The Role of the Serotonin 2 Family of Receptors in Cocaine-elicited and Cocaine-conditioned Behaviors

By

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ABSTRACT

5-HT_{2A} receptor (R) antagonists and 5-HT_{2C}R agonists attenuate reinstatement of cocaine-seeking behavior (i.e., incentive motivation). 5-HT_2Rs are distributed throughout the brain, primarily in regions involved in reward circuitry, including the prefrontal cortex (PFC), caudate putamen (CPu), and basolateral (BlA) and central (CeA) amygdala. Using animal models, we tested our hypotheses that 5-HT_{2A}Rs in the medial (m) PFC mediate the incentive motivational effects of cocaine and cocaine-paired cues; 5-HT_{2A}Rs and 5-HT_{2C}Rs interact to attenuate cocaine hyperlocomotion and functional neuronal activation (i.e, Fos protein); and 5-HT_{2C}Rs in the BlA mediate the incentive motivational effects of cocaine-paired cues and anxiety-like behavior, while 5-HT_{2C}Rs in the CeA mediate the incentive motivational effects of cocaine. In chapter 2, we infused M100907, a selective 5-HT_{2A}R antagonist, directly into the mPFC and examined its effects on reinstatement of cocaine-seeking behavior. We found that M100907 in the mPFC dose-dependently attenuated cue-primed reinstatement, without affecting cocaine-primed reinstatement, cue-primed reinstatement of sucrose-seeking behavior, or locomotor activity. In chapter 3, we used subthreshold doses of M100907 and MK212, a 5-HT_{2C}R agonist, to investigate whether these compounds interact to attenuate cocaine hyperlocomotion and Fos protein expression. Only the drug combination attenuated cocaine hyperlocomotion and cocaine-induced Fos expression in the CPu, but had no effect on spontaneous locomotion. Finally, in chapter 4 we investigated the effects of a 5-HT_{2C}R agonist in the BlA and CeA on cocaine-seeking behavior and anxiety-like behavior. We found that CP809101, a selective 5-HT_{2C}R agonist, infused into the BlA increased anxiety-like behavior on the elevated plus maze (EPM), but failed to alter

cocaine-seeking behavior. CP809101 infused into the CeA attenuated cocaine-primed reinstatement and this effect was blocked by co-administration of a 5-HT_{2C}R antagonist. Together, these results suggest that 5-HT_{2A}Rs in the mPFC are involved in cue-primed reinstatement, 5-HT_{2A} and 5-HT_{2C}Rs may interact in the nigrostriatal pathway to attenuate cocaine hyperlocomotion and Fos expression, and 5-HT_{2C}Rs are involved in anxiety-like behavior in the BlA and cocaine-primed reinstatement in the CeA. Our findings add to the literature on the localization of 5-HT_{2A}R antagonist and 5-HT_{2C}R agonist effects, and suggest a potential treatment mechanism via concurrent 5-HT_{2A}R antagonism and 5-HT_{2C}R agonism.

DEDICATION

To my mentors for being eternally patient and generous

and for teaching me so much more than science.

To my colleagues and students for their knowledge,

advice, and camaraderie.

To my parents for giving me so many opportunities in life

and always supporting me along the way.

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TABLE OF CONTENTS

Page

CHAPTER Page

CHAPTER Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

Figure Page

Chapter 1

General Overview

Drug addiction is a serious social and financial problem, with an estimated 37% of the population having used illicit drugs and over $25 billion spent in 2012 to combat this disorder (Office of National Drug Control Policy, 2013). In a recent analysis, 2.4 million Americans self-reported current cocaine abuse (SAMHSA). Despite the prevalence of cocaine addiction and decades of research, treatment remains inadequate. Psychotherapy utilized for some cocaine addicts is often insufficient for long-term abstinence (Alterman et al., 1996; K. M. Carroll et al., 2004; Kampman et al., 2001) and there are no approved pharmacological treatments available.

Typically cocaine abuse involves binges during which cocaine is repeatedly taken for its psychoactive effects, followed by a period of voluntary abstinence during which craving is usually absent initially but then gradually emerges and is thought to motivate another binge. Even after prolonged periods of abstinence, cocaine craving can be triggered by stress, exposure to cocaine-related cues, or sampling cocaine (Childress, McLellan, Ehrman, & O'Brien, 1988; Jaffe, Cascella, Kumor, & Sherer, 1989; Sinha, Catapano, & O'Malley, 1999). Though the definition is controversial, craving is thought to reflect incentive motivation for cocaine (Stewart, 1983), which in turn, is a major factor contributing to relapse.

The extinction/reinstatement model is used to measure incentive motivation for cocaine in animals trained to lever press for cocaine reinforcement (de Wit & Stewart, 1981). These animals undergo extinction sessions during which cocaine is withheld and operant responding under this condition is referred to as cocaine-seeking behavior.

Initially, animals exhibit robust cocaine-seeking behavior, which declines as extinction progresses. Once the behavior is extinguished, it can be reinstated by cocaine priming or presenting cues previously associated with cocaine. The reinstatement is thought to be a measure of the incentive motivation for cocaine elicited by these stimuli, similar to cue- or drug-primed craving in humans (de Wit & Stewart, 1981; Markou et al., 1993; Neisewander & Acosta, 2007).

Cocaine and Serotonin

In the brain, a primary action of cocaine is blockade of 5-HT transporters, resulting in enhanced synaptic levels of 5-HT (Koe, 1976; Koob, Sanna, & Bloom, 1998; Woolverton & Johnson, 1992). 5-HT plays a complex role in cocaine reinforcement and incentive motivation for cocaine. In rats, increasing 5-HT neurotransmission via indirect agonists fluoxetine and *d*-fenfluramine attenuates cue-primed reinstatement, cocaine self-administration, progressive ratio breakpoint, and reward threshold (Baker, Tran-Nguyen, Fuchs, & Neisewander, 2001; Burmeister, Lungren, Kirschner, & Neisewander, 2004; M. E. Carroll, Lac, Asencio, & Kragh, 1990; Lee & Kornetsky, 1998; Peltier & Schenk, 1993; Richardson & Roberts, 1991), and chronic fluoxetine administration decreases sensitivity to the rewarding effects of cocaine (Baker, et al., 2001; Lee & Kornetsky, 1998) and decreases cocaine-seeking behavior during extinction (Baker, et al., 2001). Interestingly, 5-HT depletion with a tryptophan hydroxylase inhibitor para-chlorophenylalanine (p-CPA) or the 5-HT-selective neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) also attenuates cocaine-seeking behavior (Tran-Nguyen, Baker, Grote, Solano, & Neisewander, 1999; Tran-Nguyen, et al., 2001). Similarly in humans, increasing 5-HT with fluoxetine (Batki, Manfredi, Jacob, & Jones, 1993; Walsh, Preston,

Sullivan, Fromme, & Bigelow, 1994) or decreasing 5-HT via tryptophan depletion (Aronson et al., 1995; Batki, Washburn, Delucchi, & Jones, 1996; Satel, Krystal, Delgado, Kosten, & Charney, 1995) decreases self-reports of cocaine craving and "high" and craving. Collectively, these findings suggest that either low or high levels of 5-HT can inhibit cocaine-related behaviors.

Serotonin Receptor Subtypes

5-HT is involved in many aspects of behavior and is implicated in many mental health disorders including depression, anxiety, and addiction. The wide-ranging role of 5-HT is likely due to a complex receptor system. Since the discovery of different 5-HT receptors in the brain (Peroutka & Snyder, 1979), over 20 receptor subtypes organized into 7 families have been identified (Hoyer et al., 1994; Pytliak, Vargova, Mechirova, & Felsoci, 2011), all of which are G-protein coupled metabotropic receptors aside from the 5-HT_3R which is a ligand-gated ion channel (Barnes & Sharp, 1999).

A brief review of the 5-HTR subtypes reveals that the 5-HT_{1A}R is most implicated in anxiety (Klemenhagen, Gordon, David, Hen, & Gross, 2006), with a 5-HT_{1A}R agonist buspirone a notable treatment for generalized anxiety disorder, as well as aggression (de Boer & Koolhaas, 2005; Rickels, 1983). The 5-HT_{1B}R is also involved in aggression (Groenink, van Bogaert, van der Gugten, Oosting, & Olivier, 2003) and anxiety (Benjamin, Lal, & Meyerson, 1990), in addition to addiction (Groenink, et al., 2003; Pentkowski, Acosta, Browning, Hamilton, & Neisewander, 2009) and migraine (Buzzi & Moskowitz, 1991). 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F}Rs are less studied but thought to be involved in anxiety, memory, and vasoconstriction, respectively (Pytliak, et al., 2011). In the 5-HT_2R family, the 5-HT_{2A}R is involved in hallucinations (Glennon, 1990) and

addiction (Fletcher, Grottick, & Higgins, 2002; Nic Dhonnchadha, Fox, Stutz, Rice, & Cunningham, 2009; Pockros, Pentkowski, Swinford, & Neisewander, 2010), with 5-HT_{2A}R agonists including hallucinogenic drugs lysergic acid diethylamide (LSD) and mescaline. 5-HT_{2B}R ligands have more of an effect peripherally than in the brain (Borman et al., 2002; Ellis et al., 1995), however some findings suggest that 5-HT_{2B}Rs are involved in anxiety (Kennett et al., 1998). 5-HT_{2C}Rs are highly involved in appetite (Sargent, Sharpley, Williams, Goodall, & Cowen, 1997) and addiction (Neisewander & Acosta, 2007; Pentkowski et al., 2010), with a 5-HT_{2C}R agonist loraserin recently FDA-approved for the treatment of obesity (Halford, Harrold, Boyland, Lawton, & Blundell, 2007). The 5-HT_3R is the only ligand-gated ion channel receptor of the 7 families, and is most involved in nausea and vomiting (Gyermek, 1995), with 5-HT_3R antagonists used to treat the side effects of chemotherapy, as well as a few studies suggesting a connection to anxiety and addiction (Rodd et al., 2007; Thompson & Lummis, 2007). 5-HT_4Rs are mostly studied for their peripheral effects on the gastrointestinal tract (De Ponti & Crema, 2002), though one study suggests a role in memory and addiction (Reynolds et al., 1995). While 5-HT_5Rs have not been studied extensively, they may be involved in locomotion and sleep (Pytliak, et al., 2011; Thomas, 2006). 5-HT_6Rs have been implicated in memory, cognition, and mood (Johnson, Ahmed, & Miller, 2008; Woolley, Marsden, & Fone, 2004). Finally, 5-HT_7Rs appear to be involved in depression and sleep (Hedlund & Sutcliffe, 2004; Mnie-Filali, Lambas-Senas, Zimmer, & Haddjeri, 2007).

While several 5-HTR subtypes have been implicated in addiction, this dissertation will focus on the 5-HT_{2A} and 5-HT_{2C}Rs. Both receptors are known to play a role in cocaine-seeking behavior, with a consistent pattern suggesting that manipulations of

these receptors have opposite effects (Fletcher, et al., 2002; Neisewander & Acosta, 2007; Nic Dhonnchadha, et al., 2009). The 5-HT_{2A}R plays an excitatory role in cocaine-related behaviors while the 5-HT_{2C}R plays an inhibitory role.

Peripheral injections of the 5-HT_{2A}R selective antagonist M100907 decrease cocaine hyperlocomotion as well as cue- and cocaine-primed reinstatement of cocaine-seeking behavior, but have no effect on cocaine self-administration (Fletcher, et al., 2002; Nic Dhonnchadha, et al., 2009). Systemic administration of M100907 also attenuates cocaine discriminative stimulus effects (McMahon, Filip, & Cunningham, 2001) and stimulant-induced hyperlocomotion (Fletcher, et al., 2002; McMahon, et al., 2001). Furthermore, blocking 5-HT_{2A}Rs attenuates MDMA-induced DA release (Schmidt, Fadayel, Sullivan, & Taylor, 1992; Schmidt, Sullivan, & Fadayel, 1994) and decreases c-Fos expression in the nucleus accumbens (NAc) shell and caudate-putamen (CPu) (Szucs, Frankel, McMahon, & Cunningham, 2005).

Conversely, the 5-HT_{2C}R plays an inhibitory role in cocaine-related behaviors. For instance, a selective 5-HT_{2C}R antagonist enhances cocaine self-administration, as well as cocaine-primed reinstatement of cocaine-seeking behavior and cocaine-induced locomotor activity (Fletcher, et al., 2002; McMahon, et al., 2001). 5-HT_{2C}R agonists also inhibit cue- and cocaine-primed reinstatement of cocaine-seeking behavior, effects that are blocked by pre-administration of 5-HT_{2C}R antagonists, indicating that they are 5-HT_{2C}R-mediated (Fletcher, Rizos, Sinyard, Tampakeras, & Higgins, 2008; Neisewander & Acosta, 2007; Pentkowski, et al., 2010). Further, 5-HT_{2C}R agonists attenuate morphine-induced DA release in the NAc (Willins & Meltzer, 1998), and antagonists enhance amphetamine-induced DA release in the NAc and CPu (Porras et al., 2002).

Unpublished data from our laboratory also suggests that a 5-HT_{2C} agonist administered into the vmPFC also decreases cocaine hyperlocomotion as well as cocaine-induced Fos expression in the dorsolateral caudate-putamen.

The 5-HT_{2A} and 5-HT_{2C}R are both G-q protein coupled receptors which activate phospholipase C (PLC) and continue their signaling pathway through diacylglycerol (DAG), inositol triphosphate (IP3), and protein kinase C (PKC), to increase calcium (Ca^{2+}) release. These receptors share approximately 51% of their amino acid sequences (Hoyer, Hannon, & Martin, 2002), making them fairly homologous in structure, though there are some differences in their localization and signaling properties (Berg et al., 1994; Grotewiel & Sanders-Bush, 1999). Both receptors are found throughout the brain (Doherty & Pickel, 2000; Pompeiano, Palacios, & Mengod, 1994), but their concentrations differ and the 5-HT_{2A}R is also found peripherally, predominantly in the digestive tract (Leysen, 2004). Both primarily activate PLC, but also phospholipase A2 (PLA_2), which increases arachidonic (AA) release, and phospholipase D (PLD), which increases phosphatidic acid (PA) formation. There are some differences in the degree to which these pathways are activated by constitutive activity and ligand binding at the receptors (Cunningham et al., 2013). These differences may be due to alterations in the receptors over time, as they can undergo trafficking to relocate the receptor on a neuron and the 5-HT_{2C}R is the only 5-HT receptor to undergo RNA editing (Niswender et al., 2001), which may contribute to the different behavioral effects seen when they are activated.

5-HT$_2$R and Brain Reward Pathway

The mesolimbic DA system, which includes dopaminergic projections from the ventral tegmental area (VTA) to the NAc, plays a critical role in reward learning and is certainly involved in addiction, and the mesocortical and nigrostriatal DA systems are also implicated (Wise, 2009). The mesocortical DA pathway projects from the VTA to the prefrontal cortex (PFC), and the nigrostrial DA pathway projects from the substantia nigra (SN) to the CPu. While it was initially thought that the rewarding effects of drugs of abuse were due to increased dopamine (DA) in the nucleus accumbens (NAc), research has shown that the addiction circuitry is much more complex.

5-HT affects these DA reward pathways as well (Alex & Pehek, 2007). 5-HT$_{2A}$ and 5-HT$_{2C}$Rs are found in several regions of the reward circuitry involved in addiction (Doherty & Pickel, 2000; Pompeiano, et al., 1994). 5-HT$_2$R manipulations can directly influence DA release in the mesolimbic, mesocortical, and nigrostriatal pathways (Alex & Pehek, 2007). The nonselective 5-HT$_{2A}$R agonist, 2,5-dimethoxy-4-iodoamphetamine (DOI), has been shown to increase single unit recordings of VTA DA neuron firing as well as microdialysis measures of DA release in the PFC, effects which were reversed with co-administration of a 5-HT$_{2A}$R antagonist (Bortolozzi, Diaz-Mataix, Scorza, Celada, & Artigas, 2005; Pehek, McFarlane, Maguschak, Price, & Pluto, 2001). 5-HT$_{2A}$R antagonists also decrease cocaine- and amphetamine-induced DA release in the NAc (Auclair, Blanc, Glowinski, & Tassin, 2004; Broderick, Olabisi, Rahni, & Zhou, 2004). In the nigrostriatal pathway, 5-HT$_{2A}$R antagonists attenuate phasic DA release in the CPu (De Deurwaerdere & Spampinato, 1999; Gobert & Millan, 1999; Ichikawa & Meltzer, 1995; Lucas & Spampinato, 2000; Porras, et al., 2002; Schmidt, et al., 1992). Conversely,

5-HT_{2C}R agonists attenuate both tonic and phasic DA activity (Di Giovanni et al., 1999; Di Matteo, Di Giovanni, Di Mascio, & Esposito, 2000; Gobert et al., 2000; Porras, et al., 2002). For example, a 5-HT_{2C}R agonist decreases VTA DA neuron firing rates (Di Giovanni, Di Matteo, Di Mascio, & Esposito, 2000; Di Matteo, et al., 2000) as well as DA release in the NAc (De Deurwaerdere & Spampinato, 1999; Di Giovanni, et al., 1999; Di Matteo, Di Giovanni, Di Mascio, & Esposito, 1999). Further, a 5-HT_{2C}R antagonist increases DA release in the PFC (Pozzi, Acconcia, Ceglia, Invernizzi, & Samanin, 2002) and cocaine-induced DA in the NAc (De Deurwaerdere, Navailles, Berg, Clarke, & Spampinato, 2004). 5-HT_{2C}R inverse agonists also increase DA release in the CPu, and this can be reversed with concurrent administration of a 5-HT_{2C}R agonist (Alex, Yavanian, McFarlane, Pluto, & Pehek, 2005).

Aims of Research

The goals of the research in this dissertation were to explore the treatment potential of 5-HT_2R manipulations and to expand upon the knowledge of the localization of the effects of systemic 5-HT_2R manipulations within the brain reward pathways. In the second chapter of this dissertation, I investigated the effects of a 5-HT_{2A}R antagonist in the medial subregion of the mPFC on reinstatement of cocaine-seeking behavior. The PFC is a key region in the mesocorticolimbic DA pathway and is involved in impulsivity and decision-making (Bechara, Damasio, Damasio, & Anderson, 1994; Damasio, Grabowski, Frank, Galaburda, & Damasio, 1994; Puumala & Sirvio, 1998). In addiction, it is thought that repeated drug taking leads to dysfunction of the PFC in which non-drug rewards become less salient and drug rewards become overly salient, thus leading to a loss of control and continued drug taking despite consequences (Childress et al., 1999;

Goldstein & Volkow, 2002). The medial (m) PFC is further divided into the prelimbic (PrL) and infralimbic (IL) regions. The PrL PFC sends projections to the basolateral amygdala (BlA) and NAc core, while the IL PFC connects to the CeA via GABA inhibitory neurons in intercalated cell masses in the amygdala as well as the NAc shell (Brog, Salyapongse, Deutch, & Zahm, 1993; McFarland & Kalivas, 2001; Pare & Smith, 1993; Sesack, Deutch, Roth, & Bunney, 1989). Similar to their roles in fear conditioning (Quirk, Garcia, & Gonzalez-Lima, 2006; Vidal-Gonzalez, Vidal-Gonzalez, Rauch, & Quirk, 2006), the PrL PFC is thought to initiate conditioned cocaine-seeking (Kalivas & O'Brien, 2008; Peters, Kalivas, & Quirk, 2009), while the IL PFC is necessary for extinction of conditioned cocaine-seeking behavior (Peters, et al., 2009). Pharmacological inactivation of the PrL PFC attenuates context-, cue-, cocaine-, and stress-primed reinstatement as well as cocaine conditioned place preference (CPP) (Capriles, Rodaros, Sorge, & Stewart, 2003; Fuchs et al., 2005; Peters, et al., 2009; Zavala, Weber, Rice, Alleweireldt, & Neisewander, 2003). Inactivation of the IL PFC fails to affect reinstatement of cocaine-seeking (McFarland & Kalivas, 2001), though one study showed an increase in cocaine-seeking (Peters, LaLumiere, & Kalivas, 2008); however it has also been shown to attenuate cue-primed reinsatement of morphine-seeking behavior as well (Rocha & Kalivas, 2010).

Both the PrL and IL regions of the mPFC are densely packed with 5-HT_{2A}Rs (Doherty & Pickel, 2000; Lopez-Gimenez, Mengod, Palacios, & Vilaro, 1997; Pompeiano, et al., 1994), and 5-HT_{2C}Rs in these regions have been shown to be involved in cue- and cocaine-primed reinstatement (Pentkowski, et al., 2010). My study examined the effects of intra-mPFC injections of M100907, a selective 5-HT_{2A}R antagonist, on cue-

and cocaine-primed reinstatement as well as locomotor activity and sucrose-seeking behavior. This chapter is published in *Psychopharmacology*.

In the third chapter of this dissertation, I examined the effects of concurrent 5-HT_{2A}R antagonism and 5-HT_{2C}R agonism on cocaine hyperlocomotion and cocaine-induced Fos protein expression in the CPu. Systemically, both 5-HT_{2A}R antagonists and 5-HT_{2C}R agonists attenuate cocaine hyperlocomotion; we used subthreshold doses of each type of ligand, predicting that they would produce a greater effect when given together. Research suggests that 5-HT_{2A}R antagonists and 5-HT_{2C}R agonists may have therapeutic potential in treating addiction; however since both drugs have side-effects, this type of combination therapy may be beneficial in producing a therapeutic effect while minimizing potential side effects produced at the respective receptors.

We measured Fos protein expression in the CPu, as this is the terminal region in the nigrostriatal DA pathway involved in motor activity and habitual behaviors (Brown, Robertson, & Fibiger, 1992; Naylor & Olley, 1972; White, Doubles, & Rebec, 1998; Zimmerberg & Glick, 1974). Some researchers believe that the transition from voluntary to habitual drug use is a reflection of a shift of neural control from the ventral to dorsal striatum, which includes the CPu in rats (Belin-Rauscent, Everitt, & Belin, 2012; B. J. Everitt & Robbins, 2005). After prolonged cocaine self-administration, expected cocaine and cue administration increases DA release in the CPu, rather than the ventral striatum as in the beginning stages of drug-taking (B. J. Everitt et al., 2008; Ito, Dalley, Howes, Robbins, & Everitt, 2000; Ito, Dalley, Robbins, & Everitt, 2002). Additionally, lesions to the CPu do not affect cocaine self-administration (Roberts, Koob, Klonoff, & Fibiger, 1980), but prevent amphetamine-induced stereotypy (Creese & Iversen, 1974; Kelly &

Iversen, 1976). In humans, exposure to cues activates the dorsal striatum in experienced cocaine addicts (Garavan et al., 2000; Volkow et al., 2006). This chapter of the dissertation is published in *Synapse*.

Finally, in chapter 4 I hypothesized that 5-HT_{2C}Rs in the amygdala mediate the incentive motivational effects of cocaine and cocaine-paired cues. 5-HT_{2C}R agonists attenuate cue- and cocaine-primed reinstatement of cocaine-seeking behavior (Fletcher, et al., 2008; Neisewander & Acosta, 2007; Pentkowski, et al., 2010) and also increase anxiety-like behavior on several paradigms (Nic Dhonnchadha, Bourin, & Hascoet, 2003). 5-HT_{2C}Rs are found throughout the brain (Pompeiano, et al., 1994), including the amygdala, a part of the mesolimbic DA pathway known to be involved in reinstatement of cocaine-seeking behavior (Alleweireldt, Hobbs, Taylor, & Neisewander, 2006; McFarland, Davidge, Lapish, & Kalivas, 2004; See, 2005) as well as stress and anxiety (Blanchard & Blanchard, 1972).

Generally, the amygdala is involved in emotional learning (Bechara et al., 1995) and memory (Cahill, 2000), notably the recognition of facial expressions of emotion in humans (Adolphs, Tranel, Damasio, & Damasio, 1994; A. W. Young et al., 1995). Another role of the amygdala is in regulating fear (Kluver, 1938), in particular Pavlovian fear conditioning (Blanchard & Blanchard, 1972; Pribram, Reitz, McNeil, & Spevack, 1979) and avoidance learning (Weiskrantz, 1956). The amygdala is also involved in appetitive conditioning (Cahill & McGaugh, 1990; B. J. Everitt, Morris, O'Brien, & Robbins, 1991; Hamann, Ely, Grafton, & Kilts, 1999; Kesner, Walser, & Winzenried, 1989), which lead to discovering its role in addiction (B. J. C. Everitt, R. N.; Hall, J.; Parkinson, J. A.; Robbins, T. W., 2000).

The amygdala is made up of distinct nuclei, primarily the basolateral (BlA) and central (CeA) regions, which have been differentially implicated in addiction. The BlA is highly involved in associating environmental stimuli and reward (B. J. C. Everitt, R. N.; Hall, J.; Parkinson, J. A.; Robbins, T. W., 2000). The BlA has an important role in reinstatement of cocaine-seeking by drug-associated contextual and discrete cues (B. J. Everitt et al., 1999; Fuchs & See, 2002; Fuchs, Weber, Rice, & Neisewander, 2002; Kufahl et al., 2009; McLaughlin & See, 2003; Neisewander et al., 2000). The CeA projects to brainstem regions to control expression of emotional behaviors (Lanuza, Moncho-Bogani, & Ledoux, 2008) and is implicated in stress-reinstatement and primary reinforcement effects of cocaine (Koob & Le Moal, 2005; Koob & Nestler, 1997; Neisewander, et al., 2000; O'Dell, Sussman, Meyer, & Neisewander, 1999; Wurtz & Olds, 1963).

To investigate my hypothesis, we examined the effects of a 5-HT_{2C}R agonist CP809101 administered directly into the basolateral amygdala (BlA) and the central amygdala (CeA) on cue- and cocaine-primed reinstatement of cocaine-seeking behavior. We also evaluated the effects of CP809101 in these regions on anxiety-like behavior, as 5-HT_{2C}R agonists have been shown to affect anxiety (Heisler, Zhou, Bajwa, Hsu, & Tecott, 2007; Kimura et al., 2009; Strong, Greenwood, & Fleshner, 2009), and the amygdala is an important region in the neurocircuitry of anxiety (M. Davis, 2000).

Chapter 2

Blockade of 5-HT_{2A} Receptors in the mPFC attenuates cue-primed reinstatement of cocaine-seeking behavior in rats

(Master's thesis, published 2011)

Previous work with 5-HT_{2A} selective antagonists found that peripheral administration decreases cocaine-induced locomotor activity as well as cue- and cocaine-primed reinstatement, but has no effect on cocaine self- administration (Fantegrossi, Ullrich, Rice, Woods, & Winger, 2002; Filip, Bubar, & Cunningham, 2006; Fletcher, et al., 2002; Nic Dhonnchadha, et al., 2009; Orejarena, Lanfumey, Maldonado, & Robledo, 2010). It is believed that 5-HT action at 5-HT_{2A} receptors may oppose its action at 5-HT_{2C} receptors (Bubar & Cunningham, 2006; Higgins & Fletcher, 2003). For example, in contrast to the inhibitory effects of the 5-HT_{2A} receptor antagonist on cocaine- seeking behavior, the 5-HT_{2C} receptor antagonist was found to enhance cocaine-primed reinstatement of extinguished cocaine-seeking behavior, as well as cocaine self-administration and cocaine-induced locomotor activity (Fletcher, et al., 2002; Nic Dhonnchadha, et al., 2009). Furthermore, 5-HT_{2C} receptor agonists inhibit cue-elicited and cocaine-primed reinstatement of extinguished cocaine- seeking behavior when injected systemically (Fletcher, et al., 2002; Neisewander & Acosta, 2007) or directly into the ventral medial prefrontal cortex (vmPFC), which includes the prelimbic and infralimbic subregions (Gabbott, Warner, Jays, Salway, & Busby, 2005; Pentkowski, et al., 2010).

The vmPFC plays a critical role in cue-elicited and cocaine-primed reinstatement of extinguished cocaine- seeking behavior, as well as cocaine reinforcement (Di Pietro,

Mashhoon, Heaney, Yager, & Kantak, 2008; Goeders & Smith, 1983; McGregor, Baker, & Roberts, 1996; Olsen & Duvauchelle, 2006). Exposure to drug-associated cues causes an increase in activity-related gene expression in the infralimbic, prelimbic, anterior cingulate, and orbitofrontal subregions of the prefrontal cortex (PFC) (Hearing, Miller, See, & McGinty, 2008; Kufahl, Zavala, et al., 2009; Neisewander, et al., 2000; Zavala, Osredkar, Joyce, & Neisewander, 2008). Furthermore, excitoxic lesions or reversible pharmacological inactivation of these subregions of the PFC prevents cue-elicited reinstatement of extinguished cocaine-seeking behavior (Di Pietro, Black, & Kantak, 2006; Fuchs, Evans, Parker, & See, 2004; McLaughlin & See, 2003; Weissenborn, Robbins, & Everitt, 1997). Conversely, cocaine injections directly into the mPFC are reinforcing (Goeders & Smith, 1983; Guzman, Moscarello, & Ettenberg, 2009) and reinstate cocaine self-administration (Goeders, Dworkin, & Smith, 1986). Given our recent findings that stimulation of 5-HT_{2C} receptors in the vmPFC attenuates cue-elicited and cocaine-primed reinstatement of extinguished cocaine-seeking behavior (Pentkowski, et al., 2010) together with research demonstrating opposing roles of 5- HT_{2C} and 5-HT_{2A} receptors in modulating cocaine-seeking behavior (Fletcher, et al., 2002; Nic Dhonnchadha, et al., 2009), we hypothesized that blockade of 5-HT_{2A} receptors in the vmPFC would attenuate cue- and cocaine-primed reinstatement of extinguished cocaine-seeking behavior.

Additional rationale for this hypothesis is that 5-HT_{2A} receptors are densely distributed throughout the cortex including the vmPFC, as well as the ventral tegmental area (VTA), substantia nigra, and the striatum which have also been implicated in addiction (Doherty & Pickel, 2000; Lopez-Gimenez, et al., 1997; Pompeiano, et al.,

1994). The highly selective 5-HT_{2A} receptor antagonist, M100907, has been shown to decrease extracellular dopamine levels in the vmPFC and striatum when infused directly into these regions (Pehek, et al., 2001; Schmidt, et al., 1992; Schmidt, et al., 1994). Furthermore, elevated glutamate release in the PFC is thought to excite outputs to the nucleus accumbens (NAc) resulting in potentiation of cue- and cocaine-primed reinstatement of extinguished cocaine-seeking behavior (Di Ciano & Everitt, 2001; McFarland, Lapish, & Kalivas, 2003), and increases in glutamate in the vmPFC are attenuated by systemic injections of M100907 (Ceglia et al., 2004). These findings are consistent with the idea that 5-HT_{2A} receptors in the vmPFC may mediate the inhibitory effects of M100907 on cue- and cocaine-primed reinstatement of extinguished cocaine-seeking behavior.

This study investigated the hypothesis that 5-HT_{2A} receptor stimulation in the vmPFC contributes to the incentive motivational effects of cocaine-conditioned cues and cocaine itself. To test this hypothesis, we examined the effects of localized microinjections of M100907 on reinstatement of extinguished cocaine-seeking behavior elicited by cocaine-paired cues or cocaine-priming injections. The effects of M100907 on cocaine self-administration, cue- elicited reinstatement of sucrose-seeking behavior, and spontaneous and cocaine-induced locomotor activity were also examined in order to assess the specificity of the effects for cocaine-seeking behavior.

Methods

Animals

Adult male Sprague-Dawley rats weighing 300-325 g at the start of the experiments were used in this study. Animals were housed in a climate-controlled colony room with a 12-h reversed light/dark cycle (lights off at 7:00AM) and were cared for in accordance with the 'Guide for the Care and Use of Laboratory Animals' (Institute of Laboratory Animal Resources on Life Sciences, National Research Council 1996).

Surgery

Animals were handled for at least 6 days before implanting catheters into the right jugular vein. Catheters were connected to a bent 22 gauge metal cannula within a plastic screw connector (Plastics One, Roanoke, VA) attached to a 10 cm Silastic tube (inner diameter 0.012 x outer diameter 0.025 inches, Dow Corning, Midland, MI) with a small ball of aquarium sealant ~ 4 cm from the other end. Animals were anesthetized with approximately 3% isoflurane throughout surgery. Incisions were made in clean, shaven areas on the head to expose the skull and on the neck to expose the right jugular vein. A small incision was made in the jugular vein, the catheter was then inserted until flush with the ball of aquarium sealant, and the catheter was secured to the vein with sutures on either side of the ball. The catheter was then pulled through a burrow made subcutaneously between the two incisions and the rat was then placed into a stereotaxic instrument. Connective tissue was removed from the skull surface and four small screws were drilled into the skull to serve as an anchor. Small holes were then drilled into the skull and stainless steel guide cannulae were lowered to a point 1 mm above the targeted site of the medial prefrontal cortex (n=59) or the Cg2 region of the anterior cingulate

cortex (n=9). The coordinates for the medial prefrontal cortex were selected based on previous research (Filip & Cunningham, 2003; Pentkowski, et al., 2010) and were as follows: AP = + 2.7 and ML = +/- 0.75 mm relative to bregma; DV = -3 mm from skull surface (Paxinos, 2007). The coordinates for the Cg2 were the following: AP = + 2.0 and ML = +/- 0.75 mm relative to bregma; DV = -3 mm from skull surface (Paxinos, 2007). The guide cannulae were secured to the skull along with the metal end of the catheter and the anchor screws using dental acrylic cement. Metal stylets were inserted with the cannulae during surgery. All incisions were sutured and treated with a topical antibiotic. Catheters were flushed with a solution of 0.1 ml saline containing heparin sodium (70 U/ml; APP Pharmaceuticals, Schaumburg, IL), Abbokinase (20 mg/ml; ImaRx Therapeutics, Tucson, AZ) and Timentin (66.7 mg/ml; GlaxoSmithKline, Research Triangle Park, NC) for 5 days after surgery. Throughout the rest of self-administration training and testing, catheters were flushed daily with a solution containing only the Timentin and heparin sodium in order to maintain catheter patency. Animals were given at least 7 days of recovery from surgery before beginning self-administration training. Catheter patency was tested periodically by administering 0.05 ml Brevital (16.6 mg/ml, Jones Pharma Inc., St. Louis, MO), which briefly anesthetizes the animal only if delivered i.v.

Cocaine Self-administration Training

Cocaine self-administration training took place during daily 2-h sessions, 6 days per week. Animals were trained in operant conditioning chambers (28 x 10 x 20 cm; Med Associates, St Albans, Vermont, USA) each containing an active lever, a cue light 4 cm above the active lever, an inactive lever, a tone generator (500 Hz, 10 dB above

ground noise), and a house light on the wall opposite the levers. Upon pressing the active lever to complete a schedule of reinforcement, the light and tone cues were simultaneously activated and followed 1 s later by a 0.1 ml cocaine infusion delivered over 6 s. The house light was then activated for a 20-s timeout period, during which active lever presses were recorded but had no effects. Responses on the inactive lever were recorded but had no effects.

For the first 5 days of training, all animals began on a fixed ratio (FR) 1 schedule of reinforcement with the capability to progress to a variable ratio (VR) 2, VR3, and finally VR5 schedule. After ending the session on a VR5 schedule for 5 consecutive days, animals then began the remaining sessions on a VR5 schedule. In this experiment, all animals were starting on a VR5 schedule by day 14 and were on a VR5 schedule exclusively for at least the last 5 days of self-administration. All animals were restricted to 16 g of food to facilitate acquisition of self-administration (M. E. Carroll, France, & Meisch, 1981) and remained food-restricted until they ended on a VR5 schedule for three consecutive sessions. Animals were then given food *ad libitum* for the rest of the experiment.

Intracranial Drug Infusions

M100907 (RTI International, Research Triangle Park, NC) was dissolved in phosphate buffered saline containing hydrochloric acid, titrated to pH 6.9. Microinjections were delivered over a 1-min period using a 30-gauge injector (Plastics One) connected via polyethylene 50 tubing (Becton Dickinson, Sparks, MD) to a 25 μl syringe (Hamilton Co., Reno, NV) housed in an infusion pump (CMA Microdialysis, North Chelmsford, MA). Injection cannulae extended 2 mm below the guide cannulae for

the mPFC and 1 mm below for the Cg2. Successful infusion of the drug was confirmed by movement of an air bubble through the drug infusion line. After the infusion was complete, the injectors remained for 1 min to ensure thorough diffusion. After removing the injectors, metal stylets and caps were replaced before the animal was placed into the conditioning chamber for the test sessions.

Cocaine Self-Administration Testing

A subset of animals (n=23) with mPFC cannulae were tested for the effects of M100907 on cocaine self-administration once they reached a self-administration stability criterion of less than 15% variability of infusions per session for 3 consecutive days without any upward or downward trends. Rats were assigned to one of four dose groups (0.1, 0.3, 1.0, or 1.5 μg/0.2 μl/side). All animals were tested twice for self-administration, once with a vehicle microinjection into the mPFC and once with their assigned dose of M100907, with order counterbalanced. At least 3 additional self-administration sessions were given in between each test in order to re-establish stable self-administration baseline rates. The bilateral microinjections of M100907 or vehicle into the mPFC were administered 5-min before testing. Three additional sessions of self-administration were conducted after these tests were completed. Test sessions lasted for 2 hours; however data is presented for only the first hour as drug effects do not likely persist beyond that time.

Extinction Phase

Upon completing self-administration training, and testing if applicable, all animals began receiving daily 1-h extinction sessions. Rats were placed into the self-administration chambers as before and lever presses were recorded, but produced no

consequences (i.e., no infusions or cues were presented). Catheters were connected to the infusion lines during extinction, as well as during all reinstatement tests, even though no cocaine was infused. Extinction sessions continued for 10-14 days and until there was an 80% reduction in active lever pressing from the animals' highest response rate during extinction or to less than 20 active lever presses.

Cue Reinstatement of Cocaine-Seeking Behavior

Following extinction training, a subset of animals with mPFC (n=59) cannulae were assigned (or re-assigned if they had undergone self-administration testing) to one of four M100907 dose groups (0.1, 0.3, 1.0, or 1.5 μg/0.2 μl/side), counterbalanced based on the amount of cocaine intake during self-administration, as this has been shown to affect reinstatement response rates (Deroche et al., 1999; Baker et al, 2001). Another subset of animals with anterior cingulate cannulae (n=9) were assigned to receive 1.5μg/0.2 μl/side M100907 or vehicle. Animals underwent two tests for the effects of M100907 on cue reinstatement of extinguished cocaine-seeking behavior, receiving a vehicle microinjection prior to one test and their assigned dose of M100907 prior to the other test, with the order of these pretreatments counterbalanced. Animals were given a minimum of 3 extinction days between tests to allow extinction baseline rates to stabilize. If animals failed to meet a reinstatement criteria of doubling extinction baseline response rates and at least 10 responses on the active lever on both of the 2 test days, they were considered 'nonreinstaters' and excluded from the analysis.

Five min after receiving their assigned microinjection, animals were tested for 1 hr with the same stimulus complex as that paired with cocaine during training available response-contingently on an FR 1 schedule; however no cocaine was delivered during

cue tests. The FR 1 schedule was used in place of the VR 5 training schedule because we have previously shown that under tests for cue reinstatement the FR1 schedule yields higher response rates, and thus greater sensitivity for detecting the predicted decrease, than the training schedule (Acosta, Thiel, Sanabria, Browning, & Neisewander, 2008). A noncontingent cue presentation was delivered if the animal did not receive a response-contingent cue within the first 5 min of the session to minimize the possibility that animals would fail to press the lever leaving them unaware that cues were available.

Cocaine-Primed Reinstatement of Cocaine-Seeking Behavior

After the two cue reinstatement tests, a subset of animals with mPFC cannulae (n=55) received at least 5 extinction sessions to re-establish a stable baseline extinction rate of responding. They were then given two tests for cocaine-primed reinstatement of extinguished cocaine-seeking behavior. Prior to one test, they received the same dose of M100907 as they had received during cue reinstatement testing (0.1, 0.3, 1.0, or 1.5 μg/0.2 μl/side). For the other test, they received a vehicle microinjection. The order of the 2 pretreatments was counterbalanced within a group. Five min after the microinjection, animals received a priming injection of cocaine (10 mg/kg, i.p.) and were then immediately placed into the conditioning chamber. Lever presses were recorded, but produced no consequences (i.e., no cues or cocaine were delivered). To control for injection stress, animals were given saline i.p. injections on the day preceding their cocaine reinstatement tests and the average response rates during these sessions were used as the extinction baseline. Animals were given a minimum of 3 extinction sessions between tests to allow extinction baseline rates to stabilize. If animals failed to meet the reinstatement criteria of doubling baseline and at least 10 responses on the active lever

during both of the reinstatement tests, they were considered 'nonreinstaters' and were excluded from the analysis. All animals were tested for cue reinstatement before cocaine reinstatement.

M100907-Primed Reinstatement of Cocaine-Seeking Behavior

After cocaine reinstatement testing, a subset of animals with mPFC cannulae (n=44) was given a minimum of 5 extinction sessions to allow extinction baseline response rates to stabilize. Animals were then given two reinstatement tests with a microinjection of M100907 (0.1, 0.3, 1.0, or 1.5 μg/0.2 μl/side) prior to one test and vehicle prior to the other test, counterbalanced for order of pretreatment. Animals received the same assigned dose of M100907 that they had received for cue and cocaine-primed reinstatement testing. Animals were placed into the self-administration chambers 5 min after the microinjection for a 1-h test. Responding on neither the active nor the inactive lever had any consequences during these test sessions.

Cue Reinstatement of Sucrose-Seeking Behavior

After cocaine-primed reinstatement tests, a subset of animals with mPFC cannulae (n=10) were food-restricted to approximately 18 g of food/day for two days prior to beginning sucrose reinforcement training. The animals were also given approximately 30 sucrose pellets (45 mg, Bio-Serv, Frenchtown, NJ) in their home cage to familiarize them with the pellets. Animals were trained in a different room with a different set of operant conditioning chambers than those used for cocaine self-administration training. These chambers were each equipped with a food pellet dispenser and a food well located between 2 levers. The location of the active and inactive levers from cocaine self-administration was reversed for sucrose reinforcement training. In all

other respects, the training was similar to that used for cocaine self-administration. Upon completion of a schedule of reinforcement, a cue light was presented above the active lever that oscillated on for 1 s and off for 1 s for 7 s total and a 45 mg sucrose pellet was delivered 1 s after the onset of the light. The house light remained on during the session aside from when the cue light was on as well as a 20-s timeout period after completion of a schedule during which active lever presses had no effects. Rats were given daily 30-min sessions beginning on an FR 1 schedule of reinforcement with the capability to progress to a VR3 and then a VR5 schedule. Once animals ended the session on a VR5 schedule, they began the next session on a VR3 schedule. If they ended on a VR5 schedule again, they began the next session on a VR5 schedule and stayed on this schedule for the remainder of training. Animals remained food restricted until they ended three sessions on a VR5 schedule, at which point they were given food *ad libitum* for the rest of the experiment. All animals began on a VR5 schedule during the last 7 sessions of training and all were given a total of 14 sucrose training sessions.

Next, animals underwent a total of 14 days of 1-hr extinction training sessions, during which there was at least an 80% reduction in lever pressing from the animals' highest response rate during extinction. Subsequently, animals were tested twice for cue reinstatement. They received a 1.5 μg/0.2μl/side M100907 microinjection prior to one test and a vehicle microinjection prior to the other test, counterbalanced for order of pretreatment. 5-min after receiving a microinjection, animals were tested for 1-hr with the same stimulus complex as was paired with sucrose during training on an FR 1 schedule; however no sucrose was available. A noncontingent cue was delivered if a rat did not receive a response-contingent cue within the first 5 min of the test session.

Animals were given a minimum of 3 extinction sessions between tests to allow extinction baseline rates to stabilize.

Locomotor Activity

A subset of animals with mPFC cannulae (n=24) that had a history of cocaine intake from the previous experiments were assigned to receive a microinjection of M100907 at an effective dose from cue-primed reinstatement testing (1.0 μg/0.2μl/side) or vehicle. For two days before testing, animals received 1-h habituation sessions in the locomotor activity chambers. They were then tested twice, receiving either an injection of cocaine (10 mg/kg, i.p.) or saline, counterbalanced for order, immediately after receiving their assigned microinjection. Rats were then placed into Plexiglas locomotor chambers (44 x 24 x 20 cm high) and were tested for 90 min. A computer-automated video tracking system (Clever Systems, Reston, VA) was used to measure the distance traveled by each animal. Animals were given 5 rest days in their home cages between the two tests.

Statistical Analyses

Data were analyzed using mixed-factor analyses of variance (ANOVAs) with session (e.g. extinction baseline, vehicle test, and M100907 test) as a within-subjects factor and dosage group (0.1, 0.3, 1.0, or 1.5 μg/0.2 μl/side) as a between-subject factor. A Greenhouse-Geisser correction was used to correct for heterogeneity of variance in the data. Subsequent post-hoc comparisons were made using tests of simple main effects. In addition, planned t-tests were used to test the prediction that cocaine-seeking behavior is attenuated after M100907 relative to vehicle pretreatment. Baseline values were

calculated as the average of the two sessions that occurred before each test day (e.g. the day before cue testing with M100907 and the day before cue testing with vehicle).

Histology

Animals were deeply anesthetized with 3% isoflurane and given intracranial infusions (0.2 μl/side) of 1% methylene blue to verify cannulae placements. Animals were then decapitated and the brains were removed, cryoprotected, frozen, and stored at -20°C. Brains were sliced in coronal sections (40 μm), stained with thionin, and examined under a microscope by observers unaware of group assignment who determined the point of drug infusion.

Timeline of testing and summary of attrition

All 68 animals, 59 with mPFC and 9 with Cg2 cannulae, were trained to self-administer cocaine and underwent extinction training followed by cue reinstatement testing. Although this study was conducted using 4 different cohorts of rats, each cohort included rats tested at each of the M100907 doses, except for the highest dose which was included only in the last cohort. The cue reinstatement tests were the only tests that animals with Cg2 cannulae underwent. During cue reinstatement tests, 2 animals with mPFC cannulae and 1 animal with a Cg2 cannula failed to meet reinstatement criteria of double baseline or at least 10 lever presses, and so these animals were omitted from the data analysis for these tests. Almost all animals with mPFC cannulae (n=55) underwent cocaine-primed reinstatement testing following the cue reinstatement tests, and of these, 2 animals failed to meet the reinstatement criteria and were omitted from the data analyses for these tests. One animal with mPFC cannulae given the 1.5 μg dose of M100907 prior to cocaine-primed reinstatement was considered an outlier (3+ standard

deviations above the mean) and was also excluded from the analysis. A subset of 23 animals with mPFC cannulae underwent self-administration testing prior to extinction training, and a different subset of 10 animals with mPFC cannulae underwent testing for cue reinstatement of sucrose-seeking behavior. Finally, 24 animals with mPFC cannulae were also tested for the effects of M100907 on locomotor activity after reinstatement testing had been completed. In summary, animals with mPFC cannulae received a total of 6 to 8 microinfusions, whereas animals with anterior cingulate cannulae received a total of 2 microinfusions. The order of specific test types is summarized in Table 1.

Results

Figures 1A and 1B show the representative cannula tip placements for each region. None of the animals had misplaced cannulae. All descriptive statistics given below are presented as the mean ± SEM.

Effects of M100907 on Cocaine Self-Administration

There was no significant difference between groups for total cocaine intake before testing. The average number of infusions ± SEM across the last 5-days of self-administration training for the groups ranged from 24.7 ± 0.37 to 29.9 ± 0.72. Figure 2 illustrates the effects of mPFC M100907 infusions on the number of reinforcers obtained during self-administration testing. The ANOVA of number of reinforcers/h showed that there was a main effect of test day [$F(2,38)=5.23$, $P<0.05$] but no main effect of group or interaction with test day on self-administration behavior. When collapsed across doses (see Figure 2B) there was a significant decrease in reinforcers obtained on the M100907 test day versus baseline [$t(22)=3.29$, $P<0.05$].

Extinction

Active and inactive lever presses during the first session of extinction training are shown in Table 2. All animals had at least 13 extinction sessions before reinstatement testing began. For animals with mPFC cannulae, ANOVAs of the number of active and inactive lever presses/h on the first day of extinction versus the last day of extinction before testing showed main effects of day [$F(1,54)=144.70$ and 18.79, respectively, $P<0.01$] but no dose effect or interaction with dose. Similarly for animals with Cg2 cannulae, the ANOVA of number of active lever presses/h during the first extinction session versus the last extinction session before testing showed only a main effect of day [$F(1,7)=29.45$, $P<0.01$]. In each case, the main effects indicated a significant drop in responding across training sessions. There were no significant effects for inactive lever presses/h in animals with Cg2 cannulae, likely because initial response rates during the first sessions were low.

Effects of M100907 on Cue Reinstatement of Cocaine-Seeking Behavior

Figure 3 shows the effects of M100907 infusions into mPFC on cue-elicited reinstatement of cocaine-seeking behavior. The ANOVA of responses/h on the active lever for animals with mPFC cannulae indicated a significant interaction between test session and M100907 dosage group [$F(6,104)=2.20$, $p<0.05$]. Tests of simple main effects indicated that all groups exhibited cue reinstatement evident as an increase in responding on the vehicle pretreatment test day relative to the extinction baseline ($p<0.05$). In addition, M100907 pretreatment significantly decreased responding in animals receiving the 1.0 and 1.5 μg doses relative to their vehicle pretreatment

[t(13)=2.00, P<.05 and t(16)=3.56, P<.05, respectively], demonstrating a decrease in cue reinstatement at these doses.

Figure 4 illustrates the effects of M100907 infusions on cue reinstatement of cocaine-seeking behavior in animals with Cg2 cannulae. The ANOVA of responses/h on the active lever indicated a significant main effect of test day [$F(2,14)=9.242$, $p<0.05$]. Tests of simple main effects indicated that animals exhibited cue reinstatement evident as an increase in responding on the vehicle pretreatment test day relative to the extinction baseline ($p<0.05$), however there were no significant differences between vehicle and M100907 test days. Table 2 shows inactive lever presses on M100907 test days for all groups. There were no significant differences for inactive lever presses.

Effects of M100907 on Cocaine-Primed Reinstatement of Cocaine-Seeking Behavior

Figure 5 illustrates the effects of intra-mPFC infusions of M100907 on cocaine-primed reinstatement of cocaine-seeking behavior. There were 2 animals out of 55 that failed to meet the reinstatement criteria and were excluded from the analyses. The ANOVA of responses/h on the active lever indicated a significant main effect of test day [$F(1.6,76.6)=42.2$, $P<0.001$] but no interaction with dose or main effect of dose. The planned comparisons also failed to show any differences between vehicle and M100907 test days for any of the groups. Also shown is the main effect of M100907 on cocaine-primed reinstatement collapsed across all four doses. Tests of simple main effects indicated an increase in responding relative to the extinction baseline on both the vehicle and M100907 test days ($p<0.001$). There was also a significant decrease in responding on M100907 test day compared to vehicle test day ($p<0.05$). There were no differences in inactive lever presses (see Table 2). Because these results were contrary to our

hypothesis, we examined whether effects occurred during the first 30 min when the drug effects should be maximal. Responses/30 min showed a similar pattern across groups as the 1-h analysis (data not shown) with a main effect of test day [$F(1.6,78.6)=35.2$, $P<0.001$) but no dose or interaction effects.

Effects of M100907 on Reinstatement of Cocaine-Seeking Behavior

Figure 6 illustrates that M100907 priming injections infused into mPFC prior to testing failed to alter responding relative to extinction baseline. The ANOVA of responses/h indicated that there were no significant effects on response rates on either the active or inactive levers (see Table 2).

Effects of M100907 on Cue Reinstatement of Sucrose-Seeking Behavior

Figure 7 shows the effects of intra-mPFC infusions of 1.5 μg M100907 on reinstatement of sucrose-seeking behavior. The ANOVA of responses/h on the active lever indicated a significant main effect of test day [$F(2,18)=6.87$, $p<0.05$] but no interaction with dose or main effect of dose. Tests of simple main effects indicated that animals exhibited cue reinstatement evident as an increase in responding on the vehicle pretreatment test day relative to the extinction baseline ($p<0.05$), however reinstatement was also evident on the M100907 test day and there was no significant difference between vehicle and M100907 test days. There were no differences in inactive lever presses (see Table 2).

Effects of M100907 on Locomotor Activity

Figure 8 shows the effects of 1.0 μg/0.2 μl/side M100907 infused into mPFC on spontaneous and cocaine-induced locomotor activity. The ANOVA indicated a significant effect of test session [$F(1,22)=76.292$, $P<.05$], but no M100907 dose effect or

test by dose interaction. Tests of simple main effects indicated that animals exhibited significantly more distance traveled when given cocaine as compared to saline, but there was no difference in distance traveled in animals pretreated with vehicle versus M100907.

Discussion

The present findings are the first to demonstrate that a 5-HT_{2A} receptor antagonist infused into the mPFC dose-dependently decreases cue-elicited reinstatement of cocaine-seeking behavior. These results are consistent with the findings that peripheral injections of M100907 attenuate cue-elicited reinstatement of cocaine-seeking behavior (Nic Dhonnchadha, 2009). Furthermore, the findings support our hypothesis that stimulation of 5-HT_{2A} receptors in the mPFC modulates incentive motivational effects of cocaine-paired cues. In contrast to the effects on cue reinstatement, intra-mPFC infusions of M100907 failed to dose-dependently alter cocaine self-administration or cocaine-primed reinstatement. There appeared to be a mild attenuation of these behaviors as there was a main effect of test day in both cases; however, there was no significant difference between numbers of reinforcers obtained or number of active lever presses following vehicle pretreatment relative to M100907 pretreatment at any given dose of M100907. For this reason, we suggest that the attenuation of both cocaine self-administration and cocaine-primed reinstatement that was detected when the data were collapsed across dose (i.e., main effect of test day) is likely due to some nonspecific effect rather than antagonism of 5-HT_{2A} receptors. The lack of effect of M100907 on cocaine self-administration was expected given that systemic administration of M100907 does not affect this behavior (Nic Dhonnchadha, 2009; Fletcher et al, 2002). In contrast, we had

predicted that M100907 infusions into mPFC would attenuate cocaine-primed reinstatement, similar to its systemic effects (Nic Dhonnchadha, 2009; Fletcher et al, 2002).

Out control manipulations provide support for the anatomical and behavioral selectivity of M100907 effects on cue reinstatement. For instance, intra-mPFC infusions of M100907 did not alter spontaneous or cocaine-induced locomotor activity at the 1.0 μg dose, which was a dose that effectively reduced cue reinstatement, suggesting the latter effect was not due to motor impairment. Furthermore, the higher effective dose of 1.5 μg/side did not affect cue reinstatement of sucrose-seeking behavior. These findings further mitigate the possibility that the infusions interfered with the animals' ability to respond and also suggest that memory was intact. The 1.5 μg/side M100907 infusions into the neighboring Cg2 subregion of the anterior cingulate cortex failed to alter cue reinstatement, suggesting anatomical specificity of the mPFC infusions.

It seems likely that the attenuating effect of M100907 on cue reinstatement was due to the antagonism of 5-HT_{2A} receptors in the mPFC. M100907 has a >1000-fold selectivity for 5-HT_{2A} receptors vs. 5-HT_{2C} receptors (Kehne et al., 1996) and several studies have demonstrated that doses of 0.005-0.4 mg/kg M100907 reverse the behavioral effects of 5-HT_{2A}, but not 5-HT_{2C}, receptor agonists (Dekeyne, Girardon, & Millan, 1999; Gresch, Barrett, Sanders-Bush, & Smith, 2007; Hitchcock, Lister, Fischer, & Wettstein, 1997; McCreary, Filip, & Cunningham, 2003; Vickers et al., 2001; Wettstein, Host, & Hitchcock, 1999). We chose not to investigate the effects of a 5-HT_{2A} agonist in the present study because these drugs have hallucinogenic effects, which would cloud interpretation. If M100907 was acting on another receptor subtype, the most likely

candidate is the closely related 5-HT_{2C} receptor. This is unlikely, however, because prior research has shown that peripheral injections of the selective 5-HT_{2C} receptor antagonist SB242084 do not affect cue reinstatement (Burbassi & Cervo, 2008; Burmeister, et al., 2004), and in fact, this antagonist reverses the attenuation of cue reinstatement observed with 5-HT_{2C} receptor agonists and enhances cocaine-primed reinstatement (Burmeister, et al., 2004; Fletcher, et al., 2002; Neisewander & Acosta, 2007). Furthermore, SB242084 infused into the mPFC has no effect on cue- or cocaine-primed reinstatement, whereas the selective 5-HT_{2C} agonist MK212 attenuates both behaviors (Pentkowski, et al., 2010). Thus even though it is doubtful M100907 infusions antagonized 5-HT_{2C} receptors, if such an effect had occurred that may explain why only attenuation, and not complete reversal, of cue reinstatement was observed. Specifically, as the dose of M100907 is increased, antagonism of additional 5-HT_{2A} receptor may be accompanied by antagonism of 5-HT_{2C} receptors with the latter functionally opposing any additional reduction of cue reinstatement by 5-HT_{2A} receptor antagonism.

The region-specific effect of M100907 in attenuating cue reinstatement is consistent with literature suggesting that the mPFC plays a critical role in drug abuse-related behavior. It is thought that repeated psychostimulant administration decreases activity in the PFC, resulting in compulsive drug taking behavior (Jentsch & Taylor, 1999; Volkow, Fowler, Wang, & Goldstein, 2002). Although activation of mPFC may be reduced in drug-dependent individuals relative to controls, when drug-experienced humans or rats are exposed to drug-associated cues, the PFC exhibits increased activity (Childress, et al., 1999; Ciccocioppo, Sanna, & Weiss, 2001; Grant et al., 1996; Maas et al., 1998; Neisewander, et al., 2000). Furthermore, the mPFC has been shown to be

involved in the incentive motivational effects of drug-paired cues through other pharmacological manipulations as well (Bossert, Ghitza, Lu, Epstein, & Shaham, 2005; Kalivas & McFarland, 2003; McLaughlin & See, 2003).

Our findings that only cue and not cocaine-primed reinstatement was affected by mPFC infusion of M100907 suggest the intriguing possibility that different mechanisms within the mPFC may be involved in these types of reinstatement. It has been hypothesized that there is a "final common pathway" for the neurocircuitry involved in stress-, cocaine-, and cue-primed reinstatement (Capriles, et al., 2003; Kalivas, 2008; Neisewander, et al., 2000) that likely involves glutamateric projections from the mPFC to the NAc (Feltenstein & See, 2008; Kalivas & McFarland, 2003; Kalivas, Peters, & Knackstedt, 2006; Shaham, Shalev, Lu, De Wit, & Stewart, 2003). To our knowledge, this study is the first to find differential effects of a given manipulation of the mPFC across the different types of reinstatement, suggesting that there may be independent circuitries involving the mPFC that mediate the effects of cue and cocaine-primed reinstatement. This hypothesis is equivocal presently, however, given that the general attenuation of cocaine-primed reinstatement by M100907 does not allow us to completely rule out the possibility of mPFC 5-HT_{2A} receptor involvement in the incentive motivational effects of cocaine priming. Indeed it is possible that cocaine-primed reinstatement may be altered at different doses of either the cocaine prime or intra-mPFC M100907 infusions. It should be noted that the doses used in the present study were based on previous literature indicating behavioral effects of intracranial infusions approximating the dose range. For instance, microinjections of M100907 into the VTA attenuate cocaine-induced (10 mg/kg) locomotor activity at doses lower (0.1-0.3 μg/0.2

μl/side) than those we found to be effective (McMahon, et al., 2001). Further, infusions of 0.1-0.5 μg M100907 into the NAc were found to decrease impulsive responding on a 5-choice serial reaction time task, though the higher dose was thought to possibly impair the animals' functioning on the task (Robinson et al., 2008). Thus, the doses of M100907 that we tested were within an effective range based on localized infusions into other regions; however this drug has not been used extensively for intracranial injections so we cannot rule out the possibility that effects may occur at lower or higher doses.

Other possible reasons for the lack of M100907 effects on behaviors were considered. For instance, we believe it is unlikely that tolerance to M100907 occurred with repeated administration based on our unpublished observation that 1.0 μg M100907 significantly attenuated headshakes induced by the 5-HT_{2A} receptor agonist DOI after animals had undergone three other test phases (M100907-, cue- and cocaine-primed reinstatement testing). We also found no effect of M100907 on cocaine-primed reinstatement during the first 30 minutes of testing, so it is unlikely that M100907 effects were obscured by testing beyond a period of maximal drug levels. Finally, while we did not observe any effects of M100907 on cue reinstatement of sucrose-seeking behavior, our data cannot completely rule out the possibility that the mPFC is involved in the incentive motivational effects of other reinforcers given the difficulties of equating the motivational value of the different reinforcers. Thus, it will be important to further test the role of mPFC 5-HT_{2A} receptors in order to draw firm conclusions as to whether these receptors modulate the incentive motivational effects of cocaine priming injections and/or sucrose-associated cues. Presently it appears that at the very least, 5-HT_{2A} receptors in

mPFC may be more sensitive to modulating motivational effects of cocaine-associated cues relative to other cocaine-related behaviors.

Future research will be needed to determine the specific neuroanatomical pathways involved in the attenuation of cue-elicited reinstatement of cocaine-seeking behavior that we observed in this study. In the mPFC, 5-HT_{2A} receptors are located postsynaptically, primarily on apical dendrites of pyramidal neurons with a minority found on GABA interneurons (Cornea-Hebert, Riad, Wu, Singh, & Descarries, 1999; Hamada et al., 1998; Jakab & Goldman-Rakic, 1998; Santana, Bortolozzi, Serrats, Mengod, & Artigas, 2004; Willins, Deutch, & Roth, 1997). 5-HT has an excitatory effect on glutamate release from pyramidal cells originating in the mPFC (Aghajanian & Marek, 1997). Studies showing activation of the mPFC by cocaine-paired cues (Childress, et al., 1999; Grant, et al., 1996; Kilts et al., 2001) and attenuation of cue-primed reinstatement of cocaine-seeking behavior by an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) antagonist in the NAc core (Di Ciano & Everitt, 2001) have lead to the hypothesis that glutamate transmission from the PFC to NAc core is involved in reinstatement (McFarland, et al., 2003). Therefore, one potential effect of blocking 5-HT_{2A} receptors in the mPFC is a decrease in the activity of glutamatergic projection neurons to the VTA and NAc.

Another potential mechanism for M100907 effects is via modulation of dopamine, as 5-HT_{2A} receptor antagonism decreases mesocortical dopamine release (Alex & Pehek, 2007) and is thought to inhibit excitatory inputs from the mPFC to the VTA (Vazquez-Borsetti, Cortes, & Artigas, 2009). Furthermore, systemic injections have shown that M100907 attenuates DOI-elicited increases in dopamine in the mPFC

(Gobert & Millan, 1999; Pehek, et al., 2001) and dorsal raphe stimulated release of dopamine in the NAc (De Deurwaerdere & Spampinato, 1999). These findings lead to the conclusion that 5-HT_{2A} receptors in the mPFC modulate phasic, but not tonic, dopamine release in the mPFC and striatum (De Deurwaerdere & Spampinato, 1999; Gobert & Millan, 1999; Lucas & Spampinato, 2000; Zhang et al., 2000). Blocking 5-HT_{2A} receptors in the mPFC may decrease activation of excitatory projections to the VTA, thereby attenuating glutamate receptor stimulation in the VTA and decreasing dopamine release in the PFC and NAc (Alex & Pehek, 2007).

Overall, our results indicate that selectively blocking 5-HT_{2A} receptors in the mPFC dose-dependently reduced cue-elicited reinstatement of drug-seeking behavior. The lack of a dose-dependent effect of M100907 on cocaine self-administration and cocaine-primed reinstatement of cocaine-seeking behavior suggests that the attenuation of these behaviors regardless of M100907 dose is not likely receptor-mediated. Further, M100907 in the mPFC did not affect locomotor activity or cue reinstatement of sucrose-seeking behavior, suggesting the effects on cue reinstatement were not due to general performance or a memory deficit. Therefore, we conclude that 5-HT_{2A} receptors in the mPFC play a role in mediating the incentive motivational effects of cocaine-paired cues. The findings contribute novel information about the neural circuitry underlying the incentive motivational effects of cocaine cues and further suggest that 5-HT_{2A} receptor blockade may be a potential therapeutic mechanism to treat cocaine craving and relapse.

Chapter 3

Interaction between 5-HT_{2A} receptor blockade and 5-HT_{2C} receptor activation on cocaine hyperlocomotion and Fos protein activation in the caudate-putamen

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Structurally, 5-HT_{2C} and 5-HT_{2A}Rs are very similar (Hoyer, et al., 2002; Raymond et al., 2001), and are found to coexist in many brain regions involved in addiction circuitry (Bubar & Cunningham, 2007; Doherty & Pickel, 2000; Pompeiano, et al., 1994), including the mesolimbic pathway originating in the ventral tegmental area (VTA) and projecting to nucleus accumbens (NAc) and the nigrostriatal pathway originating in the substantia nigra (SN) and projecting to the caudate-putamen (CPu). Functionally, 5-HT_{2A} and 5-HT_{2C}Rs play opposing facilitative and inhibitory roles, respectively, in cocaine-related behaviors. For instance, peripheral injections of the 5-HT_{2A}R selective antagonist M100907 decrease cocaine hyperlocomotion (Fletcher, et al., 2002; McMahon, et al., 2001) as well as cue- and cocaine-primed reinstatement of cocaine-seeking behavior, but have no effect on cocaine self-administration (Fletcher, et al., 2002; Nic Dhonnchadha, et al., 2009). Systemic administration of M100907 also attenuates cocaine discriminative stimulus effects (McMahon, et al., 2001). Further, systemic or intra-striatal injections of M100907 attenuate MDMA- and amphetamine-induced DA release in the NAc and CPu (Porras, et al., 2002; Schmidt, et al., 1992; Schmidt, et al., 1994). Conversely, a selective 5-HT_{2C}R antagonist enhances cocaine hyperlocomotion as well as cocaine-primed reinstatement and cocaine self-administration (Fletcher, et al., 2002; McMahon, et al., 2001). 5-HT_{2C}R agonists RO-60-0175 and MK212 inhibit cue- and cocaine-primed reinstatement of cocaine-seeking behavior, effects that are blocked by pre-administration

of 5-HT_{2C}R antagonists, indicating that they are 5-HT_{2C}R-mediated (Fletcher, et al., 2008; Neisewander & Acosta, 2007; Pentkowski, et al., 2010). Systemic administration of 5-HT_{2C}R antagonists has been found to increase amphetamine-induced DA release in the NAc and CPu (Porras, et al., 2002), while agonists attenuate morphine-induced DA release in the NAc (Willins & Meltzer, 1998).

Despite the oppositional relationship between behavioral effects mediated by 5-HT_{2A} versus 5-HT_{2C}Rs, to date, no research has investigated a potential interaction between these two receptor subtypes. By contrast, behavioral pharmacology studies of the DA system have revealed that interactions between D1-like and D2-likeR families mediate effects of psychostimulants. For instance, D2R-mediated stereotypy is observed only when there is also tonic stimulation of D1Rs, even though stimulation of D1Rs alone has little, if any, effect (Missale, Nash, Robinson, Jaber, & Caron, 1998). Furthermore, D1 and D2R antagonists synergistically decrease discriminative stimulus properties of cocaine and amphetamine (Callahan, Appel, & Cunningham, 1991), and D1 and D2R agonists administered together produce qualitatively more intense stereotypy than either one alone (Feldman, 1997; Jackson & Westlind-Danielsson, 1994). Investigating whether similar interactions exist between the 5-HT_{2A} and 5-HT_{2C}Rs is an important research question that may suggest a novel approach to developing treatments for psychostimulant dependence (Whitten, 2007).

One way to examine potential interactive effects of 5-HT_{2A}R antagonists and 5-HT_{2C}R agonists is by measuring Fos activation. Fos protein expression is a commonly used measure of functional neuronal activity (Harlan & Garcia, 1998; Herrera & Robertson, 1996). Region-specific patterns of Fos expression are associated with acute

cocaine administration (Robertson, Paul, Moratalla, & Graybiel, 1991; Torres & Rivier, 1994; Zahm et al., 2010) and exposure to cocaine-paired cues (Kufahl, Zavala, et al., 2009; Neisewander, et al., 2000; Zavala, Biswas, Harlan, & Neisewander, 2007) or a cocaine-associated context (Brown, et al., 1992; Crawford, McDougall, Bolanos, Hall, & Berger, 1995; Hamlin, Clemens, & McNally, 2008; Hotsenpiller, Horak, & Wolf, 2002). The 5-HT_{2A}R antagonist, M100907, has been found to decrease cocaine-induced Fos expression in the NAc shell and CPu (Szucs, et al., 2005). On the other hand, 5-HT_{2C}R antagonists enhance Fos expression in the subthalamic nucleus and CPu (De Deurwaerdere, Le Moine, & Chesselet, 2010). We have also found that a 5-HT_{2C} agonist administered into the ventromedial PFC decreases cocaine hyperlocomotion as well as cocaine-induced Fos expression in the dorsolateral CPu (Pentkowski, et al., 2010; Pockros, Pentkowski, Weber, & Neisewander, 2011). Thus, it seems that 5-HT_{2A} antagonists and 5-HT_{2C} agonists both decrease cocaine-induced Fos expression in the CPu, as well as attenuate cocaine hyperlocomotion.

In the present study, we hypothesized that concurrent 5-HT_{2A} antagonism and 5-HT_{2C} agonism would interact to attenuate the effects of cocaine on locomotion and Fos expression. We first examined dose-dependent decreases in cocaine hyperlocomotion by M100907 and MK212 given alone. From these experiments, we identified subthreshold doses of each drug that produced no effect on cocaine hyperlocomotion when given alone. We then tested the effects of a combination of these subthreshold doses of M100907 and MK212 on cocaine-induced and spontaneous locomotion. We also conducted Fos immunohistochemistry in several brain regions to investigate the effects of

concurrent 5-HT_{2A} antagonism and 5-HT_{2C} agonism on cocaine-induced neuronal activation.

Methods

Animals

Adult male Sprague–Dawley rats weighing 250–350 g at the start of the experiments were used in this study. Animals were housed in a climate-controlled colony room with a 14-h reversed light/dark cycle (lights off at 7:00 a.m. and on at 9 p.m.) and were cared for in accordance with the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources on Life Sciences, National Research Council 1996). The animals were given food and water *ad libitum* except during the testing sessions.

Drugs

M100907 (RTI International, Research Triangle Park, NC, USA) and MK212 (Tocris Cookson Inc., Ellisville, Missouri, USA) were dissolved in a 0.9% saline containing 3% tween. Cocaine-HCl (RTI International, Research Triangle Park, NC, USA) was dissolved in 0.9% saline. Euthasol (Hospira, Lake Forest, IL, USA) was used to deeply anesthetize animals before perfusions.

General procedures

Animals were handled daily for 1 week and were given saline injections to habituate them to injection stress on each of the 2 days prior to the start of the experiments. All animals were tested in Plexiglas locomotor activity chambers (44 × 24 × 20 cm high) in a soundproof, dimly lit room for 60-min sessions. A computer-automated video tracking system (Clever Systems, Reston, VA, USA) used the orientation of the animal's body (e.g. center of body) to measure the total horizontal distance traveled. All

testing was conducted during the animals' dark cycles. In experiments testing cocaine hyperlocomotion, animals were placed into the test chambers for 1 h prior to any drug injections in order for them to habituation to the chamber, thus providing a low baseline level of locomotion from which to detect cocaine-induced increases.

Dose-dependent effects of M100907 and MK212 on cocaine hyperlocomotion

Separate cohorts of animals were randomly assigned to groups that received i.p. injections of either M100907 (0.025, 0.05, or 0.1 mg/kg) or MK212 (0.125, 0.25, or 0.5 mg/kg) (n=8/dose) prior to one test; both cohorts received vehicle prior to the other test with the order of the drug versus vehicle tests counterbalanced within groups. Following habituation on the test day, the animals were given an injection of their assigned drug, put back into their home cage for 5-min, and then given an injection of cocaine (15 mg/kg, i.p.) immediately before being placed back into the test chambers for the 1-h test.

Effects of M100907 + MK212 on cocaine hyperlocomotion

Subthreshold doses of M100907 (0.025 mg/kg, i.p.) and MK212 (0.125 mg/kg, i.p.) that failed to alter cocaine-induced locomotor activity on their own in the above experiments were then combined as a cocktail to examine potential receptor interactions. Animals were randomly assigned to receive 2 drug injections given 5 min apart following the habituation period as follows, respectively: saline + saline, saline + cocaine (15 mg/kg, i.p.), 0.025 mg/kg M100907 + cocaine, 0.125 mg/kg MK212 + cocaine, or cocktail + cocaine (n=6-7/group). Rats were placed into their home cage following the first injection and were placed back into the locomotor activity chambers for a 1-h test following the second injection. This experiment utilized a between-subjects design, as

animals were sacrificed after testing and their brains were extracted for Fos analysis as described below.

Effects of M100907 + MK212 on spontaneous locomotion

Drug-naïve rats were randomly assigned to receive saline, M100907 (0.025 mg/kg, i.p.), MK212 (0.125 mg/kg, i.p.) or a cocktail of the latter two drugs. Five min after injection of their assigned drug, the rats were placed into the locomotor activity chambers for a 1-h test. There was no habituation period in this experiment in order to avoid having a floor effect that would obviate detection of drug effects on locomotion.

Tissue preparation

To allow 90-min after drug injections for optimum Fos expression, animals remained uninterrupted in the locomotor chambers for 30-min after the 1-h test. They were then deeply anesthetized with Euthasol (100 mg/kg, i.p.) and perfused transcardially with 300 ml of ice-cold 0.1 M phosphate-buffered saline (PBS), pH 7.4, followed by 300 ml of ice-cold 4% paraformaldehyde in 0.1 M PBS, pH 7.4. The brains were removed, postfixed overnight at 4 °C in 4% paraformaldehyde, and then transferred to 15% sucrose for 24 h and then to 30% sucrose for an additional 24 h while continuously being stored at 4 °C. Coronal sections (40 μm) were collected using a freezing microtome at levels corresponding to 3.2, 1.6, 2.56, 5.6 mm relative to bregma (Paxinos and Watson, 1998). The tissue sections were then frozen and stored at 20 °C in a cryoprotectant solution comprised of 0.02 M PBS (pH 7.2), 30% sucrose, 10% polyvinyl pyrrolidone, and 30% ethylene glycol.

Fos protein immunohistochemistry

Tissue sections were first washed in 0.1 M PBS (6×10 min) to remove the cryoprotectant. Sections were then incubated in 0.3% H_2O_2 for 30 min and rinsed with 0.1 M PBS (3×10 min), followed by incubation in 0.1 M PBS containing 5% normal goat serum (NGS) (Vector Laboratories, Burlingame, CA, USA) and 0.2% Triton X-100 (Sigma, St. Louis, MO, USA) for 1 h. Sections were then incubated for 48 h at 4 °C in 0.1 M PBS containing anti-Fos rabbit polyclonal antibody (SC-52; 1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), 0.1% Triton X-100 and 1% NGS, and then rinsed in 0.1 M PBS (3×10 min). Sections were then incubated in 0.1 M PBS containing biotinylated goat anti-rabbit IgG (1:500; Vector Laboratories, Burlingame, CA, USA) and 1% NGS for 1 h, and then rinsed in 0.1 M PBS (3×10 min). Subsequently, horseradish peroxidase activity was visualized with nickel diaminobenzidine and glucose oxidase reaction as described in Dielenberg et al. (2001). This reaction was terminated after 10 min by rinsing the tissue in 0.1 M PBS (3×10 min). Sections were then mounted onto gelatin-coated slides, dried, and dehydrated before cover slipping.

Fos immunoreactivity analysis

Sections were taken at +3.2 mm, which contained the prelimbic (PrL) and infralimbic (IL) cortices; sections taken at +1.6 mm contained the NAc core (NAcC) and shell (NAcS), and the dorsolateral CPu. Care was taken to ensure that the sections that were labeled came from the same anatomical level within each plane for each subject. Quantification of Fos immunoreactivity was examined using a Nikon Eclipse E600 (Nikon Instruments, Melville, NY, USA) microscope set at 20X. For all regions, the sample area counted was 0.26 mm^2 and there were a total of six sample areas counted for

each subject (i.e., 1 sample area/2 hemispheres/3 sections) that were then averaged to provide a mean number of immunoreactive cells per sample area. An observer blind to treatment conditions identified Fos immunoreactivity as a blue-black oval-shaped nucleus distinguishable from background using size and optical density criteria set using the Image Tool software package (Version 3.0, University of Texas Health Sciences Center, San Antonio, TX, USA). Regions analyzed and representative sample areas are shown in Figure 9. These images were captured using SPOT Advanced software (Version 3.5 Sterling Heights, MI, USA) and no modifications were made to the images.

Statistical analyses

Because the purpose of the dose-effect experiments was to identify subthreshold doses of M100907 and MK212, locomotor activity data at each dose were analyzed separately using repeated measures analyses of variance (ANOVAs) with time (e.g. 15-min time bins) and test (e.g. saline or cocaine) as within-subject factors. For drug interaction experiments, spontaneous and cocaine-induced locomotor activity data were analyzed using mixed-factor ANOVAs with time as a repeated measure within-subject factor and drug group as a between-subject factor. For all tests of cocaine hyperlocomotion, the last 15-min of habituation served as a baseline. Fos data were analyzed by region using one-way ANOVAs with drug as a between-subject factor. In order to focus analyses of Fos on group differences in response to cocaine standardized across regions, raw data was converted to a percent of saline control and analyzed using one-way ANOVAs with drug as a between-subject factor. A Greenhouse–Geisser correction was used to correct for heterogeneity of variance in the data. Significant

effects were further analyzed using smaller ANOVAs and post hoc comparisons were made using Tukey's HSD test. All statistics were run using SPSS, version 20.

Results

Dose-Effect Function of M100907 on Cocaine Hyperlocomotion

Figure 10 shows the dose-effect function of M100907 on cocaine hyperlocomotion. For the lowest dose of 0.025 mg/kg, the ANOVA of distance traveled showed a main effect of time [$F(1.51,10.60)=10.92$, $p<0.01$], but no effect of test nor test by time interaction demonstrating that locomotion was similar regardless of vehicle versus M100907 treatment. Post hoc tests on the main effect of time showed that cocaine increased locomotor activity relative to baseline, and locomotor activity remained elevated for the first 30-min of testing ($p<0.05$).

For the 0.05 mg/kg dose, the ANOVA of distance traveled showed an interaction of time by test [$F(4,28)=3.30$, $p<0.05$], as well as a main effect of time [$F(1.76,12.33)=9.06$, $p<0.01$]. Post hoc tests showed a difference between baseline and the first 15-min of testing only when animals were pretreated with vehicle ($p<0.05$). On the vehicle test day, locomotor activity remained elevated at the 30-min time point compared to baseline ($p<0.05$). On the M100907 test day, there was no difference between any of the time points, consistent with a failure to observe cocaine hyperlocomotion. However, the M100907 attenuation of cocaine hyperlocomotion was not due to a change at a particular time as there were no differences between test days any time points.

For the 0.1 mg/kg dose, the ANOVA of distance traveled showed a main effect of time [$F(4,28)=14.69$, $p<0.01$] as well as a main effect of test [$F(1,7)=9.06$, $p<0.05$], with

the latter indicating less distance traveled overall during the M100907 test than during the vehicle test. Post hoc tests on the main effect of time showed that cocaine increased locomotor activity relative to baseline for the first 15-min of testing regardless of pretreatment ($p<0.01$). Collectively, these findings suggest that cocaine hyperlocomotion was slightly attenuated at this dose of M100907.

Dose-Effect Function of MK212 on Cocaine Hyperlocomotion

Figure 11 shows the effects of MK212 on cocaine hyperlocomotion. For the lowest dose of 0.125 mg/kg, the ANOVA of distance traveled showed a main effect of time [$F(4,28)=35.95$, $p<0.01$], but no effect of test nor test by time interaction demonstrating that locomotion was similar regardless of vehicle versus MK212 treatment. Post hoc tests on the main effect of time showed that cocaine increased locomotor activity relative to baseline, and locomotor activity remained elevated for 45 min regardless of test day ($p<0.05$).

For the 0.25 mg/kg dose, the ANOVA of distance traveled showed an interaction of time by test [$F(4,28)=3.40$, $p<0.05$], as well as a main effect of time [$F(4,28)=23.17$, $p<0.01$], and a main effect of test [$F(1,7)=17.07$, $p<0.01$). Post hoc tests showed a difference between baseline and the first 15 min of testing on both test days ($p<0.05$). On the vehicle test day only, locomotor activity remained elevated at the 30-min time point compared to baseline ($p<0.05$). There was also a significant difference between vehicle and MK212 test days at the 30-min time point ($p<0.05$).

For the 0.5 mg/kg dose, the ANOVA of distance traveled showed an interaction of time by test [$F(4,28)=11.77$, $p<0.01$], as well as a main effect of time [$F(4,28)=37.35$, $p<0.01$], and a main effect of test [$F(1,7)=9.98$, $p<0.05$]. Post hoc tests showed a

difference between baseline and the first 15 min of testing on the vehicle test day ($p<0.05$), and locomotor activity remained elevated compared to baseline ($p<0.05$) until the last 15-min of the test. On the MK212 test day, there was no difference between baseline and the first 15-min of testing, however there was a significant decrease in activity compared to baseline at all other time points ($p<0.05$). There were also significant differences between vehicle and MK212 test days at the last three time points ($p<0.05$).

M100907/MK212 Interaction Effects on Cocaine Hyperlocomotion

The effects of the M100907/MK212 cocktail on cocaine-induced locomotor activity are shown in Figure 12. The ANOVA of distance traveled showed a significant time by drug interaction [$F(6.95,45.20)=2.49$, $p<0.05$] as well as a main effect of time [$F(1.74, 45.20)=40.29$, $p<0.01$] and a main effect of drug [$F(4,26)=4.97$, $p<0.01$]. Post-hoc comparisons indicated a significant difference between the saline + saline and saline + cocaine groups ($p<0.05$, Tukey HSD), as well as the saline + cocaine and cocktail + cocaine groups ($p<0.01$, Tukey HSD). There were no differences between the cocaine + saline and cocaine + M100907 or cocaine + MK212 groups ($p=0.474$ and $p=0.463$, respectively).

At each 15-min time-bin during testing, the one-way ANOVAs of distance traveled showed a significant effect of drug group [$Fs(4,30)=5.491$-3.751, $p<0.05$]. Across all post-cocaine time bins, the cocktail + cocaine group exhibited less locomotion than the saline + cocaine group ($p<0.05$, Tukey HSD), whereas the saline + saline group exhibited less locomotion at the 30 ($p<0.01$, Tukey HSD) and 60 min time bins only ($p<0.05$, Tukey HSD). In contrast, the M100907 + cocaine and MK212 + cocaine groups

did not differ from either the saline + saline or saline + cocaine groups at any time point. There was no difference in baseline between groups.

M100907/MK212 Interaction Effects on Spontaneous Locomotion

The effects of the M100907/MK212 cocktail on spontaneous locomotor activity are shown in Figure 13. The ANOVA of distance traveled showed a main effect of time [$F(3,84)=138.551$, $p<0.01$], but no effect of drug or drug by time interaction, suggesting that none of the drug treatments altered spontaneous locomotion. Post-hoc tests showed a difference between the first 15-min and each of the 30-min, 45-min, and 60-min time points ($p<0.01$), as well as a difference between the 30-min and 45-min time points ($p<0.01$).

M100907/MK212 Interaction Effects on Cocaine-induced Fos Activation

The effects of the M100907/MK212 cocktail on cocaine-induced Fos activation in striatal subregions are shown in Figure 14. Fos data are from the same animals whose behavioral data are shown in Figure 12. The ANOVA of percent control of Fos-positive nuclei in the dorsal CPu (Panel a) showed a significant between-group effect [$F(4,30)=3.859$, $p<0.05$]. Post-hoc comparisons showed significant differences between the saline + saline and saline + cocaine ($p<0.05$, Tukey HSD) groups, indicating that cocaine increased Fos expression in this region. There were also significant differences between saline + saline and MK212 + cocaine ($p<0.05$, Tukey HSD) and saline + saline and M100907 + cocaine ($p<0.05$, Tukey HSD) groups, suggesting neither drug alone reversed the effect of cocaine on Fos expression. In contrast, there was no significant difference between the saline + saline and cocktail + cocaine groups, indicating that the cocktail significantly attenuated cocaine-induced Fos expression. ANOVAs of percent

control of Fos-positive nuclei in the NAc core (Panel b) and shell (Panel c) and the infralimbic (Panel d) and prelimbic (Panel e) PFC failed to reveal any between-group effects.

Discussion

Results from the present study support our hypothesis that 5-HT_{2A} and 5-HT_{2C}Rs interact to decrease cocaine-induced locomotor activity and Fos expression. We determined from dose-response experiments that the doses of 0.025 mg/kg M100907 and 0.125 mg/kg MK212 had no effect on cocaine hyperlocomotion when given alone but these doses given in combination significantly attenuated cocaine hyperlocomotion, consistent with receptor interaction effects. The interaction effect was specific for cocaine hyperlocomotion as this dose combination had no effect on spontaneous locomotor activity. This dose combination also region-specifically attenuated cocaine-induced Fos expression in the dorsolateral CPu. It is likely that the effects of M100907 and MK212 observed in the present study were in fact due to actions at 5-HT_{2A} and 5-HT_{2C} receptors, respectively. M100907 has >1000-fold selectivity for 5-HT_{2A} vs. 5-HT_{2C} receptors (Kehne, et al., 1996) and several studies have demonstrated that doses of 0.005-0.4 mg/kg reverse the behavioral effects of 5-HT_{2A} agonists, but not those of 5-HT_{2C}R agonists (Dekeyne, et al., 1999; Gresch, et al., 2007; Hitchcock, et al., 1997; McCreary, et al., 2003; Vickers, et al., 2001; Wettstein, et al., 1999). MK212 binds to 5-HT_{2C}Rs with the highest affinity compared to other receptors, but it does have affinity for 5-HT_{2A}, 5-HT_{2B} and 5-HT_3Rs (Cussac, Newman-Tancredi, Duqueyroix, Pasteau, & Millan, 2002; Glennon et al., 1989; Porter et al., 1999). It is unlikely that 5-HT_{2B} receptors were involved in the effects observed in this study since previous research has found that

neither 5-HT_{2B}R agonists nor antagonists have any effect on cocaine hyperlocomotion (Filip, Bubar, & Cunningham, 2004). Further, we have shown that the locomotor activity effects of even higher doses of MK212 (0.32-1.0 mg/kg), which should be less selective for 5-HT_{2C}Rs than the low doses used in the present study, are reversed by a selective 5-HT_{2C}R antagonist, SB242084 (Neisewander & Acosta, 2007; Pentkowski, et al., 2009; Pentkowski, et al., 2010). The hypolocomotive effects of MK212 itself are also reversed by SB242084 (Stiedl et al., 2007). Thus it is likely that the low dose effects observed here are 5-HT_{2C}R-mediated.

Our findings fit with the existing literature, which clearly shows that both 5-HT_{2A}R antagonists and 5-HT_{2C}R agonists given alone decrease cocaine hyperlocomotion, as well as reinstatement of cocaine-seeking behavior (Grottick, Fletcher, & Higgins, 2000; McMahon, et al., 2001; Neisewander & Acosta, 2007; Nic Dhonnchadha, et al., 2009; Pentkowski, et al., 2010; Pockros, et al., 2011). Similar results have been found in the nicotine literature as well, with 5-HT_{2A}R antagonists decreasing nicotine self-administration (Fletcher et al., 2012; Levin et al., 2008) and 5-HT_{2C}R agonists attenuating nicotine self-administration as well as nicotine-induced locomotion, sensitization, conditioned place preference, and discriminative stimulus effects (Fletcher, et al., 2012; Grottick, Corrigall, & Higgins, 2001; Zaniewska, McCreary, Przegalinski, & Filip, 2007). 5-HT_{2A}R antagonists and 5-HT_{2C}R agonists have also been shown to attenuate premature responding on a five-choice serial reaction time test with and without cocaine, suggesting a role in drug-induced impulsivity (Fletcher, Tampakeras, Sinyard, & Higgins, 2007). While there is ample evidence that 5-HT_{2A} antagonists and 5-HT_{2C} agonists have

opposing effects, to our knowledge the present study is the first to demonstrate an interaction between these two serotonin receptor subtypes.

Interestingly, the middle dose (0.05 mg/kg) of M100907 appeared to have a stronger effect than the highest dose (0.1 mg/kg). Previous research has shown that a higher dose of 0.5 mg/kg attenuates cocaine hyperlocomotion (Fletcher, et al., 2002), suggesting that there may be an inverted U-shaped dose-effect function for M100907 effects on this behavior. The dose-effect function of M100907 on methamphetamine hyperlocomotion is similar, with a higher dose producing less robust attenuation than an intermediate dose (Steed, Jones, & McCreary, 2011). Some studies have shown dose-dependent effects of M100907 on impulsivity (Agnoli & Carli, 2012) and reinstatement of nicotine-seeking behavior (Fletcher, et al., 2012), whereas M100907 effects on cue-primed reinstatement of cocaine-seeking behavior (Nic Dhonnchadha, et al., 2009) do not appear to vary dose-dependently.

The effects of M100907 and MK212 on cocaine-induced Fos expression in the dorsolateral CPu mimicked the behavioral data, where low doses of M100907 and MK212 had no effect on cocaine-induced Fos expression when given alone, but produced a significant decrease when given in combination. Although this study did not include control groups for possible effects of M100907 or MK212 alone on Fos expression, it is unlikely that these drugs altered Fos expression on their own given previous findings. For instance, it has been shown that M100907 (0.2-0.8 mg/kg) has no significant effect on Fos activation in the CPu after saline pretreatment (Szucs, et al., 2005). The effects of MK212 on Fos expression have not been examined, although another 5-HT_{2C}R agonist, RO-60-1057, fails to alter Fos expression in the CPu at doses (1-3 mg/kg) that attenuate

cocaine-primed reinstatement (Beyeler et al., 2010; Grottick, et al., 2000). Furthermore, studies have previously found that cocaine-induced Fos expression in the CPu is altered by 5-HT_2R manipulations. For instance systemic injections of M100907 attenuate cocaine-induced Fos in the CPu (Szucs, et al., 2005), and our laboratory has found that intra-mPFC infusions of MK212 also attenuate cocaine-induced Fos in the CPu (Pockros, et al., 2011). Thus, it is unlikely that these drugs induce Fos expression on their own, but instead selectively attenuate cocaine-induced Fos expression, similar to their selective attenuation of cocaine-induced hyperlocomotion without producing any effect on spontaneous locomotion.

It is surprising that cocaine hyperlocomotion was associated with increased Fos expression only in the CPu and not in the NAc or PFC because several previous studies have found that acute injection of cocaine produces hyperlocomotion and increases Fos protein expression in all of these regions (Graybiel, Moratalla, & Robertson, 1990; Szucs, et al., 2005; S. T. Young, Porrino, & Iadarola, 1991). However, cocaine-induced Fos expression in the striatum exhibits a rostral to caudal increasing gradient. For instance, Szucs et al. (2005) found no effects of cocaine on Fos expression in the anterior NAc core or shell at +1.7 mm from Bregma, consistent with the lack of cocaine-induced Fos expression in the present study at +1.6 mm from bregma. However, in contrast to Szucs et al. (2005) who found only a nonsignificant trend toward an effect of cocaine on Fos expression in the CPu at +1.7 mm from Bregma, we found that cocaine significantly increased Fos in the CPu at +1.6mm from Bregma. This difference may be due to different slicing angles or staining or counting techniques. It is possible that we may have observed cocaine-induced Fos expression if we had analyzed tissue from the caudal

regions of the NAc core and shell because Szucs et al. (2005) only found significant effects at a more caudal level (i.e., +1.0 mm from Bregma). Contrary to our results, several studies have shown that cocaine (2 mg/kg, i.v. or 25 mg/kg) increases Fos expression in the medial PFC (Graybiel, et al., 1990; Kufahl, Pentkowski, Heintzelman, & Neisewander, 2009). However, differences in cocaine dose or different routes of administration may account for differences across studies.

The similar pattern of changes in locomotion and Fos expression in the CPu suggests that this region is involved in the observed behavioral changes. Although, site-specific injections will be necessary to determine the brain regions and cellular mechanisms involved in the functional receptor interaction, the CPu is likely involved given its role in stimulant-induced motor activities, including locomotion, repetitive stereotypic and habitual behaviors (Brown, et al., 1992; Naylor & Olley, 1972; White, et al., 1998; Zimmerberg & Glick, 1974). The dose of cocaine used in the present study (15 mg/kg, i.p.) does not typically produce stereotypic behaviors, however slightly higher doses (20 mg/kg, i.p.) have been found to produce stereotopies typically manifesting as headbobbing (Bhattacharyya & Pradhan, 1979; Budygin, 2007; O'Dell, Khroyan, & Neisewander, 1996; White, et al., 1998). Repetitive stereotypic behaviors may compete with expression of cocaine hyperlocomotion; however, if stereotypy occurred in this study, the drugs co-administered with cocaine would likely have attenuated rather than exacerbated this behavior. Indeed 5-HT_{2A}R antagonists, including M100907, have been found to attenuate stereotopy produced by other drugs (Barwick, Jones, Richter, Hicks, & Young, 2000; Higgins, Enderlin, Haman, & Fletcher, 2003; Ninan & Kulkarni, 1998). Similarly, 5-HT_{2C}R mutant mice exhibit enhanced DAT antagonist-induced stereotypic

behavior (Abdallah et al., 2009), suggesting that stimulation of these receptors inhibits dopamine-induced stereotypy. Thus, it is unlikely that the reduction of cocaine hyperlocomotion was due to competing stereotypic behavior.

Although the cellular mechanisms by which M100907 and MK212 produced their combined effect on cocaine hyperlocomotion and Fos expression remain to be elucidated, one possibility is via a decrease in DA release in the nigrostriatal pathway. Acute injection of cocaine has been shown to stimulate Fos expression (Neisewander, et al., 2000; Zahm, et al., 2010) and increase DA in the CPu (Hurd & Ungerstedt, 1989; Hurd, Weiss, Koob, & Ungerstedt, 1990). 5-HT_{2A} and 5-HT_{2C}Rs have been found to regulate amphetamine- and morphine-induced DA release in the CPu in opposing manners; 5-HT_{2A}R blockade attenuates phasic DA release (De Deurwaerdere & Spampinato, 1999; Gobert & Millan, 1999; Ichikawa & Meltzer, 1995; Lucas & Spampinato, 2000; Porras, et al., 2002; Schmidt, et al., 1992), while 5-HT_{2C}R activation decreases both tonic and phasic DA activity (Di Giovanni, et al., 1999; Di Matteo, et al., 2000; Gobert, et al., 2000; Porras, et al., 2002). Cellular localization of 5-HT_{2A} and 5-HT_{2C}Rs in the dorsal CPu has yet to be determined, although the majority of 5-HT_{2A}R-labeled cells in this region contain parvalbumin, indicative of γ-Aminobutyric acid (GABA) interneurons (Bubser, Backstrom, Sanders-Bush, Roth, & Deutch, 2001). In the mesolimbic pathway, 5-HT_{2A} and 5-HT_{2C}Rs are expressed on both DA and GABA neurons in the VTA, and on GABA neurons in the NAc. In the SN pars compacta, 5-HT_{2C}Rs are found on GABAergic neurons and 5-HT_{2C}R agonists stimulate GABA release in the SN (Eberle-Wang, Mikeladze, Uryu, & Chesselet, 1997; Invernizzi et al., 2007). Intra-CPu administration of a 5-HT_{2C}R inverse agonist increases DA release in the CPu, an effect

that can be reversed by concurrent administration of a 5-HT_{2C}R agonist mCPP (1.0 mg/kg, i.p.) (Alex, et al., 2005). Thus, the 5-HT_{2A} and 5-HT_{2C}R interactive effects on cocaine hyperlocomotion and Fos expression in the CPu may be due to direct modulation of DA release from nigrostriatal neurons, or indirect modulation of DA release via an increase in GABA inhibition either within the dorsal CPu itself or within the SN. While we did not observe effects of M100907 and/or MK212 on Fos activation in the terminal regions of the mesocorticolimbic dopamine pathway, this does not rule out the possibility that this pathway is involved in the interaction effects.

We chose to test our hypothesis by examining effects of subthreshold doses of M100907 and MK212 on cocaine-induced locomotion to establish proof or principle that 5-HT_{2A} and 5-HT_{2C}Rs interact. Although this method of combining subthreshold doses is an approach that has been used to detect synergistic interactions (Brown, Finlay, Wong, Damsma, & Fibiger, 1991; Gotoh et al., 2006; Thiel, Sanabria, & Neisewander, 2009), there are some limitations. First, although statistically our findings are consistent with a synergistic interaction, the non-significant tendency of both drugs to attenuate cocaine hyperlocomotion when given alone raises the possibility that their interactive effect may be additive rather than synergistic. Thus, more sophisticated isobolographic analyses will be needed to precisely determine the nature of the 5-HT_{2A} and 5-HT_{2C}R interaction. Second, it remains unclear whether pharmacokinetic interactions between these drugs are involved in the interaction effect observed. Future research will be needed to address these issues.

In conclusion, the findings from this study provide support for the idea that a combination of 5-HT_{2A} antagonist and 5-HT_{2C} agonist may offer potential therapeutic

advantages for development of treatment for cocaine dependence. For instance, relatively low doses of two drugs could be used instead of a high dose of a single drug. These lower doses would likely be less disruptive than a high dose to various systems throughout the body involving these receptors, resulting in fewer side-effects. In support of this idea, the present findings indicate that combined subthreshold doses that decreased cocaine hyperlocomotion without disturbing spontaneous locomotion. 5-HT_{2C}R agonists with greater selectivity have recently been developed that may improve upon therapeutic efficacy and used at a lower dose range in combination with 5-HT_{2A}R antagonists. Clinical trials are currently investigating the effectiveness of 5-HT_{2A}R antagonists in treating depression and insomnia and 5-HT_{2C}R agonists in treating obesity (NIH, 2010), supporting the potential clinical utility of these drugs for treating addiction. Ideally, pharmacological treatments aimed at cocaine dependence should not only curtail the reinforcing effects of cocaine, but also drug craving and relapse (Washton, 1988). To further explore the potential clinical utility of a combination treatment with a 5-HT_{2A}R antagonist and a 5-HT_{2C}R agonist, future research is needed to determine whether the combination would reduce cocaine self-administration and drug-seeking behavior while not interfering with unconditioned behaviors (i.e. locomotion and feeding).

Chapter 4

Effects of a 5-HT_{2C}R agonist in the amygdala on reinstatement of cocaine-seeking behavior and anxiety on the elevated plus maze

Increasing or decreasing 5-HT levels in the brain has been found to attenuate craving and cocaine-seeking behavior in humans (Aronson, et al., 1995; Batki, et al., 1993; Satel, et al., 1995; Walsh, et al., 1994) and animals (Baker, et al., 2001; Burmeister, Lungren, & Neisewander, 2003; Tran-Nguyen, et al., 1999), respectively. This paradoxical relationship is thought to be due to activation of different 5-HT receptors in the brain that are involved in addiction, including the 5-HT_{2C} receptor (R). 5-HT_{2C}R agonists attenuate cue- and cocaine-primed reinstatement of cocaine-seeking behavior, (Fletcher, et al., 2008; Neisewander & Acosta, 2007; Pentkowski, et al., 2010) while 5-HT_{2C}R antagonists enhance cocaine hyperlocomotion as well as cocaine-primed reinstatement and cocaine self-administration (Fletcher, et al., 2002; McMahon, et al., 2001).

5-HT_{2C}Rs are found throughout the mesolimbic DA pathway (Doherty & Pickel, 2000; Pompeiano, et al., 1994), including the amygdala, which is a key region in the neurocircuitry of cocaine addiction (Alleweireldt, et al., 2006; O'Dell, et al., 1999; See, 2005). Generally, the amygdala is involved in emotional learning (Bechara, et al., 1995) and memory (Cahill, 2000), fear conditioning (Blanchard & Blanchard, 1972; M. Davis, 2000; Lieblich, Yitzhaky, & Cohen, 1976; Pribram, et al., 1979), avoidance learning (Weiskrantz, 1956), and appetitive conditioning (B. J. Everitt, Cardinal, Parkinson, & Robbins, 2003; Parkinson, Robbins, & Everitt, 2000). The role of the amygdala in emotional conditioning and memory using natural reinforcers led researchers to

investigate involvement of the amygdala in drug abuse (B. J. C. Everitt, R. N.; Hall, J.; Parkinson, J. A.; Robbins, T. W., 2000; Grant, et al., 1996).

The amygdala is a heterogenous region composed of subnuclei, including the basolateral and central amygdaloid nuclei (i.e., BlA and CeA respectively, (Gloor, 1955; Wood, Schottelius, Frost, & Baldwin, 1958). The BlA is responsible for assigning incentive value to a conditioned stimulus (CS) based on its association with an unconditioned stimulus (US, (B. J. C. Everitt, R. N.; Hall, J.; Parkinson, J. A.; Robbins, T. W., 2000), as well as for processing fear and anxiety (M. Davis, 2000; Sananes & Davis, 1992). For instance, the BlA has been implicated in processing the incentive motivational effects of drug-associated contextual and discrete cues (B. J. Everitt, et al., 1999; Fuchs & See, 2002; Fuchs, et al., 2002; McLaughlin & See, 2003). 5-HT_{2C}Rs are found throughout the amygdala, with higher levels in the BlA than CeA (Clemett, Punhani, Duxon, Blackburn, & Fone, 2000; Lopez-Gimenez, et al., 1997; Pompeiano, et al., 1994). Although it is presently unclear whether 5-HT_{2C}Rs in the BlA play a role in processing the significance of cocaine-associated cues, they are involved in anxiety-like behavior. Transgenic mice overexpressing 5-HT_{2C}R mRNA show enhanced anxiety on the elevated plus maze (EPM, (Kimura, et al., 2009), while 5-HT_{2C}R knockout mice exhibit a reduction in anxiety on the EPM (Heisler, Zhou, et al., 2007). Systemic pretreatment with a 5-HT_{2C}R agonist induces learned helplessness behaviors (Strong, et al., 2009), and 5-HT_{2C}R agonists infused into the BlA potentiate anxiety-like behavior in open-field and social exploration tests (Campbell & Merchant, 2003; Christianson et al., 2010). Further, the anxiogenic effects of a 5-HT_{2C}R agonist can be reversed by intra-BlA infusion of a 5-HT_{2C}R antagonist (de Mello Cruz et al., 2005).

The CeA is a part of the extended amygdala (Alheid & Heimer, 1988; Heimer & Alheid, 1991; Johnston, 1923), which is implicated in some of the unconditioned effects of drugs of abuse, including reward (O'Dell, et al., 1999), reinforcement (Caine, Heinrichs, Coffin, & Koob, 1995), stress, and drug withdrawal (Koob & Le Moal, 2005; Koob & Nestler, 1997). Inactivation of the CeA eliminates stress-primed reinstatement in both CPP (Ma, Xu, Yang, & Yang, 2008; Wang, Luo, Ge, Fu, & Han, 2002) and self-administration models (McFarland, et al., 2004). Some studies suggest a role for the CeA in responding for cocaine-paired cues (Kruzich & See, 2001; Thiel et al., 2010), though selective inactivation of the CeA does not affect acquisition of (Kruzich & See, 2001), or responding with (Burns, Annett, Kelley, Everitt, & Robbins, 1996; Robledo, Robbins, & Everitt, 1996), conditioned reinforcement. The CeA appears to amplify conditioned responses, including the "incubation effect" in which cue-primed reinstatement intensifies during abstinence (Grimm & See, 2000; Y. Q. Li et al., 2008; Lu et al., 2005; Tran-Nguyen et al., 1998). Although the distribution of 5-HT_{2C}Rs in the CeA is not well characterized, it has been shown that 5-HT_{2C}R knockout mice exhibit lower levels of Fos activation in the CeA following social-defeat stress (Heisler, Zhou, et al., 2007) and that 5-HT_{2C}R agonists increase Fos expression in the CeA (Singewald, Salchner, & Sharp, 2003; Somerville, Horwood, Lee, Kennett, & Clifton, 2007). Further, there is a positive correlation between 5-HT_{2C}R levels in the CeA and anxiety-like behavior on the EPM (Q. Li, Luo, Jiang, & Wang, 2012); however, other studies failed to find effects of 5-HT_{2C}R agonists in the CeA on anxiety-like behavior (Campbell & Merchant, 2003; Christianson, et al., 2010).

In the present study, we hypothesized that 5-HT_{2C}Rs in the BlA play a role in the inhibitory effects of systemic 5-HT_{2C}R agonists on cue reinstatement of extinguished cocaine-seeking behavior based on the known involvement of the BlA in the incentive motivational effects of cocaine-paired cues and the presence of 5-HT_{2C}R in the BlA. We further hypothesized that 5-HT_{2C}Rs in the CeA play a role in the inhibitory effects of systemic 5-HT_{2C}R agonists on cocaine-primed reinstatement based on the known involvement of the CeA in unconditioned effects of cocaine. To examine this hypothesis, we used the selective 5-HT_{2C}R agonist, CP890101. This agonist has >500-fold selectivity for 5-HT_{2C} over other 5-HT_2Rs and EC50 values of 0.11, 153, and 65.3 nM for 5-HT_{2C}, 5-HT_{2A}, and 5-HT_{2B}Rs, respectively (Fletcher et al., 2009; Siuciak et al., 2007). We predicted that CP809101 microinfused into the BlA would attenuate cue-primed reinstatement of cocaine-seeking behavior and increase anxiety-like behavior on the elevated plus maze (EPM), while having no effect on cocaine-primed reinstatement or extinction responding. Conversely, we predict that CP809101 infused into the CeA would attenuate cocaine-primed reinstatement of cocaine-seeking behavior, and may affect cue-primed reinstatement or anxiety-like behavior on the EPM.

Methods

Animals

Adult male Sprague–Dawley rats weighing 275–325 g at the start of the experiments were used in this study. Animals were housed in a climate-controlled colony room with a 14-h reversed light/dark cycle (lights off at 7:00 a.m.) and cared for in accordance with the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources on Life Sciences, National Research Council 2011).

Surgery

Animals were handled daily for at least 6 days before implanting catheters into the right jugular vein. Catheters were implanted using the same protocol as Pockros et al., 2011. Stainless steel guide cannulae were lowered through small holes drilled into the skull to a depth 2.5 mm above the targeted site of the BlA and 2.7 mm above the targeted site of the CeA. The coordinates were selected based on previous research (Christianson, et al., 2010; Fuchs & See, 2002; Thiel, et al., 2010) as well as our own pilot surgeries, and were as follows: for the BlA the AP=-2.5, the ML= 5.0 mm to the left and 4.9 mm to the right relative to bregma, and the DV=-8.2 mm from the skull surface; for the CeA the AP=-2.5, the ML= 4.2 mm to the left and 4.1 mm to the right relative to bregma, and the DV=-8.2 mm from the skull surface (Paxinos, 2007). The guide cannulae and the metal end of the catheter were secured to the skull using dental acrylic cement and anchor screws. Metal stylets were inserted into the cannulae to maintain patency. All incisions were sutured and treated with a topical antibiotic. Catheters were flushed daily with a solution of 0.1 ml saline containing heparin sodium (70 U/ml; APP Pharmaceuticals, Schaumburg, IL, USA) and Timentin (66.7 mg/ml; GlaxoSmithKline, Research Triangle

Park, NC, USA) for 5 days after surgery and throughout the rest of self-administration training and testing. Animals were given at least 7 days of recovery from surgery before beginning self-administration training. Catheter patency was tested periodically by administering 0.05 ml Brevital (16.6 mg/ml, Jones Pharma Inc., St. Louis, MO, USA), which briefly anesthetizes the animal only if delivered i.v.

Intracranial drug infusions

CP809101 (Tocris, Ellisville, MI, USA) was dissolved in artificial cerebral spinal fluid (aCSF). Microinjections were delivered over a 1-min period using a 30-gauge injector (Plastics One) connected via polyethylene 50 tubing (Becton Dickinson, Sparks, MD, USA) to a 25-µl syringe (Hamilton Co., Reno, NV, USA) housed in an infusion pump (CMA Microdialysis, North Chelmsford, MA, USA). Injection cannulae extended 2.5 mm below the guide cannulae for the BlA and 2.7 mm below the guide cannulae for the CeA. Movement of an air bubble the proper distance through the drug infusion line confirmed successful infusion of the drug. After the infusion was complete, the injectors remained in place for 1 min to ensure thorough diffusion. After removing the injectors, metal stylets and caps were replaced before the animal was placed into the conditioning chamber for the test sessions.

Self-administration

Cocaine self-administration training took place daily for 2 h, 6 days per week. Animals were trained in operant conditioning chambers (28 × 10 × 20 cm; Med Associates, St Albans, VT, USA), each containing an active lever, a cue light 4 cm above the active lever, an inactive lever, a tone generator (500 Hz, 10 dB above ground noise), and a house light on the wall opposite the levers. Upon pressing the active lever to

complete a schedule of reinforcement, the light and tone cues were simultaneously activated and followed 1 s later by a 0.1-ml cocaine (0.75 mg/kg, i.v.) infusion delivered over 6 s. The house light was then activated for a 20-s timeout period, during which active lever presses were recorded but had no effects. Responses on the inactive lever were recorded but had no effects.

For the first 5 days of training, all animals began on a fixed ratio (FR) 1 schedule of reinforcement with the capability to progress to a variable ratio (VR) 3, and finally VR5 schedule within a session. After ending the session on a VR5 schedule for five consecutive days, animals then began the next session on a VR3 schedule. Once animals began on a VR3 schedule and ended on a VR5 schedule, they began on a VR5 schedule for the rest of the sessions. All animals were starting on a VR5 schedule by day 14 and were on a VR5 schedule exclusively for at least the last 5 days of self-administration. All animals were restricted to 16 g of food to facilitate acquisition of self-administration (M. E. Carroll, et al., 1981) and remained food-restricted until they ended on a VR5 schedule for three consecutive sessions. Animals were then given food ad libitum for the rest of the experiment.

Extinction phase

Extinction training began once rats had completed at least 15 self-administration sessions and had received food ad libitum for at least the last 5 sessions. Extinction training occurred daily for 1 h/day. Rats were placed into the self-administration chambers as before and lever presses were recorded, but produced no consequences (i.e., no infusions or cues were presented). Catheters were connected to the infusion lines during extinction, as well as during all reinstatement tests, even though the infusion lines

were not connected to a syringe. Extinction sessions continued for 10–14 days and until there was an 80% reduction in active lever pressing from the animals' highest response rate during extinction or to less than 20 active lever presses for three consecutive days.

Experiments

Upon meeting the extinction criterion, animals with BlA and CeA cannulae (experiments 1 and 2, respectively) underwent CP809101-primed reinstatement testing, cue-primed reinstatement testing, cocaine-primed reinstatement testing, and elevated plus maze (EPM) testing for the effects of CP809101 (0, 0.01, 0.1, or 1.0μg/0.2μg/side). Assignment to dosage groups was counterbalanced based on the amount of cocaine intake during self-administration, as this has been shown to affect reinstatement response rates (Baker, et al., 2001; Deroche, Le Moal, & Piazza, 1999). Animals received their assigned dose of agonist prior to each type of reinstatement test. In experiment 3, a new cohort of animals with CeA cannulae underwent CP809101-primed reinstatement testing for the effects of CP809101 (0.01μg/0.2μL/side) or SB242082 (0.1μg/0.2μL/side), cocaine-primed reinstatement testing for the effects of CP809101 (0.01μg/0.2μL/side) or CP809101+SB242084 (0.01ug and 0.1μg/0.2μL/side, respectively), and a subset of animals underwent EPM testing for the effects of vehicle and CP809101 (0 and 0.01μg/0.2μL/side).

CP809101-primed reinstatement testing

Following extinction training, animals were assigned to one of three CP809101 dose groups (0.01, 0.1, or 1.0 μg/side), counterbalanced based on the amount of cocaine intake during self-administration as this has been shown to affect reinstatement responses (Baker, et al., 2001; Deroche, et al., 1999). Animals underwent two tests for CP809101-

primed reinstatement of extinguished cocaine-seeking behavior, receiving a vehicle microinjection prior to one test and their assigned dose of CP809101 prior to the other test, with the order of these pretreatments counterbalanced. Immediately after receiving their assigned microinjection, animals were given a 1 h extinction session where responding on either lever had no consequences.

Cue-primed reinstatement testing

Animals underwent two tests for cue reinstatement of extinguished cocaine-seeking behavior, receiving a vehicle microinjection prior to one test and their assigned dose of CP809101 prior to the other test, with the order of these pretreatments counterbalanced. Animals were given a minimum of three extinction days between tests to allow extinction baseline rates to stabilize. If animals failed to meet the reinstatement criteria of doubling extinction baseline response rates and at least twenty responses on the active lever on either of the two test days, they were considered "nonreinstaters" and excluded from the analysis. Immediately after receiving their assigned microinjection, animals were tested for 1 h with the same stimulus complex as that paired with cocaine during training available response-contingently on an FR1 schedule; however, no cocaine was delivered during cue tests. The FR1 schedule was used in place of the VR5 training schedule because we have previously shown that under tests for cue reinstatement the FR1 schedule yields higher response rates, and thus greater sensitivity for detecting the predicted decrease, than the training schedule (Acosta, et al., 2008). A noncontingent cue presentation was delivered if the animal did not receive a response-contingent cue within the first 5 min of the session to minimize the possibility that animals would fail to press the lever leaving them unaware that cues were available.

Cocaine-primed reinstatement testing

After the two cue reinstatement tests, animals received at least three extinction sessions to re-establish a stable baseline extinction rate of responding. They were then given two tests for cocaine-primed reinstatement of extinguished cocaine-seeking behavior. Prior to one test, they received the same dose of CP809101 as they had received during cue reinstatement testing. For the other test, they received a vehicle microinjection. The order of the two pretreatments was counterbalanced within a group. Immediately after the microinjection, animals received a priming injection of cocaine (10 mg/kg) and were then immediately placed into the conditioning chamber. Lever presses were recorded, but produced no consequences (i.e., no cues or cocaine were delivered). To control for injection stress, animals were given mock i.p. injections immediately before the extinction session preceding each of their cocaine-reinstatement tests and the average response rates during these sessions was used as the extinction baseline. Animals were given a minimum of three extinction sessions between tests to allow extinction baseline rates to stabilize. If animals failed to meet the reinstatement criteria of doubling baseline and at least twenty responses on the active lever during at least one of the reinstatement tests, they were considered "nonreinstaters" and were excluded from the analysis.

Elevated plus maze testing

Following the completion of reinstatement testing, animals were tested for the effects of CP809101 on anxiety-like behavior on the EPM following at least a 5-day washout period from their last CP809101 treatment. Animals all received one test on the EPM and were assigned to receive the same dose of CP809101 from reinstatement testing

or vehicle. The EPM apparatus consisted of four Plexiglas arms arranged in a cross, elevated 75 cm above the floor. Each arm was 10 cm wide and 50 cm long, and each arm was joined at the center by a 10 cm square platform. The two opposite 'open' arms contained no walls, while the other two 'closed' arms had 40 cm high sides. Animals received their assigned microinjection 5-minutes prior to testing, and then were individually placed in the center of the apparatus facing one of the two closed arms (File, Lippa, Beer, & Lippa, 2004). The 10-min test was conducted under dim lighting, and behaviors were scored using Observer 5.0 software (Noldus Information Technology BV, Wageningen, The Netherlands) from videotapes by a highly trained observer blind to group assignment. The following behaviors were scored: total time spent in open arms, closed arms, and the middle of the maze; and total number of entries into open arms, closed arms, and the middle of the maze. Locomotor activity was also recorded and analyzed with a computer-automated video tracking system (Clever Systems, Reston, VA). The apparatus was cleaned with 0.05% ethanol between each test.

Histology

Animals were deeply anesthetized with 3% isoflurane and given intracranial infusions (0.2 μl/side) of 1% methylene blue to verify cannulae placements. Animals were then decapitated, brains were removed, frozen, and stored at -20°C. Brains were later sliced in coronal sections (40 μm), stained with cresyl violet, and examined under a microscope by observers unaware of group assignment who determined the point of drug infusion. Animals with incorrect placement of the drug infusions were excluded from the analyses. A schematic of accurate cannulae placements is shown in Figure 15.

Statistical Analyses

For self-administration/reinstatement, data were analyzed using mixed-factor analyses of variance (ANOVAs) with session (e.g. extinction baseline, vehicle test, and CP809101 test) as a within-subjects factor and dosage group (0.01, 0.1 and 1.0 μg/0.2 μl/side) as a between-subject factor. Effect size (η^2) was also calculated for significant interactions. A Greenhouse-Geisser correction of degrees of freedom was used to correct for heterogeneity of variance in the data. Subsequent post-hoc comparisons were made using tests of simple main effects. In addition, planned t-tests were used to test the predictions that cocaine-seeking behavior would increase after cocaine priming or cue presentation relative to baseline. Baseline values were calculated as the average of the sessions that occurred before each test day (i.e., the day before testing with agonist and the day before testing with vehicle).

For EPM testing, data were analyzed using one-way ANOVAs of percent of time spent on the open arms with dosage group as a between-subjects factor. In addition, planned t-tests were used to test the prediction that anxiety-like behavior would increase after CP809101 relative to vehicle pretreatment. We also calculated an anxiety index score described previously (Huynh, Krigbaum, Hanna, & Conrad, 2011) as follows:

Anxiety Index = 1 – [(open arm time/10 min) + (open arm entry/10 min)] / 2

Results

Experiment 1

Intra-BlA infusion effects on extinction

Active and inactive lever presses during the first session of extinction training are shown in Table 3. All animals had at least 10 extinction sessions before reinstatement

testing began. The ANOVA of the number of active lever presses/h on the first day of extinction versus the last day of extinction before testing showed a main effect of day [$F(1,19)=87.18$, $P<0.001$] but no dose effect or interaction with dose. The main effect indicated a significant drop in responding across training sessions. There were no differences in inactive lever presses.

Effects of CP809101 in the BlA on reinstatement of cocaine-seeking behavior

Figure 16 illustrates that CP809101 priming injections infused into BlA prior to testing failed to alter responding relative to extinction baseline. The ANOVA of responses/h indicated that there were no significant effects on response rates on either the active or inactive levers.

Effects of CP809101 in the BlA on cue reinstatement of cocaine-seeking behavior

Figure 17 shows the effects of CP809101 infusions into the BlA on cue-elicited reinstatement of cocaine-seeking behavior. There was 1 rat out of 27 that failed to meet the reinstatement criteria and was excluded from the analyses. The ANOVA of responses/h on the active lever showed a main effect of test day [$F(1.30,24.77)=20.51$, $P<0.001$, $\eta^2=0.510$] but no interaction with dose or main effect of dose. Tests of simple main effects indicated that when collapsed across dose, all animals showed cue reinstatement evident as an increase in responding on the vehicle and CP809101 pretreatment test days relative to the extinction baseline ($p<0.05$). Table 3 shows inactive lever presses on CP809101 test days for all groups. There were no significant differences in inactive lever presses.

Effects of CP809101 in the BlA on cocaine-primed reinstatement of cocaine-seeking behavior

Figure 18 illustrates the effects of intra-BlA infusions of CP809101 on cocaine-primed reinstatement of cocaine-seeking behavior. There were 4 animals out of 27 that failed to meet the reinstatement criteria and were excluded from the analyses. The ANOVA of responses/h on the active lever indicated a significant main effect of test day [$F(1.45,24.71)=6.84$, $P<0.01$, $\eta^2=0.276$] but no interaction with dose or main effect of dose. Tests of simple main effects indicated that when collapsed across dose, all animals showed cocaine-primed reinstatement evident as an increase in responding on the vehicle and CP809101 pretreatment test days relative to the extinction baseline ($p<0.05$). There were no differences in inactive lever presses (see Table 3).

Effects of CP809101 in the BlA on anxiety-like behavior on the elevated plus maze

Figure 19 shows the effects of CP809101 infused into the BlA on the percent of time spent on the open arms of the EPM. The ANOVA failed to show a significant effect of CP809101 dose, however planned comparisons indicated a significant difference between the vehicle and 1.0μg/μL groups ($p<0.05$). Figure 20 shows the effects of CP809101 infused into the BlA on an anxiety index score which accounts for percent of time spent in the open arms as well as number of open arm entries (Huynh, et al., 2011). The ANOVA did not show a significant effect of CP809101 dose, however planned comparisons indicated a marginally significant difference between the vehicle and 1.0 μg/μL groups ($p=0.052$).

Effects of CP809101 in the BlA on locomotor activity

Figure 21 shows the effects of CP809101 infused into the BlA on spontaneous locomotor activity. The ANOVA of total distance traveled failed to show a significant effect of dose.

Experiment 2

Extinction behavior of animals with CeA cannulae

Active and inactive lever presses during the first session of extinction training are shown in Table 3. All animals had at least 10 extinction sessions before reinstatement testing began. ANOVAs of the number of active and inactive lever presses/h on the first day of extinction versus the last day of extinction before testing showed significant main effects of day [$F(1,20)=90.92$, $P<0.001$ and $F(1,20)=4.63$, $P<0.05$, respectively] but no dose effect or interaction with dose. The main effects indicated a significant drop in responding across training sessions.

Effects of CP809101 in the CeA on reinstatement of cocaine-seeking behavior

Figure 22 illustrates that CP809101 priming injections infused into CeA prior to testing failed to alter responding relative to extinction baseline. The ANOVA of responses/h indicated that there were no significant effects on response rates on either the active or inactive levers.

Effects of CP809101 in the CeA on cue reinstatement of cocaine-seeking behavior

Figure 23 shows the effects of CP809101 infusions into the CeA on cue-elicited reinstatement of cocaine-seeking behavior. The ANOVA of responses/h on the active lever showed a significant main effect of test day [$F(1.33,26.60)=23.78$, $P<0.001$, $\eta^2=0.543$] but no interaction with dose. There was a nonsignificant trend toward a main

effect of dose of CP809101 [F(2,20)=3.22, P=0.061, η^2=0.244], however there were no statistical differences between CP809101 and vehicle test days at any specific dose. Tests of simple main effects indicated that when collapsed across dose, all animals showed cue reinstatement evident as an increase in responding on the vehicle and CP809101 pretreatment test days relative to the extinction baseline ($p<0.05$). Table 3 shows inactive lever presses on CP809101 test days for all groups. There were no significant differences in inactive lever presses.

Effects of CP809101 in the CeA on cocaine-primed reinstatement of cocaine-seeking behavior

Figure 24 illustrates the effects of intra-CeA infusions of CP809101 on cocaine-primed reinstatement of cocaine-seeking behavior. There were 3 animals out of 23 that failed to meet the reinstatement criteria and were excluded from the analyses. The ANOVA of responses/h on the active lever indicated a significant day by dose interaction [F(4,34)=4.06, $p<0.01$, η^2=0.323] as well as a main effect of test day [F(2,34)=12.33, $P<0.001$, η^2=0.420]. Post hoc comparisons indicated an increase in responding relative to the extinction baseline on all vehicle test days and CP809101 test days at the 0.1 and 1.0μg/μL doses (t-test, $p<0.05$) and a significant decrease in lever pressing at the 0.01μg/μL dose of CP809101 compared to vehicle (t-test, $p<0.05$). Although the vehicle test days appear to have a fair amount of variance by dosage group, there were no significant differences between them. Further, there were no differences between animals that received their vehicle test day first compared to those that received their vehicle test day second. There were no differences in inactive lever presses (see Table 3).

Effects of CP809101 in the CeA on anxiety-like behavior on the elevated plus maze

Figure 25 shows the effects of CP809101 infused into CeA on the percent of time spent on the open arms of the EPM. The ANOVA failed to show a significant effect of CP809101 dose although planned comparisons indicated a trend toward a difference between the vehicle and 0.1μg/μL groups ($p=0.071$). Figure 26 shows that CP809101 infused into the CeA had no effect on anxiety index score.

Effects of CP809101 in the CeA on locomotor activity

Figure 27 shows the effects of CP809101 infused into CeA on spontaneous locomotor activity. The ANOVA of total distance traveled failed to show a significant between-group effect of dose.

Experiment 3

Extinction behavior of animals with CeA cannulae

Active and inactive lever presses during the first session of extinction training are shown in Table 3. All animals had at least 10 extinction sessions before reinstatement testing began. ANOVAs of the number of active and inactive lever presses/h on the first day of extinction versus the last day of extinction before testing showed significant main effects of day [$F(1,14)=82.99$, $P<0.001$ and $F(1,14)=10.26$, $P<0.01$, respectively] but no dose effect or interaction with dose. The main effects indicated a significant drop in responding across training sessions.

Effects of CP809101 and SB242084 in the CeA on reinstatement of cocaine-seeking behavior

Figure 28 illustrates the effects of CP809101 or SB242084 priming infusions into CeA prior to testing on responding relative to extinction baseline. The ANOVA of

responses/h indicated that there were no significant effects on response rates on either the active or inactive levers.

Effects of CP809101+SB242084 in the CeA on cocaine-primed reinstatement of cocaine-seeking behavior

Figure 29 illustrates the effects of intra-CeA infusions of CP809101 or CP809101+SB242084 on cocaine-primed reinstatement of cocaine-seeking behavior. There were 3 animals that failed to meet the reinstatement criteria and 1 animal that was an outlier (i.e., more than 2 standard deviations above the mean without data from the animal in question), and all 4 were excluded from the analyses. The ANOVA of responses/h on the active lever indicated a significant day by condition interaction [$F(2,20)=9.30$, $p<0.01$, $\eta^2=0.482$] as well as a main effect of test day [$F(2,20)=24.53$, $P<0.001$, $\eta^2=0.710$]. Post hoc comparisons indicated an increase in responding relative to the extinction baseline on all vehicle test days and on CP809101 or CP809101+SB242084 test days (t-test, $p<0.05$) as well as a significant decrease in lever pressing when animals received CP809101 compared to vehicle (t-test, $p<0.01$). There was a significant difference between vehicle test days for animals that received CP809101 compared to those that received CP809101+SB242084 (t-test, $p<0.05$). There were no differences in inactive lever presses (see Table 3).

Discussion

Results from the present study partially support our hypothesis that 5-HT_{2C}Rs in the BlA are involved in cue-primed reinstatement and anxiety-like behavior, whereas those in the CeA are involved in cocaine-primed reinstatement. We found that the 5-HT_{2C}R agonist, CP809101, infused into the BlA increased anxiety-like behavior on the

EPM. This result is consistent with findings that intra-BlA infusions of 5-HT_{2C}R agonists, including CP809101, increase anxiety-like behavior in other anxiety paradigms (Campbell & Merchant, 2003; Christianson, et al., 2010; Q. Li, et al., 2012). Intra-CeA infusions of CP809101 also consistently attenuated cocaine-primed reinstatement at the lowest dose, and this effect was reversed with co-administration of a 5-HT_{2C}R antagonist. CP809101 in the BlA or CeA had no effect on locomotor activity, so the anxiogenic effects in the BlA were likely not great enough to produce freezing behavior nor were the decreases in cocaine-seeking behavior likely due to nonspecific effects on motor function.

Surprisingly, we found no support for our hypothesis that actions at 5-HT_{2C}Rs via CP809101 in the BlA attenuate cue-primed reinstatement. This hypothesis was based on previous findings demonstrating a role of the BlA in cue-primed reinstatement (B. J. Everitt, et al., 1999; Fuchs & See, 2002), an abundance of 5-HT_{2C}Rs in this region (Clemett, et al., 2000), and consistent attenuation of cue-primed reinstatement by 5-HT_{2C}R agonists (Burbassi & Cervo, 2008; Higgins & Fletcher, 2003; Neisewander & Acosta, 2007; Pentkowski, et al., 2010). It is possible that the doses of CP809101 tested were outside the range of effective doses for cue-primed reinstatement, however we observed significant effects at both the lowest and highest doses on other behavioral tests, which mitigates this explanation. Since the highest dose of CP809101 in the BlA increased anxiety-like behavior on the EPM and moderate acute anxiety increases cue-primed reinstatement (Feltenstein, Henderson, & See, 2011), it is possible that there were opposing effects of cue incentive motivation on cocaine-seeking behavior and stress-induced attenuation of behavior, resulting in a null effect.

Interestingly, the effects of CP809101 in the CeA on cocaine-primed reinstatement were only seen at the lowest dose. It is not uncommon to observe a U-shaped dose-response effect where only intermediate doses are effective (J. M. S. Davis, D. J., 1990; Neisewander, O'Dell, & Redmond, 1995). It is likely that as the dose of CP809101 decreases lower than the effective dose of 0.01 μg/μL/side in the CeA, the effects on cocaine-primed reinstatement diminish, resulting in an inverted U-shaped dose-response function. Unfortunately, we may not have captured the full CP809101 dose-response function in this study, however our results as well as Christianson et al. (2010) found that 1.0 μg/μL/side in the BlA effectively increased anxiety-like behavior while 0.01 μg/μL/side was ineffective. One explanation for the lack of effect at higher doses is that these doses may produce nonspecific effects at 5-HT_{2A}Rs, which would have an opposite effect on cocaine-primed reinstatement from 5-HT_{2C}R agonist inhibition effect (Fletcher, et al., 2002; Nic Dhonnchadha, et al., 2009). CP809101 is highly selective for 5-HT_{2C}Rs over 5-HT_{2A} and 5-HT_{2B}Rs (Barnes & Sharp, 1999; Fletcher, Sinyard, & Higgins, 2010; Fletcher, et al., 2009; Siuciak, et al., 2007), however with administration directly into the brain it is possible that the higher doses in this study were high enough to activate 5-HT_{2A}Rs. Further, as the CeA is not densely packed with 5-HT_{2C}Rs, the ratio of 5-HT_{2C}R:5-HT_{2A}R occupancy may be lower than in other regions with higher levels of 5-HT_{2C}Rs, such as the BlA (Clemett, et al., 2000; Q. Li, et al., 2012). 5-HT_{2A}R antagonists have been found to produce anxiogenic effects (Graeff, Guimaraes, De Andrade, & Deakin, 1996; Graeff, Netto, & Zangrossi, 1998), so this may be responsible for the trend toward an intra-CeA CP809101 effect on the EPM as well. In any case, the effect of the lowest dose of CP809101 in the CeA on cocaine-primed

reinstatement was replicated and reversed with co-administration of a 5-HT_{2C}R antagonist, providing strong evidence that this is a 5-HT_{2C}R-mediated effect.

There was an unusual amount of variability in our vehicle test results for cocaine-primed reinstatement with infusions into the CeA. When CP809101 was injected into the CeA in experiment 2, the lowest dose consistently attenuated cocaine-primed reinstatement but we observed unusually high responding on the vehicle test day for this group. Statistically, however, there were no significant differences in vehicle test days across the dose groups, nor was there an order effect between animals that had their vehicle test first compared to second. In experiment 3, we again found a significant difference between vehicle and CP809101, as well as vehicle and SB242084, groups. Furthermore, there was again no order effect between animals that had their vehicle test first compared to second. The lack of order effects is likely due to the fact that there is a high amount of variability in lever pressing and the n=2-4 when split by drug and testing order, which reduces power to detect a potential order effect. Thus we speculate that order of drug versus vehicle contributes to the high variance. Specifically, when the low dose of CP809101 was tested before vehicle, the suppression of cocaine-primed reinstatement may have resulted in a higher amount of lever pressing on the subsequent vehicle reinstatement test. By contrast, when the highest dose of CP809101 was tested first in experiment 2, there was no suppression of responding so extinction learning during that test likely resulted in lower responding on the subsequent vehicle test. While the variance in responding on vehicle test days in experiments 2 and 3 is curious, it does not detract from the reliability of our findings that 0.01 μg/0.2μL CP809101 in the CeA attenuated cocaine-primed reinstatement.

Other issues that are important to consider when using intracranial drug administration are spread of the drug to neighboring regions and potential damage or tolerance from repeated drug infusions. Because the BlA and CeA border each other, the region specificity of the effects suggests that the drug effects are not due to spread to a neighboring region. Animals underwent EPM testing at the end of the experiment when they had already received two to three injections of CP809101, and we still observed a significant effect of CP809101 in the BlA on this test. This finding mitigates the idea that tolerance to CP809101 developed or that there was a nonspecific effect of repeated infusion. Further, histological verification of cannula placements showed no evidence of excessive tissue damage from repeated infusions.

We targeted the rostral section of the BlA. The rostral (rBlA) and caudal (cBlA) subsections of the BlA have divergent projections to the NAc core and dorsal agranular insular PFC, and NAc shell and prelimbic PFC, respectively (Groenewegen, Berendse, Wolters, & Lohman, 1990; Kita & Kitai, 1990; Shinonaga, Takada, & Mizuno, 1994). Inactivation of the rBlA using excitotoxic lesions, tetrodotoxin (TTX), and lidocaine inactivation has been consistently shown to disrupt cue-primed reinstatement (Grimm & See, 2000; Kantak, Black, Valencia, Green-Jordan, & Eichenbaum, 2002; Meil & See, 1997), while cBlA lidocaine inactivation reduces drug-seeking behavior during cocaine self-administration (Kantak, et al., 2002). Other studies have found effects of pharmacological manipulations in the cBlA on both cue- and cocaine-primed reinstatement (Alleweireldt, et al., 2006; Berglind, Case, Parker, Fuchs, & See, 2006), and in the rBlA on cue-primed reinstatement (Alleweireldt, et al., 2006; Mashhoon, Tsikitas, & Kantak, 2009). Thus, if 5-HT_{2C}Rs in the BlA are involved in cue-primed

reinstatement, we likely would have observed an effect of CP809101 as the injections were in the rBlA.

Based on previous literature, we expected CP809101 in the BlA, but not the CeA to affect anxiety-like behavior. We did observe a non-significant increase in anxiety-like behavior from CP809101 in the CeA, though the previous literature on 5-HT_{2C}Rs in the CeA is inconsistent (Campbell & Merchant, 2003; Christianson, et al., 2010; Heisler, Zhou, et al., 2007; Q. Li, et al., 2012), so 5-HT_{2C}Rs in this region may play are less prominent role in anxiety than the BlA 5-HT_{2C}Rs. Accordingly, Campbell and Merchant (2003) found that a 5-HT_{2C}R agonist, mCPP, in the CeA did not affect ultrasonic vocalizations or exploratory behavior on a novel-object task, though in the BlA it increased ultrasonic vocalizations and decreased exploratory behavior on a novel-object task, which is consistent with an anxiogenic effect. Similarly, Christianson et al. (2010) found that a 5-HT_{2C}R antagonist, SB242084, in the CeA did not affect anxiety-like behavior on a juvenile social exploration task, though in the BlA the antagonist decreased anxiety-like behavior. The elevated plus maze is a well-accepted test of anxiety in rodents where animals spend more time in the two closed arms of the maze when anxious than in the two open arms. Time spent in the open arms is also correlated with higher levels of the stress hormone, CORT, and is thought to be a measure of anxiety-like behavior (Pellow, Chopin, File, & Briley, 1985). 5-HT activates the hypothalamic-pituitary-adrenal (HPA) axis (Fuller & Snoddy, 1980), an effect that is thought to involve 5-HT_{2C}R stimulation (Heisler et al., 2007). Findings from our study and others suggest that the BlA is likely involved in the anxiogenic behavioral effects of 5-HT_{2C}R stimulation.

While we can only speculate about the neurocircuitry underlying the effects of CP809101 on anxiety-like behavior in the BlA and cocaine-primed reinstatement in the CeA, we hypothesize that the mechanism is via modulation of GABA release. In the BlA, 5-HT_{2C}Rs activate GABA interneurons (Stein, Davidowa, & Albrecht, 2000), however it is hypothesized that this effect is overshadowed by activation of projection neurons at higher doses (Rainnie, 1999) and leads to the anxiogenic effects of 5-HT_{2C}R agonists (Campbell & Merchant, 2003). It is also possible that 5-HT_{2C}R-induced GABA activation may inhibit other GABA interneuron inhibition of projection neurons, thus resulting in net disinhibition and anxiety-like behavior. To our knowledge, the cellular localization of 5-HT_{2C}Rs in the CeA is not known, however it is possible that they are located on GABA interneurons and thus 5-HT_{2C}R agonists in this region would increase GABA inhibition. Accordingly, GABA receptor agonists in the CeA attenuate footshock-primed reinstatement of cocaine-seeking behavior (McFarland, et al., 2004). While we did not assess the effects of CP809101 on stress-primed reinstatement, the CeA is involved in both stress and primary reinforcing effects of cocaine (Cain, Denehy, & Bardo, 2008; McFarland, et al., 2004; O'Dell, et al., 1999). However, since we did not observe an anxiogenic effect of CP809101 in the CeA, involvement of 5-HT_{2C}Rs in the CeA in stress-primed reinstatement would likely be due to the motivational impact of the stressor rather than an anxiogenic effect.

In conclusion, our results suggest that 5-HT_{2C}R activation in the CeA reduces cocaine-primed reinstatement, an effect that was reversed with concurrent administration of a 5-HT_{2C}R antagonist. We also found an anxiety-like effect with 5-HT_{2C}R activation in the BlA, but no effect on cue-primed reinstatement. The lack of effects of CP809101 in

the BlA on cue-primed reinstatement was surprising and warrants additional investigation, perhaps with approaches that alter expression of 5-HT_{2C}Rs in this region to verify our findings. CP809101 in the CeA did not significantly affect anxiety-like behavior on the EPM, and CP809101 in either region of the amygdala had no effect on locomotor activity. From these data, we conclude that 5-HT_{2C}Rs in the BlA play a role in anxiety and 5-HT_{2C}Rs in the CeA mediate the incentive motivational effects of cocaine priming.

Chapter 5

Concluding Remarks

The purpose of this dissertation was to examine the role of 5-HT_2Rs in brain regions involved in reward on cocaine abuse-related behaviors and to explore the use of 5-HT_2R drugs for the treatment of cocaine abuse and dependence. Specifically, Chapter 2 investigated the effects of a 5-HT_{2A}R antagonist localized to the mPFC on cocaine-seeking behavior; Chapter 3 examined a combination of a 5-HT_{2A}R antagonist and 5-HT_{2C}R agonist on cocaine hyperlocomotion and neuronal activation in the dorsal striatum; Chapter 4 examined the effects of a 5-HT_{2C}R agonist localized to either the BlA or CeA on anxiety-like and cocaine-seeking behaviors.

We hypothesized that a 5-HT_{2A}R antagonist in the mPFC would attenuate the incentive motivational effects of cocaine and cocaine-paired cues based on previous findings that 5-HT in the mPFC plays a role in cocaine-seeking behavior (Pentkowski, et al., 2010), 5-HT_{2A}R antagonists attenuate cocaine-seeking behavior when given systemically (Fletcher, et al., 2002; Nic Dhonnchadha, et al., 2009), and the PFC plays a role in impulsivity and decision-making (Bechara, et al., 1994; Damasio, et al., 1994; Puumala & Sirvio, 1998), which contribute to drug abuse (Capriles, et al., 2003; Childress, et al., 1999). We found that the selective 5-HT_{2A}R antagonist, M100907, microinfused into the mPFC dose-dependently attenuated cue-primed reinstatement of cocaine-seeking behavior, consistent with its effects when given systemically (Nic Dhonnchadha, et al., 2009). Surprisingly, M100907 infusions in the mPFC had no effect on cocaine-primed reinstatement in contrast to its systemic effects (Fletcher, et al., 2002; Nic Dhonnchadha, et al., 2009). The effects of M100907 in the mPFC were specific to

cue-induced motivation for cocaine, as the drug failed to affect cue-primed reinstatement of sucrose-seeking behavior. This finding together with a lack of effect on spontaneous or cocaine-induced locomotion suggests that the decrease in cue reinstatement of cocaine-seeking behavior is not due to locomotor activity deficits. Further, M100907 infused into a neighboring region of the cortex failed to affect cue-primed reinstatement, supporting the anatomical specificity of the effect in the mPFC.

Our findings from this study and Pentkowski et al, 2010 support the role of the PFC in cocaine-seeking behavior. After extended drug use, addicts exhibit "hypofrontality," or deficits in PFC activation, which is thought to contribute to their lack of control over continued drug use (Childress, et al., 1999; Goldstein & Volkow, 2002). Despite this reduction in baseline PFC functioning, when exposed to drug-associated cues, animals and humans with drug history actually exhibit increased activity in the PFC, likely due to activation of the mesocorticolimbic DA pathway (Childress, et al., 1999; Ciccocioppo, et al., 2001; Grant, et al., 1996; Maas, et al., 1998; Neisewander, et al., 2000). While we have not determined the neuroanatomical pathways underlying the attenuation of cocaine-seeking behavior by 5-HT_{2A}R antagonists and 5-HT_{2C}R agonists, they likely involve inhibition of the mesocorticolimbic DA pathway via increases in GABA or decreases in glutamate in the mPFC to reduce excitatory outputs to the VTA and NAc (Di Ciano & Everitt, 2001; McFarland, et al., 2003). An important future direction is to examine the circuitry of these localized effects of a 5-HT_{2A}R antagonist and 5-HT_{2C}R agonist in the mPFC.

In Chapter 3 we discovered an interaction between a 5-HT_{2A}R antagonist and 5-HT_{2C}R agonist. 5-HT_{2A}R antagonists and 5-HT_{2C}R agonists have been investigated for

their treatment potential in several mental health disorders, including schizophrenia, obesity, and addiction (Barnes & Sharp, 1999; Sargent, et al., 1997). However, both have side effects including sleep and appetite disturbances (Morairty, Hedley, Flores, Martin, & Kilduff, 2008; Sargent, et al., 1997), which may reduce their effectiveness in a clinical setting. Ideally, we may be able to use lower doses of these ligands to avoid side effects, while still maintaining therapeutic effects. We hypothesized that concurrent 5-HT_{2A}R antagonism and 5-HT_{2C}R agonism using subthreshold doses may interact to reduce the effects of cocaine. Accordingly, we found that ineffective doses of M100907 and MK212, a 5-HT_{2C}R agonist, attenuated cocaine hyperlocomotion and cocaine-induced Fos activation in the dorsal striatum only when given together. The reduction of Fos in the CPu is fitting as this region is highly involved in stimulant-induced locomotion, stereotypy, and habitual behaviors (Brown, et al., 1992; Naylor & Olley, 1972; White, et al., 1998; Zimmerberg & Glick, 1974). Similarly, another recent study found that a combination of subthreshold doses of M100907 and WAY163909, another 5-HT_{2C}R agonist, attenuated cocaine hyperlocomotion, cocaine-induced impulsivity, and cue- and cocaine-primed reinstatement (Cunningham, et al., 2013). Further, M100907 and Ro60-0175, another 5-HT_{2C}R agonist, were shown to decrease Zif protein activation in the dorsal striatum (Burton, Rizos, Diwan, Nobrega, & Fletcher, 2013). The findings from these studies reinforce our conclusion that a 5-HT_{2A}R antagonist and 5-HT_{2C}R agonist interact and involve the dorsal striatum to attenuate cocaine-related behaviors.

A future direction is to clarify the role of the CPu in the interaction between a 5-HT_{2A}R antagonist and 5-HT_{2C}R agonist. This study suggests the nigrostriatal pathway rather than the mesolimbic pathway may be a point of 5-HT_{2A}R and 5-HT_{2C}R interaction

effects. We have conducted an unpublished follow-up study examining the effects of intra- CPu administration of M100907 and another 5-HT_{2C}R agonist, CP809101, on cocaine hyperlocomotion, however this combination treatment had no effect on systemically administered cocaine (15 mg/kg, i.p.). However, we did find that systemic administration of M100907 and CP809101 attenuated intra-CPu administration of cocaine (100μg/0.5μL/side). These findings lead us to hypothesize that systemic administration of M100907 and CP809101 may attenuate DA release in the CPu via 5-HT_{2A}R blockade and 5-HT_{2C}R activation in the substantia nigra pars compacta (SNpc). While we have not directly tested this hypothesis yet, 5-HT_{2A} and 5-HT_{2C}Rs are found in the SNpc (Cornea-Hebert, et al., 1999; Lopez-Gimenez, et al., 1997; Pasqualetti et al., 1999) and this region sends a rich DA projection to the CPu, so this is a plausible explanation. We plan to further test this idea by administering M100907 and CP809101 directly into the SNpc to examine the effects on cocaine hyperlocomotion and Fos activation in the CPu.

Another future direction to this study mentioned by Cunningham et al, 2013 is to develop a single ligand to act as a dual 5-HT_{2A}R antagonist and 5-HT_{2C}R agonist. Some studies suggest that this type of treatment may be ideal for using low doses, thus increasing compliance by reducing side effects (Kiessling, Gestwicki, & Strong, 2006; Zhou et al., 2006). This is an important avenue to explore for pharmacological treatment of addiction, however to our knowledge a dual 5-HT_{2A}R antagonist and 5-HT_{2C}R agonist has not yet been developed.

Finally, the last set of experiments in Chapter 4 sought to localize the effects of a 5-HT_{2C}R agonist in the BlA and CeA. 5-HT_{2C}R agonists attenuate cocaine-seeking

behavior (Fletcher, et al., 2008; Neisewander & Acosta, 2007; Pentkowski, et al., 2010), and also increase anxiety-like behavior when given systemically or directly into the BlA (Campbell & Merchant, 2003; Christianson, et al., 2010; Heisler, Pronchuk, et al., 2007). Since the BlA and CeA are highly involved in cue- and cocaine-primed reinstatement, respectively, and 5-HT_{2C}Rs are found in these regions, we hypothesized that localized 5-HT_{2C}R agonism in the BlA may attenuate cue-primed reinstatement and increase anxiety-like behavior, while CP809101 in the CeA would attenuate cocaine-primed reinstatement. We found that CP809101 in the BlA increased anxiety-like behavior on the EPM, but had no effect on cue- or cocaine-primed reinstatement. CP809101 in the CeA attenuated cocaine-primed reinstatement, and this effect was reversed with concurrent administration of a 5-HT_{2C}R antagonist, but had no effect on cue-primed reinstatement or anxiety-like behavior on the EPM. We were surprised that CP809101 in the BlA did not attenuate cue-primed reinstatement due to the important role of the BlA in cue-primed reinstatement (B. J. Everitt, et al., 1999; Fuchs, et al., 2002) and the consistent attenuation of cue-primed reinstatement by 5-HT_{2C}R agonists (Fletcher, et al., 2008; Neisewander & Acosta, 2007; Pentkowski, et al., 2010). We explored possible limitations to this finding in the discussion from Chapter 4 and suggest that increased anxiety from CP809101 in the BlA may have interfered with cue-primed reinstatement or that nonspecific effects of the drug at 5-HT_{2A}Rs may oppose the hypothesized action at 5-HT_{2C}Rs.

A future direction for the Chapter 4 study is to examine the effects of a 5-HT_{2A}R antagonist in the amygdala. 5-HT_{2A}Rs are found in the BlA, on both excitatory and inhibitory neurons (Bombardi, 2011; McDonald & Mascagni, 2007). It has been shown that 5-HT_{2A}R agonism increases GABA inhibition from the BlA, but this effect is

attenuated after repeated stress (Jiang et al., 2009). 5-HT_{2A}Rs are also located in the CeA (Bombardi, 2011; Cornea-Hebert, et al., 1999; Wright, Seroogy, Lundgren, Davis, & Jennes, 1995), though the specific cellular localization has not yet been reported. Due to the opposing effects of 5-HT_{2A}R antagonists and 5-HT_{2C}R agonists on cocaine-related behaviors, it is possible that a 5-HT_{2A}R antagonist in the BlA and CeA may have similar effects to a 5-HT_{2C}R agonist in Chapter 4.

An important issue to discuss when conducting pharmacological studies such as those in this dissertation is selectivy of the ligands being used. In Chapters 2 and 3, we used M100907 which is a highly selective 5-HT_{2A}R antagonist. Due to the similarity of the 5-HT_{2A} and 5-HT_{2C}Rs, the 5-HT_{2C}R is the most likely receptor, aside from 5-HT_{2A}, to be blocked by a non-selective 5-HT_{2A}R antagonist. M100907 has greater than a 1000-fold selectivity for 5-HT_{2A}Rs over 5-HT_{2C}Rs (Kehne, et al., 1996), and reverses the effects of 5-HT_{2A}R agonists but not 5-HT_{2C}R agonists (Dekeyne, et al., 1999; Gresch, et al., 2007; Hitchcock, et al., 1997; McCreary, et al., 2003; Vickers, et al., 2001; Wettstein, et al., 1999). In Chapter 3, we used MK212, a moderately-selective 5-HT_{2C}R agonist. While MK212 has affinity for the 5-HT_{2A}, 5-HT_{2B}, and 5-HT_3Rs, it has a 150-fold selectivity for the 5-HT_{2C}R relative to the other 5-HT_2Rs (Cussac, et al., 2002; Glennon, et al., 1989; Porter, et al., 1999). Further, the effects of MK212 have been shown to be reversed with a 5-HT_{2C}R antagonist (Neisewander & Acosta, 2007; Pentkowski, et al., 2010; Stiedl, et al., 2007). In Chapter 4 we used a newer 5-HT_{2C}R agonist, CP809101, which is more selective for the 5-HT_{2C}R than MK212 and another older 5-HT_{2C}R agonist, mCPP (Barnes & Sharp, 1999; Fletcher, et al., 2009). Additionally, we reversed the effects of

CP809101 with a 5-HT_{2C}R antagonist, which greatly strengthens the argument that the effects of CP809101 were due to 5-HT_{2C}R stimulation.

In conclusion, this dissertation built upon the literature localizing the effects of 5-HT_{2A}R antagonists and 5-HT_{2C}R agonists on cocaine-related behaviors and investigated a potential treatment mechanism via concurrent 5-HT_{2A}R antagonism and 5-HT_{2C}R agonism. There is a growing area of research on 5-HT_2Rs and addiction, with the ultimate goal of identifying potential pharmacotherapeutic techniques to curb craving and motivation to seek addictive drugs. Studies such as those in this dissertation are essential in understanding the circuitry underlying the effects of 5-HT_2R manipulations on cocaine-related behaviors, which, in turn, will aid in the use these mechanisms to treat addiction. Our findings, as well as others, offers strong support for the use of 5-HT_{2A}R antagonists and 5-HT_{2C}R agonists to attenuate the incentive motivational effects of cocaine and cocaine-associated cues, which contribute to relapse (Childress, et al., 1988; Jaffe, et al., 1989; Sinha, et al., 1999).

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Table 1 Order of M100907 testing.

Test 1	Test 2	Test 3	Test 4
Self-Administration Testing (n=23)[1]	Cue Reinstatement (n=42)	Cocaine-Primed Reinstatement (n=39)	M100907 Reinstatement (n=22)
Cue Reinstatement (n=22)	Cocaine-Primed Reinstatement (n=14)	M100907 Reinstatement (n=14)	Cue Reinstatement-Sucrose (n=10)
M100907 Reinstatement (n=17)			Locomotor Activity (n=24)

[1] The number of animals shown for each test excludes those that did not reinstate or were outliers. Each animal received no more than 4 types of tests, and for each test type they received a vehicle infusion prior to one test and their assigned dose of M100907 prior to the other test, with order counterbalanced, resulting in a maximum of 8 microinfusions total.

Table 2 Lever presses/h (mean ± SEM) during the first day of extinction and during the M100907 reinstatement tests.

Brain Region and Dose Assignments	**Active Lever Presses**	**Inactive Lever Presses**			
	First Day Extinction	First Day Extinction	Cue Reinstatement	Cocaine Reinstatement	M100907 Reinstatement
PFC					
0.1 µg/side	109.2 ± 16.5	24.4 ± 6.9	8.8 ± 4.1	10.2 ± 5.1	2.7 ± 0.8
0.3 µg/side	106.2 ± 11.5	23.8 ± 4.7	8.4 ± 1.9	3.7 ± 1.5	5.7 ± 1.6
1.0 µg/side	104.9 ± 18.3	27.5 ± 10.0	4.9 ± 1.0	10.0 ± 7.1	6.2 ± 2.6
1.5 µg/side	83.8 ± 12.9	20.9 ± 4.0	4.7 ± 1.0	12.6 ± 8.1	2.8 ± 1.4
Cg2					
1.5 µg/side	70.8 ± 13.3	24.8 ± 6.3	12.7 ± 3.0		

Table 3 Lever presses/h (mean ± SEM) during the first day of extinction and during the CP809101 and SB242084 reinstatement tests.

Brain region and CP809101 Dose	**Active Lever Presses**	**Inactive Lever Presses**			
	First Day Extinction	First Day Extinction	Cue Reinstatement	Cocaine Reinstatement	CP809101 Reinstatement
BlA					
0.01 µg/side	90.5 ± 17.0	32.3 ± 14.2	11.3 ± 4.4	7.0 ± 3.0	14.3 ± 7.0
0.1 µg/side	84.9 ± 13.0	15.0 ± 5.3	15.3 ± 3.7	26.3 ± 12.0	9.9 ± 2.9
1.0 µg/side	81.0 ± 12.5	29.6 ± 14.5	7.0 ± 1.3	34.8 ± 26.0	9.0 ± 2.2
CeA					
0.01 µg/side	102.2 ± 11.6	29.2 ± 8.1	11.2 ± 5.3	15.7 ± 7.7	9.8 ± 3.6
0.1 µg/side	69.9 ± 13.0	16.0 ± 5.8	7.0 ± 1.6	4.4 ± 1.3	12.0 ± 2.0
1.0 µg/side	83.6 ± 18.3	66.6 ± 31.3	25.5 ± 11.1	13.3 ± 3.8	12.9 ± 5.2
0.01 µg/side +SB242084*	103.0 ± 13.5	25.5 ± 5.3		27.6 ± 15.4	8.9 ± 1.4

* 0.01 µg/side was co-infused with 0.1 µg/side SB242084 in the CeA in Experiment 3.

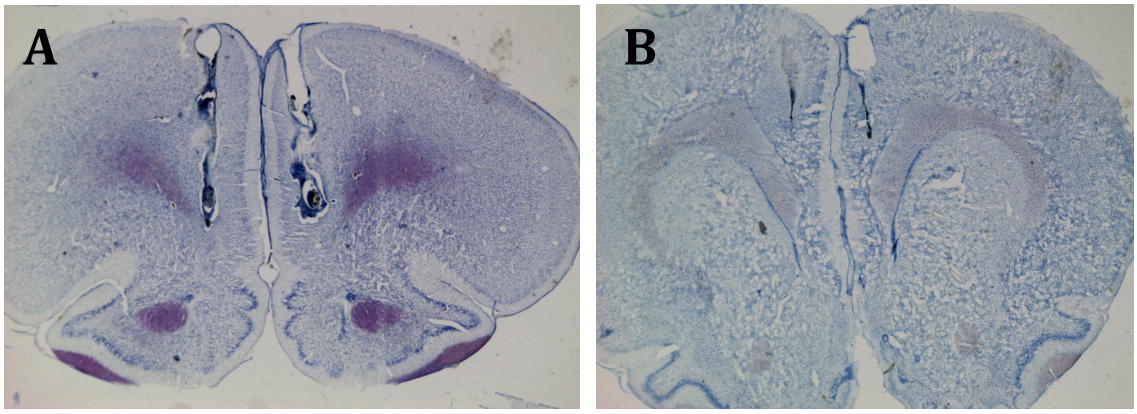


Figure 1 Thionin-stained sections taken in the coronal plane demonstrating representative cannula placements in mPFC (A) and Cg2 (B).

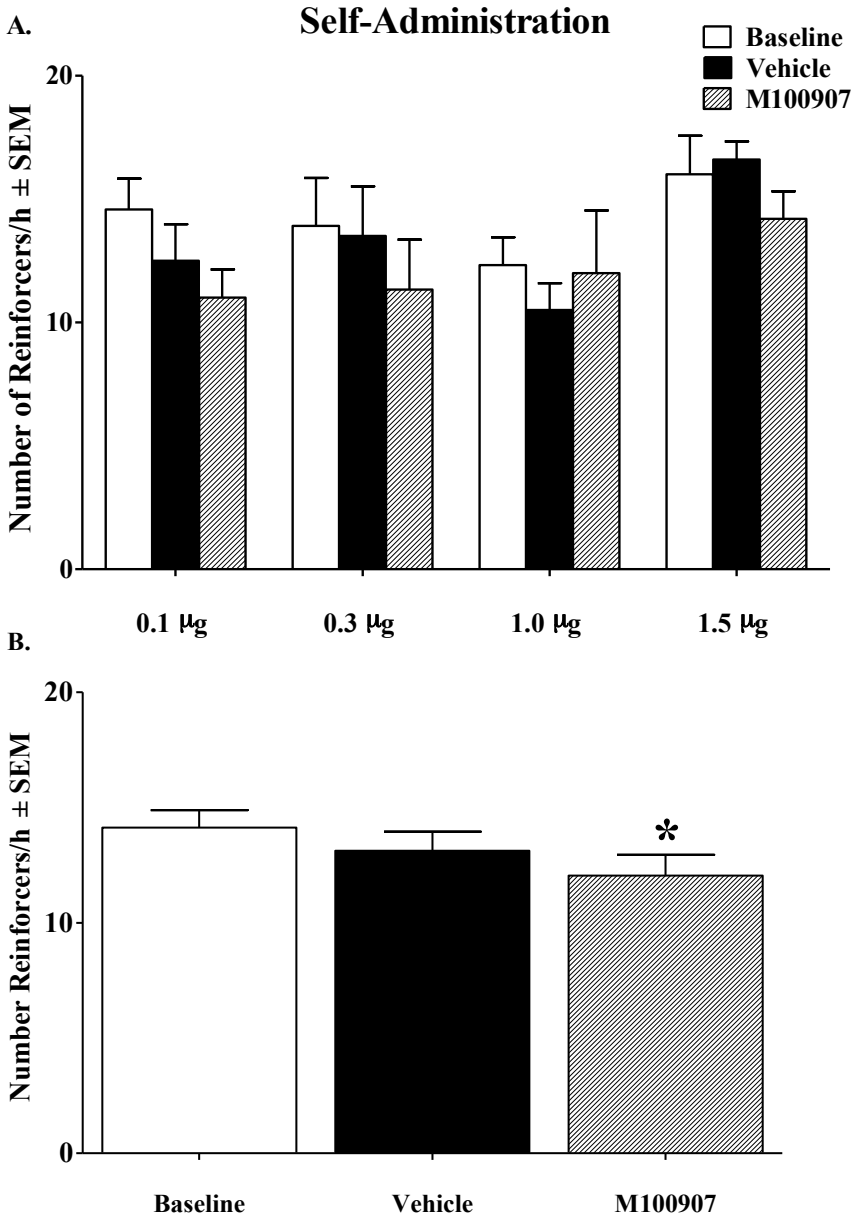


Figure 2 Effects of M100907 on cocaine self-administration, expressed as the mean ± SEM number of reinforcers (infusions of cocaine with cues) received over a 1-h test session in each dosage group (A) and collapsed across dosage groups (B). Animals assigned to receive 0.1 (n=6), 0.3 (n=6), 1.0 (n=6), or 1.5 (n=5) μg/0.2 μl/side M100907 into the mPFC were tested on one day with their assigned dose (striped bar) and on another day with the vehicle (black bar), with order counterbalanced. Baselines (white bar) were calculated as the average number of reinforcers obtained during the first h of the self-administration sessions immediately preceding each test. There was a small, but significant decrease in responding on the M100907 test day relative to baseline when collapsed across dose (i.e., main effect of test day). The asterisk (*) represents a significant difference from extinction baseline, test of simple main effects, $p<0.05$.

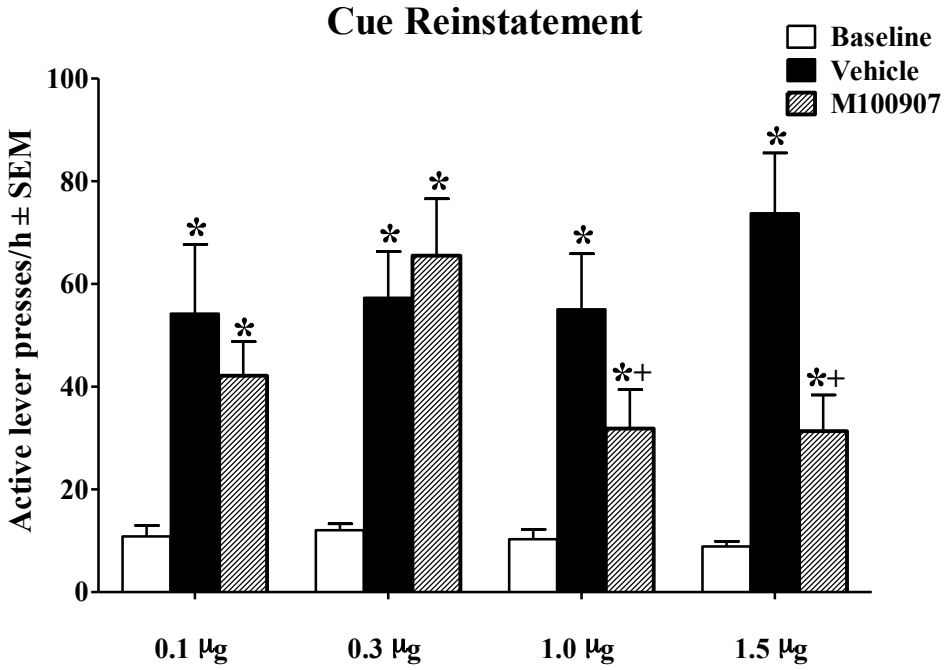


Figure 3 Effects of M100907 pretreatment on cue-elicited reinstatement of cocaine-seeking behavior when injected directly into the mPFC, expressed as mean responses/h ± SEM on the active lever. Animals assigned to receive 0.1 (n=13), 0.3 (n=12), 1.0 (n=14), or 1.5 (n=17) μg/0.2 μl/side M100907 into the mPFC were tested on one day with their assigned dose (striped bar) and on another day with the vehicle (black bar), with order counterbalanced. These pretreatments were infused within 1 min before placing the animals into the self-administration chambers, where light and tone cues were available response-contingently on an FR1 schedule. Baselines (white bar) were calculated as the average number active lever presses during the extinction sessions immediately preceding each test. The asterisk (*) represents a significant difference from extinction baseline, test of simple main effects, $p<0.05$. The plus sign (+) represents a significant difference from vehicle pretreatment session, planned t-tests, $p<0.05$.

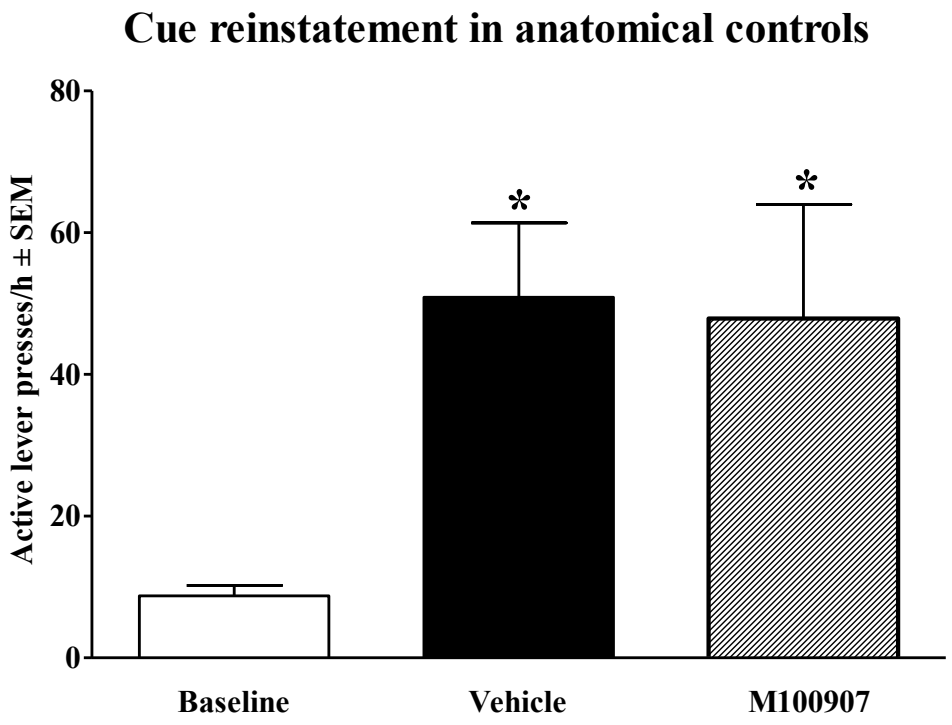


Figure 4 The effects of 1.5 μg/0.2 μl/side M100907 on cue-elicited reinstatement of cocaine-seeking behavior when injected directly into the Cg2 region of the anterior cingulate cortex (n=8), which served as an anatomical control site. Animals received 1.5 μg/0.2 μl/side M100907 and were tested on one day with their assigned dose (striped bar) and on another day with the vehicle (black bar), with order counterbalanced. These pretreatments were infused within 1 min before placing the animals into the self-administration chambers, where light and tone cues were available response-contingently on an FR1 schedule. Baselines (white bar) were calculated as the average number active lever presses during the extinction sessions immediately preceding each test. The asterisk (*) represents a significant difference from extinction baseline, test of simple main effects, $p<0.05$.

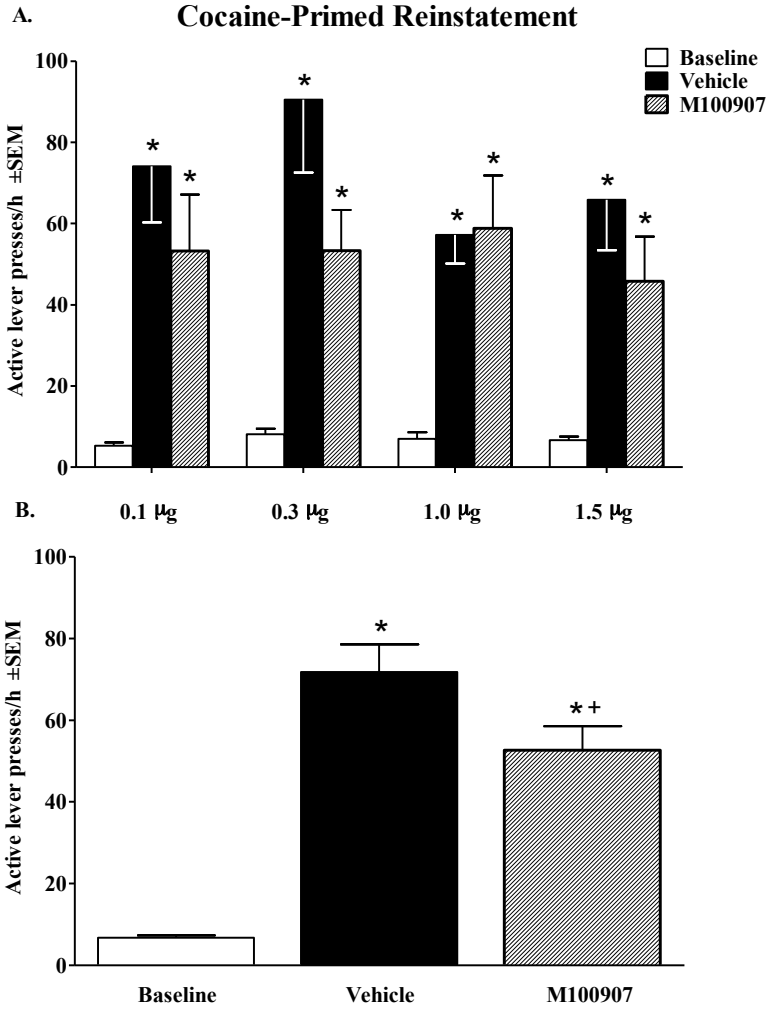


Figure 5 Effects of M100907 pretreatment on cocaine-primed reinstatement of cocaine-seeking behavior, expressed as mean responses/h ± SEM on the active lever in each dosage group (A) and collapsed across dosage groups (B). Animals assigned to receive 0.1 (n=13), 0.3 (n=13), 1.0 (n=13), or 1.5 (n=14) μg/0.2 μl/side M100907 into the mPFC were tested on one day with their assigned dose (striped bar) and on another day with the vehicle (black bar), with order counterbalanced. These pretreatments were infused, and immediately after the animals received the cocaine prime (10 mg/kg, i.p.), and were then immediately placed into the self-administration chambers. No cues were presented during the test sessions. Baselines (white bar) were calculated as the average number of active lever presses during the extinction sessions immediately preceding each test. When collapsed across doses (B) there was a significant increase in responding on both the vehicle and M100907 test days relative to extinction baseline and a significant decrease in responding on the M100907 test day relative to the vehicle test day. The asterisk (*) represents a significant difference from extinction baseline, test of simple main effects, $p<0.05$. The plus sign (+) represents a significant difference from vehicle test day, test of simple main effects, $p<0.05$.

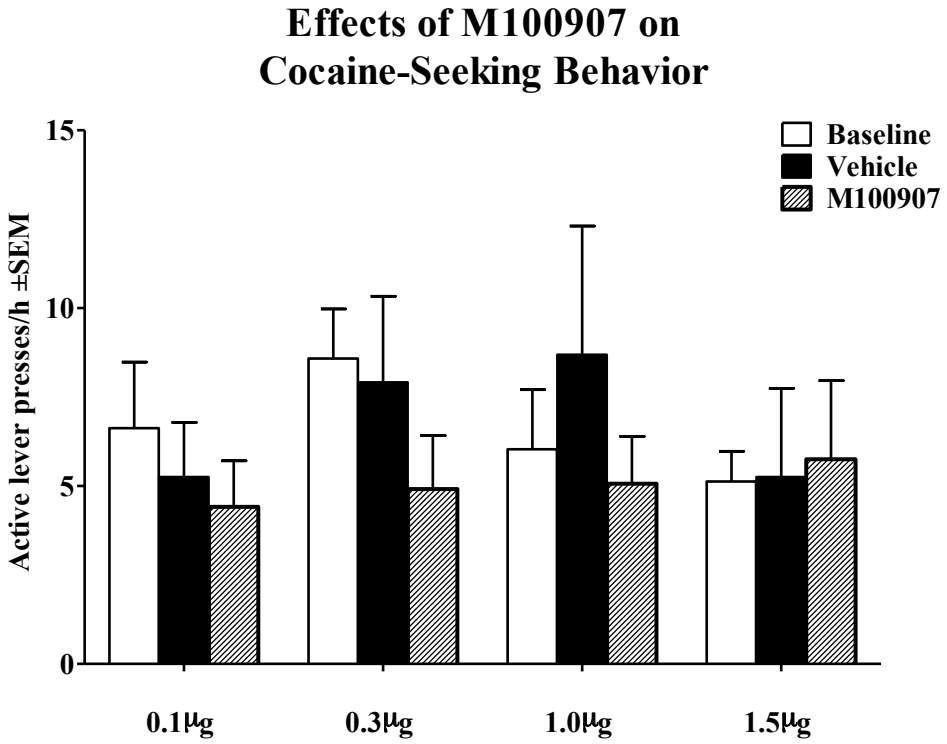


Figure 6 Effects of M100907 priming injections on reinstatement of cocaine-seeking behavior, expressed as mean responses/h ± SEM on the active lever. Animals received 0.1 (n=13), 0.3 (n=13), 1.0 (n=14), or 1.5 (n=13) μg/0.2 μl/side M100907 infused into the mPFC on one test day (striped bar) and vehicle on another day (black bar), with order counterbalanced. They were placed into the self-administration chambers immediately after these pretreatments. Baselines (white bar) were calculated as the average number of active lever presses obtained during the extinction sessions immediately preceding each test. No cues were presented during the test sessions.

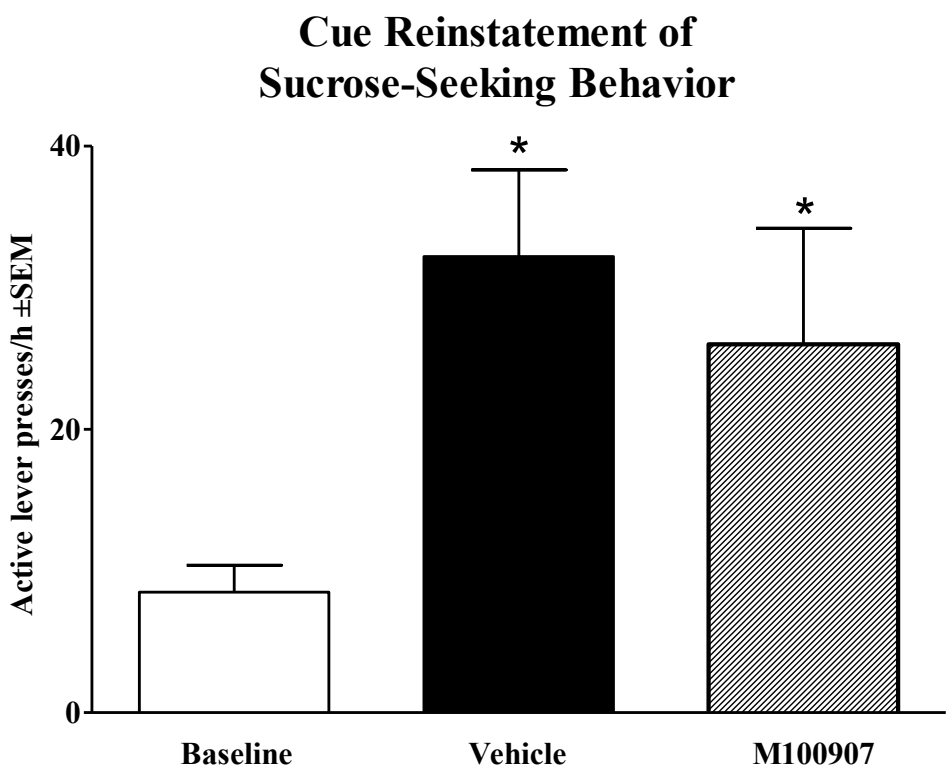


Figure 7 Effects of M100907 pretreatment on cue reinstatement of sucrose-seeking behavior (n=10), expressed as mean responses/h ± SEM on the active lever. Animals were tested on one day with 1.5 μg/side M100907 (striped bar) and on another day with the vehicle (black bar), with order counterbalanced. These pretreatments were infused within 1 min before placing the animals into the self-administration chambers, where light and tone cues were available response-contingently on an FR1 schedule. Baselines (white bar) were calculated as the average number of active lever presses obtained during the extinction sessions immediately preceding each test. There was a significant increase in responding on both the vehicle and M100907 test days relative to extinction baseline. The asterisk (*) represents a significant difference from extinction baseline, test of simple main effects, $p<0.05$.

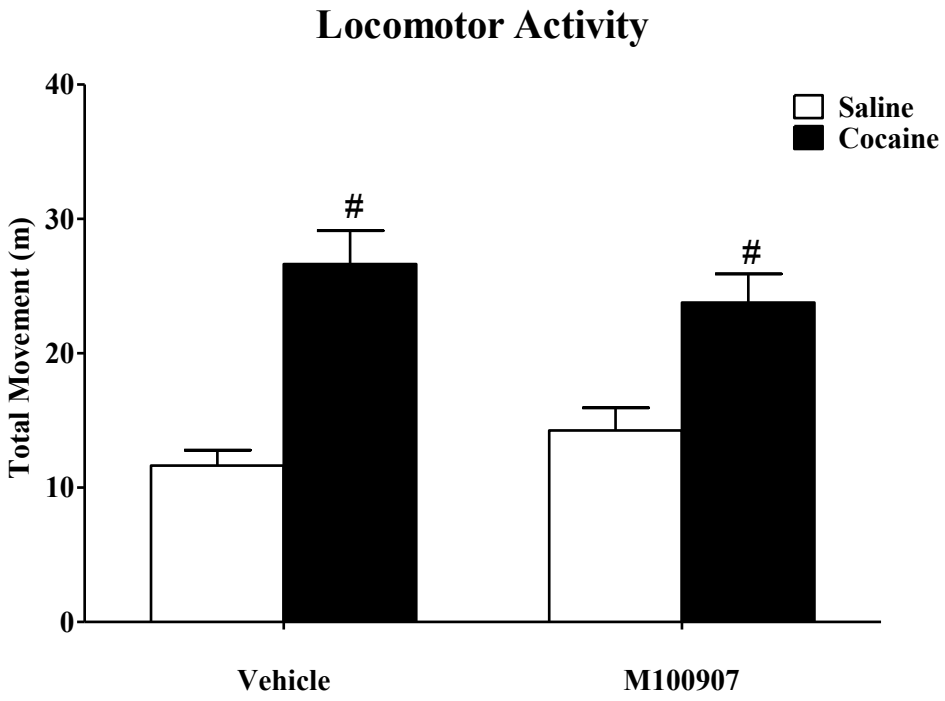


Figure 8 Effects of M100907 pretreatment on locomotor activity, expressed as total movement (m) during a 90-min test session. Animals received either vehicle or 1.0 μg/side M100907 into the mPFC (n=12/group) and were given an injection 5 min later of cocaine (10 mg/kg, i.p.) for one test and saline (10 mg/kg, i.p.) for the other test, with order counterbalanced. They were then immediately placed into the test chambers. A pound sign (#) indicates a difference from saline test day, ANOVA main effect, $P<0.05$.

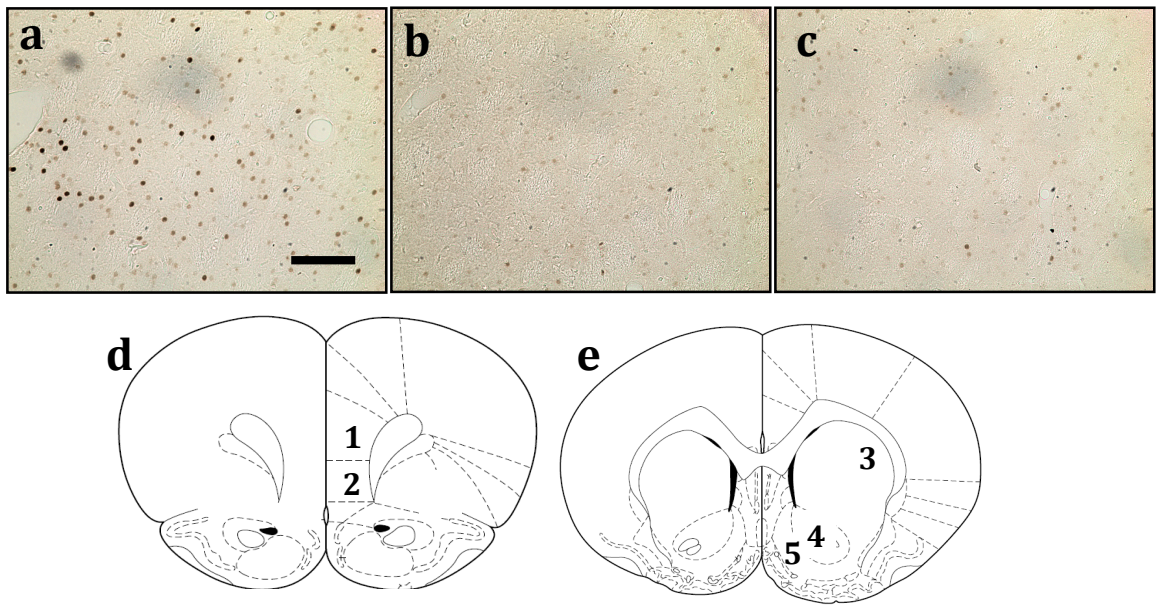
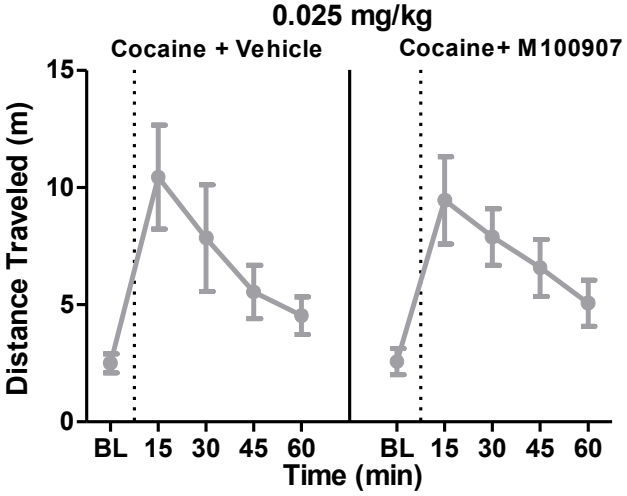
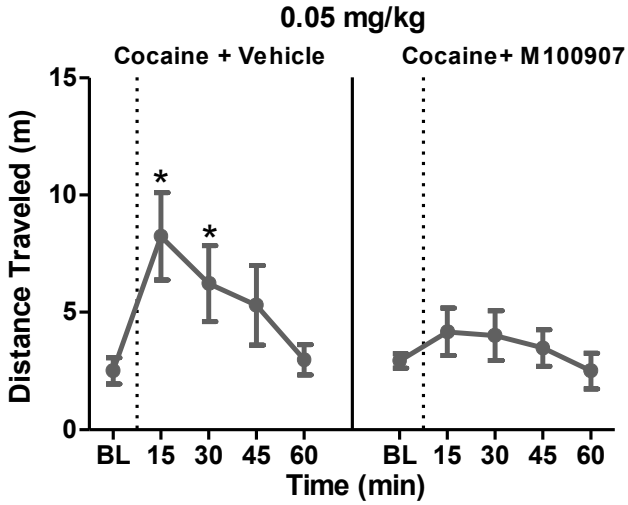


Figure 9 Representative images of Fos positive nuclei in the dorsolateral CPu for animals that received either cocaine (a), saline (b), or cocaine + M100907 and MK 212 cocktail (c) and schematic representation of coronal sections of the rat brain taken at +3.2 (d) and +1.6 mm from Bregma (e; Paxinos and Watson, 1998). Numbers in the sections represent the regions analyzed for Fos as follows: (1) prelimbic cortex (PrL); (2) infralimbic cortex (IL); (3) dorsolateral CPu; (4) NAcC; (5) NAcSh. Scale bar on the first image (a) is equal to 100 µm.


0.025 mg/kg
Cocaine + Vehicle
Cocaine+ M100907
Distance Traveled (m)
15
10
5
0
BL 15 30 45 60
BL 15 30 45 60
Time (min)


0.05 mg/kg
Cocaine + Vehicle
Cocaine+ M100907
Distance Traveled (m)
15
10
5
0
*
*
BL 15 30 45 60
BL 15 30 45 60
Time (min)

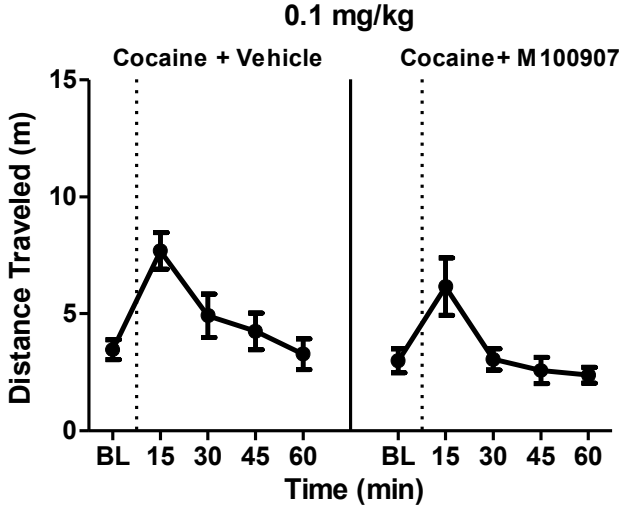

0.1 mg/kg
Cocaine + Vehicle
Cocaine+ M100907
Distance Traveled (m)
15
10
5
0
BL 15 30 45 60
BL 15 30 45 60
Time (min)

Figure 10 Effects of 0.025 (a), 0.05 (b), and 0.1 (c) mg/kg M100907 (n=8/dose group) on cocaine hyperlocomotion, expressed as the mean ± SEM total distance traveled in meters across 15-min time bins relative to the last 15-min of habituation (baseline; BL), or for the entire 1-h session (insert). Dose was a between-subjects factor, while test day (vehicle or M100907) was a within-subjects factor. In between habituation and cocaine hyperlocomotion testing, rats were injected subcutaneously with vehicle on one test day (left) and their assigned dose of M100907 on the other test day (right), with order counterbalanced; 5 min later they were injected with cocaine (15 mg/kg, i.p.), indicated by the dotted vertical lines. Only the 0.025 mg/kg dose failed to have any effect on cocaine hyperlocomotion. The *asterisk* (*) represents a significant difference from baseline, $P<0.05$. The plus sign (+) represents a significant difference from the vehicle test day, $P<0.05$.

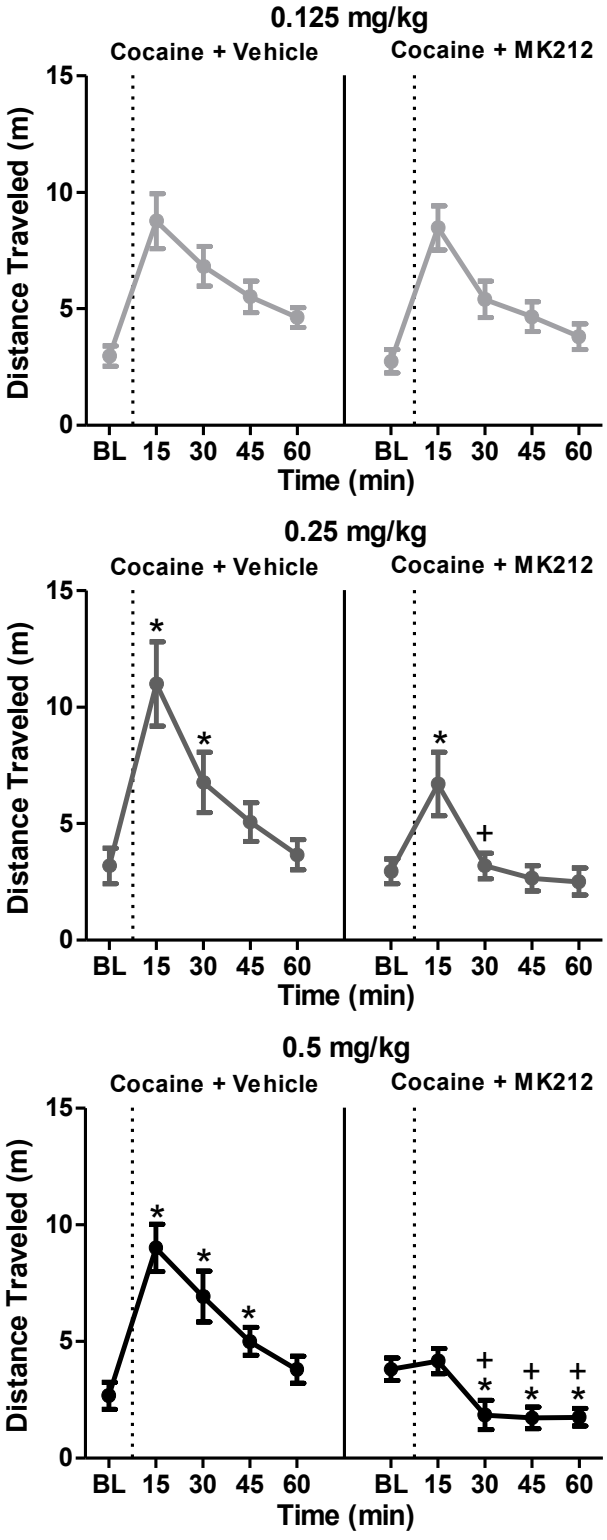

0.125 mg/kg
Cocaine + Vehicle
Cocaine + MK212
Distance Traveled (m)
0
5
10
15
BL 15 30 45 60
BL 15 30 45 60
Time (min)
0.25 mg/kg
Cocaine + Vehicle
Cocaine + MK212
Distance Traveled (m)
0
5
10
15
*
*
*
+
BL 15 30 45 60
BL 15 30 45 60
Time (min)
0.5 mg/kg
Cocaine + Vehicle
Cocaine + MK212
Distance Traveled (m)
0
5
10
15
*
*
*
+
*
+
*
+
*
BL 15 30 45 60
BL 15 30 45 60
Time (min)

Figure 11 Effects of 0.125 (a), 0.25 (b), and 0.5 (c) mg/kg MK212 (n=8/ dose group) on cocaine hyperlocomotion, expressed as the mean ± SEM total distance traveled in meters across 15-min time bins relative to the last 15-min of habituation (baseline; BL). Dose was a between-subjects factor, while test day (vehicle or M100907) was a within-subjects factor. In between habituation and cocaine hyperlocomotion testing, animals were injected subcutaneously with vehicle on one test day (left) and their assigned dose of MK212 on the other test day (right), with order counterbalanced; 5 min later they were injected with cocaine (15 mg/kg, i.p.), indicated by the dotted vertical lines. Only the 0.125 mg/kg dose failed to have any effect on cocaine hyperlocomotion. The *asterisk* (*) represents a significant difference from baseline, $P<0.05$. The plus sign (+) represents a significant difference from respective time point on the vehicle test day, $P<0.05$.

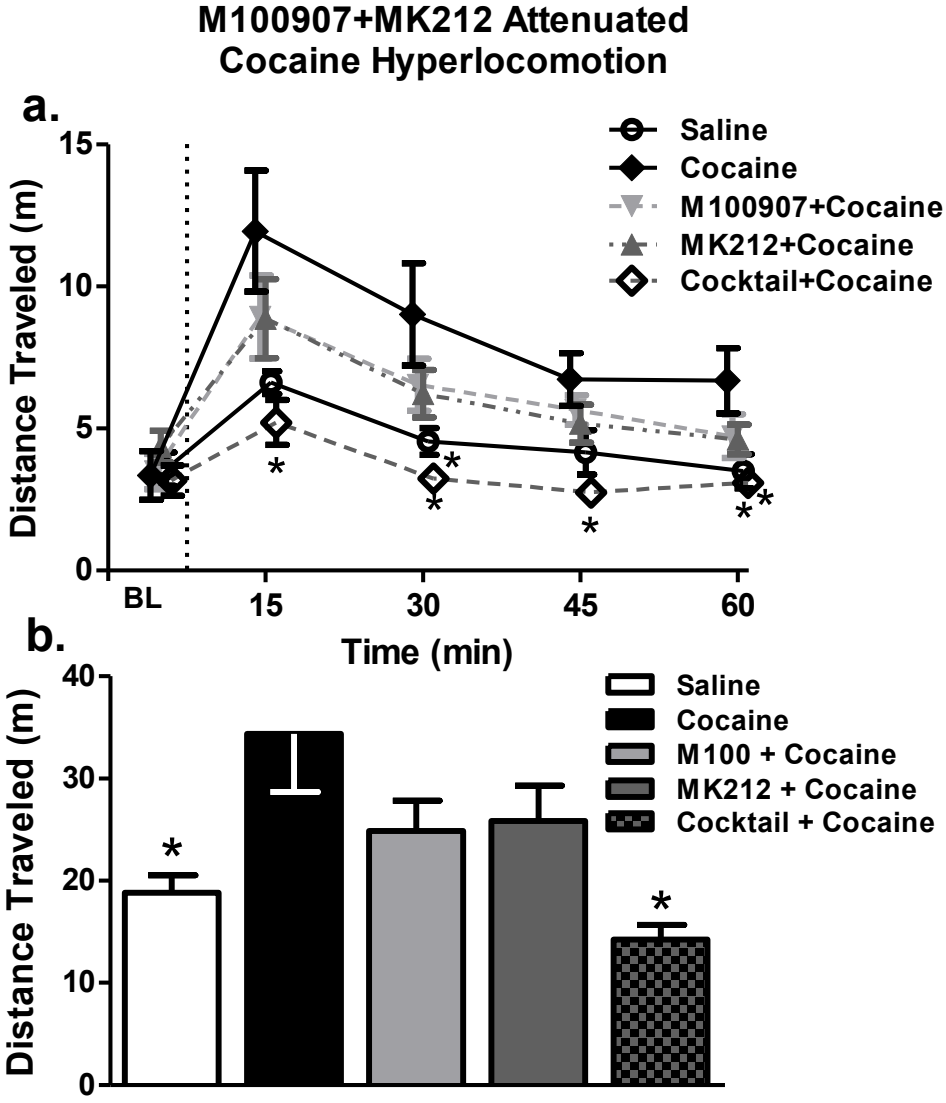


Figure 12 Panel a shows the effects of cocaine, saline, 0.025 mg/kg M100907 + cocaine, 0.125 mg/kg MK212 + cocaine, and M100907 + MK212 cocktail + cocaine (n=8/group) on cocaine hyperlocomotion, expressed as the mean ± SEM total distance traveled in meters across 15-min time bins relative to the last 15-min of habituation (baseline; BL). Panel b shows the cumulative data for the entire 60-min session. In between habituation and cocaine hyperlocomotion testing, animals were injected subcutaneously with vehicle on one test day (left) and their assigned dose of MK212 on the other test day (right), with order counterbalanced; 5 min later they were injected with cocaine (15 mg/kg, i.p.), indicated by the dotted vertical lines. In both graphs, only the saline and the M100907 + MK212 cocktail + cocaine groups showed a significant difference from the cocaine alone group, indicating that M100907 and MK212 only had an effect when given in combination. The *asterisk* (*) represents a significant difference from the cocaine group, $P<0.05$.

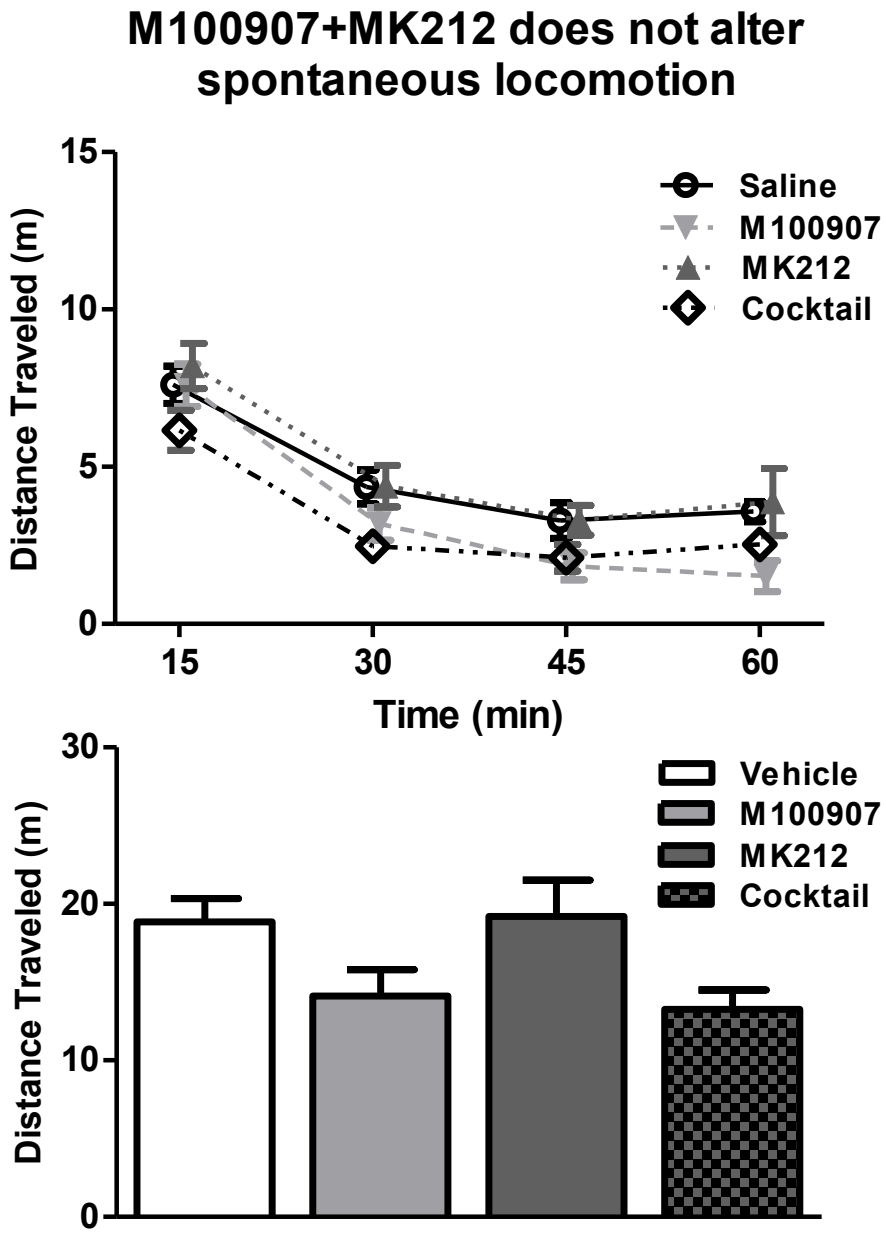


Figure 13 Effects of saline, 0.025 mg/kg M100907, 0.125 mg/kg MK212, and M100907/MK212 cocktail (n=8/group) on spontaneous locomotion, expressed as the mean ± SEM total distance traveled in meters across 15-min time bins (a) and as total locomotion for the 1-h test (b). Animals were injected subcutaneously with their assigned drug, and 5-min later were given a 1-hr test for locomotor activity. There were no differences in locomotion between groups.

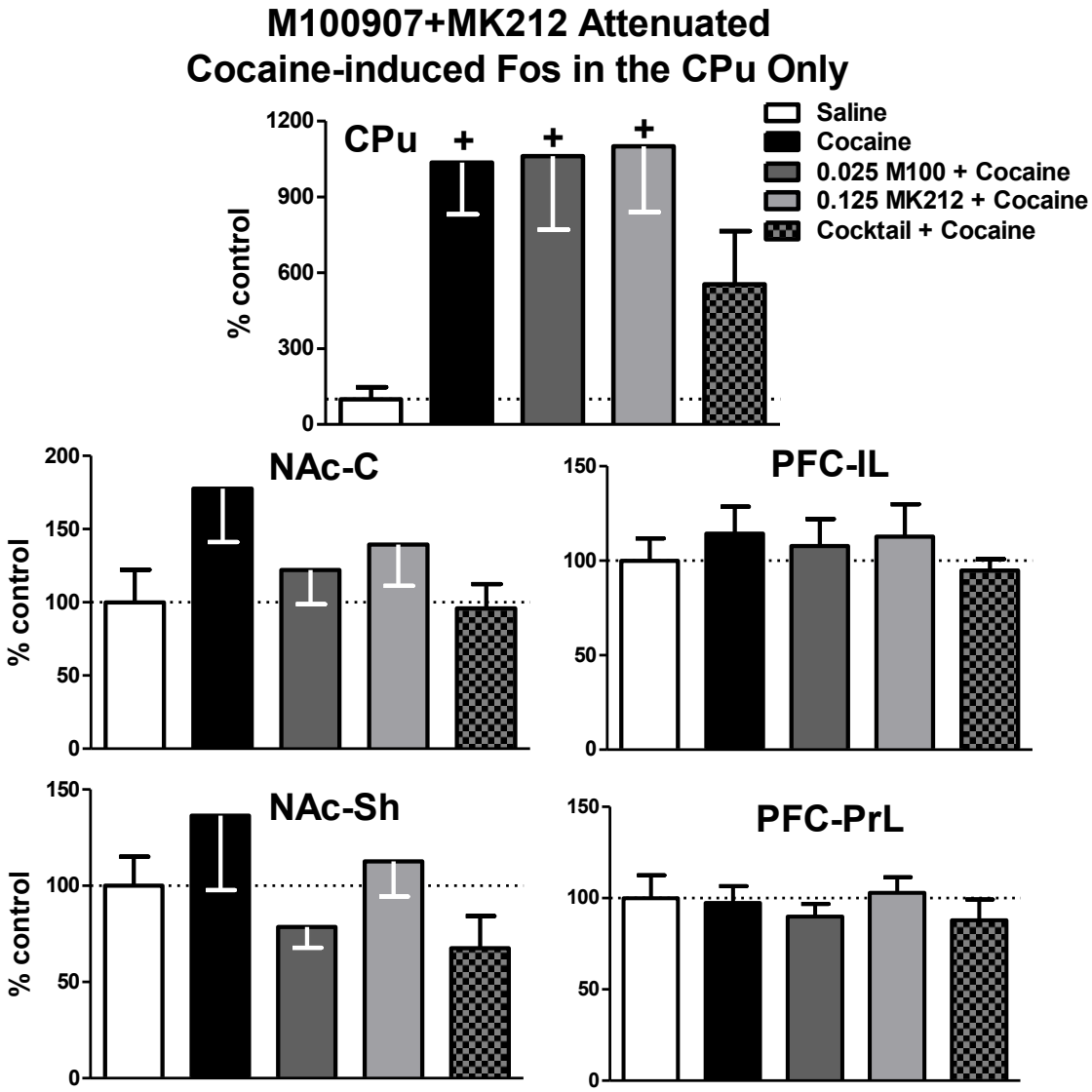


Figure 14 Effects of saline, 15 mg/kg cocaine, 0.025 mg/kg M100907 + cocaine, 0.125 mg/kg MK212 + cocaine, and M100907 + MK212 cocktail + cocaine (n=8/group) on Fos activation in the dorsolateral CPu (a), NAc core (b) and shell (c), and infralimbic (d) and prelimbic (e) PFC, expressed as the mean ± SEM of percent of control (saline alone group). The dotted line indicates reference point for change from saline control (i.e., 100%,). All animals underwent locomotor activity testing and were sacrificed 90 min after drug injections. Animals were perfused and brains were harvested for Fos immunohistochemistry. There was a significant difference between saline and cocaine groups, indicating that cocaine increased Fos expression in the CPu. There was also a difference between the M100 + cocaine and MK212 + cocaine groups, which shows that these drugs alone had no effect on cocaine-induced Fos activation. There was no difference between the saline and cocktail + cocaine groups, indicating that the cocktail decreased Fos activation levels back to baseline. There were no differences between any of the groups in any regions of the NAc or PFC. The *asterisk* (*) represents a significant difference from the saline group, $P<0.05$.

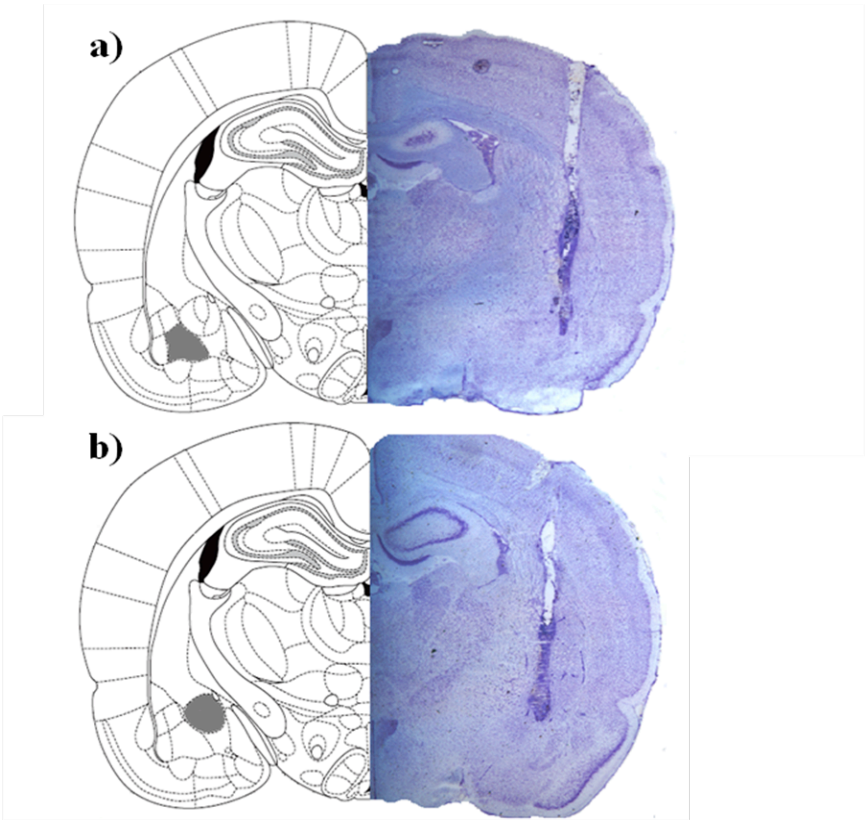


Figure 15 Histological reconstructions (left) with shaded regions showing where cannulae tips were considered correctly placed for the BlA (A) and CeA (B). The right sides show pictographs of cresyl violet stained tissue samples with methylene blue microinfusions for the BlA (A) and CeA (B).

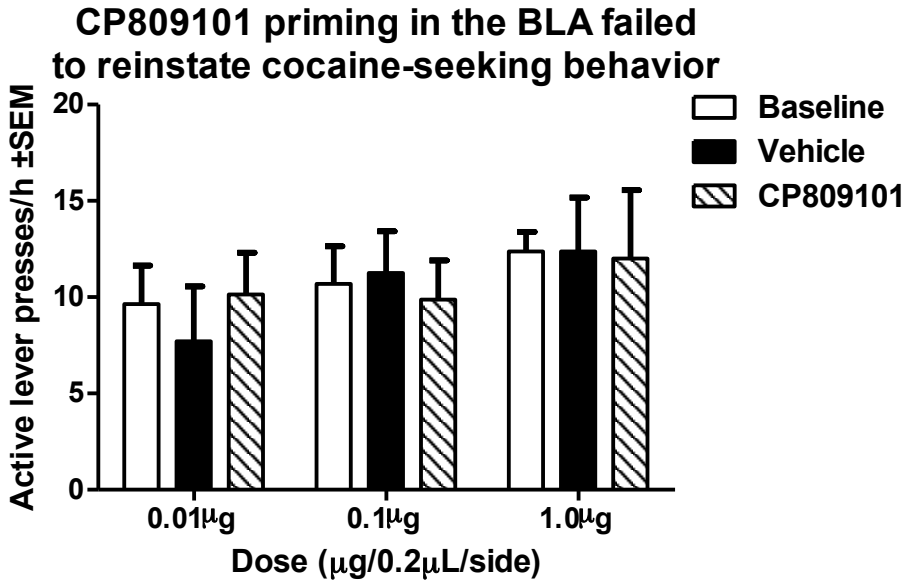


Figure 16 Effects of CP809101 priming injections in the BlA on reinstatement responding, expressed as the mean ± SEM number of active lever presses received over a 1-h test session in each dosage group. Animals assigned to receive 0.01 (n=7), 0.1 (n=8) or 1.0 (n=8) μg/0.2μL/side CP809101 into the BlA were tested on one day with their assigned dose (striped bar) and on another day with the vehicle (BlAck bar), with order counterbalanced. Baselines (white bars) were calculated as the average number of active lever presses during the extinction sessions immediately preceding each test. There was no effect of vehicle or CP809101 pretreatment on reinstatement responding.

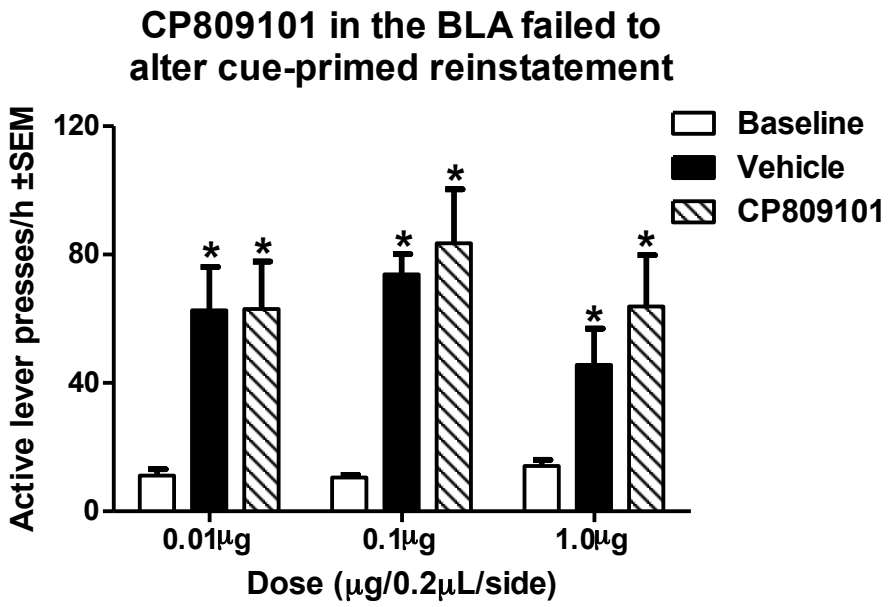


Figure 17 Effects of CP809101 in the BlA on cue-primed reinstatement, expressed as the mean ± SEM number of active lever presses received over a 1-h test session in each dosage group. Animals assigned to receive 0.01 (n=6), 0.1 (n=8) or 1.0 (n=8) µg/0.2µL/side CP809101 into the BlA were tested on one day with their assigned dose (striped bar) and on another day with the vehicle (BlAck bar), with order counterbalanced. These pretreatments were infused within 1 min before placing the animals into the self-administration chambers, where light and tone cues were available response-contingently on an FR 1 schedule. Baselines (white bars) were calculated as the average number of active lever presses during the extinction sessions immediately preceding each test. The asterisk (*) represents a significant difference from extinction baseline, test of simple main effects, $P<0.05$. All groups showed significant reinstatement, but CP809101 had no effect on cue-primed reinstatement relative to vehicle.

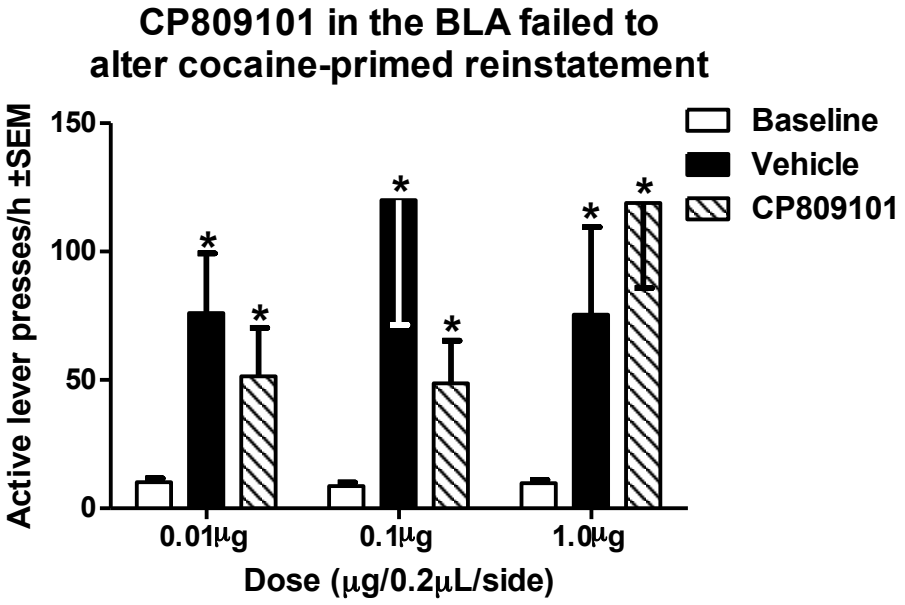


Figure 18 Effects of CP809101 in the BlA on cocaine-primed reinstatement, expressed as the mean ± SEM number of active lever presses received over a 1-h test session in each dosage group. Animals assigned to receive 0.01 (n=5), 0.1 (n=7) or 1.0 (n=8) μg/0.2μL/side CP809101 into the BlA were tested on one day with their assigned dose (striped bar) and on another day with the vehicle (BlAck bar), with order counterbalanced. These pretreatments were infused immediately before the animals received the cocaine prime (10 mg/kg, i.p.) and were then immediately placed into the self-administration chambers. No cues were presented during the test sessions. Baselines (white bars) were calculated as the average number of active lever presses during the extinction sessions immediately preceding each test. The asterisk (*) represents a significant difference from extinction baseline, test of simple main effects, $P<0.05$. All groups showed significant reinstatement, but CP809101 had no effect on cue-primed reinstatement relative to vehicle.

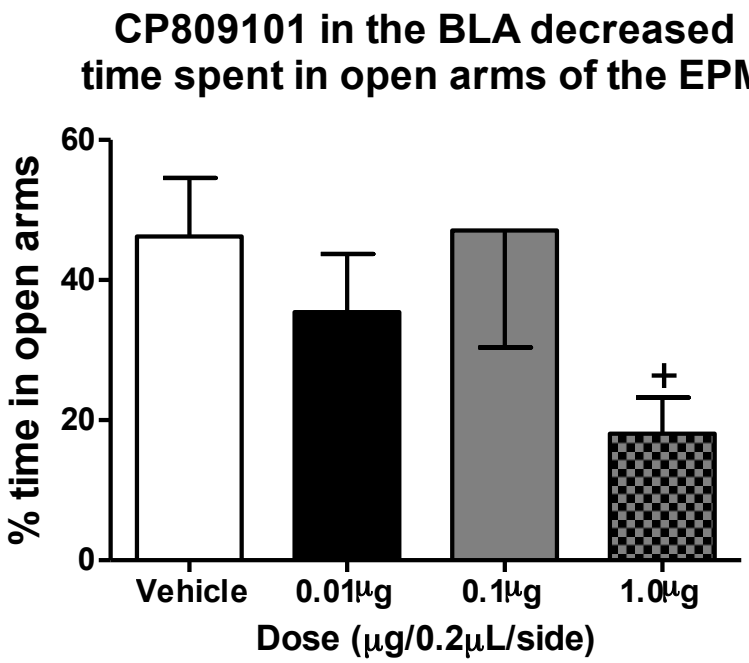


Figure 19 Effects of CP809101 in the BlA on the percent of time spent on the open arms of the EPM. Animals assigned to receive vehicle (n=3), 0.01 (n=7), 0.1 (n=4) or 1.0 (n=8) μg/0.2μL/side CP809101 into the BlA were given one 10-min test on the EPM. These pretreatments were infused 5-min before animals were placed on the center of the EPM. The plus sign (+) indicates a significant difference from the vehicle group, planned comparison, ($P<0.05$).

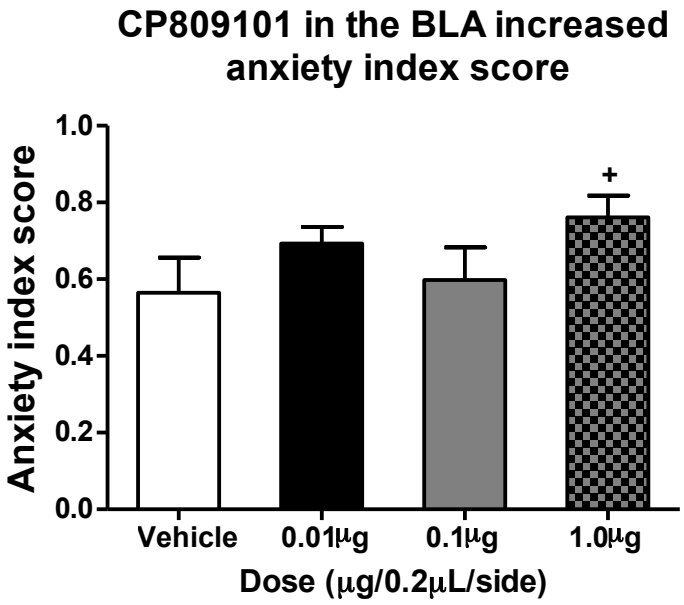


Figure 20 Effects of CP809101 in the BlA on an anxiety-index score, which accounts for percent of time spent in open arms as well as open arm entries on the EPM. Animals assigned to receive vehicle (n=3), 0.01 (n=7), 0.1 (n=4) or 1.0 (n=8) μg/0.2μL/side CP809101 into the BlA were given one 10-min test on the EPM. These pretreatments were infused 5-min before animals were placed on the center of the EPM. The plus sign (+) indicates a marginally significant difference from the vehicle group, planned comparison, (P=0.052).

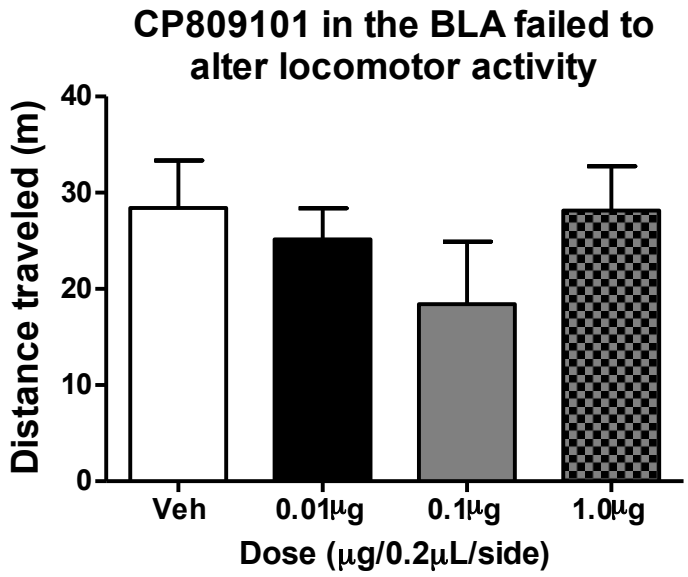


Figure 21 Effects of CP809101 in the BlA on spontaneous locomotor activity. Animals assigned to receive vehicle (n=3), 0.01 (n=7), 0.1 (n=4) or 1.0 (n=8) μg/0.2μL/side CP809101 into the BlA were given one 10-min test on the EPM. These pretreatments were infused 5-min before animals were placed on the center of the EPM. There were no effects of CP809101 on locomotor activity.

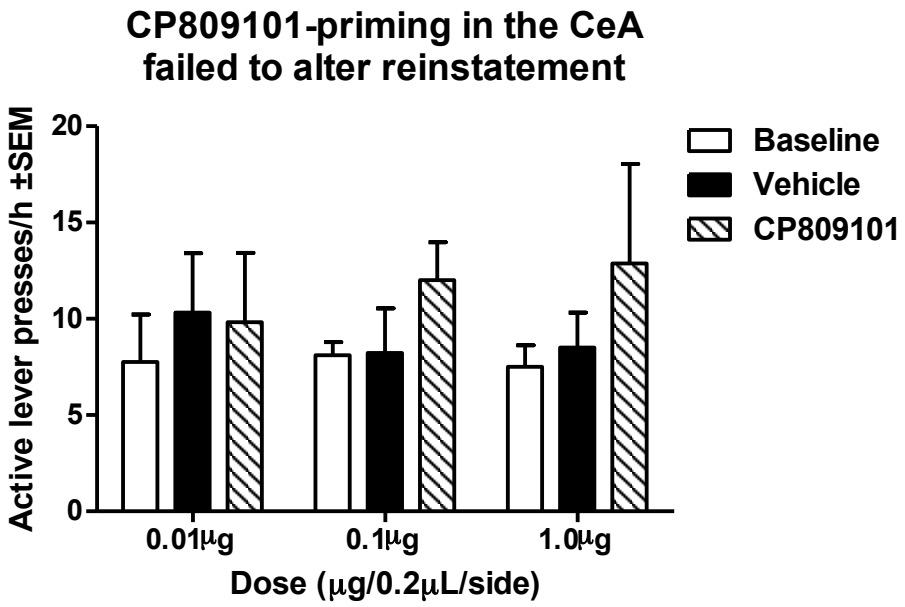


Figure 22 Effects of CP809101 priming injections in the CeA on reinstatement responding, expressed as the mean ± SEM number of active lever presses received over a 1-h test session in each dosage group. Animals assigned to receive 0.01 (n=6), 0.1 (n=9) or 1.0 (n=8) μg/0.2μL/side CP809101 into the CeA were tested on one day with their assigned dose (striped bar) and on another day with the vehicle (BlAck bar), with order counterbalanced. Baselines (white bars) were calculated as the average number of active lever presses during the extinction sessions immediately preceding each test. There was no effect of vehicle or CP809101 pretreatment on reinstatement responding.

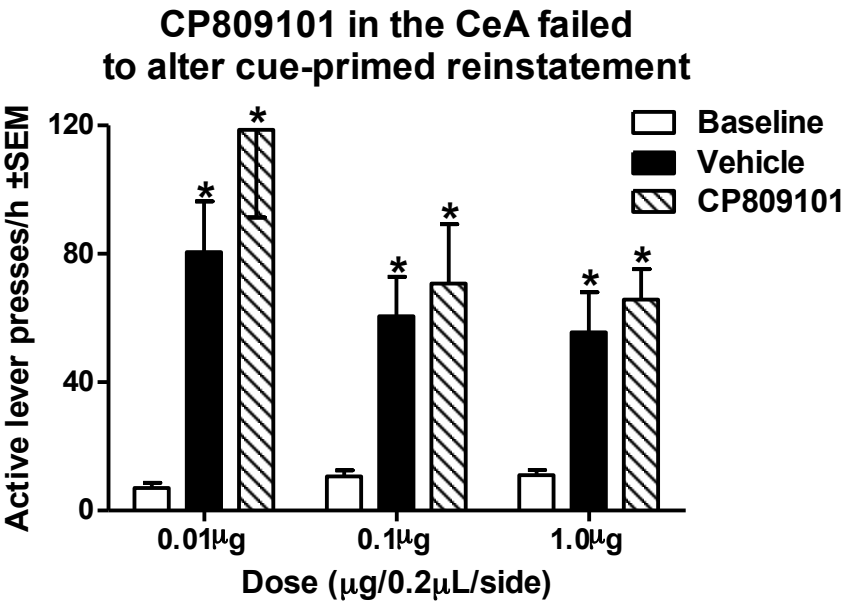


Figure 23 Effects of CP809101 in the CeA on cue-primed reinstatement, expressed as the mean ± SEM number of active lever presses received over a 1-h test session in each dosage group. Animals assigned to receive 0.01 (n=6), 0.1 (n=9) or 1.0 (n=8) µg/0.2µL/side CP809101 into the CeA were tested on one day with their assigned dose (striped bar) and on another day with the vehicle (BlAck bar), with order counterbalanced. These pretreatments were infused within 1 min before placing the animals into the self-administration chambers, where light and tone cues were available response-contingently on an FR 1 schedule. Baselines (white bars) were calculated as the average number of active lever presses during the extinction sessions immediately preceding each test. The asterisk (*) represents a significant difference from extinction baseline, test of simple main effects, $P<0.05$. All groups showed significant reinstatement, but CP809101 had no effect on cue-primed reinstatement relative to vehicle.

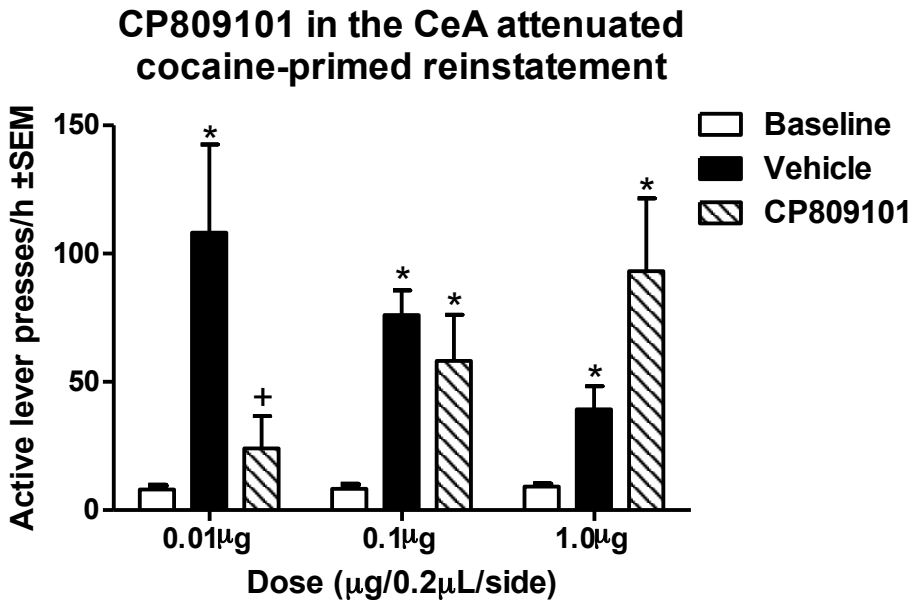


Figure 24 Effects of CP809101 in the CeA on cocaine-primed reinstatement, expressed as the mean ± SEM number of active lever presses received over a 1-h test session in each dosage group. Animals assigned to receive 0.01 (n=6), 0.1 (n=7) or 1.0 (n=7) µg/0.2µL/side CP809101 into the BlA were tested on one day with their assigned dose (striped bar) and on another day with the vehicle (BlAck bar), with order counterbalanced. These pretreatments were infused immediately before the animals received the cocaine prime (10 mg/kg, i.p.) and were then immediately placed into the self-administration chambers. No cues were presented during the test sessions. Baselines (white bars) were calculated as the average number of active lever presses during the extinction sessions immediately preceding each test. The asterisk (*) represents a significant difference from extinction baseline, test of simple main effects, $P<0.05$. The plus sign (+) represents a significant difference from vehicle test day, $P<0.05$. All groups showed significant reinstatement except for the 0.01 µg dose group, which was also significant different from the corresponding vehicle test day.

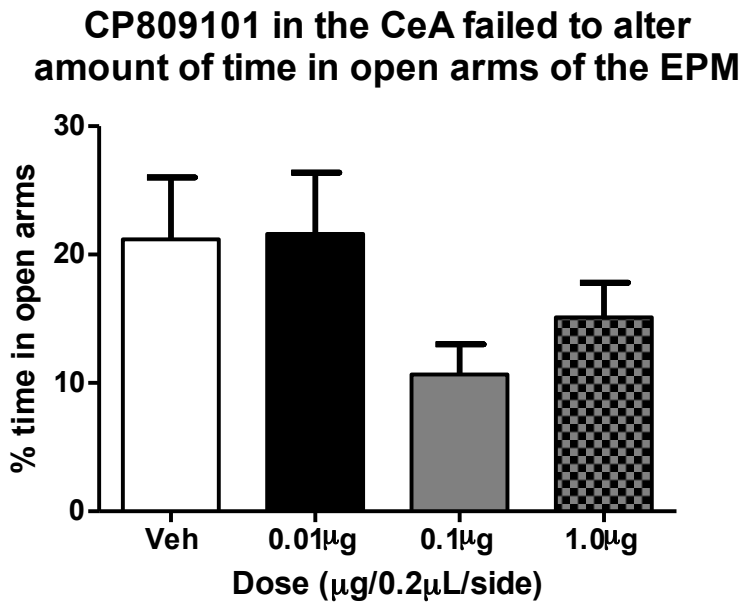


Figure 25 Effects of CP809101 in the CeA on amount of time spent in the open arms of the EPM. Animals assigned to receive vehicle (n=10), 0.01 (n=8), 0.1 (n=7) or 1.0 (n=6) µg/0.2µL/side CP809101 into the CeA were given one 10-min test on the EPM. These pretreatments were infused 5-min before animals were placed on the center of the EPM. There was no effect of CP809101 in the CeA on amount of time spent in the open arms of the EPM.

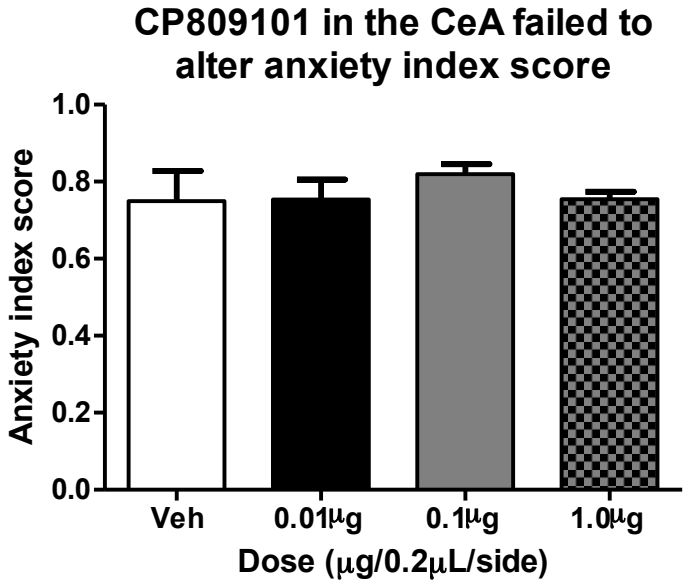


Figure 26 Effects of CP809101 in the CeA on an anxiety-index score, which accounts for percent of time spent in open arms as well as open arm entries on the EPM. Animals assigned to receive vehicle (n=10), 0.01 (n=8), 0.1 (n=7) or 1.0 (n=6) μg/0.2μL/side CP809101 into the CeA were given one 10-min test on the EPM. These pretreatments were infused 5-min before animals were placed on the center of the EPM. There was no effect of CP809101 in the CeA on anxiety-index score.

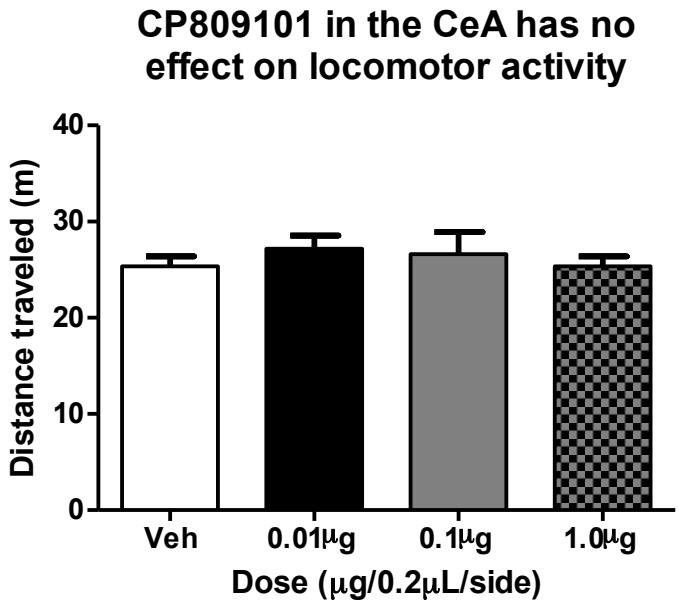


Figure 27 Effects of CP809101 in the CeA on spontaneous locomotor activity. Animals assigned to receive vehicle (n=10), 0.01 (n=8), 0.1 (n=7) or 1.0 (n=6) μg/0.2μL/side CP809101 into the CeA were given one 10-min test on the EPM. These pretreatments were infused 5-min before animals were placed on the center of the EPM. There were no effects of CP809101 on locomotor activity.

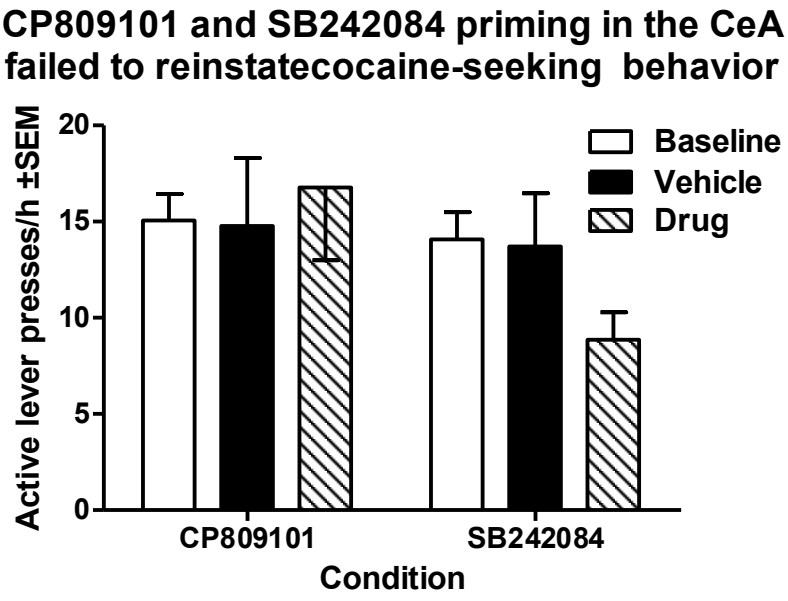


Figure 28 Effects of CP809101 priming injections in the CeA on reinstatement responding, expressed as the mean ± SEM number of active lever presses emitted over a 1-h test session in each dosage group. Animals assigned to receive either 0.01 μg/0.2μL/side CP809101 (n=9) or 0.1 μg/0.2μL/side SB242084 (n=7) into the CeA were tested on one day with their assigned dose (striped bar) and on another day with the vehicle (BlAck bar), with order counterbalanced. Baselines (white bars) were calculated as the average number of active lever presses during the extinction sessions immediately preceding each test. There was no effect of vehicle, CP809101, or SB242084 pretreatment on reinstatement responding.

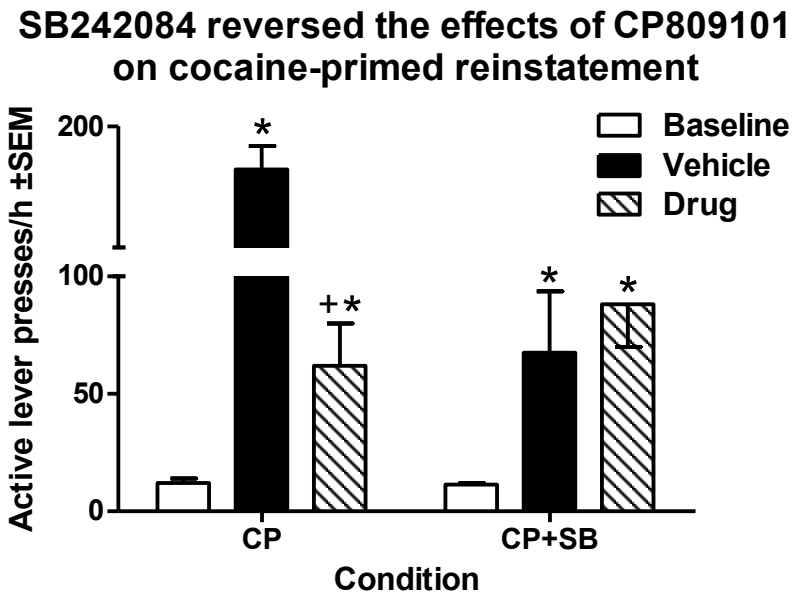


Figure 29 Effects of CP809101 and SB242082 in the CeA on cocaine-primed reinstatement, expressed as the mean ± SEM number of active lever presses received over a 1-h test session in each dosage group. Animals assigned to receive either 0.01 μg/0.2μL/side CP809101 (n=7) or 0.01 μg/0.2μL/side CP809101 + 0.1 μg/0.2μL/side SB242084 (n=5) into the CeA were tested on one day with their assigned dose (striped bar) and on another day with the vehicle (BlAck bar), with order counterbalanced. These pretreatments were infused immediately before the animals received the cocaine prime (10 mg/kg, i.p.) and were then immediately placed into the self-administration chambers. No cues were presented during the test sessions. Baselines (white bars) were calculated as the average number of active lever presses during the extinction sessions immediately preceding each test. The asterisk (*) represents a significant difference from extinction baseline, test of simple main effects, $P<0.05$. The plus sign (+) represents a significant difference from vehicle test day, $P<0.05$. All groups showed significant reinstatement, however the 0.01 μg CP809101 group showed significantly lower responding on CP809101 test day relative to vehicle.

APPENDIX A

CURRICULUM VITAE

CURRICULUM VITAE
Lara Ann Pockros

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ISTB 1 Room 429
Tempe 85287
Tel (480) 965-7253
Email: Lara.Pockros@asu.edu

Education

2013 Ph.D., Behavioral Neuroscience (in progress), Arizona State University, Tempe, AZ

2010 M.A., Psychology, Behavioral Neuroscience program, Arizona State University, Tempe, AZ

2008 B.A., Psychology, University of California San Diego, La Jolla, CA

Professional Research Experience

Present: *Graduate Research Assistant*, Behavioral Neuroscience Program
Arizona State University, Tempe, AZ
Drug Addiction Laboratory
Supervisor: Dr. Janet Neisewander

Duties Project head on several experiments examining the role of serotonin receptors in the neural basis of drug addiction using rodent animal models. Primary duties include coordination and management of projects, training undergraduate research assistants, literary searches, surgery, statistical analyses, and publication of findings.

Qualifications Skillful in conducting a number of behavioral techniques including intravenous drug self-administration, intracranial microinfusions, intraperitoneal injections, subcutaneous injections, locomotor activity testing, sucrose self-administration, perfusions, and brain extractions. Proficient in surgical techniques such as jugular vein catheterization, head piece mounts, and stereotaxic surgery (intracranial cannulation). Also experienced with cell staining (cresyl violet and thionine) and Fos immunohistochemistry. Proficient in statistical analyses using SPSS and Prism graphing software.

1/07-7/07: *Undergraduate Research Assistant*, Committee on the Neurobiology of Addictive Disorders
The Scripps Research Institute, La Jolla, CA
Supervisors: Dr. George Koob, Dr. Eric Zorrilla, Dr. Pietro Cottone

Duties Independently ran food self-administration on rats to investigate the role of Corticotropin-releasing Factor on eating patterns in rats. Primary duties included daily use of operant boxes and computer programs to monitor animals, data input, weighing food intake, assistance with injections, and attendance of laboratory meetings.

1/08-6/08: *Laboratory Assistant*, Molecular and Integrative Neurosciences Department
The Scripps Research Institute, La Jolla, CA
Supervisor: Dr. Amanda Roberts

Duties Independently ran behavioral test batteries (two-bottle choice, Barnes Maze, Y-Maze, Novel Object, etc) on different genetic strains of mice, recorded and analyzed data, attended laboratory meetings.

4/07-4/08: *Peer Counselor/Educator,* SAFE Program
University of California San Diego Psychological Services, La Jolla, CA
Supervisor: Dr. Jerry Phelps

Duties Conducted one-on-one counseling sessions with students about alcohol and substance abuse problems, organized campus events such as National Alcohol Screening Day and Alcohol Awareness Week, met weekly with supervisors to discuss cases. Trained in counseling skills, group facilitation, crisis management, ethics, substance abuse issues, and Motivational Interviewing technique.

9/07-12/07: *Student Intern*, Project Options Laboratory
University of California San Diego Psychology Department, La Jolla, CA
Supervisor: Dr. Sandra Brown

Duties Conducted weekly visits to different high schools for interactive information sessions about substance abuse problems. Distributed surveys and assessed data for a longitudinal study investigating the effectiveness of different methods used to dissuade underage alcohol use.

3/07-6/07: *Independent Study*
University of California San Diego Psychology Department, La Jolla, CA
Supervisor: Dr. Amanda Roberts

Duties Designed a research project investigating sex differences in body image and motivation of physical appearance. Constructed and administered surveys, assessed data, wrote manuscript, and presented findings of project.

2005-2006: *Student Intern*, Hepatology Department
Scripps Clinic Torrey Pines, La Jolla, CA
Supervisor: Dr. Christian Vallejos

Duties Reviewed patient charts and input data for investigative study on the link between narcotic analgesic use and chronic Hepatitis C.

Awards & Honors

2008 Arizona State University, *Research Enhancement Award*

2008 University of California San Diego, *Provost's Honors*

2007 University of California San Diego, *Provost's Honors*

Publications

1. **Pockros, L.**, Pentkowski, N., Der-Ghazarian, T., Neisewander, J. (in prep). Effects of 5-HT_{2C} receptor agonist in the amygdala on reinstatement of cocaine-seeking behavior and anxiety on the elevated plus maze.

2. Der-Ghazarian, T., **Pockros, L.**, Pentkowski, N., Mirando, R., Brunwasser, S., Neisewander, J. (in prep). Effects of 5-HT_{2A} receptor antagonist and 5-HT_{2C} receptor agonist on intrastriatal cocaine-induced hyperlocomotion.

3. Mach, R., Xu, J., **Pockros, L**., Sun, J., Cohen, S., Conway, S., Ullman, T., Neisewander, J. (in prep). Dopamine D1, D2, D3 receptor and vesicular monoamine transporter type-2 (VMAT2) density changes and neuroinflammation in rats receiving cocaine challenge.

4. **Pockros, L.A.,** Pentkowski, N., Conway, S., Neisewander, J. (2012). 5-HT_{2A} receptor blockade and 5-HT_{2C} receptor activation interact to reduce cocaine hyperlocomotion and Fos protein expression in the caudate-putamen. *Synapse*. 66(12):989-1001.

5. **Pockros, L.A.**, Pentkowski, N., Swinford, S., Neisewander, J. (2011). Blockade of 5-HT_{2A} Receptors in the Medial Prefrontal Cortex Attenuates Reinstatement of Cue-elicited Cocaine-seeking Behavior in Rats. *Psychopharmacology*. 213(2-3):307-20.

6. Pentkowski, N., Duke, F., Weber, S., **Pockros, L.A.**, Teer, A., Hamilton, E., Thiel, K., Neisewander, J. (2010). Stimulation of Medial Prefrontal Cortex 5-HT_{2C} Receptors Attenuates Cocaine-Seeking Behavior mPFC 5-HT_{2C}Rs Modulate Cocaine-seeking Behavior. *Neuropsychopharmacoloyg. 35*(10):2037-48.

7. Cottone, P., Sabino, V., Roberto, M., Bajo, M., **Pockros, L.**, Frihauf, J., Fekete, E., Steardo, L., Rice, K., Grigoriadis, D., Conti, B., Koob, G., Zorrilla, E. (2009). CRF System Recruitment Mediates Dark Side of Compulsive Eating. *Proc Natl Acad Sci U S A. 106*(47):20016-20.

8. Vallejos, C., Bordin-Wosk, T., **Pockros, L.**, Feng, A., Pockros, P. (2009). Narcotic Analgesics and Progression of Fibrosis in Patients with Chronic Hepatitis C. *J Clin Gastroenterol 43*(4):357-61.

Presentations

1. **Pockros, L.**, Pentkowski, N., Berger, A., Ostos, M., Conway, S., Neisewander J. (2011) Interaction between 5-HT2A receptor blockade and 5-HT2C receptor activation on spontaneous and cocaine-induced locomotion. *The College on Problems of Drug Dependence*, Hollywood, FL.

2. **Pockros, L.**, Pentkowski, N., Swinford, S., Neisewander J. (2010) Blockade of 5-HT2A Receptors in the Medial Prefrontal Cortex Attenuates Cue-elicited Reinstatement of Cocaine-seeking Behavior in Rats. *The College on Problems of Drug Dependence*, Scottsdale, AZ.

Abstracts

1. Mach, R., Xu, J., **Pockros, L**., Sun, J., Cohen, S., Conway, S., Ullman, T., Neisewander, J. (Oct 2012). Dopamine D1, D2, D3 receptor and vesicular monoamine transporter type-2 (VMAT2) density changes and neuroinflammation in rats receiving cocaine challenge. *Society for Neuroscience*, New Orleans, LA.

2. **Pockros, L**. Der Ghazarian, T., Pentkowski, S., Conway, S., Zwick, K., Harder, B., Neisewander, J. (Oct 2012). Effects of 5-HT2C receptor stimulation in the BLA on reinstatement of cocaine-seeking behavior and anxiety-like behavior in the elevated plus maze. *Society for Neuroscience*, New Orleans, LA.

3. **Pockros, L.**, Pentkowski, N., Weber, S., Neisewander, J. (Nov, 2011). Stimulation of serotonin 2C receptors in the mPFC attenuates cocaine-induced hyperlocomotion and alters Fos expression in brain regions containing dopamine neurons. *Society for Neuroscience*, Washington, D.C.

4. **Pockros, L.**, Berger, A., Pentkowski, N., Neisewander, J. (Nov, 2010). Synergistic Effects of 5-HT2A Receptor Blockade and 5-HT2C Receptor Activation on Inhibition of Spontaneous Locomotion. *Society for Neuroscience*, San Diego, CA.

5. **Pockros, L.**, Pentkowski, N., Swinford, S., Shepard, A., Neisewander, J. (Nov, 2009). Blockade of 5-HT2A Receptors in the Prefrontal Cortex Attenuates Cue- and Cocaine-primed Reinstatement of Cocaine-seeking Behavior in Rats. *Society for Neuroscience,* Chicago, IL.

6. Cottone, P., Sabino, V., Roberto, M., Bajo, M., **Pockros, L.**, Frihauf, J., Fekete, E., Steardo, L., Rice, K., Grigoriadis, D., Conti, B., Koob, G., Zorrilla, E. (2009). CRF System Recruitment Mediates Dark Side of Compulsive Eating. *Society for Neuroscience,* Chicago, IL.

Colloquia

Jan 2013 Speaker, Behavioral Neuroscience Seminar Series, Psychology Dept., Arizona State University. "The Role of Serotonin 2 Receptors in Cocaine Related Behaviors."

Oct 2011 Speaker, Behavioral Neuroscience Seminar Series, Psychology Dept., Arizona State University. "The Role of 5-HT2A and 5-HT2C Receptors in Cocaine Addiction."

Feb 2011 Speaker, Behavioral Neuroscience Seminar Series, Psychology Dept., Arizona State University. "Synergistic Effects of 5-HT2A Receptor Blockade and 5-HT2C Receptor Activation on Inhibition of Spontaneous Locomotion."

April 2010 Speaker, Behavioral Neuroscience Seminar Series, Psychology Dept., Arizona State University. "Blockade of 5-HT2A Receptors in the Medial Prefrontal Cortex Attenuates Cue-primed Reinstatement of Cocaine-seeking Behavior."

May 2009 Speaker, Behavioral Neuroscience Seminar Series, Psychology Dept., Arizona State University. "Blockade of 5-HT2A Receptors in the Prefrontal Cortex Attenuates Cue- and Cocaine-primed Reinstatement of Cocaine-seeking Behavior in Rats."

Professional Service & Affiliations

Brain Research- journal reviewer

Society for Neuroscience- student member

Community Work-Related Services

2013 *Science Fair Judge*: Judge for Loma Linda High School Science fair

2010-2011 *Brain Awareness Week:* Assisted in Arizona State University's Brain Awareness Fair. Showed various brain samples, assisted in brain related art projects, and answered questions about neuroscience.

Teaching Experience

2013 *Teaching assistant (PSY290), Arizona State University:* Independently taught laboratory class for Dr. Eva Szeli's research methods course for undergraduates majoring in Psychology. Developed curriculum and syllabus, prepared and gave lectures on topics of research methods (experimental design, APA format, etc), graded research papers, and managed Blackboard site.

2012 *Teaching assistant (PSY290), Arizona State University:* Independently taught laboratory section and assisted Dr. Donald Homa with a research methods course for undergraduates majoring in Psychology. Gave lectures on topics of research methods, graded homework and exams and managed Blackboard site.

2012 *Teaching assistant (PSY320), Arizona State University:* Assisted Dr. Christa Lynch with an online Learning and Motivation course for undergraduates majoring in Psychology. Graded homework and exams and managed Blackboard site.

2011-2012 *Teaching assistant (PSY230), Arizona State University:* Assisted Dr. Julie Patock-Peckham with a statistics course for undergraduates majoring in Psychology. Attended lectures, graded homework and exams, proctored exams, and held office hours.

2009 *Teaching Assistant (PSY101), Arizona State University*: Assisted Dr. Robert Short in organizing an introductory level psychology course. Prepared, administered and graded exams, ran Blackboard course website, and held office hours.

2009 *Guest Lecture (PSY101), Arizona State University*: Guest lecture on abnormal psychology in introductory level psychology course.

2008 *Exam Proctor, Arizona State University:* Proctored exams for various introductory level psychology courses.

2008 *Teaching Assistant (PSY134), University of California San Diego:* Assisted Dr. Amanda Roberts with an upper division psychology course on eating disorders. Attended lectures, assisted with exams, held office hours, and ran group lecture and review sessions for students.

Mentoring & Supervision

Undergraduate Student Research Training/Supervision (technique):
1. Kimberly Zwick (Fos immunohistochemistry, cell counting, data analyses, microinjections, elevated plus maze testing)
2. Sineadh Conway (self-administration, locomotor testing, injections, brain slicing, statistical analyses, microinjections, elevated plus maze testing, writing)
3. Teresa Ulman (self-administration, locomotor testing, injections, perfusions, brain extractions, statistical analyses)
4. Anthony Shepard (self-administration, microinfusions, catheter surgery)
5. Sarah Swinford (self-administration, microinfusions, locomotor testing, catheter surgery, brain slicing and staining, statistical analyses)
6. Julie Lukas (self-administration, microinfusions)
7. Marisa Ostos (self-administration, microinfusions, sucrose self-administration)
8. Bryan Harder (microinfusions, elevated plus maze testing)
9. Anthony Berger (locomotor testing, injections, statistical analyses, writing)
10. Samuel Brunwasser (microinfusions, elevated plus maze testing)
11. Alex Medawar (self-administration, microinfusions)
12. Julianna Goenaga (self-administration, microinfusions)
13. Jeffrey Lynn (self-administration, microinfusions)

Research Technician Training/Supervision (technique):
1. Suzanne Weber (catheter surgery)
2. Lindsey Robertson (microinfusions, catheter surgery)

References

Janet Neisewander: Janet.Neisewander@asu.edu, (480)965-0209

M. Foster Olive: Foster.Olive@asu.edu (480)965-7598

Julie Patock-Peckham: jpp01@asu.edu (480)965-9246