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Incubation of cocaine cue reactivity associates with neuroadaptations in the cortical serotonin (5-HT) 5-HT_{2C} receptor (5-HT_{2C}R) system

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Abstract

Intensification of craving elicited by drug-associated cues during abstinence occurs over time in human cocaine users while elevation of cue reactivity ("incubation") is observed in rats exposed to extended forced abstinence from cocaine self-administration. Incubation in rodents has been linked to time-dependent neuronal plasticity in the medial prefrontal cortex (mPFC). We tested the hypothesis that incubation of cue reactivity during abstinence from cocaine self-administration is accompanied by lower potency and/or efficacy of the selective 5-HT_{2C}R agonist WAY163909 to suppress cue reactivity and a shift in the subcellular localization profile of the mPFC 5-HT_{2C}R protein. We observed incubation of cue reactivity (measured as lever presses reinforced by the discrete cue complex) between Day 1 and Day 30 of forced abstinence from cocaine relative to sucrose self-administration. Pharmacological and biochemical analyses revealed that the potency of the selective 5-HT_{2C}R agonist WAY163909 to suppress cue reactivity, the expression of synaptosomal 5-HT_{2C}R protein in the mPFC, and the membrane to cytoplasmic expression of the 5-HT_{2C}R in mPFC were lower on Day 30 *vs.* Day 1 of forced abstinence from cocaine self-administration. Incubation of cue reactivity assessed during forced abstinence from sucrose self-administration did not associate with 5-HT_{2C}R protein expression in the mPFC. Collectively, these outcomes are the first indication that neuroadaptations in the 5-HT_{2C}R system may contribute to incubation of cocaine cue reactivity.

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Conflict of Interest

Dr. Cunningham is a consultant for Arena Pharmaceuticals. All other authors report no biomedical financial interests or potential conflicts of interest.

Author Contributions

SESJ, NCA and KAC designed experiments. SESJ, NCA, RGF and SJS performed experiments. SESJ and NCA analyzed the data. SESJ, NCA and KAC wrote the manuscript. All authors reviewed the manuscript and approved the final version.

Keywords

5-HT_{2C} receptor; cocaine; cue reactivity; incubation; medial prefrontal cortex; sucrose

INTRODUCTION

Cocaine use disorder is characterized by cycles of use, abstinence and relapse (Volkow et al., 2010). Exposure to the environmental contexts and stimuli previously associated with the drug experience (cue reactivity) can precipitate relapse (Carter and Tiffany, 1999; O'Brien et al., 1998). Cue reactivity can be defined as the attentional orienting response to drug-associated stimuli that predict reward, and exposure to cocaine-associated cues includes physiological responses (e.g., elevated heart rate), subjective reactions (e.g., craving), appetitive approach behaviors (e.g., cocaine seeking) as well as neural circuit activation in humans (Carter and Tiffany, 1999; Field and Cox, 2008; Garavan et al., 2000; Maas et al., 1998). Abstinent drug abusers are reported to exhibit a time-dependent increase in craving elicited by drug-associated cues (Bedi et al., 2010; Wang et al., 2013). In rodents, cue reactivity (lever presses reinforced by the discrete drug-paired cue complex) escalates over the first months following cessation of self-administration of cocaine (Grimm et al., 2001; Neisewander et al., 2000), other abused drugs (Abdolahi et al., 2010; Li et al., 2015) as well as non-drug rewards such as sucrose (Grimm et al., 2006; Grimm et al., 2002); escalation of cue reactivity during abstinence has been termed "incubation" (for review, Pickens et al., 2011). Plasticity of neuronal signaling within the medial prefrontal cortex (mPFC) (Koya et al., 2009; LaLumiere et al., 2012; Whitfield et al., 2011), in concert with other nodes within the limbic-corticostriatal circuit (Conrad et al., 2008; Lu et al., 2003; Lu et al., 2005; Ma et al., 2014; Terrier et al., 2015), plays a fundamental role in the generation of cue reactivity and incubation phenomena.

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

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

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

METHODS

Animals

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

Drugs

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

Apparatus

Both cocaine and sucrose self-administration studies employed standard operant conditioning chambers (Med-Associates, Inc., St. Albans, VT, USA) housed in ventilated, sound-attenuating cubicles with fans (Med-Associates, Inc.). Each chamber was outfitted

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

Cocaine self-administration and cue reactivity analyses

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

Cocaine self-administration training consisted of 14 daily 180-min sessions during which rats were trained to lever press for cocaine infusions (0.75 mg/kg/0.1 mL infusion) (Anastasio et al., 2014a; Anastasio et al., 2014b; Cunningham et al., 2013; Cunningham et al., 2011). Schedule completions on the active lever resulted in delivery of a cocaine infusion over a 6-sec period paired simultaneously with illumination of the house and stimulus lights and activation of the infusion pump (discrete cue complex paired with cocaine delivery); inactive lever presses produced no scheduled consequences. Following reinforcer delivery, the stimulus light as well as the infusion pump were inactivated; the house light remained on for an additional 20 sec to indicate a timeout period during which lever presses had no scheduled consequences. Rats were trained on a fixed ratio (FR) 1 schedule of reinforcement and progressed to an FR5 schedule after achieving seven infusions/hr with less than 10% variability for three consecutive days. Upon achieving stability on the FR5 schedule (less than 10% variability for a minimum of three consecutive days), rats were pseudorandomly assigned to either FA Day 1 or FA Day 30, and returned to their home cages for the appropriate FA period.

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

behavioral experience during the cue reactivity session in *ex vivo* neurochemical studies (Anastasio et al., 2014a).

Two cohorts of rats were trained to self-administer cocaine and assessed for cue reactivity. In the first cohort (n=149), pharmacological analyses were employed to test the hypothesis that incubation of cue reactivity during abstinence from cocaine self-administration is accompanied by lower potency and/or efficacy of the selective 5-HT_{2C}R agonist WAY163909 to suppress cue reactivity. After meeting the criterion for stable cocaine self-administration, rats received an injection of vehicle (0.9% NaCl, 1 mL/kg; i.p.) or WAY163909 (0.05 mg/kg, 0.2 mg/kg, or 1.0 mg/kg; i.p.) 15 min prior to the cue reactivity session on FA Day 1 or FA Day 30. The doses were chosen from our previous studies with WAY163909 (Anastasio et al., 2014a; Anastasio et al., 2014b) (Cunningham et al., 2013; Cunningham et al., 2011). The second cohort of rats (n=20) was trained to self-administer cocaine as above and treated with vehicle 15 min prior to the cue reactivity session on FA Day 1 or FA Day 30. The vehicle-treated rats from both cohorts were employed in *ex vivo* analyses (see below).

Sucrose self-administration and cue reactivity analyses

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

5-HT_{2C}R protein analysis

The 5-HT_{2C}R protein expression profiles were assessed using Western blot analyses of tissue harvested from FA Day 1 or FA Day 30 “cue test” and “no test” cocaine- or sucrose-trained rats (Anastasio et al., 2014a) or naïve rats. Naïve rats were included as an additional control to interrogate levels of 5-HT_{2C}R protein expression and subcellular distribution independent of experimental manipulation. Rats were anesthetized (400 mg/kg chloral hydrate solution), decapitated, and brains were cut in 2 mm coronal sections, rapidly microdissected with a scalpel on a cool tray (4°C) (Heffner et al., 1980), frozen in liquid nitrogen and stored at −80°C. Tissue encompassing the mPFC (cingulate cortex 1, prelimbic cortex and infralimbic cortex) was extracted at 3.00 mm from bregma (Paxinos and Watson, 1998). Protein fractionation techniques were employed to assess the synaptosomal expression profile (Anastasio et al., 2010; Liu et al., 2007) or the membrane *vs.* cytoplasmic protein expression profile of the 5-HT_{2C}R (Anastasio et al., 2013; Anastasio et al., 2014b).

The crude synaptosomal protein fraction is enriched for pre- and postsynaptic proteins [i.e., presynaptic terminals, postsynaptic membranes, postsynaptic density, synaptic protein complexes (Breukel et al., 1997)] and was prepared as described previously (Anastasio et al., 2010; Liu et al., 2007). Individual mPFC tissues were homogenized in 10 times w/v ice cold Krebs buffer (125 mM NaCl, 1.2 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 22 mM Na_2CO_3, 1 mM NaH_2PO_4, 10 mM glucose) containing 0.32 M sucrose plus protease

inhibitor cocktail and phosphatase inhibitor 2 and 3 cocktails (10 μL/mL; Sigma-Aldrich, St. Louis, MO). The homogenate was centrifuged at 1000 g for 10 min at 4°C to pellet the nuclear fraction. The supernatant was collected and centrifuged at 16,000 g for 20 min at 4°C to pellet the crude synaptosome. The pellet was re-suspended in Krebs buffer with 1% dodecyl maltoside or 0.5% NP40.

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

Equal amounts of crude synaptosomal protein (30 μg) were reduced with Laemmli sample buffer and heated for 20 min at 70°C then separated by SDS-PAGE using 10% Bis-Tris gels (Invitrogen, San Diego, CA) for 2–3 hrs at 110V. Proteins were transferred to a PVDF membrane (BioRad, Hercules, CA) via a wet-transfer electroblotting apparatus (BioRad) overnight at 60–70V (Anastasio et al., 2010). Membranes were blocked with Odyssey blocking buffer [LI-COR® Biosciences; 1:1 in Tris Buffered Saline (TBS), pH 7.4] followed by incubation with primary antibody [mouse monoclonal 5-HT_{2C}R (D-12, sc-17797, Santa Cruz; 1:100), mouse monoclonal pan-cadherin (ab6528, Abcam; 1:5000)] (Anastasio et al., 2010; Anastasio et al., 2015; Fink, 2015). Membranes were rinsed in TBS + 0.1% Tween-20 (TBS-T), incubated with secondary antibody [infrared-labeled goat anti-mouse IRDye™800CW (926-32210) or IRDye™680RD (926-68070; LI-COR® Biosciences, Lincoln, NE, 1:10000)], then rinsed in TBS-T. PVDF membranes were imaged using the Odyssey® Infrared Imaging System (LI-COR® Biosciences, Lincoln, NE). The integrated intensity of immunoreactive bands normalized to the housekeeping protein cadherin were analyzed with the Odyssey® software.

The subcellular localization (synaptosomal; membrane *vs.* cytoplasmic) profile of the mPFC 5-HT_{2C}R protein was assessed via the Wes™ automated Western blotting system (ProteinSimple, San Jose, CA) which utilizes capillary electrophoresis-based immunodetection for higher resolution, sensitivity, and reproducibility (even at low sample concentrations) relative to traditional immunoblotting techniques (Anastasio et al., 2015; Fink, 2015; Liu et al., 2013). Wes™ reagents (biotinylated molecular weight marker, streptavidin-HRP fluorescent standards, luminol-S, hydrogen peroxide, sample buffer, DTT,

stacking matrix, separation matrix, running buffer, wash buffer, and matrix removal buffer, secondary antibodies, antibody diluent, and capillaries) were obtained from the manufacturer (ProteinSimple) and used according to the manufacturer's recommendations with minor modifications (Anastasio et al., 2015; Fink, 2015). The mouse monoclonal 5-HT_{2C}R (D-12, sc-17797, Santa Cruz; 1:50), mouse monoclonal pan-cadherin (ab6528, Abcam; 1:10000), and mouse monoclonal GAPDH antibody (6C5, Advanced Immunochemicals; 1:50000) were diluted with ProteinSimple antibody diluent. Equal amounts of protein (3 μg) were combined with 0.1X sample buffer and 5X master mix (200 mM DTT, 5X sample buffer, 5X fluorescent standards), gently mixed, and then denatured at 95°C for 5 min. The denatured samples, biotinylated ladder, antibody diluent, primary antibodies, HRP-conjugated secondary antibodies, chemiluminescent substrate, and wash buffer were dispensed to designated wells in a pre-filled microplate (ProteinSimple). Separation electrophoresis (375 V, 31 min, 25°C) and immunodetection in the capillaries were fully automated using the following settings: separation matrix load for 200-s, stacking matrix load for 14-s, sample load for 7-s, antibody diluent for 30 min, primary antibody incubation for 60 min, secondary antibody incubation for 30 min, and chemiluminescent signal exposure for 5-s, 15-s, 30-s, 60-s, 120-s, 240-s, and 480-s. Data analyses were performed using the Compass Software (ProteinSimple). The area under the curve of the 5-HT_{2C}R peak was normalized to the area under the curve of the cadherin peak (synaptosomal and membrane fractions) or area under the curve of the GAPDH peak (cytoplasmic fraction). Representative "virtual blot" electrophoretic images were automatically generated by the Compass Software (ProteinSimple).

5-HT_{2C}R mRNA analysis

We employed RT-PCR analyses to determine 5-HT_{2C}R mRNA levels on FA Day 30 *vs.* FA Day 1 from cocaine self-administration. Immediately following the cue reactivity test session, rats were sacrificed and the mPFC was harvested as described above. Samples of mPFC were homogenized in Trizol Reagent® and RNA was isolated using the Trizol Reagent Protocol (Life Technologies, Grand Island, NY) (Anastasio et al., 2014b). Reverse transcription was performed on 250 ng RNA using SuperScript III Reverse Transcriptase (Life Technologies) with random hexamer primers. RT-PCR reactions were assayed in triplicate on a 7500 Fast RT PCR System using TaqMan Fast Advanced Master Mix and TaqMan® gene specific primer/probes [*Htr2c*: Rn00562748_m1 (spans boundary of exons 4 and 5); Cyclophilin A (*Ppia*): Rn00690933_m1; Life Technologies]. Data are presented in terms of Crossing Threshold (Ct), where $\Delta Ct = Ct(Htr2c) - Ct(Cyclophilin)$.

Statistical analyses

Student's t-test was used to analyze total intake during acquisition and maintenance of cocaine or sucrose self-administration. A two-way ANOVA for the factors of FA Day (FA Day 1, FA Day 30) and WAY163909 treatment (vehicle, 0.05, 0.2 and 1.0 mg/kg) was used to analyze previously-active and inactive lever presses and latency to the first response during the cue reactivity test session; planned comparisons were subsequently made with a Dunnett's test (Keppel, 1973). The four parameter logistic nonlinear regression (Sigma Plot, Version 12.3, Systat Software, Inc., Chicago, IL) was used to estimate the dose of WAY163909 estimated to decrease cue reactivity by 50% of the maximum suppression by

WAY163909 (ID_{50}) on FA Day 1 *vs.* FA Day 30 from cocaine self-administration (Ratkowsky and Reedy, 1986; Tallarida and Murray, 1987). A one-way ANOVA was used to analyze previously-active and inactive lever presses and latency to the first response on FA Day 1 *vs.* FA Day 30 following sucrose or cocaine (second cohort) self-administration. A two-way ANOVA for the factors of FA (FA Day 1, FA Day 30) and group ("cue test", "no test") was used to analyze synaptosomal 5-HT_{2C}R protein expression; planned comparisons were made with a Tukey's test (Keppel, 1973). Student's t-test was used to analyze 5-HT_{2C}R mRNA levels on FA Day 1 *vs.* FA Day 30. A one-way ANOVA (FA Day 1, FA Day 30, naïve) was used to analyze synaptosomal, membrane, cytoplasmic or membrane:cytoplasmic 5-HT_{2C}R protein expression; planned comparisons were made with a Tukey test (Keppel, 1973). A Pearson's correlation was used to analyze the relationship between previously-active lever presses on the cue reactivity test session and the membrane:cytoplasmic ratio of 5-HT_{2C}R protein expression (Keppel, 1973). The experiment-wise error rate for all analyses was set at α=0.05.

RESULTS

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

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

We tested the hypothesis that rats assessed for cue reactivity (previously-active lever presses; mean ± SEM) on FA Day 1 and FA Day 30 from cocaine self-administration would display lower potency and/or efficacy of the selective 5-HT_{2C}R agonist WAY163909 to suppress cue reactivity (Anastasio et al., 2014a). Rats were injected with vehicle (0.9% NaCl) or WAY163909 (0.05 mg/kg, 0.2 mg/kg, or 1.0 mg/kg; i.p.) 15 min prior to the cue reactivity session on FA Day 1 or FA Day 30. A main effect of FA Day ($F_{1,132}$=74.28; p<0.05), WAY163909 treatment ($F_{3,132}$=21.81; p<0.05), and an FA Day x WAY163909 treatment interaction ($F_{3,132}$=4.38; p<0.05) were observed for previously-active lever presses for the discrete cue complex. Planned comparisons indicated that cue reactivity was significantly elevated in the vehicle-treated rats on FA Day 30 *vs.* FA Day 1 from cocaine self-administration (Fig. 1b), consistent with previous observations that cue reactivity incubates during forced abstinence from cocaine-taking (Grimm et al., 2001; Neisewander et al., 2000). Cue reactivity in vehicle-treated rats is replotted (Fig. 1c) for FA Day 1 (dotted line) and FA Day 30 (dashed line). Planned comparisons for FA Day 1 indicated that rats treated with the intermediate (0.2 mg/kg) or high dose (1.0 mg/kg) of WAY163909 exhibited lower previously-active lever presses *vs.* rats treated with vehicle (p<0.05); the low dose (0.05 mg/kg) of WAY163909 had no effect *vs.* rats treated with vehicle (Fig. 1c; n.s.). Planned comparisons for FA Day 30 indicated that only the high dose (1.0 mg/kg) of WAY163909 significantly attenuated previously-active lever presses *vs.* rats treated with vehicle (p<0.05); the intermediate (0.2 mg/kg) and low (0.05 mg/kg) doses of WAY163909 had no effect *vs.*

rats treated with vehicle (Fig. 1c). The dose of WAY163909 estimated to decrease previously-active lever presses by 50% of the maximum suppression by WAY163909 (ID_{50}) was 0.12 mg/kg on FA Day 1 and 0.39 mg/kg on FA Day 30 (~3-fold rightward shift). There was no difference in the maximum efficacy of 1 mg/kg of WAY163909 (previously active lever presses expressed as percent suppression *vs.* vehicle) on FA Day 1 (32.8 ± 8.8%) *vs.* FA Day 30 (48.0 ± 4.1%; $t_{1,34}$=2.24; n.s.). Thus, incubation was associated with lower potency of WAY163909, but no change in efficacy, to suppress cue reactivity on FA Day 30 *vs.* FA Day 1.

Inactive lever presses in vehicle-treated rats are replotted (Fig. 1d) for FA Day 1 (dotted line) and FA Day 30 (dashed line). For inactive lever presses, no main effect of FA Day ($F_{1,132}$=0.94; n.s.), a main effect of WAY163909 treatment ($F_{3,132}$=3.66; p<0.05), and no FA Day x WAY163909 treatment interaction ($F_{3,132}$=0.22; n.s.) were observed during the cue reactivity test session. Planned comparisons indicated that WAY163909 did not significantly alter inactive lever presses on either FA Day 1 or FA Day 30 at any dose tested (Fig. 1d). No main effect of FA Day ($F_{1,132}$=1.85; n.s.), a main effect of WAY163909 treatment ($F_{3,132}$=5.57; p<0.05), and no FA Day x WAY163909 treatment interaction ($F_{3,132}$=0.04; n.s.) were observed for latency to the first press on the previously-active lever during the cue reactivity test session. On FA Day 1, planned comparisons indicated that the latency to first press (mean ± SEM) following WAY163909 treatment at 0.05 mg/kg (27.4 ± 4.6 sec; n.s.), 0.2 mg/kg (39.7 ± 6.6 sec; n.s.) and 1.0 mg/kg (57.7 ± 10.6 sec; n.s) did not differ from vehicle (32.4 ± 10.9). On FA Day 30, the latency to first press following WAY163909 treatment at 0.05 mg/kg (18.9 ± 5.5 sec; n.s.) or 0.2 mg/kg (34.0. ± 10.6 sec; n.s.) did not differ from vehicle (21.0 ± 4.9 sec). The latency to first press following 1.0 mg/kg (50.8 ± 11.3; p<0.05) was significantly higher relative to vehicle (21.0 ± 4.9 sec). Thus, WAY163909 suppressed cue reactivity with a limited impact on additional measures of behavioral performance.

Cue reactivity incubates during forced abstinence from sucrose self-administration

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

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

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

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

Figure 2 illustrates the qualitative (**inset**) and quantitative analyses (**bars**) of mPFC 5-HT_{2C}R synaptosomal protein expression at FA Day 1 and FA Day 30 from cocaine (Fig. 2b) or sucrose self-administration for both "cue test" and "no test" rats (Fig. 2c). For cocaine cue reactivity, a main effect of FA Day ($F_{1,21}$=13.19; p<0.05), but no main effect of cue group ($F_{1,21}$=3.75; n.s.) or FA Day x cue group interaction ($F_{1,21}$=0.93; n.s.) on mPFC 5-HT_{2C}R protein expression was observed. Planned comparisons indicated that synaptosomal 5-HT_{2C}R protein expression in the mPFC was lower on FA Day 30 *vs.* FA Day 1 regardless of cue group ("cue test" or "no test"; Fig. 2b). We performed RT-PCR to assess 5-HT_{2C}R mRNA levels in the mPFC from rats sacrificed immediately following the cue reactivity test session on FA Day 1 or FA Day 30 from cocaine self-administration. Expression of 5-HT_{2C}R mRNA (mean ± SEM.) was identical between FA Day 1 (4.82 ± 0.13 arbitrary units, A.U.) and FA Day 30 (4.80 ± 0.30 A.U.; $t_{1,4}$=0.00; n.s.). For sucrose cue reactivity, there was no main effect of FA Day ($F_{1,27}$=0.21; n.s.) or cue group ($F_{1,27}$=0.02; n.s.) and no FA Day x cue group interaction ($F_{1,27}$=0.70; n.s.). Synaptosomal 5-HT_{2C}R protein expression in the mPFC was identical on FA Day 30 vs. FA Day 1 under both "cue test" and "no test" conditions (Fig. 2c). Together, these data demonstrate that lower 5-HT_{2C}R expression in the mPFC is specific to incubation of cocaine cue reactivity and that the profile of 5-HT_{2C}R protein expression is potentially regulated by post-transcriptional mechanisms (e.g., trafficking and/or recycling processes).

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

We tested the hypothesis that incubation of cocaine cue reactivity is associated with differential subcellular distribution of 5-HT_{2C}R protein expression in the mPFC as an indicator of post-transcriptional mechanisms including receptor trafficking and/or recycling processes. Rats from the second cohort readily acquired cocaine self-administration to stability (i.e., seven infusions/hr on an FR 5 schedule for at least three sessions) and displayed <10% variation in the number of infusions earned during the maintenance sessions (data not shown). There was no difference in total cocaine intake across the self-administration phase between rats assigned to FA Day 1 (366.7 ± 18.8 mg/kg) or FA Day 30 (385.6 ± 30.0 mg/kg; $t_{1,18}$=0.29; n.s.). Cue reactivity was significantly higher on FA Day 30 *vs.* FA Day 1 (Fig. 3a; $F_{1,18}$=25.38; p<0.05) as shown for the first cohort (Fig. 1b).

Analyses of synaptosomal (Fig. 3b), membrane (Fig. 3c), and cytoplasmic (Fig. 3d) mPFC 5-HT_{2C}R protein expression in these rats are presented. A cohort of naïve rats were included as an additional neurochemical control. The horizontal bars represent the mean (solid line) ± SEM (dotted lines and shading) of 5-HT_{2C}R protein expression in the mPFC of naïve rats. A main effect of group (naïve, FA Day 1, FA Day 30) was detected for synaptosomal 5-HT_{2C}R protein expression in the mPFC ($F_{2,13}$=5.33; p<0.05). Planned comparisons indicated that synaptosomal 5-HT_{2C}R protein expression (Fig. 3b, **inset**) was lower on FA Day 30 *vs.* FA Day 1 (Fig. 3b; p<0.05), replicating observations presented in Fig. 2b. Synaptosomal 5-HT_{2C}R protein expression on FA Day 1 and FA Day 30 did not differ from naïve (Fig. 3b; n.s.). A main effect of group was detected for membrane 5-HT_{2C}R protein expression ($F_{2,14}$=6.98; p<0.05). Membrane expression of 5-HT_{2C}R (Fig. 3c, **inset**) was lower on FA day 30 *vs.* FA Day 1 (Fig. 3c; p<0.05) and *vs.* naïve (Fig. 3c; p<0.05); membrane 5-HT_{2C}R protein expression on FA Day 1 did not differ from naïve (Fig. 3c; n.s.). A main effect of group was detected for cytoplasmic 5-HT_{2C}R protein expression ($F_{2,14}$=4.04; p<0.05). Cytoplasmic expression of 5-HT_{2C}R (Fig. 3d, **inset**) did not differ between FA Day 1 and FA Day 30 or naïve (Fig. 3d; n.s.). A main effect of group was detected for the ratio of membrane to cytoplasmic 5-HT_{2C}R protein expression ($F_{2,14}$=13.27, p<0.05). The ratio of membrane to cytoplasmic 5-HT_{2C}R expression was lower on FA Day 30 *vs.* FA Day 1 (Fig. 3e; p<0.05), but did not differ from naïve (Fig. 3e; n.s.). There was an inverse correlation between previously-active lever presses during the cue reactivity test session and the membrane:cytoplasmic ratio of 5-HT_{2C}R protein expression in individual rats (Fig. 3f; r= −0.8922; p<0.05). Together, these data suggest that incubation of cocaine cue reactivity is manifested by adaptations in trafficking and/or recycling processes that dictate the subcellular localization of the 5-HT_{2C}R protein.

DISCUSSION

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

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

need only occupy a fraction of available receptors to activate a maximal response (Kenakin, 2002; Strange, 2008); this concept is supported here by the fact that the maximal efficacy of WAY163909 (1 mg/kg) is retained in prolonged forced abstinence. The subcellular localization of the 5-HT_{2C}R is a key feature of receptor readiness to signal and respond to agonists (Kenakin, 2002; Strange, 2008). In the present study, the ratio of membrane to cytoplasmic 5-HT_{2C}R expression in the mPFC was significantly lower at prolonged *vs.* early abstinence, in the absence of differences in 5-HT_{2C}R mRNA levels, suggesting that post-transcriptional mechanisms govern the neuroadaptations in the 5-HT_{2C}R system that contribute to elevated cocaine cue reactivity.

Neuroimaging studies in humans (Garavan et al., 2000; Maas et al., 1998) and pharmacological and behavioral studies in rodents (Koya et al., 2009; LaLumiere et al., 2012; Ma et al., 2014; Pentkowski et al., 2010; Whitfield et al., 2011) have implicated the mPFC as a key neuronal locus that controls the persistence of cocaine-associated cues to generate susceptibility to relapse, including the 5-HT_{2C}R (Anastasio et al., 2014a; Anastasio et al., 2014b). The observations described herein support the hypothesis that disruption of a population of synaptically-localized 5-HT_{2C}R (Anastasio et al., 2010) and a reduced 5-HT_{2C}R tone in mPFC is an integral mechanism in the expression of cue reactivity during abstinence from cocaine self-administration (for review, Cunningham and Anastasio, 2014). The 5-HT_{2C}R interfaces in a functionally-coordinated manner with synaptic proteins to promote or inhibit receptor readiness and subsequent signaling capacity (Gavarini et al., 2006), most likely a reactive process to the dynamic microenvironment. A shift in receptor expression from the membrane fraction to the cytoplasmic fraction indicates that post-transcriptional processes which govern 5-HT_{2C}R trafficking and subcellular localization (e.g., RNA editing, alternative splicing) (Marion et al., 2004; Martin et al., 2013) may also be subject to regulation during periods of abstinence from cocaine. It is intriguing to propose that the neurochemical adaptations, including fluctuations in 5-HT neurotransmission promoted by cocaine-taking and periods of withdrawal (Parsons and Justice, 1993; Parsons et al., 1995), may be responsible for shuttling 5-HT_{2C}R protein between subcellular compartments. Future analyses are required to determine the importance of other factors likely to contribute to the 5-HT_{2C}R functional capacity and responsivity to agonist *in vivo*, including receptor density, affinity, reserve and/or coupling efficiency (Kenakin, 2002; Sanders-Bush and Breeding, 1990). Our primary objective in the present studies was to uncover the late-emerging changes in 5-HT_{2C}R expression that may be involved in incubation, but shifts in 5-HT_{2C}R expression may also occur during cocaine self-administration and/or in the first 24 hours following the cessation of cocaine-taking. Interestingly, the profile of mPFC 5-HT_{2C}R membrane expression in naïve rats mirrored that observed at early forced abstinence from cocaine self-administration. Without a comprehensive understanding of 5-HT levels and 5-HT_{2C}R function during abstinence from cocaine (Parsons and Justice, 1993; Parsons et al., 1995), concise conclusions about the functional status of the 5-HT_{2C}R system relative to basal conditions are difficult to formulate. Nonetheless, while similar between naïve and early abstinence, 5-HT_{2C}R membrane expression in prolonged abstinence was lower than naïve controls and early abstinence, findings that support the interpretation that the subcellular distribution of 5-HT_{2C}R expression is dysregulated at prolonged abstinence from cocaine-taking. A greater

appreciation of the subcellular distribution of the 5-HT_{2C}R and the specific mechanisms which regulate its postsynaptic functionality will advance the development of therapeutics designed to harmonize 5-HT_{2C}R signaling in the mPFC to minimize heightened cue reactivity.

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

We found that dysregulation of the mPFC 5-HT_{2C}R system does not appear to be involved in incubation following forced abstinence from sucrose self-administration which is consistent with prior reports that have identified disparate mechanisms involved in incubation following cocaine *vs.* sucrose self-administration in the mPFC (Koya et al., 2009) and NAc (Counotte et al., 2014; Lu et al., 2003). In contrast, convergent mechanisms for incubation following cocaine and sucrose self-administration have been described within limbic areas of the brain, such as the amygdala (Lu et al., 2007; Uejima et al., 2007), which may implicate different neurocircuitry in incubation following abstinence from drug *vs.* non-drug reinforcers. Of note, 5-HT_{2C}R populations within the NAc (Filip and Cunningham, 2002; Navailles et al., 2008) and the ventral tegmental area (Bubar and Cunningham, 2007; Bubar et al., 2011; Navailles et al., 2008) also control corticostriatal circuitry (Pozzi et al., 2002), but have yet to be fully investigated for their role in incubation following either cocaine or sucrose self-administration. Collectively, neuroadaptations in the 5-HT_{2C}R and other neurobiological systems within the corticoaccumbens circuit could aggregately prime the neural network for an augmented response to reward-related cues in prolonged abstinence from cocaine self-administration.

An improved understanding of the mechanisms which mediate the incubation phenomenon could provide insight into treatment modalities that may be differentially effective to curb

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

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Abbreviations

5-HT	serotonin
5-HT_{2C}R	serotonin 2C receptor
ANOVA	analysis of variance
BDNF	brain derived neurotrophic factor
ERK	extracellular signal-regulated kinase
FA	forced abstinence
FR	fixed ratio
GABA	γ-aminobutyric acid
GPCR	G protein-coupled receptor
i.p	intraperitoneal
mPFC	medial prefrontal cortex
NAc	nucleus accumbens

References

Abdolahi A, Acosta G, Breslin FJ, Hemby SE, Lynch WJ. Incubation of nicotine seeking is associated with enhanced protein kinase A-regulated signaling of dopamine-and cAMP-regulated phosphoprotein of 32 kDa in the insular cortex. Eur J Neurosci. 2010; 31:733–741. [PubMed: 20384816]

Anastasio NC, Gilbertson SR, Bubar MJ, Agarkov A, Stutz SJ, Jeng YJ, Bremer NM, Smith TD, Fox RG, Swinford SE, et al. Peptide inhibitors disrupt the serotonin 5-HT_{2C} receptor interaction with phosphatase and tensin homolog to allosterically modulate cellular signaling and behavior. Journal of Neuroscience. 2013; 33:1615–1630. [PubMed: 23345234]

Anastasio NC, Lanfranco MF, Bubar MJ, Seitz PK, Stutz SJ, McGinnis AG, Watson CS, Cunningham KA. Serotonin 5-HT_{2C} receptor protein expression is enriched in synaptosomal and post-synaptic compartments of rat cortex. J Neurochem. 2010; 113:1504–1515. [PubMed: 20345755]

Anastasio NC, Liu S, Maili L, Swinford SE, Lane SD, Fox RG, Hamon SC, Nielsen DA, Cunningham KA, Moeller FG. Variation within the serotonin (5-HT) 5-HT_{2C} receptor system aligns with vulnerability to cocaine cue reactivity. Transl Psychiatry. 2014a; 4:e369. [PubMed: 24618688]

Anastasio NC, Stutz SJ, Fink LH, Swinford-Jackson SE, Sears RM, DiLeone RJ, Rice KC, Moeller FG, Cunningham KA. Serotonin (5-HT) 5-HT_{2A} receptor (5-HT_{2A}R):5-HT_{2C}R imbalance in medial prefrontal cortex associates with motor impulsivity. ACS Chem Neurosci. 2015; 6:1248–1258. [PubMed: 26120876]

Anastasio NC, Stutz SJ, Fox RG, Sears RM, Emeson RB, DiLeone RJ, O'Neil RT, Fink LH, Li D, Green TA, et al. Functional status of the serotonin 5-HT_{2C} receptor (5-HT_{2C}R) drives interlocked phenotypes that precipitate relapse-like behaviors in cocaine dependence. Neuropsychopharmacology. 2014b; 39:370–382. [PubMed: 23939424]

Bedi G, Preston KL, Epstein DH, Heishman SJ, Marrone GF, Shaham Y, de Wit H. Incubation of cue-induced cigarette craving during abstinence in human smokers. Biol Psychiatry. 2010

Breukel AI, Besselsen E, Ghijsen WE. Synaptosomes. A model system to study release of multiple classes of neurotransmitters. Methods Mol Biol. 1997; 72:33–47. [PubMed: 9249736]

Bubar MJ, Cunningham KA. Distribution of serotonin 5-HT_{2C} receptors in the ventral tegmental area. Neuroscience. 2007; 146:286–277. [PubMed: 17367945]

Bubar MJ, Stutz SJ, Cunningham KA. 5-HT(2C) receptors localize to dopamine and GABA neurons in the rat mesoaccumbens pathway. PLoS One. 2011; 6:e20508. [PubMed: 21687728]

Burbassi S, Cervo L. Stimulation of serotonin(2C) receptors influences cocaine-seeking behavior in response to drug-associated stimuli in rats. Psychopharmacology (Berl). 2008; 196:15–27. [PubMed: 17899022]

Buydens-Branchey L, Branchey M, Fergeson P, Hudson J, McKernin C. The meta-chlorophenylpiperazine challenge test in cocaine addicts: Hormonal and psychological responses. Biol Psychiat. 1997; 41:1071–1086. [PubMed: 9146818]

Carr DB, Cooper DC, Ulrich SL, Spruston N, Surmeier DJ. Serotonin receptor activation inhibits sodium current and dendritic excitability in prefrontal cortex via a protein kinase C-dependent mechanism. J Neurosci. 2002; 22:6846–6855. [PubMed: 12177182]

Carter BL, Tiffany ST. Meta-analysis of cue-reactivity in addiction research. Addiction. 1999; 94:327–340. [PubMed: 10605857]

Choi KH, Edwards S, Graham DL, Larson EB, Whisler KN, Simmons D, Friedman AK, Walsh JJ, Rahman Z, Monteggia LM, et al. Reinforcement-related regulation of AMPA glutamate receptor subunits in the ventral tegmental area enhances motivation for cocaine. J Neurosci. 2011; 31:7927–7937. [PubMed: 21613507]

Clemett DA, Punhani T, Duxon MS, Blackburn TP, Fone KC. Immunohistochemical localisation of the 5-HT2C receptor protein in the rat CNS. Neuropharmacology. 2000; 39:123–132. [PubMed: 10665825]

Conrad KL, Tseng KY, Uejima JL, Reimers JM, Heng LJ, Shaham Y, Marinelli M, Wolf ME. Formation of accumbens GluR2-lacking AMPA receptors mediates incubation of cocaine craving. Nature. 2008; 454:118–121. [PubMed: 18500330]

Counotte DS, Schiefer C, Shaham Y, O'Donnell P. Time-dependent decreases in nucleus accumbens AMPA/NMDA ratio and incubation of sucrose craving in adolescent and adult rats. Psychopharmacology (Berl). 2014; 231:1675–1684. [PubMed: 24114427]

Cunningham KA, Anastasio NC. Serotonin at the nexus of impulsivity and cue reactivity in cocaine addiction. Neuropharmacology. 2014; 76(Pt B):460–478. [PubMed: 23850573]

Cunningham KA, Anastasio NC, Fox RG, Stutz SJ, Bubar MJ, Swinford SE, Watson CS, Gilbertson SR, Rice KC, Rosenzweig-Lipson S, et al. Synergism between a serotonin 5-HT_{2A} receptor (5-HT_{2A}R) antagonist and 5-HT_{2C}R agonist suggests new pharmacotherapeutics for cocaine addiction. ACS Chemical Neuroscience. 2013; 4:110–121. [PubMed: 23336050]

Cunningham KA, Fox RG, Anastasio NC, Bubar MJ, Stutz SJ, Moeller FG, Gilbertson SR, Rosenzweig-Lipson S. Selective serotonin 5-HT2C receptor activation suppresses the reinforcing efficacy of cocaine and sucrose but differentially affects the incentive-salience value of cocaine- vs. sucrose-associated cues. Neuropharmacology. 2011; 61:513–523. [PubMed: 21575646]

Dunlop J, Sabb AL, Mazandarani H, Zhang J, Kalgaonker S, Shukhina E, Sukoff S, Vogel RL, Stack G, Schechter L, et al. WAY-163909 ((7bR,10aR)-1,2,3,4,8,9,10,10a-octahydro-7bH-cyclopenta-[b][1,4]diazepino[6,7,1hi]indole): A novel 5-HT2C receptor selective agonist with anorectic activity. J Pharmacol Exp Ther. 2005; 313:862–869. [PubMed: 15705738]

Edwards S, Bachtell RK, Guzman D, Whisler KN, Self DW. Emergence of context-associated GluR(1) and ERK phosphorylation in the nucleus accumbens core during withdrawal from cocaine self-administration. Addiction biology. 2011; 16:450–457. [PubMed: 21309958]

Field M, Cox WM. Attentional bias in addictive behaviors: a review of its development, causes, and consequences. Drug Alcohol Depend. 2008; 97:1–20. [PubMed: 18479844]

Filip M, Cunningham KA. Serotonin 5-HT(2C) receptors in nucleus accumbens regulate expression of the hyperlocomotive and discriminative stimulus effects of cocaine. Pharmacol Biochem Behav. 2002; 71:745–756. [PubMed: 11888566]

Filip M, Cunningham KA. Hyperlocomotive and discriminative stimulus effects of cocaine are under the control of serotonin(2C) (5-HT(2C)) receptors in rat prefrontal cortex. J Pharmacol Exp Ther. 2003; 306:734–743. [PubMed: 12721337]

Fink LHLA, NC, Fox RG, Rice KC, Moeller FG, Cunningham KA. Individual differences in impulsive action reflect variation in the cortical serotonin 5-HT_{2A} receptor system. Neuropsychopharmacol. 2015 in press.

Fletcher PJ, Rizos Z, Sinyard J, Tampakeras M, Higgins GA. The 5-HT(2C) receptor agonist RO 60-0175 reduces cocaine self-administration and reinstatement induced by the stressor yohimbine and contextual cues. Neuropsychopharmacology. 2008; 33:1402–1412. [PubMed: 17653111]

Gabbott PL, Dickie BG, Vaid RR, Headlam AJ, Bacon SJ. Local-circuit neurones in the medial prefrontal cortex (areas 25, 32 and 24b) in the rat: morphology and quantitative distribution. J Comp Neurol. 1997; 377:465–499. [PubMed: 9007187]

Garavan H, Pankiewicz J, Bloom A, Cho JK, Sperry L, Ross TJ, Salmeron BJ, Risinger R, Kelley D, Stein EA. Cue-induced cocaine craving: neuroanatomical specificity for drug users and drug stimuli. Am J Psychiatry. 2000; 157:1789–1798. [PubMed: 11058476]

Gavarini S, Becamel C, Altier C, Lory P, Poncet J, Wijnholds J, Bockaert J, Marin P. Opposite effects of PSD-95 and MPP3 PDZ proteins on serotonin 5-hydroxytryptamine2C receptor desensitization and membrane stability. Molecular Biology of the Cell. 2006; 17:4619–4631. [PubMed: 16914526]

Grimm JW, Buse C, Manaois M, Osincup D, Fyall A, Wells B. Time-dependent dissociation of cocaine dose-response effects on sucrose craving and locomotion. Behav Pharmacol. 2006; 17:143–149. [PubMed: 16495722]

Grimm JW, Hope BT, Wise RA, Shaham Y. Neuroadaptation. Incubation of cocaine craving after withdrawal. Nature. 2001; 412:141–142. [PubMed: 11449260]

Grimm JW, Shaham Y, Hope BT. Effect of cocaine and sucrose withdrawal period on extinction behavior, cue-induced reinstatement, and protein levels of the dopamine transporter and tyrosine hydroxylase in limbic and cortical areas in rats. Behav Pharmacol. 2002; 13:379–388. [PubMed: 12394414]

Harvey-Lewis C, Li Z, Higgins GA, Fletcher PJ. The 5-HT2C receptor agonist lorcaserin reduces cocaine self-administration, reinstatement of cocaine-seeking and cocaine induced locomotor activity. Neuropharmacology. 2016; 101:237–245. [PubMed: 26427596]

Heffner TG, Hartman JA, Seiden LS. Rapid method for the regional dissection of the rat brain. Pharmacol Biochem Behav. 1980; 13:453–456. [PubMed: 7422701]

Herrick-Davis K, Grinde E, Lindsley T, Teitler M, Mancia F, Cowan A, Mazurkiewicz JE. Native Serotonin 5-HT2C Receptors Are Expressed as Homodimers on the Apical Surface of Choroid Plexus Epithelial Cells. Mol Pharmacol. 2015; 87:660–673. [PubMed: 25609374]

Higgins GA, Desnoyer J, Van Niekerk A, Silenieks LB, Lau W, Thevarkunnel S, Izhakova J, DeLannoy IA, Fletcher PJ, DeLay J, et al. Characterization of the 5-HT2C receptor agonist lorcaserin on efficacy and safety measures in a rat model of diet-induced obesity. Pharmacol Res Perspect. 2015; 3:e00084. [PubMed: 25692009]

Kenakin T. Drug efficacy at G protein-coupled receptors. Annual review of pharmacology and toxicology. 2002; 42:349–379.

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

Koya E, Uejima JL, Wihbey KA, Bossert JM, Hope BT, Shaham Y. Role of ventral medial prefrontal cortex in incubation of cocaine craving. Neuropharmacology. 2009; 56(Suppl 1):177–185. [PubMed: 18565549]

LaLumiere RT, Smith KC, Kalivas PW. Neural circuit competition in cocaine-seeking: roles of the infralimbic cortex and nucleus accumbens shell. Eur J Neurosci. 2012; 35:614–622. [PubMed: 22321070]

Lappalainen J, Zhang L, Dean M, Oz M, Ozaki N, Yu DH, Virkkunen M, Weight F, Linnoila M, Goldman D. Identification, expression, and pharmacology of a Cys23-Ser23 substitution in the human 5-HT2c receptor gene (HTR2C). Genomics. 1995; 27:274–279. [PubMed: 7557992]

Lee MA, Meltzer HY. Blunted oral body temperature response to MK-212 in cocaine addicts. Drug Alcohol Depend. 1994; 35:217–222. [PubMed: 7956751]

Li X, Rubio FJ, Zeric T, Bossert JM, Kambhampati S, Cates HM, Kennedy PJ, Liu QR, Cimbro R, Hope BT, et al. Incubation of methamphetamine craving is associated with selective increases in expression of Bdnf and trkb, glutamate receptors, and epigenetic enzymes in cue-activated fos-expressing dorsal striatal neurons. J Neurosci. 2015; 35:8232–8244. [PubMed: 26019338]

Liu S-B, Sardi S, Sonom B, Zocco D, McSweeney R, Fraser AD, Halleck AE, Li H, Smeljkal GB, Munevar S, et al. The application of a novel nanovolume capillary electrophoresis-based protein analysis system in personalized and translational medicine research. J Bioanal Biomed. 2013; S3

Liu S, Bubar MJ, Lanfranco MF, Hillman GR, Cunningham KA. Serotonin$_{2C}$ receptor localization in GABA neurons of the rat medial prefrontal cortex: Implications for understanding the neurobiology of addiction. Neuroscience. 2007; 146:1667–1688.

Lopez-Gimenez JF, Mengod G, Palacios JM, Vilaro MT. Regional distribution and cellular localization of 5-HT2C receptor mRNA in monkey brain: comparison with [3H]mesulergine binding sites and choline acetyltransferase mRNA. Synapse. 2001; 42:12–26. [PubMed: 11668587]

Lu L, Grimm JW, Shaham Y, Hope BT. Molecular neuroadaptations in the accumbens and ventral tegmental area during the first 90 days of forced abstinence from cocaine self-administration in rats. J Neurochem. 2003; 85:1604–1613. [PubMed: 12787079]

Lu L, Hope BT, Dempsey J, Liu SY, Bossert JM, Shaham Y. Central amygdala ERK signaling pathway is critical to incubation of cocaine craving. Nat Neurosci. 2005; 8:212–219. [PubMed: 15657599]

Lu L, Uejima JL, Gray SM, Bossert JM, Shaham Y. Systemic and central amygdala injections of the mGluR(2/3) agonist LY379268 attenuate the expression of incubation of cocaine craving. Biol Psychiatry. 2007; 61:591–598. [PubMed: 16893525]

Ma YY, Lee BR, Wang X, Guo C, Liu L, Cui R, Lan Y, Balcita-Pedicino JJ, Wolf ME, Sesack SR, et al. Bidirectional modulation of incubation of cocaine craving by silent synapse-based remodeling of prefrontal cortex to accumbens projections. Neuron. 2014; 83:1453–1467. [PubMed: 25199705]

Maas LC, Lukas SE, Kaufman MJ, Weiss RD, Daniels SL, Rogers VW, Kukes TJ, Renshaw PF. Functional magnetic resonance imaging of human brain activation during cue-induced cocaine craving. American Journal of Psychiatry. 1998; 155:124–126. [PubMed: 9433350]

Marion S, Weiner DM, Caron MG. RNA editing induces variation in desensitization and trafficking of 5-hydroxytryptamine 2c receptor isoforms. J Biol Chem. 2004; 279:2945–2954. [PubMed: 14602721]

Markram H, Toledo-Rodriguez M, Wang Y, Gupta A, Silberberg G, Wu C. Interneurons of the neocortical inhibitory system. Nature reviews Neuroscience. 2004; 5:793–807. [PubMed: 15378039]

Martin CB, Ramond F, Farrington DT, Aguiar AS Jr, Chevarin C, Berthiau AS, Caussanel S, Lanfumey L, Herrick-Davis K, Hamon M, et al. RNA splicing and editing modulation of 5-HT(2C) receptor function: relevance to anxiety and aggression in VGV mice. Mol Psychiatry. 2013; 18:656–665. [PubMed: 23247076]

Navailles S, Moison D, Cunningham KA, Spampinato U. Differential regulation of the mesoaccumbens dopamine circuit by serotonin2C receptors in the ventral tegmental area and the nucleus accumbens: an in vivo microdialysis study with cocaine. Neuropsychopharmacology. 2008; 33:237–246. [PubMed: 17429406]

Neisewander JL, Acosta JI. Stimulation of 5-HT2C receptors attenuates cue and cocaine-primed reinstatement of cocaine-seeking behavior in rats. Behav Pharmacol. 2007; 18:791–800. [PubMed: 17989517]

Neisewander JL, Baker DA, Fuchs RA, Tran-Nguyen LT, Palmer A, Marshall JF. Fos protein expression and cocaine-seeking behavior in rats after exposure to a cocaine self-administration environment. J Neurosci. 2000; 20:798–805. [PubMed: 10632609]

Nocjar C, Alex KD, Sonneborn A, Abbas AI, Roth BL, Pehek EA. Serotonin-2C and -2a receptor co-expression on cells in the rat medial prefrontal cortex. Neuroscience. 2015; 297:22–37. [PubMed: 25818050]

O'Brien CP, Childress AR, Ehrman R, Robbins SJ. Conditioning factors in drug abuse: can they explain compulsion? J Psychopharmacol. 1998; 12:15–22. [PubMed: 9584964]

Okada M, Northup JK, Ozaki N, Russell JT, Linnoila M, Goldman D. Modification of human 5-HT(2C) receptor function by Cys23Ser, an abundant, naturally occurring amino-acid substitution. Mol Psychiatry. 2004; 9:55–64. [PubMed: 14699441]

Parsons LH, Justice JB Jr. Serotonin and dopamine sensitization in the nucleus accumbens, ventral tegmental area, and dorsal raphe nucleus following repeated cocaine administration. J Neurochem. 1993; 61:1611–1619. [PubMed: 7693863]

Parsons LH, Koob GF, Weiss F. Extracellular serotonin is decreased in the nucleus accumbens during withdrawal from cocaine self-administration. Behav Brain Res. 1995; 73:225–228. [PubMed: 8788507]

Patkar AA, Mannelli P, Peindl K, Hill KP, Gopalakrishnan R, Berrettini WH. Relationship of disinhibition and aggression to blunted prolactin response to meta-chlorophenylpiperazine in cocaine-dependent patients. Psychopharmacology (Berl). 2006; 185:123–132. [PubMed: 16418827]

Paxinos, G.; Watson, C. The Rat Brain in Stereotaxic Coordinates. 4. Sydney: Academic Press; 1998.

Pentkowski NS, Duke FD, Weber SM, Pockros LA, Teer AP, Hamilton EC, Thiel KJ, Neisewander JL. Stimulation of medial prefrontal cortex serotonin 2C 5-HT_{2C} receptors attenuates cocaine-seeking behavior. Neuropsychopharmacology. 2010; 35:2037–2048. [PubMed: 20520599]

Pickens CL, Airavaara M, Theberge F, Fanous S, Hope BT, Shaham Y. Neurobiology of the incubation of drug craving. Trends Neurosci. 2011; 34:411–420. [PubMed: 21764143]

Piva F, Giulietti M, Baldelli L, Nardi B, Bellantuono C, Armeni T, Saccucci F, Principato G. Bioinformatic analyses to select phenotype affecting polymorphisms in HTR2C gene. Hum Psychopharmacol. 2011; 26:365–372. [PubMed: 21717509]

Pozzi L, Acconcia S, Ceglia I, Invernizzi RW, Samanin R. Stimulation of 5-hydroxytryptamine (5-HT(2C)) receptors in the ventrotegmental area inhibits stress-induced but not basal dopamine release in the rat prefrontal cortex. J Neurochem. 2002; 82:93–100. [PubMed: 12091469]

Ratkowsky DA, Reedy TJ. Choosing near-linear parameters in the four-parameter logistic model for radioligand and related assays. Biometrics. 1986; 42:575–582. [PubMed: 3567290]

Rockstroh M, Muller SA, Jende C, Kerzhner A, Von Bergen M, Tomm JM. Cell fractionation - an important tool for compartment proteomics. J Integr Omics. 2011; 1:135–143.

Sanders-Bush E, Breeding M. Serotonin1c receptor reserve in choroid plexus masks receptor subsensitivity. J Pharmacol Exp Ther. 1990; 252:984–988. [PubMed: 2319481]

Smiley JF, Goldman-Rakic PS. Serotonergic axons in monkey prefrontal cerebral cortex synapse predominantly on interneurons as demonstrated by serial section electron microscopy. J Comp Neurol. 1996; 367:431–443. [PubMed: 8698902]

Strange PG. Agonist binding, agonist affinity and agonist efficacy at G protein-coupled receptors. Br J Pharmacol. 2008; 153:1353–1363. [PubMed: 18223670]

Suski JM, Lebiedzinska M, Wojtala A, Duszynski J, Giorgi C, Pinton P, Wieckowski MR. Isolation of plasma membrane-associated membranes from rat liver. Nature protocols. 2014; 9:312–322. [PubMed: 24434800]

Tallarida, RJ.; Murray, RB. Manual of Pharmacologic Calculations with Computer Programs. 2. New York: Springer-Verlag; 1987.

Terrier J, Luscher C, Pascoli V. Cell-Type Specific Insertion of GluA2-Lacking AMPARs with Cocaine Exposure Leading to Sensitization, Cue-Induced Seeking, and Incubation of Craving. Neuropsychopharmacology. 2015

Uejima JL, Bossert JM, Poles GC, Lu L. Systemic and central amygdala injections of the mGluR2/3 agonist LY379268 attenuate the expression of incubation of sucrose craving in rats. Behav Brain Res. 2007; 181:292–296. [PubMed: 17537525]

Volkow ND, Wang GJ, Fowler JS, Tomasi D, Telang F, Baler R. Addiction: decreased reward sensitivity and increased expectation sensitivity conspire to overwhelm the brain's control circuit. Bioessays. 2010; 32:748–755. [PubMed: 20730946]

Vysokanov A, Flores-Hernandez J, Surmeier DJ. mRNAs for clozapine-sensitive receptors co-localize in rat prefrontal cortex neurons. Neuroscience Letters. 1998; 258:179–182. [PubMed: 9885960]

Walstab J, Steinhagen F, BRUSS M, Gothert M, BONISCH H. Differences between human wild-type and C23S variant 5-HT2C receptors in inverse agonist-induced resensitization. Pharmacol Rep. 2011; 63:45–53. [PubMed: 21441610]

Wang G, Shi J, Chen N, Xu L, Li J, Li P, Sun Y, Lu L. Effects of length of abstinence on decision-making and craving in methamphetamine abusers. PLoS One. 2013; 8:e68791. [PubMed: 23894345]

Whitfield TW Jr, Shi X, Sun WL, McGinty JF. The suppressive effect of an intra-prefrontal cortical infusion of BDNF on cocaine-seeking is Trk receptor and extracellular signal-regulated protein kinase mitogen-activated protein kinase dependent. J Neurosci. 2011; 31:834–842. [PubMed: 21248106]

Zacharias DA, Violin JD, Newton AC, Tsien RY. Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. Science. 2002; 296:913–916. [PubMed: 11988576]

Highlights

- Cue reactivity incubates during forced abstinence from cocaine- or sucrose-taking.
- Incubation aligns with lower potency of 5-HT_{2C}R agonist to suppress cocaine cue reactivity.
- Incubation of cocaine cue reactivity associates with lower 5-HT_{2C}R expression in mPFC.
- Membrane to cytoplasmic 5-HT_{2C}R ratio inversely correlates with cocaine cue reactivity.

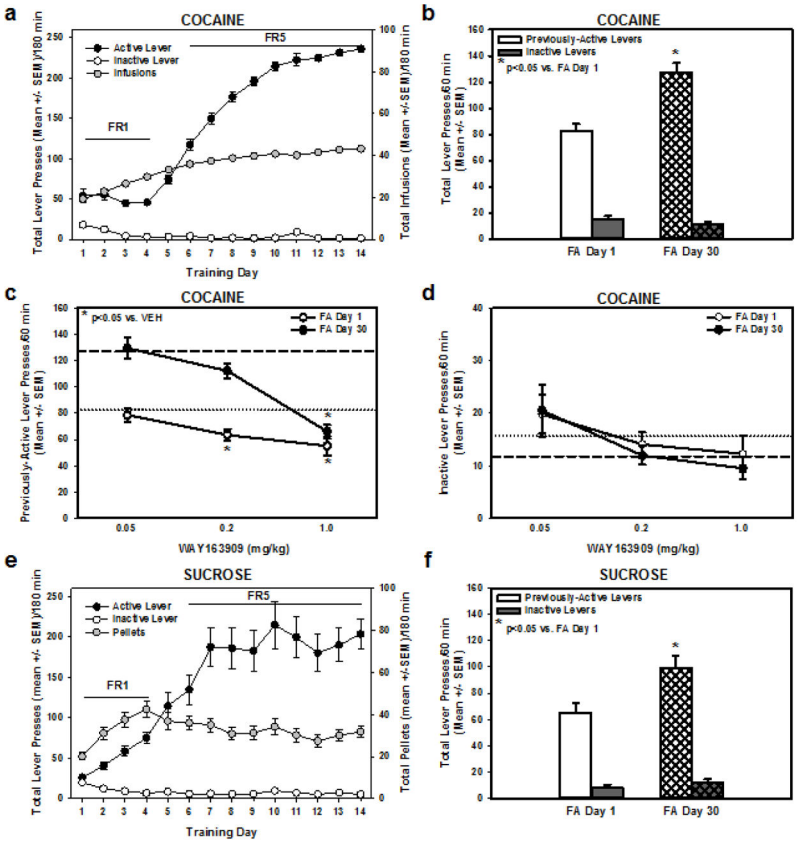

Figure 1. Cue reactivity incubates during forced abstinence from cocaine and sucrose self-administration, and incubation aligns with lower potency of a selective 5-HT$_{2C}$R agonist to blunt cocaine cue reactivity

(a) Mean responses (± SEM) on the active (black circles) or inactive lever (white circles), and total number of cocaine infusions earned (± SEM; gray circles) are presented for the acquisition and maintenance phase of cocaine self-administration. **(b)** Mean (± SEM) previously-active lever presses and inactive lever presses are presented for the cue reactivity test session in vehicle-treated rats on FA Day 1 and FA Day 30 from cocaine self-administration. Cue reactivity is significantly elevated on FA Day 30 *vs.* FA Day 1 from cocaine self-administration (*p<0.05; n=19–20/group). **(c)** Mean (± SEM) previously-active lever presses are presented for the cue reactivity test session on FA Day 1 and FA Day 30 from cocaine self-administration. On FA Day 1, WAY163909 at 0.2 mg/kg (n=16; p<0.05) and 1.0 mg/kg (n=19; p<0.05), but not 0.05 mg/kg (n=16; n.s.), suppressed cue reactivity *vs.* vehicle (dotted line). On FA Day 30, WAY163909 at 1.0 mg/kg (n=17; p<0.05), but not 0.05 mg/kg (n=17; n.s.) or 0.2 mg/kg; n=16; n.s.), suppressed cue reactivity *vs.* vehicle (dashed line). **(d)** Mean (± SEM) inactive lever presses are presented for the cue reactivity test session on FA Day 1 and FA Day 30 from cocaine self-administration. Treatment with WAY163909 did not alter inactive lever presses on either FA Day 1 (n.s.) or FA Day 30 (n.s.). **(e)** Mean responses (± SEM) on the active (black circles) or inactive lever (white circles), and total number of sucrose pellets earned (± SEM; gray circles) are presented for the acquisition and maintenance phase of sucrose self-administration. **(f)** Mean (± SEM) previously-active lever presses and inactive lever presses are presented for the cue reactivity

test session on FA Day 1 and FA Day 30 from sucrose self-administration. Cue reactivity is significantly elevated on FA Day 30 *vs.* FA Day 1 from sucrose self-administration (*p<0.05; n=8–10/group).

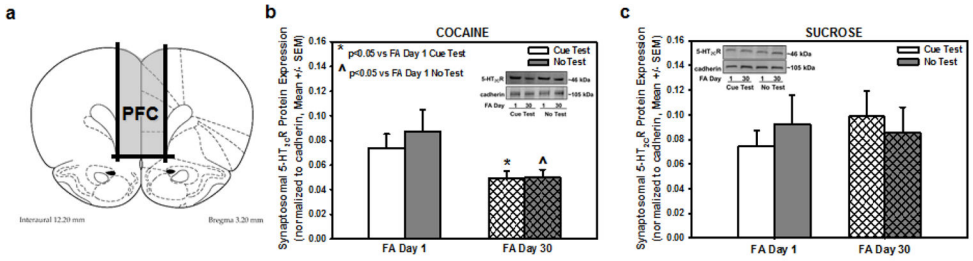

Figure 2. Incubation of cocaine, but not sucrose, cue reactivity associates with lower synaptosomal 5-HT$_{2C}$R protein expression in the mPFC

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

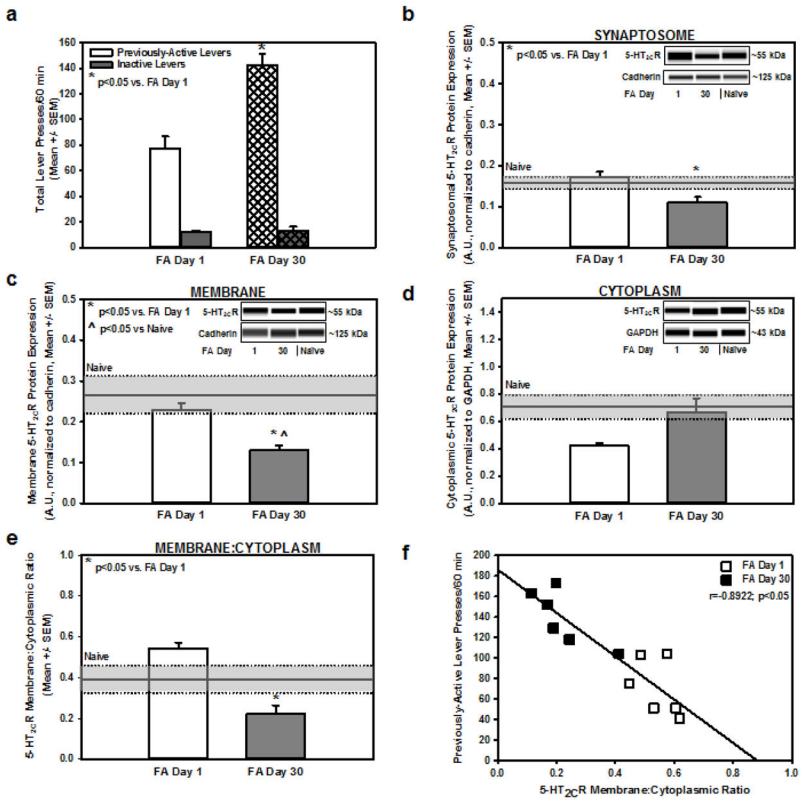

Figure 3. Incubation of cocaine cue reactivity associates with altered subcellular distribution of 5-HT$_{2C}$R protein in the mPFC

(a) Mean (± SEM) previously-active and inactive lever presses are presented for the cue reactivity test session on FA Day 1 and FA Day 30 from cocaine self-administration in the second cohort. Cue reactivity is significantly elevated in vehicle-treated rats on FA Day 30 *vs.* FA Day 1 (*p<0.05; n=10/group). **(b–e)** The horizontal bars represent the mean (solid line) ± SEM (dotted lines and shading) 5-HT$_{2C}$R protein expression in the mPFC of naïve rats. **(b)** Synaptosomal 5-HT$_{2C}$R protein expression (normalized to cadherin) in the mPFC is lower on FA Day 30 *vs.* FA Day 1 from cocaine self-administration (*p<0.05; n=4–6/group); representative electrophoretic bands are located in inset. Synaptosomal 5-HT$_{2C}$R protein expression on FA Day 1 and FA Day 30 did not differ from naïve (n.s.). **(c)** Membrane expression of 5-HT$_{2C}$R (normalized to cadherin) is lower on FA Day 30 *vs.* FA Day 1 (*p<0.05) and lower on FA Day 30 *vs.* naïve (^p<0.05; n=5–6/group); representative electrophoretic bands are located in inset. Membrane 5-HT$_{2C}$R protein expression on FA Day 1 did not differ from naïve (n.s.) **(d)** Cytoplasmic expression of 5-HT$_{2C}$R (normalized to GAPDH) did not differ between FA Day 1 and FA Day 30 or naïve (n.s.); representative electrophoretic bands are located in inset. **(e)** The ratio of membrane to cytoplasmic 5-HT$_{2C}$R expression in the mPFC is lower on FA Day 30 relative to FA Day 1 (*p<0.05), but did not differ from naïve (n.s.). **(f)** There was an inverse correlation between active lever presses in the cue reactivity test session and the membrane:cytoplasmic ratio of 5-HT$_{2C}$R protein expression for individual rats (r=−0.8922; p<0.05).