration and met these criteria for three consecutive days prior to each test session (progressive ratio, cue reactivity).

Progressive ratio responding for cocaine was evaluated in a single session on day 12 of cocaine self-administration training. A progressive ratio test was performed in which the number of responses on the active lever required to earn an infusion of cocaine (0.75 mg/kg/inf) increased exponentially (i.e., 1, 2, 4, 6, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, etc.) as an indicator of motivation to obtain the reinforcer (Richardson

and Roberts, 1996). The break point, or the last schedule completed for a cocaine infusion, was recorded as the dependent measure. The session was terminated when 1 hr since the last infusion or 5 h total had elapsed; all rats completed the session in less than 5 h. Immediately following the progressive ratio test session, a standard cocaine self-administration was initiated in which rats lever pressed on an FR5 schedule for infusions of cocaine (0.75 mg/kg/inf) to re-establish self-administration performance and maintain stable levels of daily cocaine intake.

On FA Day 1 from cocaine self-administration, rats were assessed in a 60-min cue reactivity test session in which presses on the previously-active lever were reinforced by the discrete cue complex (stimulus light illuminated, infusion pump activated) on an FR1 schedule; presses on the inactive lever were recorded but produced no scheduled consequences.

5-HT_{2C}R protein analysis

Knockdown of the 5-HT_{2C}R protein in the VTA was assessed through immunohistochemistry and immunoblotting. A subset of rats (n=12) were anesthetized (sodium pentobarbital; 100 mg/kg, i.p.) and perfused transcardially with 3% paraformaldehyde for immunohistochemical analyses (Bubar et al., 2011). Brains were removed, post fixed (2 h), and cryoprotected in 30% sucrose solution. Free-floating coronal sections at the level of the VTA (30 μm) were incubated in 0.5% sodium borohydride to reduce autofluorescence. Sections were blocked (3% normal donkey serum in 0.4% triton-PBS) before incubation with a goat polyclonal 5-HT_{2C}R antibody (Ab32887; Abcam; 1 : 100; 2 h 25 °C, 18 h 4 °C) followed by AlexaFluor 555 to goat IgG

(A21432, 1 : 2000; Life Technologies). Slides were coverslipped with Vectashield fluorescent mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Slides were viewed with a Leica confocal microscope.

A subset of rats (n=26) were anesthetized (400 mg/kg chloral hydrate solution), decapitated, and brains were cut in 1 mm coronal sections containing the VTA. Sections were placed on cold glass slides and GFP was rapidly visualized using DFP-1 Dual Fluorescent Protein Flashlight by the investigator wearing a pair of VG2 barrier filter glasses (Nightsea, Bedford, MA) (Anastasio et al., 2015; Anastasio et al., 2014b; Li and Wolf, 2011). Photomicrographs of coronal sections were taken with a DSLR camera equipped with a macro lens and yellow filter (Anastasio et al., 2015; Anastasio et al., 2014b; Li and Wolf, 2011). Fluorescent regions of the VTA were then rapidly microdissected with a scalpel on a cool tray (4°C) (Heffner et al., 1980), frozen in liquid nitrogen and stored at -80°C for Western blot. Tissue was homogenized in 10 times w/v extraction buffer [20 mM HEPES, 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, protease inhibitor cocktail and phosphatase inhibitor 2 and 3 cocktails (10 μL/mL)]. The homogenate was centrifuged at 20,000 g for 5 min at 4°C. The supernatant was collected and NP40 was added to attain a final concentration of 0.5% NP40.

Ex vivo neurochemical studies assessed knockdown of 5-HT_{2C}R protein in the VTA using the Wes™ automated Western blotting system (ProteinSimple, San Jose, CA) which utilizes capillary electrophoresis-based immunodetection for higher resolution, sensitivity, and reproducibility (even at low sample concentrations) relative to traditional immunoblotting techniques (Anastasio et al., 2015; Fink, 2015; Liu et al., 2013a). 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, and matrix removal buffer, secondary antibodies, antibody diluent, and capillaries) were obtained from the manufacturer (ProteinSimple) and used according to the manufacturer's recommendations with minor modifications (Anastasio et al., 2015; Fink, 2015). The 5-HT_{2C}R antibody (D-12; sc-17797; Santa Cruz; 1:50) was diluted with ProteinSimple antibody diluent.

Equal amounts of protein (3 μg) were combined with 0.1X sample buffer and 5X master mix (200 mM DTT, 5X sample buffer, 5X fluorescent standards), gently mixed, and then denatured at 95^{o}C for 5 min. The denatured samples, biotinylated ladder, antibody diluent, primary antibodies, HRP-conjugated secondary antibodies, chemiluminescent substrate, and wash buffer were dispensed to designated wells in a pre-filled microplate (ProteinSimple). Separation electrophoresis (375 V, 31 min, 25^{o}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 30-s, 60-s, 120-s, and 240-s. Data analyses were performed using the Compass Software (ProteinSimple).

Statistical analyses

Student's t-test was used to analyze time spent per compartment and entries and explorations in the elevated plus maze. A two-way repeated measures ANOVA for the factors of AAV (NSC shRNA-eGFP or 5-HT_{2C}R shRNA-eGFP) and time (5 min bins or 30 min blocks) was used to analyze horizontal and vertical locomotor activity; planned

comparisons were subsequently made with a Tukey's test (Keppel, 1973). Student's t-test was used to analyze total basal locomotor activity. A two-way repeated measures ANOVA for the factors of AAV (NSC shRNA-eGFP or 5-HT_{2C}R shRNA-eGFP) and self-administration day was used to analyze active lever presses, inactive lever presses, and cocaine infusions earned in the acquisition and maintenance of cocaine self-administration; planned comparisons were subsequently made with a Tukey's test (Keppel, 1973). Student's t-test was used to analyze total intake during acquisition and maintenance of cocaine self-administration. Student's t-test was used to analyze break point in the progressive ratio test session. Student's t-test was used to analyze active and inactive lever presses and latency to the first response in the cocaine cue reactivity test session. Student's t-test (SAS for Windows, Version 9.4, SAS Institute, Inc., Cary, NC) was used to analyze 5-HT_{2C}R protein expression in the VTA. The experiment-wise error rate for all analyses was set at α=0.05.

Results

Anxiety-like behavior does not differ between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats

We evaluated rats in the elevated plus maze to test the hypothesis that knockdown of the 5-HT_{2C}R in the VTA alters anxiety-like behavior. Performance on the elevated plus maze can be influenced by cocaine experience (Craige et al., 2015; Yang et al., 1992) so this task was performed first in drug- and experimentally-naïve rats. There was no difference in the time spent in the open arms ($t_{1,36}$=0.06; n.s.), closed arms ($t_{1,36}$=0.02; n.s.), or junction ($t_{1,36}$=0.12; n.s.) of the maze between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats (**Fig. 4.1a**). There was no difference in entries into the open arms ($t_{1,36}$=0.00; n.s.) or closed arms ($t_{1,36}$=0.23; n.s.) of the maze between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats (**Fig. 4.1b**). There was no difference in explorations of the open arms ($t_{1,36}$=0.01; n.s.) or closed arms ($t_{1,36}$=0.00; n.s.) of the maze between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats (**Fig. 4.1c**). Intra-VTA 5-HT_{2C}R shRNA-eGFP AAV administration does not alter anxiety-like behavior, and any differences in subsequent cocaine-related behaviors are not impacted by levels of anxiety.

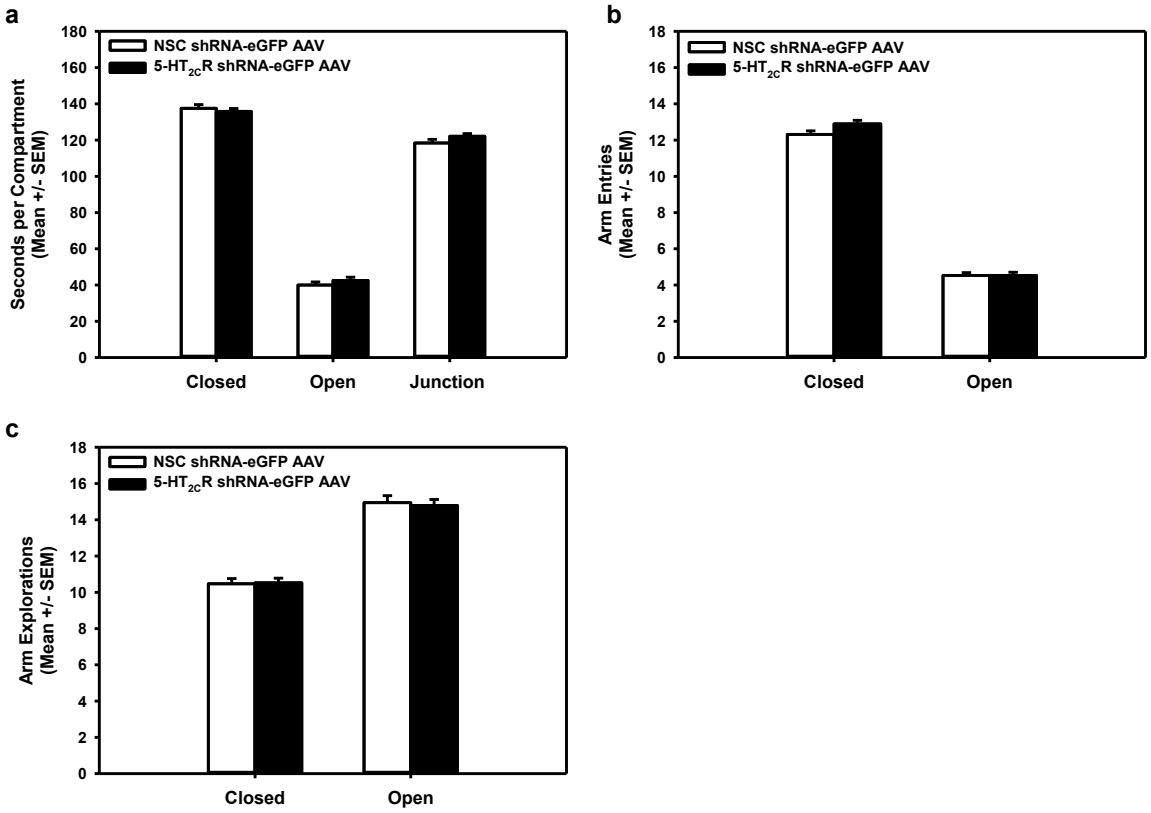

Figure 4.1: Anxiety-like behavior in the elevated plus maze does not differ between intra-VTA 5-HT$_{2C}$R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats.

(a) Mean ± SEM seconds in the closed arms, open arms, and junction is presented. There was no difference in the time spent in the open arms (n.s.; n=19/group), closed arms (n.s.), or junction (n.s.) of the maze between intra-VTA 5-HT$_{2C}$R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats. (b) Mean ± SEM entries into the closed arms and open arms is presented. There was no difference in entries into the open arms (n.s.) or closed arms (n.s.) between intra-VTA 5-HT$_{2C}$R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats. (c) Mean ± SEM explorations of the closed arms and open arms is presented. There was no difference in explorations of the open arms (n.s.) or closed arms (n.s.) of the maze between intra-VTA 5-HT$_{2C}$R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats.

Cocaine-evoked hyperactivity does not differ between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats

We evaluated basal locomotor activity in a 60 min habituation to ensure that there were no differences in general motor activity. No main effect of AAV ($F_{1,384}$=0.10; n.s.), a main effect of time ($F_{11,384}$=129.33; p<0.05), and no AAV x time interaction ($F_{11,384}$=0.88; n.s.) was observed for horizontal activity during the habituation session (**Fig. 4.2a)**. Planned comparisons indicated that horizontal activity did not differ between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats in any 5 min bin. Total horizontal activity in the 60 min habituation session did not differ in intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats relative to NSC shRNA-eGFP AAV rats (**Fig. 4.2b**; $t_{1,36}$=0.00; n.s). No main effect of AAV ($F_{1,384}$=0.32; n.s.), a main effect of time ($F_{11,384}$=62.09; p<0.05), and no AAV x time interaction ($F_{11,384}$=1.13; n.s.) was observed for vertical activity during the habituation session (**Fig. 4.2c)**. Planned comparisons indicated that vertical activity did not differ between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats in any 5 min bin. Total vertical activity in the 60 min habituation session did not differ in intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats relative to NSC shRNA-eGFP AAV rats (**Fig. 4.2d**; $t_{1,36}$=0.04; n.s). Intra-VTA 5-HT_{2C}R shRNA-eGFP AAV and NSC shRNA-eGFP AAV rats displayed identical basal locomotor activity.

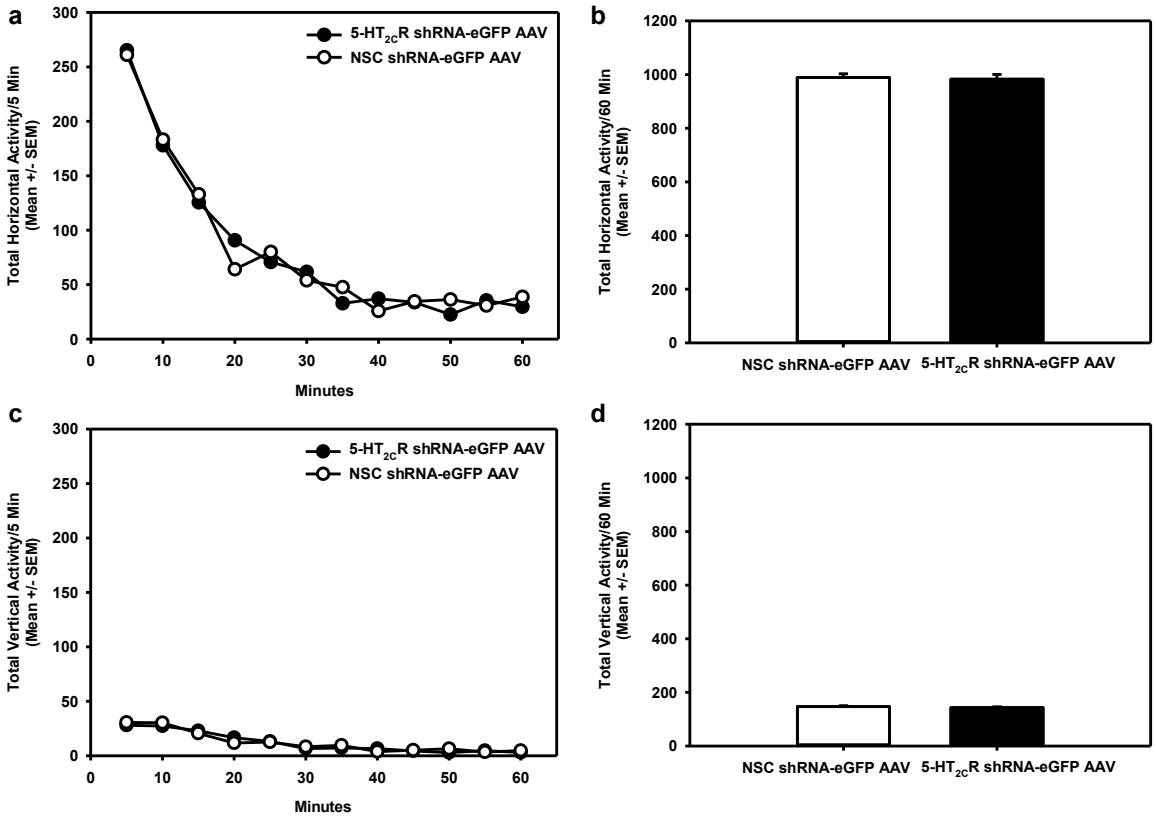

Figure 4.2: Basal locomotor activity is not altered in intra-VTA 5-HT$_{2C}$R shRNA-eGFP AAV rats relative to NSC shRNA-eGFP AAV rats.

Mean ± SEM horizontal activity counts are presented for **(a)** 5 min bins and **(b)** the sum total in a 60 min habituation session. Horizontal activity did not differ between intra-VTA 5-HT$_{2C}$R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats (n.s.; n=19/group). Mean ± SEM vertical activity counts are presented for **(c)** 5 min bins and **(d)** the sum total in a 60 min habituation session. Vertical activity did not differ between intra-VTA 5-HT$_{2C}$R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats (n.s.).

We then assessed cocaine-evoked hyperactivity in a cumulative dosing paradigm. We hypothesized that intra-VTA 5-HT$_{2C}$R knockdown would disinhibit VTA dopamine output and shift the dose-response curve for cocaine-evoked hyperactivity leftward such that the hyperlocomotive effect of cocaine would be potentiated at subthreshold doses (e.g., 10 mg/kg) but unaltered at superthreshold doses (e.g., 20 mg/kg). Locomotor activity was assessed in a single session comprised of four, 30 min consecutive blocks:

habituation (no treatment), saline (1 mL/kg; i.p.) treatment, cocaine (10 mg/kg; i.p.) treatment (Coc 10), and a second cocaine (10 mg/kg; i.p.) treatment (Coc 10 + Coc 10). No main effect of AAV ($F_{1,139}$=0.39; n.s.), a main effect of block ($F_{1,139}$=13.61; p<0.05), and no AAV x block ($F_{1,139}$=0.09; n.s.) was observed for horizontal activity during the cumulative dosing session (**Fig. 4.3a**; n=17-18/group**)**. Planned comparisons indicated that horizontal activity did not differ between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats on each of the session blocks. Horizontal activity in the second cocaine treatment block (Coc 10 + Coc 10) was significantly higher than the saline treatment block in both intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats (p<0.05) and NSC shRNA-eGFP AAV rats (p<0.05). No main effect of AAV ($F_{1,139}$=0.83; n.s.), a main effect of block ($F_{1,139}$=8.48; p<0.05), and no AAV x block ($F_{1,139}$=0.56; n.s.) was observed for vertical activity during the cumulative dosing session (**Fig. 4.3c)**. Planned comparisons indicated that vertical activity did not differ between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats on each of the session blocks. In intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats only, vertical activity in the second cocaine treatment block (Coc 10 + Coc 10) was significantly higher than the saline treatment block (p<0.05). Overall, these data suggest that the cumulative dosing paradigm can be employed to assess cocaine-evoked hyperactivity but intra-VTA 5-HT_{2C}R shRNA-eGFP AAV delivery did not potentiate the hyperlocomotive effects of cocaine.

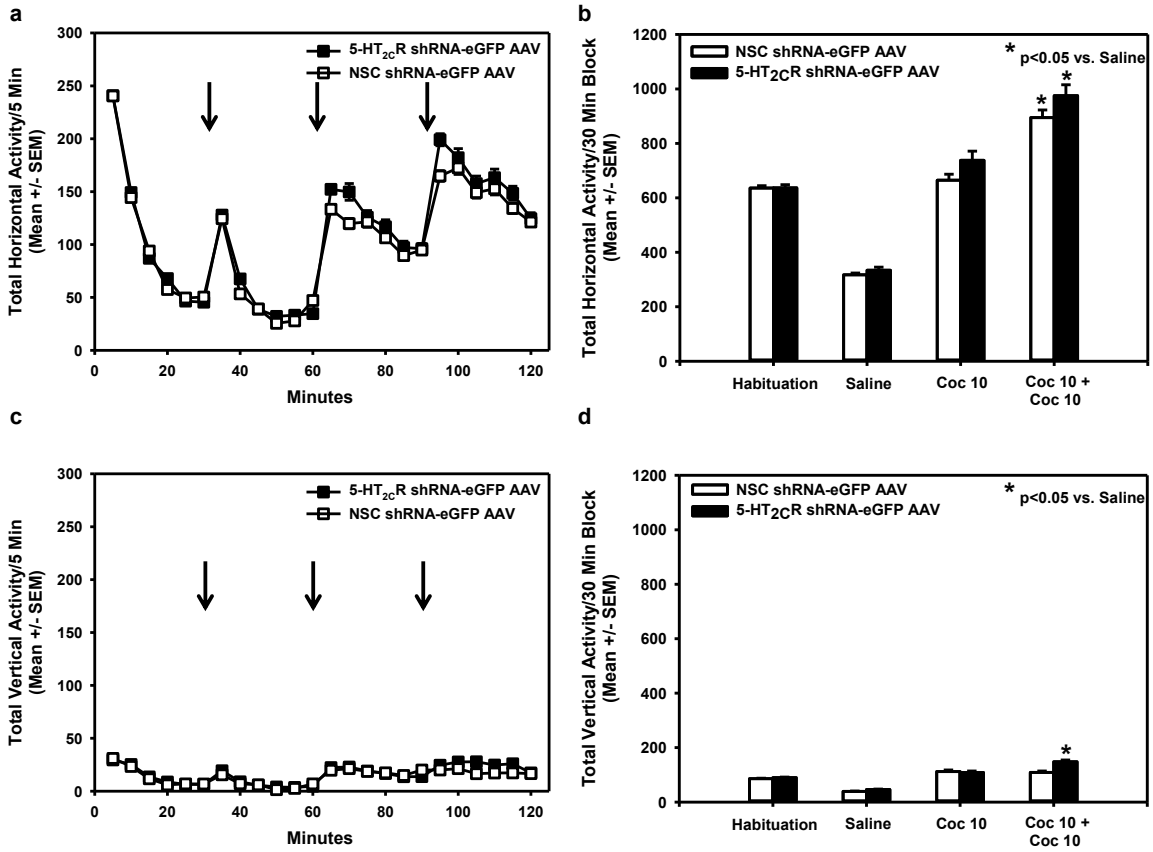

Figure 4.3: Cocaine-evoked hyperactivity does not differ between intra-VTA 5-HT$_{2C}$R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats.

(a) Mean ± SEM horizontal activity counts are presented for (a) the timecourse in 5 min bins and (b) sum total across each 30 min session block. Arrows indicate injections of saline (1 mL/kg; i.p.), cocaine (10 mg/kg; i.p.), and a second cocaine injection (10 mg/kg; i.p.). Horizontal activity did not differ between intra-VTA 5-HT$_{2C}$R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats on each of the session blocks (n.s.; n=17-18/group). Horizontal activity in the second cocaine treatment block (Coc 10 + Coc 10) was significantly higher than the saline treatment block in both intra-VTA 5-HT$_{2C}$R knockdown rats (*p<0.05) and control rats (*p<0.05). Mean ± SEM vertical activity counts are presented for (c) the timecourse in 5 min bins and (d) sum total across each 30 min session block. Vertical activity did not differ between intra-VTA 5-HT$_{2C}$R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats on each of the session blocks (n.s.). Vertical activity in the second cocaine treatment block (Coc 10 + Coc 10) was significantly higher than the saline treatment block in intra-VTA 5-HT$_{2C}$R shRNA-eGFP AAV rats (*p<0.05).

Cocaine self-administration does not differ between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats

We tested the hypothesis that the acquisition and/or maintenance of cocaine self-administration would differ between in intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats. Rats readily acquired cocaine self-administration (0.75 mg/kg/0.1 mL infusion) to stability (i.e., seven infusions/hr on an FR5 schedule for at least three sessions) and displayed <10% variation in the number of infusions earned during the maintenance sessions (**Fig. 4.4**). For active lever presses, no main effect of AAV ($F_{1,362}$=0.99; n.s.), a main effect of self-administration day ($F_{12,362}$=72.35; p<0.05), and no AAV x self-administration day interaction ($F_{12,362}$=0.90; n.s.) was observed during cocaine self-administration. Planned comparisons indicated that active lever presses did not differ between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats (**Fig. 4.4a**) and NSC shRNA-eGFP AAV rats (**Fig. 4.4b**) on any self-administration day. For inactive lever presses, a main effect of AAV ($F_{1,362}$=5.14; p<0.05), a main effect of self-administration day ($F_{12,362}$=8.90; p<0.05), and no AAV x self-administration day interaction ($F_{12,362}$=0.85; n.s.) was observed during cocaine self-administration. Planned comparisons indicated that inactive lever presses did not differ between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats (**Fig. 4.4a**) and NSC shRNA-eGFP AAV rats (**Fig. 4.4b**) on any self-administration day. For cocaine infusions earned, no main effect of AAV ($F_{1,362}$=1.27; n.s.), a main effect of self-administration day ($F_{12,362}$=15.19; p<0.05), and no AAV x self-administration day interaction ($F_{12,362}$=1.42; n.s.) was observed during cocaine self-administration. Planned comparisons indicated that cocaine infusions earned did not differ between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats (**Fig. 4.4a**) and NSC

shRNA-eGFP AAV rats (**Fig. 4.4b**) on any self-administration day. There was no difference in total cocaine intake across the self-administration phase between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats (335.6 ± 3.4 mg/kg) and NSC shRNA-eGFP AAV rats (336.5 ± 3.3 mg/kg; $t_{1,26}$=0.00; n.s.). The acquisition or maintenance of cocaine self-administration was unaltered by intra-VTA 5-HT_{2C}R shRNA-eGFP AAV administration, thus, any differences in the motivational effects of cocaine and cocaine cue reactivity are not due to differences in cocaine self-administration training.

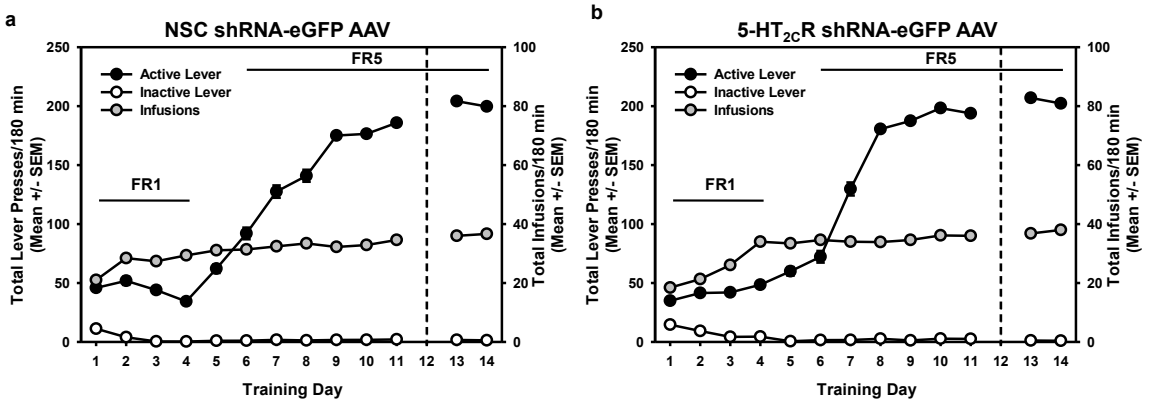

Figure 4.4: Acquisition and maintenance of cocaine self-administration does not differ between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats.

Mean responses (± SEM) on the active (black circles) or inactive lever (white circles), and total number of cocaine infusions earned (± SEM; gray circles) are presented for the acquisition and maintenance phase of cocaine self-administration (0.75 mg/mL/inf) in (a) intra-VTA NSC shRNA-eGFP AAV rats and (b) intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats. Active lever presses, inactive lever presses, and cocaine infusions earned did not differ in intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats vs. NSC shRNA-eGFP AAV rats (n.s.; n=14/group). On day 12, rats were evaluated in a progressive ratio test session and then placed back into a self-administration session to maintain stable daily cocaine intake, so acquisition data are not presented (indicated by dashed line).

Motivation for cocaine does not differ between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats

Responding on a progressive ratio schedule is an indicator of motivation to obtain a reinforcer, in this case, an infusion of cocaine (0.75 mg/kg/inf). Progressive ratio responding for cocaine was evaluated in a single session on self-administration day 12 prior to the cocaine self-administration maintenance session. We tested the hypothesis that motivation to self-administer cocaine [break point (last schedule completed for a cocaine infusion); mean ± SEM] would be higher in intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats relative to NSC shRNA-eGFP AAV rats. Break point did not differ between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats (**Fig. 4.5**; $t_{1,26}$=0.14; n.s.). The progressive ratio session was terminated 1 hr after the last schedule completion and cocaine infusion. The time to completion of the progressive ratio test session did not differ between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats (195.8 ± 2.1 min) and NSC shRNA-eGFP AAV rats (193.3 ± 3.1 min; $t_{1,26}$=0.03; n.s.). In this single-test snapshot approach, intra-VTA 5-HT_{2C}R shRNA-eGFP AAV delivery does not appear to affect the motivation to self-administer cocaine at this unit dose; it is unclear whether the motivation to self-administer cocaine would be altered in rats trained to self-administer cocaine on the progressive ratio schedule.

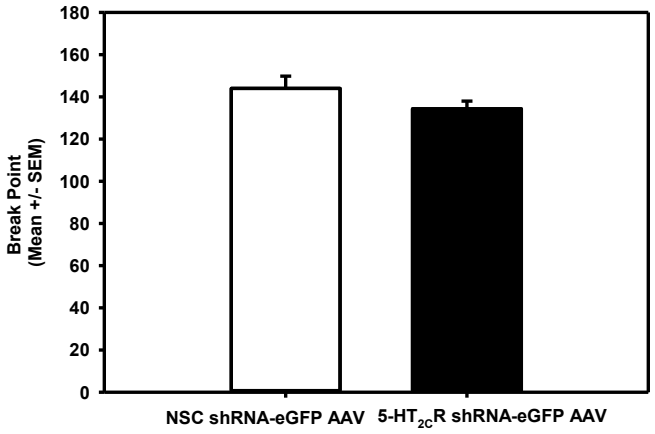

Figure 4.5: Progressive ratio responding for cocaine does not differ between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats.

Mean ± SEM break point (active lever presses on the last completed progressive ratio schedule) for self-administered cocaine (0.75 mg/kg/inf) in a single progressive ratio test session did not differ between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats (n.s.; n=14/group).

Cocaine cue reactivity does not differ between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats

We tested the hypothesis that cue reactivity (previously-active lever presses; mean ± SEM) on FA Day 1 from cocaine self-administration would be higher in intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats relative to NSC shRNA-eGFP AAV rats. Cocaine cue reactivity did not differ in intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats vs. NSC shRNA-eGFP AAV rats (**Fig. 4.6**; $t_{1,26}$=0.16; n.s.). Inactive lever presses in the cue reactivity test session did not differ between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats (**Fig. 4.6**; $t_{1,26}$=0.01; n.s.). There was no difference in latency to first press (mean ± SEM) in intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats (44.2 ± 4.0 sec) and NSC shRNA-eGFP AAV rats (40.1 ± 1.8 sec; $t_{1,26}$=0.06; n.s.). Intra-VTA 5-HT_{2C}R

shRNA-eGFP AAV delivery did not alter cocaine cue reactivity in early abstinence from cocaine self-administration.

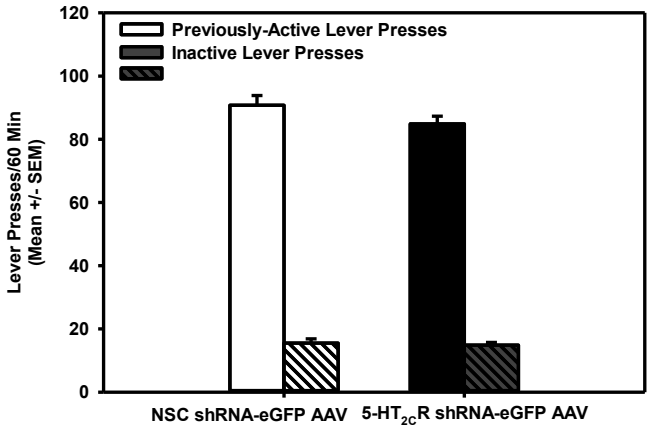

Figure 4.6: Cocaine cue reactivity does not differ between intra-VTA 5-HT$_{2C}$R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats.

Mean (± SEM) previously-active lever presses and inactive lever presses are presented for the cue reactivity test session in intra-VTA 5-HT$_{2C}$R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats on FA Day 1 from cocaine self-administration (0.75 mg/kg/inf). Cue reactivity did not differ between intra-VTA 5-HT$_{2C}$R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats (n.s.; n=14/group).

5-HT$_{2C}$R protein expression analyses in intra-VTA 5-HT$_{2C}$R shRNA-eGFP AAV rats *vs.* NSC shRNA-eGFP AAV rats

We aimed to assess 5-HT$_{2C}$R protein expression levels to confirm knockdown in intra-VTA 5-HT$_{2C}$R shRNA-eGFP AAV rats vs. NSC shRNA-eGFP AAV rats by immunohistochemistry and immunoblotting techniques. In the immunohistochemistry experiments, the GFP expressed in each AAV construct emitted an intense fluorescent signal that could be visualized under both the "green" and "red" optical filters; unfortunately, it was not possible to reliably distinguish between the "bleed through"

from the GFP signal and the punctate immunofluorescence from the Alexa Fluor 555 (red) secondary antibody which recognized the 5-HT_{2C}R primary antibody, so 5-HT_{2C}R expression in AAV-infected neurons could not be determined. These sections were used to verify correct placement of the AAV infusions (see diagram in **Fig. 4.7a**) and examine the spread of viral infection along rostral-caudal and dorsal-ventral gradients. We observed neurons expressing GFP in the VTA (**Fig. 4.7b**), which indicates that the AAV did infect neurons in this region; however, we also observed viral spread which extended more than 1 mm in the rostral and caudal directions from the mid-VTA in some cases as well as dorsal spread which was not limited to the proximity of the needle track. This pattern of viral infection matched the GFP signal which was visualized and in fresh coronal brain sections (**Fig. 4.7c**); the fluorescent region of the VTA was microdissected for biochemical analyses. A subset of samples (n=4/group) with accurate bilateral virus infusions and the most favorable viral spread (i.e., expressed throughout the VTA but limited infection outside this region) based on GFP signal were selected for immunoblotting to assess 5-HT_{2C}R protein expression. Expression of 5-HT_{2C}R protein did not differ between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats (**Fig. 4.7d**; $t_{1,6}$=0.07; n.s.). It appears that intra-VTA delivery of the 5-HT_{2C}R shRNA-eGFP AAV did not confer knockdown of the 5-HT_{2C}R protein; however, there are several technical limitations which limit the strength of this interpretation.

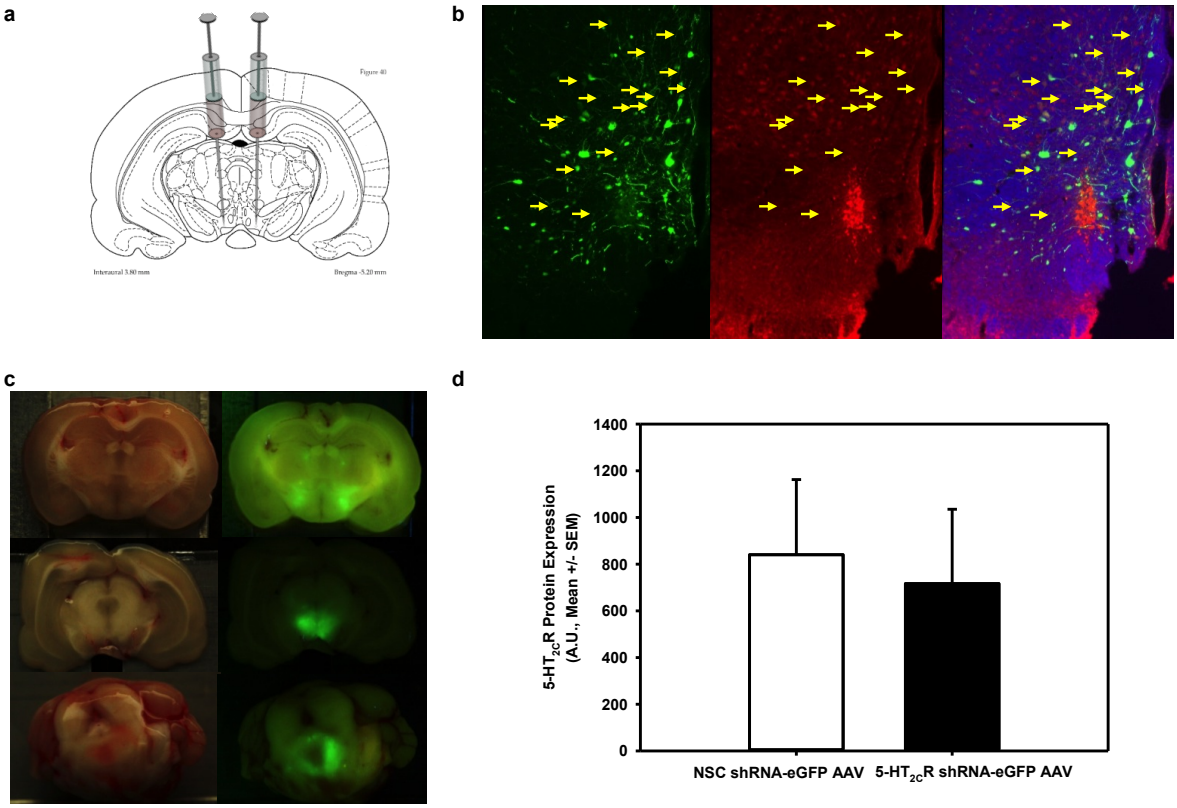

Figure 4.7: Expression of 5-HT$_{2C}$R protein in the VTA does not differ between intra-VTA 5-HT$_{2C}$R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats.

(a) The diagram demonstrates the region of the VTA targeted for AAV microinfusion (Paxinos and Watson 1998). (b) Tilescan images of intra-VTA AAV infection are shown. Green is GFP fluorescence (left); red is 5-HT$_{2C}$R immunofluorescence or GFP bleed through (middle); the overlay is also shown with DAPI immunofluorescence in blue (right). Yellow arrows denote a portion of the infected neurons (visible at this scale) expressing GFP which is clearly visualized under both green and red filters. The AAV was infused into the VTA but also exhibits viral infection in regions dorsal to the VTA and rostrally and caudally from the VTA (not shown). (c) The multi-panel image shows representative photomicrographs of coronal brain sections from a single rat under regular light (left) and using the fluorescent flashlight to visualize GFP (right). The AAV infected regions rostral to the VTA (top), the VTA target region (middle), and caudal to the VTA (bottom). Some rats also expressed GFP along the dorsal plane (not shown). (d) Mean (± SEM) 5-HT$_{2C}$R protein expression is presented for intra-VTA 5-HT$_{2C}$R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats. Expression of 5-HT$_{2C}$R protein in the VTA did not differ between intra-VTA 5-HT$_{2C}$R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats (n.s.; n=4/group).

Discussion

The present studies were designed to investigate the mechanistic role of the 5-HT_{2C}R localized to the VTA in cocaine-related behaviors. Anxiety-like behavior did not differ between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV and NSC shRNA-eGFP AAV rats and did not impact cocaine self-administration. There was no difference in the hyperlocomotive, reinforcing, or motivational properties of cocaine and no effect on cocaine cue reactivity in intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats relative to NSC shRNA-eGFP AAV rats. A significant difference in 5-HT_{2C}R protein expression was not detected between intra-VTA 5-HT_{2C}R shRNA-eGFP AAV and NSC shRNA-eGFP AAV rats. The absence of a behavioral effect is consistent with no observed knockdown of the 5-HT_{2C}R in the VTA. Significant technical limitations of the present study as well as important considerations of the genetic knockdown design did not allow effective testing of our hypothesis and a definitive role for the 5-HT_{2C}R in the VTA as a driver of cocaine-related behaviors could not be determined.

The virally-mediated 5-HT_{2C}R knockdown strategy employed in the current study generated approximately 50% reduction in 5-HT_{2C}R protein expression in the mPFC (Anastasio et al., 2015; Anastasio et al., 2014b) and NAc shell (Cunningham laboratory, unpublished observations); however, knockdown of the 5-HT_{2C}R in the VTA was not observed in the present study. One possible explanation is that VTA 5-HT_{2C}R expression was lower in knockdown vs. control rats in initial experiments, but repeated periods of cocaine administration and withdrawal downregulated 5-HT_{2C}R expression in control rats such that a difference between the groups could no longer be detected at the conclusion of the study. Cocaine cue reactivity was purposefully assessed at an early period of

forced abstinence because prolonged abstinence from cocaine self-administration was associated with lower membrane 5-HT_{2C}R expression in the VTA, but the temporally-dynamic regulation of 5-HT_{2C}R expression following acute and/or chronic cocaine administration and varied periods of withdrawal is unknown. Future studies should include intra-VTA 5-HT_{2C}R shRNA-eGFP AAV rats and NSC shRNA-eGFP AAV rats with no cocaine experience as a control in biochemical analyses.

The expansive spread of viral infection throughout the midbrain was an obvious limitation. The 5-HT_{2C}R mRNA and protein have been found in regions which were infected by the 5-HT_{2C}R knockdown virus (Clemett et al., 2000; Molineaux et al., 1989; Pompeiano et al., 1994), but the functional impact of the 5-HT_{2C}R within these regions on general or cocaine-related behaviors is entirely unknown. If 5-HT_{2C}R protein expression was decreased by the microinfusion of the 5-HT_{2C}R shRNA-eGFP AAV, the behavioral consequence of loss of the 5-HT_{2C}R within other midbrain regions may have countered the loss of the 5-HT_{2C}R within the VTA to generate no net effect on cocaine-related behaviors. Viral infection outside the target region has not been previously observed for the mPFC (Anastasio et al., 2015; Anastasio et al., 2014b) or NAc shell (Cunningham laboratory, unpublished observations), and is likely due to unique properties of the VTA rather than experimenter error, viral titer, or infusion volume. In fact, the infusion volume used for intra-VTA viral vector delivery (0.3 µl/side) based on preliminary studies was lower than the volume infused into the mPFC or NAc shell (1.0 µl/side). Targeting the infusion slightly more ventrally within the VTA or diluting stocks to infuse a lower viral titer could have minimized the spread of viral infection, but the same virus stock was microinfused into the NAc shell in a parallel experiment and

infection was contained within the NAc shell. It is also possible that the microinfusion needle traversed an unknown structure (i.e., fiber of passage, blood vessel) that promoted widespread viral infection; a review of the rat brain atlas (Paxinos and Watson, 1998) did not identify any obvious common pathway or structure. High rates of blood flow in the VTA may contribute to increased permeability of the blood brain barrier (Alavijeh et al., 2005), so leakage of the viral vector into the microvasculature could contribute to the spread of infection which appears to be specific to the VTA.

Technical difficulties may have also limited our ability to detect an existing difference in protein expression. Immunohistochemistry experiments could not be interpreted because the fluorescence from GFP was visible across microscope filters and masked the punctate 5-HT_{2C}R immunofluorescence. The 5-HT_{2C}R immunoblotting experiments were performed using the Wes^{TM} automated Western blotting system (ProteinSimple), which typically offers improved sensitivity and reproducibility compared to traditional Western blotting methods (Anastasio et al., 2015; Fink, 2015; Liu et al., 2013a); however, unbeknownst to us at the time, the proprietary buffer components in the microplates supplied by the company and used in these experiments were changed, which greatly reduced the sensitivity of the assay. Perhaps the sensitivity of the assay at the time was too low to detect relatively small differences in protein expression.

A genetic approach captures the long-term consequences of shifts in 5-HT_{2C}R functional capacity, but it is conceivable that this approach is not well-suited to interrogate the role of VTA 5-HT_{2C}R expression in cocaine-related behaviors. Intracranial pharmacology experiments identified a role for the 5-HT_{2C}R in the VTA to modulate cocaine-evoked hyperactivity and the reinforcing and motivational properties of

self-administered cocaine (Fletcher et al., 2004). There may be discrepancies between pharmacological and genetic manipulations of VTA 5-HT_{2C}R function due to differential effects on the firing pattern of dopamine neurons. Dopamine neurons fire in two distinct patterns: tonic firing occurs slowly and spontaneously in what is essentially a maintenance state, whereas phasic firing is rapid, burst firing which occurs in response to stimuli like cocaine or cocaine-associated cues (reviewed in Marinelli and McCutcheon, 2014; Wanat et al., 2009). Blockade of the 5-HT_{2C}R increased phasic dopamine firing in the VTA (Gobert et al., 2000), and activation of the 5-HT_{2C}R in the VTA may decrease phasic firing in response to administration of cocaine or cocaine-paired cues to suppress cocaine-taking and –seeking behaviors. Chronic loss of 5-HT_{2C}R tone on GABA neurons in the VTA could potentiate tonic dopamine release without altering phasic dopamine firing and may thus not alter cocaine-related behaviors. Dopamine output from the VTA is critically important for proper neuronal function and species survival, so naturally it is highly regulated by a number of mechanisms. Loss of 5-HT_{2C}R in the VTA may prompt compensatory upregulation or downregulation of other receptors to normalize dopamine output. For example, the 5-HT_{2C}R and 5-HT_{2A}R exist in a balance, and knockdown of the 5-HT_{2C}R in the mPFC upregulates 5-HT_{2A}R expression (Anastasio et al., 2015). Alternately, the expression of dopamine receptors in terminal regions (i.e., NAc, mPFC) may shift to maintain homeostasis within the dopamine system. There may also be a balance between 5-HT_{2C}R localized to dopaminergic and GABAergic neurons in the VTA (Bubar and Cunningham, 2007; Bubar et al., 2011). The AAV serotype type 2 will infect both neuron subtypes, though it expresses tropism for GABAergic neurons (Burger et al., 2004; Klein et al., 1998). Loss of the 5-HT_{2C}R in dopaminergic neurons may offset

the effect of knockdown of the 5-HT_{2C}R in GABAergic neurons and vice versa, so driving viral vector expression to one neuronal subtype using specific promoters (i.e., tyrosine hydroxylase) is an important future direction.

These studies did not identify altered 5-HT_{2C}R expression in the VTA as a neurobiological driver of cocaine-related behaviors, but the tools available and the virally-mediated knockdown approach contained inherent limitations that should not diminish consideration of 5-HT_{2C}R expression in the VTA as a contributor to the hyperlocomotive and reinforcing effects of cocaine and cocaine cue reactivity. Rather, a combined approach of intracranial pharmacology and techniques to assess the pattern dopamine firing (i.e., fast-scan cyclic voltammetry) should be used to further explore lower 5-HT_{2C}R expression in the VTA as a regulator of the incubation of cocaine cue reactivity. The interaction between serotonergic and dopaminergic systems, particularly via the 5-HT_{2C}R in the VTA, is a promising target for treating cocaine use disorder and merits additional investigation (Howell and Cunningham, 2015).

Chapter 5: Conclusions

Our overall objective was to elucidate the involvement of the 5-HT_{2C}R expressed within distinct regions of the mesocorticolimbic neurocircuitry to modulate cocaine cue reactivity. In the translationally-relevant rodent cocaine self-administration and forced abstinence model, the incubation of cue reactivity during prolonged abstinence from cocaine-taking was associated with lower synaptosomal 5-HT_{2C}R protein expression selectively within regions in the mesocorticoaccumbens pathway (PFC, VTA), but not in other analyzed cortical (OFC) or limbic areas (dHipp) which mediate distinct aspects of cue reactivity in rodents (Fuchs et al., 2007; Kalivas and Volkow, 2005; Pickens et al., 2011; Weiss, 2005). Abstinence-induced changes in the subcellular localization of the 5-HT_{2C}R in the mPFC implicate several potential mechanisms which may regulate 5-HT_{2C}R expression to govern cue reactivity. Understanding the precise regional and molecular regulation of the 5-HT_{2C}R could suggest strategies to augment the lower potency of a 5-HT_{2C}R agonist to suppress cocaine cue reactivity at prolonged periods of abstinence.

The functional capacity of the 5-HT_{2C}R is tightly controlled by a variety of factors which could shift the subcellular localization of the 5-HT_{2C}R in the PFC toward intracellular compartments and away from the plasma membrane at prolonged abstinence from cocaine self-administration (Chapter 2). Association with different protein partners (Gavarini et al., 2006), increased alternative splicing (Martin et al., 2013), and decreased RNA editing (Marion et al., 2004) can alter receptor trafficking and/or recycling processes to decrease responsivity to 5-HT_{2C}R ligands, including 5-HT. In Chapter 3, we also discovered lower 5-HT_{2C}R protein expression at the membrane in the VTA which

was concomitant with a shift in the subcellular distribution of $pERK_{1/2}$, a downstream effector in 5-HT_{2C}R signal transduction (Labasque et al., 2010; Werry et al., 2005; Werry et al., 2008) and a key responsive element in cocaine addiction models (Lu et al., 2006). It is conceivable that the 5-HT_{2C}R sequesters $pERK_{1/2}$ to the cytoplasmic compartment through β-arrestin-dependent signaling, and lower 5-HT_{2C}R expression removes a brake on nuclear localization of $pERK_{1/2}$ (through translocation of $pERK_{1/2}$ to the nucleus or activation of $ERK_{1/2}$ in the nuclear compartment); nuclear $pERK_{1/2}$ may contribute to long-lasting neuroadaptations via enhanced gene transcription (Mattson et al., 2005; Valjent et al., 2000), although a direct association between 5-HT_{2C}R and $pERK_{1/2}$ expression cannot be determined *ex vivo* using currently available protein biochemistry methods. It is not known whether 5-HT_{2C}R expression and signaling are controlled by uniform mechanisms across brain regions; this would be an important avenue for future investigation if these mechanisms were to be developed as therapeutic targets (e.g., disrupters of protein:protein interactions).

Expression of the 5-HT_{2C}R specifically within the mesocorticoaccumbens pathway was associated with cocaine cue reactivity levels (Chapter 2, 3), but the impact of 5-HT_{2C}R signaling on the neural connections between the mPFC, NAc, and VTA is highly complex. Alterations in 5-HT_{2C}R expression in the mPFC and VTA may ultimately modulate cocaine cue reactivity through increased excitatory and modulatory output to the NAc, especially given that neuroplasticity in the NAc is required for incubation (Conrad et al., 2008; Ma et al., 2014; Terrier et al., 2015). In the mPFC, the 5-HT_{2C}R is localized to GABA interneurons (Liu et al., 2007; Vysokanov et al., 1998) and glutamatergic pyramidal neurons (Carr et al., 2002; Clemett et al., 2000; Liu et al., 2007);

glutamate projections neurons from the mPFC innervate regions including the NAc and VTA to control the responsivity to drug-associated cues in rodents (Gabbott et al., 2005; George and Koob, 2010; Kalivas, 2008). Activation of the 5-HT_{2C}R in the mPFC is predicted to inhibit output from PFC through activation of GABA interneurons (Liu et al., 2007). Moreover, constitutive activity of the 5-HT_{2C}R in the mPFC indirectly enhances dopamine outflow in the NAc (Leggio et al., 2009). We were unable to successfully test our hypothesis that knockdown of the 5-HT_{2C}R in the VTA would increase cocaine cue reactivity (Chapter 4), but a role for the 5-HT_{2C}R activity in the VTA to modulate cocaine behaviors is strongly supported. In the VTA, the 5-HT_{2C}R is expressed on both GABA and dopamine neurons (Bubar and Cunningham, 2007; Bubar et al., 2011), but the inhibitory effect of 5-HT_{2C}R activity on VTA dopamine output is thought to be mediated by 5-HT_{2C}R GABA neurons (Di Giovanni et al., 2001; Di Matteo et al., 2000; Theile et al., 2009). Intra-VTA activation of the 5-HT_{2C}R attenuated cocaine-evoked dopamine outflow in the NAc (Navailles et al., 2008). It is possible that lower 5-HT_{2C}R expression in mPFC strengthens glutamate output and modulates dopamine output to the NAc; concurrently, lower 5-HT_{2C}R expression in the VTA may result in enhanced dopamine release in the NAc. Preclinical studies in individual brain areas are useful for identifying molecular underpinnings, and advanced techniques which allow manipulation of activity within specific neural pathways in rats [e.g., dual viral vector approach which uses a modified Cre-flox system to drive virally-mediated receptor expression of designer receptors exclusively activated by designer drugs (DREADDs) selectively to neurons which project to a predetermined output region (injected with a retrograde virus expressing Cre) to allow temporal activation or inhibition of a specific pathway (Krashes

et al., 2011; Nair et al., 2013)] or neuroimaging techniques that identify the temporal connectivity between regions in humans [e.g., dynamic causal modeling (Ma et al., 2015; Ma et al., 2012)] reveal the pattern(s) of neural activity which mediate cue reactivity; bridging the findings from these strategies will be essential to fully appreciate the neurobiology of cue reactivity.

The selective 5-TH_{2C}R agonist WAY163909 was effective to suppress cocaine cue reactivity at early and prolonged periods of abstinence, although the potency of WAY163909 was lower at prolonged abstinence from cocaine self-administration (Chapter 2). The maximum suppression of cue reactivity evoked by WAY163909 was approximately 50% of vehicle responding, which raises an important question: what magnitude of suppression of cue reactivity is required for a pharmacotherapy to be considered potentially therapeutically relevant? A review of the literature to investigate the maximal effect of systemic pharmacological interventions routinely found ~50-75% suppression of cue reactivity in the cocaine self-administration and FA model, regardless of mechanism targeted or protocol employed (for examples, Conrad et al., 2008; Koya et al., 2009; Lu et al., 2007; Whitfield et al., 2011). Non-pharmacological methods [e.g., optogenetics (Ma et al., 2014)] could achieve greater suppression of cue reactivity but are not feasible therapeutic options and are thus not included in this assessment. The consensus between these studies may reflect a "floor" in the ability to attenuate cue reactivity through a single mechanism or at doses that do not disrupt general behaviors; combination therapies could possibly attenuate cue reactivity further, but this has not been evaluated.

Cue reactivity as a construct is an aggregate of components including craving, liking/wanting, motivation, attention, learning and memory, and physiological responsiveness, among others (Carter and Tiffany, 1999; Drummond, 2001; Field and Cox, 2008; Robinson and Berridge, 1993). To expect a medication to block each of these processes appears unrealistic, and it may also be unnecessary. A seemingly incremental reduction in cue reactivity could have a profound impact on maintaining abstinence. For example, a 50% reduction in an individual's cue reactivity may be sufficient to prevent a relapse to drug-taking. At present, the primary outcome measure in clinical trials for treatments of substance use disorders is abstinence (Penberthy et al., 2010), but perhaps cue reactivity should be incorporated as an additional outcome measure. Experimental models of cue reactivity [e.g., cocaine-word Stroop task (Hester et al., 2006; Liu et al., 2013b; Liu et al., 2012; Liu et al., 2011)] provide a quantitative assessment of cue reactivity and can be employed to overcome issues of reproducibility in other models of cue reactivity (Modesto-Lowe and Kranzler, 1999); evidence from systematic investigation would be required to determine the utility of these models to measure cue reactivity as a clinical outcome. Addiction is a chronic disease, but abstinence as an outcome measure resembles that of an acute disease with a cure. In contrast, chronic diseases like cancer accept improvement in quality of life as an outcome measure. It could be argued that reduced cue reactivity is akin to improved quality of life, particularly if abstinence can be more easily and readily maintained (Laudet, 2011).

Overall, these studies highlight the potential for manipulations of 5-HT_{2C}R function, such as activation by the FDA-approved selective 5-HT_{2C}R agonist lorcaserin, to (at least partially) suppress cocaine cue reactivity. The nuanced, intricate, and

interconnected web of mechanisms which control 5-HT_{2C}R function to modulate complex behaviors remain to be fully untangled, but the pathway-specific regulation of the 5-HT_{2C}R to modulate cocaine cue reactivity underscores the urgency for multi-prong approaches which target multiple receptor systems (e.g., a 5-HT_{2C}R agonist plus a 5-HT_{2A}R antagonist; Anastasio et al., 2015; Cunningham et al., 2013) or multiple aspects of the same receptor [e.g., an agonist combined with an allosteric modulator (Ding et al., 2012) or a disrupter of the interaction with a protein partner (Anastasio et al., 2013)]. The development of efficacious, FDA-approved therapeutics for cocaine use disorder could be aided by careful evaluation of cue reactivity in preclinical and clinical models.

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Vita

Sarah E. Swinford-Jackson was born on October 23, 1987 to Sheryl Swinford and Mark C. Swinford. Ms. Swinford-Jackson moved from Las Vegas, Nevada to attend Arizona State University where she obtained a Bachelor of Science degree in Psychology (2010). She completed her undergraduate honors thesis research in the laboratory of Dr. Janet L. Neisewander, and matriculated to UTMB to continue investigating the role of the serotonergic system in cocaine addiction in the laboratory of Dr. Kathryn A. Cunningham. Ms. Swinford-Jackson was selected as a member of the inaugural class of Presidential Scholars at UTMB. Ms. Swinford-Jackson has been active in campus organizations, including reactivating the Society for Neuroscience Galveston Chapter and serving as Vice President for two years (2011-2013), serving as Student Representative to the Neuroscience Graduate Program (2013-2015), and mentoring students through the Summer Undergraduate Research Program (2014) and the Bench Tutorials Program (2012-2013). During her tenure, Ms. Swinford-Jackson has received a number of awards, including a NIDA National Student Research Award Predoctoral Fellowship (F31). Ms. Swinford-Jackson presented her research at nine national and international meetings, hosted an invited webinar, and has been primary authored or co-authored five manuscripts.

Awards

GSBS Associates Scholarship, UTMB, 2015
Jen Chieh and Katherine Huang Scholarship, UTMB, 2014
Dr. & Mrs. Seymour Fisher Academic Excellence Award in Neuroscience, UTMB, 2014
NIDA Director's Travel Award, College on Problems of Drug Dependence, 2014
Bohdan R. Nechay Scholarship, UTMB, 2013
The George Sealy Research Award in Neurology, UTMB, 2013
Michael Tacheeni Scott Endowed Scholarship Award, UTMB, 2012
Bromberg Scholar, UTMB, 2012-2013
NIDA Travel Award to the 25th Anniversary Meeting of the Serotonin Club, 2012
Leroy Olsen, Ph.D. Endowed Scholarship, UTMB, 2011

Frances Adoue Lynch Center for Addiction Research Endowment Award, UTMB, 2011
Travel Award, "Behavior, Biology, and Chemistry", 2011, 2013

Publications

Swinford-Jackson, S.E., Anastasio, N.C., Fox, R.G., Stutz, S.J., Cunningham, K.A. Incubation of cocaine cue reactivity associates with neuroadaptations in the cortical serotonin (5-HT) 5-HT_{2C} receptor (5-HT_{2C}R) system. *Neuroscience*, *324*, pp. 50-61, 2016. doi: 10.1016/j.neuroscience.2016.02.052 (Epub ahead of print).

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