Effects of the 5-HT2C receptor agonist CP809101 in the amygdala on reinstatement of cocaine-seeking behavior and anxiety-like behavior

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Effects of the 5-HT2C receptor agonist CP809101 in the amygdala on reinstatement of cocaine-seeking behavior and anxiety-like behavior

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Abstract
Serotonin 2C receptor (5-HT2C-R) agonists attenuate reinstatement of cocaine-seeking behavior. These receptors are found throughout the limbic system, including the basolateral amygdala (BlA), which is involved in forming associations between emotional stimuli and environmental cues, and the central amygdala (CeA), which is implicated in the expression of conditioned responding to emotional stimuli. This study investigated whether 5-HT2C-Rs in the amygdala are involved in cue and cocaine-primed reinstatement of cocaine-seeking behavior.

Rats were trained to self-administer cocaine (0.75 mg/kg, i.v.) which was paired with light and tone cues, and then subsequently they underwent daily extinction training. Rats then received bilateral microinfusions of the 5-HT2C-R agonist CP809101 (0.01–1.0 μg/0.2 μl/side) into either the BlA or CeA prior to tests for cue or cocaine-primed (10 mg/kg, i.p.) reinstatement. Rats were also tested for CP809101 effects on anxiety-like behavior on the elevated plus-maze (EPM). Surprisingly, intra-BlA CP809101 had no effect on cue reinstatement, though it did increase anxiety-like behavior on the EPM. Intra-CeA infusions of CP809101 attenuated cocaine-primed reinstatement, an effect that was prevented with concurrent administration of the 5-HT2C-R antagonist SB242084 (0.1 μg/0.2 μl/side). CP809101 had no effect on cue reinstatement or anxiety-like behavior on the EPM. These findings suggest that 5-HT2C-Rs in the BlA modulate anxiety, whereas those in the CeA modulate incentive motivational effects induced by cocaine priming injections.

Key words: Addiction, elevated plus-maze, incentive motivation, relapse, serotonin.

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Introduction
Serotonin 2C receptors (5-HT2C-Rs) modulate cocaine effects. For instance, 5-HT2C-R agonists attenuate cue and cocaine-primed reinstatement of cocaine-seeking behavior (Neisewander and Acosta, 2007; Fletcher et al., 2008; Pentkowski et al., 2010), while 5-HT2C-R antagonists enhance cocaine hyperlocomotion, cocaine-primed reinstatement, and cocaine self-administration (McManus et al., 2001; Fletcher et al., 2002). The effects of 5-HT2C-R agonists and indirect serotonin agonists on cue reinstatement of cocaine seeking are blocked by 5-HT2C-R antagonists, demonstrating 5-HT2C-R mediation (Burmeister et al., 2004; Pentkowski et al., 2010). 5-HT2C-Rs also modulate anxiety as anxiety-like behavior on the elevated plus-maze (EPM) is enhanced in transgenic mice overexpressing 5-HT2C-Rs (Kimura et al., 2009a) but reduced in 5-HT2C-R knockout mice (Heisler et al., 2007). 5-HT2C-Rs are found throughout the mesolimbic dopamine (DA) system (Pompeiano et al., 1994; Doherty and Pickel, 2000), including the basolateral (BlA) and central (CeA) nuclei of the amygdala, key regions in the neurocircuitry of cocaine addiction (O’Dell et al., 1999; See, 2005; Allweieird et al., 2006) and anxiety disorders (LeDoux, 2003). Indeed, the amygdala is involved in emotional learning (Bechera et al., 1995) and memory (Cahill, 2000), including fear conditioning (Blanchard and Blanchard, 1972; Lieblich et al., 1976; Pribram et al., 1979; Davis, 2000), avoidance learning (Weiskrantz, 1956), and appetitive conditioning (Parkinson et al., 2000; Everitt et al., 2003). The ability of cocaine and cocaine-conditioned cues to prime motivation for cocaine relies on memory circuits involving the amygdala (Grant et al., 1996; Everitt et al., 2000). Specifically, the BlA has been implicated in processing and modifying the incentive motivational value of drug-associated contextual and discrete cues (Everitt et al., 1999; Fuchs et al., 2002; McLaughlin and See, 2003). Although the role of
amygdala 5-HT₂CRs in processing the significance of cocaine-associated cues has not been investigated, there is evidence that they mediate anxiety-like behavior (Campbell and Merchant, 2003; de Mello Cruz et al., 2005; Heisler et al., 2007; Kimura et al., 2009b). For instance, intra-BLA 5-HT₂CR agonists potentiate anxiety-like behavior in open-field and social exploration tests (Campbell and Merchant, 2003; Christianson et al., 2010). Further, the anxiogenic effects of a 5-HT₂CR agonist can be reversed by intra-BLA infusion of a 5-HT₂CR antagonist (de Mello Cruz et al., 2005).

The CeA is implicated in the unconditioned effects of drugs of abuse, including reward (O’Dell et al., 1999), reinforcement (Caine et al., 1995), stress, and drug withdrawal (Koob and Nestler, 1997; Koob and Le Moal, 2005). Inactivation of the CeA eliminates stress-primed reinstatement in both conditioned place preference (CPP) (Wang et al., 2002; Ma et al., 2008) and self-administration models (McFarland et al., 2004). Studies also suggest a role for the CeA in responding for cocaine-paired cues (Kruzich and See, 2001; Thiel et al., 2010), though selective inactivation of the CeA does not affect acquisition of (Kruzich and See, 2001), or responding with (Burns et al., 1996; Robledo et al., 1996), conditioned reinforcement. However, the CeA appears to amplify conditioned responses and is likely involved in the ‘incubation effect’ in which cue reinstatements intensify during abstinence (Robledo et al., 1996; Tran-Nguyen et al., 1998; Lu et al., 2005; Li et al., 2008; Thiel et al., 2012). Although the distribution of 5-HT₂CRs in the CeA is not well characterized, it has been shown that 5-HT₂CR knockout mice exhibit lower levels of Fos activation in the CeA following social-defeat stress (Heisler et al., 2007) and 5-HT₂CR agonists increase Fos expression in the CeA (Singewald et al., 2003; Somerville et al., 2007). Furthermore, there is a positive correlation between 5-HT₂CR levels in the CeA and anxiety-like behavior on the EPM (Li et al., 2012); however, other studies failed to find effects of intra-CeA 5-HT₂CR agonists on anxiety-like behavior (Campbell and Merchant, 2003; Christianson et al., 2010).

In the present study, we hypothesized that BIA 5-HT₂CRs contribute to the inhibitory effects of systemic 5-HT₂CR agonists on cue reinstatement of extinguished cocaine-seeking behavior based on the known involvement of the BIA in the incentive motivational effects of cocaine-paired cues and the presence of 5-HT₂CRs in the BIA. We further hypothesized that CeA 5-HT₂CRs mediate the inhibitory effects of systemic 5-HT₂CR agonists on cocaine-primed reinstatement based on the known involvement of the CeA in unconditioned effects of cocaine. To examine these hypotheses, we used the selective 5-HT₂CR agonist CP890101, which has >500-fold selectivity for 5-HT₂CR over other 5-HT₂Rs with EC₅₀ values of 0.11, 153, and 65.3 μM for 5-HT₂A, 5-HT₂A, and 5-HT₂B, respectively (Siuciak et al., 2007; Fletcher et al., 2009). We predicted that intra-BIA CP890101 would attenuate cue reinstatement of cocaine seeking and increase anxiety-like behavior on the EPM, while having no effect on cocaine-primed reinstatement. Conversely, we predicted that intra-CeA CP890101 would attenuate cocaine-primed reinstatement and anxiety-like behavior on the EPM.

Method

Animals

Adult male Sprague–Dawley rats weighing 275–325 g at the start of the experiments were housed in a climate-controlled colony room with a 14h reversed light/dark cycle (lights off at 07:00 hours) 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 d before implanting catheters into the right jugular vein as described by Pockros et al. (2011). During surgery, stainless steel guide cannulae were also implanted to a depth 2.5 mm above the BIA and 2.7 mm above the CeA. The coordinates, selected based on previous research (Fuchs and See, 2002; Christianson et al., 2010; Thiel et al., 2010), were: AP=−2.5, ML=5.0 mm to the left and 4.9 mm to the right relative to bregma, and DV =−8.2 mm from the skull surface for BIA; AP=−2.5, ML=4.2 mm to the left and 4.1 mm to the right relative to bregma, and DV =−8.2 mm from the skull surface for CeA (Paxinos, 2007). The guide cannulae and the metal end of the catheter were secured to the skull and anchor screws using dental acrylic cement. Metal stylets were inserted into the cannulae to maintain patency. Catheters were flushed daily with a solution of 0.1 ml saline containing heparin sodium (70 U/ml; APP Pharmaceuticals, USA) and Timentin (66.7 mg/ml; GlaxoSmithKline, USA). Animals were given at least 7 d 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., USA), which briefly anesthetizes the animal only if delivered i.v.

Intracranial drug infusions

CP809101 (Tocris, USA) was dissolved in artificial cerebral spinal fluid. Microinjections were delivered over a 1 min period using a 30 gauge injector (Plastics One) connected via polyethylene 50 tubing (Becton Dickinson, USA) to a 25 μl syringe (Hamilton Co., USA) housed in an infusion pump (CMA Microdialysis, USA). Movement of an air bubble the proper distance through the drug infusion line confirmed successful drug infusion. After the infusion, the injectors remained
in place for 1 min to ensure thorough diffusion. After removing the injectors, metal styles and caps were replaced before the animal was placed into the conditioning chamber for the test sessions.

Self-administration

Cocaine self-administration training sessions took place for 2 h, 6 d/wk in operant conditioning chambers (28×10×20 cm; Med Associates, 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 reinforcement schedule, the light and tone cues were simultaneously activated, followed 1 s later by 0.1 ml cocaine (0.75 mg/kg, i.v.) infused over 6 s. The infusion pump, light and tone were then inactivated with the onset of the house light which signaled a 20 s time-out 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 d of training, all rats 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, rats began the next sessions on a VR3 schedule. Once rats began on a VR3 schedule and ended on a VR5 schedule, thereafter they began on a VR5 schedule. All rats started on a VR5 schedule by day 14 and were on a VR5 schedule exclusively for at least the last 5 d of self-administration. All rats were initially restricted to 16 g of food to facilitate acquisition of self-administration (Carroll et al., 1981) and remained food-restricted until they ended on a VR5 schedule for three consecutive sessions. After meeting this criterion, food rations were gradually increased to ad libitum access which remained in effect throughout the rest of the experiment.

Extinction

Extinction training began once rats completed at least 15 self-administration sessions and had received food ad libitum for at least the last five sessions. Extinction occurred daily for 1 h/d. Rats were placed into the self-administration chambers and lever presses were recorded, but produced no consequences (i.e. no infusions or cues were presented). Extinction sessions continued for 10–14 d based on the criteria of either an 80% reduction in active lever pressing from the rats’ highest response rate during extinction or less than 20 active lever presses for three consecutive days.

Experiments

Upon meeting the extinction criterion, rats with BlA and CeA cannulae (experiments 1 and 2, respectively) underwent CP809101-primed reinstatement, cue reinstatement, cocaine-primed reinstatement, and EPM testing. Rats received their assigned dose of CP809101 (0, 0.01, 0.1, or 1.0 μg/0.2 μl/side; final n/group=5–8) prior to each type of reinstatement test. In experiment 3, a new cohort of rats with CeA cannulae underwent CP809101-primed reinstatement testing for the effects of CP809101 (0.01 μg/0.2 μl/side; n=6) or SB242082 (0.1 μg/0.2 μl/side; n=5), cocaine-primed reinstatement testing for the effects of CP809101 (0.01 μg/0.2 μl/side, n=6) or CP809101+SB242084 (0.01 and 0.1 μg/0.2 μl/side, respectively, n=5), and a subset of rats underwent EPM testing for the effects of CP809101 (0 and 0.01 μg/0.2 μl/side; n=6–10). Group assignments were counterbalanced for cocaine intake during self-administration.

CP809101-primed reinstatement

Following extinction, rats were assigned to CP809101 dose groups, counterbalanced for cocaine intake during self-administration as this has been shown to affect reinstatement (Deroche et al., 1999; Baker et al., 2001). Rats underwent two tests for CP809101-primed reinstatement of extinguished cocaine-seeking behavior, receiving vehicle 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, rats were tested for 1 h during which responding on either lever had no consequences.

Cue reinstatement

Next, rats were given a minimum of three extinction sessions to restabilize extinction response rates. Then they underwent two tests for cue reinstatement, 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, rats were tested for cue reinstatement for 1 h. During the test the same stimulus complex as that paired with cocaine during self-administration was presented response-contingently on an FR1 schedule; no cocaine was delivered during cue tests. The FR1 schedule was used instead of the VR5 training schedule because we have found that the FR1 schedule yields greater sensitivity for detecting the predicted decrease in responding (Acosta et al., 2008). A noncontingent cue presentation was delivered if the rat did not receive a response-contingent cue within the first 5 min of the test session.

Cocaine-primed reinstatement

After the two cue reinstatement tests, rats received at least three extinction sessions to restabilize baseline extinction response rate. They were then given two tests for cocaine-primed reinstatement. Prior to one test, they received the
same dose of CP809101 as they had received during cue reinstatement testing, and vehicle prior to the other test. The order of the two pretreatments was counterbalanced. Immediately after the microinjection, rats received a priming injection of cocaine (10 mg/kg, i.p.) and were immediately placed into the conditioning chamber. Lever presses were recorded, but produced no consequences. To control for injection stress, rats 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. Rats were given a minimum of three extinction sessions between tests to restabilize baseline response rates.

**Elevated plus-maze**

Following reinstatement testing, rats were tested for the effects of CP809101 on anxiety-like behavior on the EPM at least 5 d after their last CP809101 treatment. Rats received one test on the EPM and were assigned to receive either the same dose of CP809101 from reinstatement testing or vehicle. EPM testing was performed as detailed previously (Pentkowski et al., 2009). Briefly, rats received their assigned microinjection and 5 min later they were individually placed in the center of the apparatus facing one of the two closed arms (File et al., 2004). Behavior was video recorded for 10 min under dim lighting, and later scored using Observer 5.0 software (Noldus Information Technology BV, The Netherlands) by a trained observer blind to group assignment. The behaviors scored were entries and time spent in the open arms, closed arms, and the middle of the maze. Locomotor activity was also analyzed by computer-automated video tracking (Clever Systems, USA). The apparatus was cleaned with 5% ethanol between each test.

**Histology**

Rats were anesthetized with 3% isoflurane, given intracranial infusions (0.2 ml/side) of 1% methylene blue, and then decapitated. Harvested brains were stored at −80 °C and later sliced coronally (40 μm). Sections were stained with cresyl violet and cannula placements were determined under a microscope by observers unaware of group assignment (Fig. 1). Rats with incorrect placements were excluded from the analyses.

**Statistical analyses**

For self-administration/reinstatement, data were analyzed using mixed-factor analyses of variance (ANOVA) 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. A Greenhouse–Geisser correction was used to correct for heterogeneity of variance. 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 days before agonist vs. vehicle testing). For cue and cocaine-primed reinstatement, if rats failed to meet the reinstatement criteria of doubling the extinction baseline response rate and at least twenty responses on the active lever on either of the two test days, they were considered ‘non-reinstaters’ and were excluded from the analysis (see results for details). Outliers were identified and excluded from analyses if they were more than 2 s.d. from the mean.

Percent of time in the open arms of the EPM was analyzed using ANOVAs 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 (Huynh et al., 2011) as follows: Anxiety Index = 1 − [(open arm time/10 min)+(open arm entry/total entries)]/2.
Table 1. The average number of cocaine infusions, and active and inactive lever presses/2 h (mean±S.E.M) during the last five cocaine self-administration training sessions

<table>
<thead>
<tr>
<th>Brain region and CP809101 dose</th>
<th>Cocaine infusions</th>
<th>Active lever presses</th>
<th>Inactive lever presses</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIA 0.01 μg/side</td>
<td>27.2±2.8</td>
<td>163.2±29.1</td>
<td>17.9±11.8</td>
</tr>
<tr>
<td>0.1 μg/side</td>
<td>27.7±4.0</td>
<td>162.4±38.4</td>
<td>14.2±6.2</td>
</tr>
<tr>
<td>1.0 μg/side</td>
<td>28.7±4.5</td>
<td>158.1±32.2</td>
<td>3.7±1.8</td>
</tr>
<tr>
<td>CeA 0.01 μg/side</td>
<td>23.3±2.5</td>
<td>113.8±11.9</td>
<td>2.2±1.2</td>
</tr>
<tr>
<td>0.1 μg/side</td>
<td>27.3±2.5</td>
<td>154.2±24.8</td>
<td>1.9±0.9</td>
</tr>
<tr>
<td>1.0 μg/side</td>
<td>26.6±1.4</td>
<td>130.4±12.1</td>
<td>428.6±425.6</td>
</tr>
<tr>
<td>CeA+SB242084*</td>
<td>26.0±4.2</td>
<td>180.0±78.9</td>
<td>75.7±73.0</td>
</tr>
</tbody>
</table>

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

Results

Cocaine self-administration

Table 1 shows the average number of cocaine infusions, and active and inactive lever presses/2 h (mean±S.E.M) during the last five cocaine self-administration training sessions for each group. While counterbalancing was slightly affected by exclusion at the end of the studies due to misplaced cannulae, there were no group differences in cocaine intake during self-administration.

Inactive lever presses

There were no differences in inactive lever presses between groups for the first or last days of extinction, nor for any reinstatement test in any experiment (Table 2).

Extinction

The ANOVAs of the number of active lever presses/h on the first day of extinction vs. the last day of extinction before testing showed main effects of day ($F_{1,19}=87.18$, $p<0.001$ and $F_{1,20}=90.92$, $p<0.001$) for rats with BLA and CeA cannulae, respectively, but no dose effect or interaction with dose. The main effects indicated a significant drop in responding across training sessions (Table 2).

Effects of CP809101 and SB242084 primes

Figure 2 shows the effects of CP809101 priming injections into the BLA (2a) or CeA (2b), and SB242084 priming injections into the CeA (2c). None of these drug primes altered responding relative to extinction baseline.

Effects of CP809101 on cue reinstatement

Figure 3 shows the effects of CP809101 infusions into the BLA (3a) or CeA (3b) on cue reinstatement. One rat out of 27 tested for BLA infusion effects was excluded for failure to meet the reinstatement criterion. The ANOVAs of responses/h on the active lever showed main effects of test day ($F_{1,30,24.77}=20.51$ for the BLA and $F_{1,33,26.60}=23.78$ for the CeA, $p<0.001$) but no interactions with dose or main effects of dose. There was a trend toward a main effect of dose of intra-CeA CP809101 infusions ($F_{2,30}=3.22$, $p=0.061$), however there were no differences between CP809101 and vehicle test days at any specific dose. Tests of simple main effects indicated that when collapsed across dose, rats in both experiments showed cue reinstatement evident as an increase in responding on both vehicle and CP809101 pretreatment test days relative to the extinction baseline ($p<0.05$). The increase in responding relative to extinction baseline was confirmed for individual dosage groups on both vehicle and drug pretreatment tests using planned comparisons (across both experiments, $t$ values ranged from 2.87 to 10.54, $p<0.05$).

Effects of CP809101 with or without SB242084 on cocaine-primed reinstatement

Figure 4a illustrates the effects of intra-BLA infusions of CP809101 on cocaine-primed reinstatement. Four out of 27 animals were excluded for failure to meet the reinstatement criterion. The ANOVA of responses/h on the active lever indicated a significant main effect of test day ($F_{1,45,24.71}=6.84$, $p<0.01$) 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 ($p<0.05$). The increase in responding relative to extinction baseline was confirmed for individual dosage groups on both vehicle and drug pretreatment tests using planned comparisons ($t$ values ranged from 2.14 to 5.80, $p<0.05$).

Figure 4b illustrates the effects of intra-CeA infusions of CP809101 on cocaine-primed reinstatement. Three out of 23 animals were excluded for failure to meet the reinstatement criterion. The ANOVA of responses/h on the active lever indicated a significant day by dose interaction ($F_{2,34}=4.06$, $p<0.01$) as well as a main effect of test day ($F_{2,34}=12.33$, $p<0.001$). 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 1.0 μg/μl doses ($t$-test, $p<0.05$) and a significant decrease in lever pressing at the 0.01 μg/μl dose of CP809101 compared to vehicle ($t$-test, $p<0.05$). There were no differences on the vehicle test day across dosage groups; however, there was a high amount of variance in these measures. Thus, it is noteworthy that there were no differences between rats that received their vehicle test day first compared to those that received their vehicle test day second.

Figure 4c illustrates the effects of intra-CeA infusions of CP809101 or CP809101+SB242084 on cocaine-primed...
reinstatement. Three rats out of 16 were excluded for failure to meet the reinstatement criterion and another was excluded as an outlier (i.e. more than 2 s.d. above the mean). The ANOVA of responses/h on the active lever indicated a significant day by drug interaction ($F_{2,20}=9.30$, $p<0.01$) as well as a main effect of test day ($F_{2,20}=24.53$, $p<0.001$). Post-hoc comparisons indicated an increase in responding relative to the extinction baseline on all vehicle test days, on CP809101 or CP809101+SB242084 test days (t-test, $p<0.05$), as well as a significant decrease in responding when rats received CP809101 alone compared to vehicle (t-test, $p<0.01$). Unexpectedly, there was a significant difference between vehicle test days for animals that received CP809101 compared to those that received CP809101+SB242084 (t-test, $p<0.05$).

**Effects of CP809101 on the elevated plus-maze behavior**

Figure 5a shows the effects of CP809101 infused into the BlA on the percent of time spent on the open arms of the EPM. The ANOVA showed no significant effect of CP809101 dose, however planned comparisons indicated a significant difference between the vehicle and 1.0 $\mu$g/ml groups ($p<0.05$). CP809101 infused into the BlA also increased the 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 anxiety index score ANOVA showed no significant effect of CP809101 dose ($F_{3,18}=1.81$, $p=0.18$); however, planned comparisons indicated a trend between the vehicle and 1.0 $\mu$g/ml groups ($p=0.052$).

Figure 5b shows the effects of CP809101 infused into CeA on the percent of time on the open arms of the EPM. The ANOVA showed no significant effect of CP809101 dose, although planned comparisons indicated a trend toward a difference between the vehicle and 0.1 $\mu$g/ml groups ($p=0.071$). CP809101 infused into the CeA had no effect on the anxiety index score ($F_{3,18}=0.67$, $p=0.582$).

**Effects of CP809101 on locomotor activity**

CP809101 infusion into the BlA or CeA had no effect on locomotor activity during EPM testing. The ANOVAs of total distance traveled failed to show a significant effect of dose for either BlA ($F_{3,13}=0.82$, $p=0.51$) or CeA ($F_{3,22}=0.40$, $p=0.76$) infusions.

**Discussion**

Results supported our hypothesis that 5-HT$_{2CR}$Rs in the BlA are involved in anxiety-like behavior, whereas those in the CeA are involved in cocaine-primed reinstatement. The 5-HT$_{2CR}$R agonist, CP809101, infused into the BlA, increased anxiety-like behavior on the EPM, effects consistent with findings that intra-BlA infusions of 5-HT$_{2CR}$R agonists, including CP809101, increase anxiety-like behavior in other paradigms (Campbell and Merchant, 2003; Christianson et al., 2010; Li et al., 2012). Intra-CeA infusions of CP809101 also consistently attenuated cocaine-primed reinstatement at the lowest dose (Fig. 4b, c), and this effect was reversed with co-administration of a 5-HT$_{2CR}$R antagonist (Fig. 4c), indicating 5-HT$_{2CR}$R-mediation. CP809101 infusions into the BlA or CeA had no effect on locomotor activity, suggesting that the anxiogenic effects of intra-BlA infusions were not great enough to interfere with locomotion, nor were the decreases in cocaine-seeking behavior likely due to nonspecific motor effects.

Surprisingly, we found no support for our hypothesis that activating 5-HT$_{2CR}$Rs in the BlA would inhibit cue reinstatement. This hypothesis was based on previous findings that the BlA plays a role in cue reinstatement.
(Everitt et al., 1999; Fuchs and See, 2002; Alleweireldt et al., 2006), that there is a dense population of 5-HT2C receptors in this region (Clemett et al., 2000), and that systemic administration of 5-HT2C agonists attenuates cue reinstatement (Higgins and Fletcher, 2003; Neisewander and Acosta, 2007; Burbasi and Cervo, 2008). It is possible that doses of CP809101 other than those tested may attenuate cue reinstatement; however, we observed effects at both the lowest and highest doses on other behavior, which mitigates this explanation. Since intra-BlA CP809101 increased anxiety-like behavior on the EPM and acute anxiety increases cue reinstatement (Feltenstein et al., 2011), opposing effects of CP809101 on cue incentive motivation and anxiety may have resulted in a null effect.

Interestingly, intra-CeA CP809101 effects on cocaine-primed reinstatement were only seen at the lowest dose. If we had tested lower doses of CP809101, we would have likely observed an inverted U-shaped dose–response effect, which is not uncommon (Davis, 1990; Neisewander...
et al., 1995). The lack of effect on cocaine-primed reinstatement at higher CP809101 doses may be due to non-specific effects, perhaps at 5-HT2ARs. Indeed, 5-HT2AR agonists are thought to play an opposite role in cocaine seeking from 5-HT2CR agonists (Fletcher et al., 2002; Burmeister et al., 2004; Nic Dhonnchadha et al., 2009). CP809101 is highly selective for 5-HT2CRs over 5-HT2A and 5-HT2BRs (Barnes and Sharp, 1999; Siuciak et al., 2007; Fletcher et al., 2009; Fletcher et al., 2010), however nonspecific effects may occur with administration directly into the CeA, especially given the relatively low 5-HT2CR density in this region (Clemett et al., 2000; Li et al., 2012). At high concentrations, CP809101 act as a partial agonist at 5-HT2ARs (Siuciak et al., 2007). Thus it is possible that the high intra-CeA dose causes some 5-HT2AR agonism that opposes the 5-HT2CR agonism effect, resulting in a lack of effect. In any case, the effect of the lowest CP809101 dose in the CeA on cocaine-primed reinstatement was replicated and blocked with co-administration of a 5-HT2CR antagonist, providing strong evidence for a 5-HT2CR-mediated effect.

Fig. 4. Effects of CP809101 infusions into the basolateral amygdala (BLA) (a) or central amygdala (CeA) (b) or CP809101 and/or SB242084 infusions into the CeA (c) on cocaine-primed reinstatement, expressed as the mean number of active lever presses (±SEM) during the 1-h test session. Rats assigned to receive 0.01 (n=6–7), 0.1 (n=7–8) or 1.0 (n=7–8) µg/0.2 µl/side CP809101 or 0.01 µg/0.2 µl/side CP809101+0.1 µg/0.2 µl/side SB242084 (n=5) 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 rats received the cocaine prime (10 mg/kg, i.p.), after which they were 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, planned comparisons, p<0.05. The plus sign (+) represents a significant difference from vehicle test day, p<0.05. The pound sign (#) in graph c represents a significant difference from vehicle test days in the CP and CP+SB groups, p<0.05.

Fig. 5. Effects of CP809101 infusions into the basolateral amygdala (BLA) (a) or central amygdala (CeA) (b) on the percent of time spent on the open arms of the elevated plus-maze (EPM) (±SEM) during the 10-min test session. Rats assigned to receive vehicle (n=3–10), 0.01 (n=7–8), 0.1 (n=4–7) or 1.0 (n=6–8) µg/0.2 µl/side CP809101 into the BLA or CeA were given one 10-min test on the EPM. These pretreatments were infused 5-min before rats were placed on the center of the EPM. The plus sign (+) indicates a significant difference from the vehicle group, planned comparison, (p<0.05).
There was an unusual amount of variability in responses during intra-CeA vehicle tests for cocaine-primed reinstatement. When CP809101 was injected into the CeA in experiment 3, the lowest dose attenuated cocaine-primed reinstatement consistent with the findings in experiment 2; however, we observed unusually high responding on the vehicle test day in experiment 3 compared to experiment 2. Statistically, there were no significant differences in vehicle tests across the groups in experiment 2. We also did not observe an order effect between rats that had their vehicle test first compared to second. In experiment 3, we did find a significant difference between vehicle groups, and although there was again no significant order effect detected, likely due to low n/condition, we suspect that order of drug vs. vehicle contributed to the high variance in response rates. 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 between-group variance in responding on vehicle test days in experiments 2 and 3 is curious, it does not detract from the reliability of our within-subjects comparisons showing that 0.01 μg/0.2 μl CP809101 infusions into the CeA attenuated cocaine-primed reinstatement relative to vehicle, and this effect was not seen with the addition of a 5-HT2C-R antagonist. There was an inconsistency in that the between group analyses revealed no effect of CP809101, however the within-subjects comparison showed that the lowest dose of CP809101 significantly decreased cocaine-seeking behavior relative to vehicle, while CP809101 + SB242084 had no effect. The lack of consistency of an antagonist reversal effect across these two types of comparisons is likely due to the high amount of variance in vehicle response rates. Given the variance and n/group in this experiment, it stands to reason that only the within-subjects comparison indicates an antagonist effect because it is a more powerful test than the between-group comparison.

Anatomical specificity and potential damage from repeated drug infusions are important to consider when using intracranial drug administration. The region specific effects of the BlA and CeA infusions mitigates effects of drug spread since these regions border each other. Furthermore, rats underwent cocaine-primed reinstatement and EPM testing as their last tests, yet we observed an effect of intra-BlA CP809101 on this test, suggesting that tissue damage was not likely. Moreover, histological verification of cannula placements showed no evidence of excessive tissue damage from repeated infusions.

We targeted the rostral part of the BlA. The rostral (rBlA) and caudal (cBlA) subsections of the BlA have divergent projections. The rBlA projects to the nucleus accumbens (NAc) core and dorsal agranular insular cortex, whereas the cBlA projects to the NAc shell and prelimbic cortex (Groenewegen et al., 1990; Kita and Kitai, 1990; Shinonaga et al., 1994). Inactivation of the rBlA using excitotoxic lesions, tetrodotoxin (TTX) or lidocaine consistently disrupts cue reinstatement (Meil and See, 1997; Grimm and See, 2000; Kantak et al., 2002), while cBlA lidocaine inactivation reduces drug-seeking behavior during cocaine self-administration on a second order schedule (Kantak et al., 2002). Other studies have found effects of pharmaceutical manipulations in the cBlA on both cue and cocaine-primed reinstatement (Alleweireldt et al., 2006; Berglind et al., 2006), and in the rBlA on cue reinstatement (Alleweireldt et al., 2006; Mashhoon et al., 2009). Thus, if 5-HT2C-Rs in the BlA are involved in cue reinstatement, it seems likely that we would have observed an effect with intra-rBlA infusion of CP809101.

Based on previous literature, we expected CP809101 in the BlA, but not the CeA, to affect anxiety-like behavior. Our results support this prediction, as the slight increase in anxiety-like behavior from intra-CeA CP809101 was not statistically significant. Together with the inconsistencies among previous studies examining 5-HT2C-Rs in the CeA on anxiety-like behavior (Campbell and Merchant, 2003; Heisler et al., 2007; Christianson et al., 2010; Li et al., 2012), our results suggest that 5-HT2C-Rs in this region may play a less prominent role in anxiety than those in the BlA. Accordingly, Campbell and Merchant (2003) found that a 5-HT2C-R agonist, mCPP, in the CeA did not affect ultrasonic vocalizations or exploratory behavior on a novel-object task, though in the BlA mCPP increased anxiety-like behaviors during this task. Similarly, Christianson et al. (2010) found that a 5-HT2C-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.

We hypothesize that the mechanism underlying the effects of CP809101 in the BlA on anxiety-like behavior and in the CeA on cocaine-primed reinstatement involves modulation of GABA release. In the BlA, 5-HT2C-R agonists activate GABA interneurons at low doses (Stein et al., 2000), however it is hypothesized that high doses of 5-HT2C-R agonists activate excitatory pyramidal projection neurons (Rainnie, 1999), which overshadows GABA activation leading to the anxiogenic effects of 5-HT2C-R agonists (Campbell and Merchant, 2003). 5-HT2C-R-induced GABA activation may inhibit GABA interneurons that inhibit projection neurons, resulting in net disinhibition and anxiety-like behavior. To our knowledge, the cellular localization of 5-HT2C-Rs in the CeA is unknown, however they may be located on GABA interneurons and thus 5-HT2C-R agonists in this region would increase GABA inhibition. Consistent with this idea, GABA receptor agonists in the CeA attenuate footshock-primed reinstatement of cocaine seeking (McFarland...
5-HT2CR agonists have potential as treatments for cocaine-seeking behavior and support the idea that approaches that alter 5-HT2CR expression. These findings are the first to show that 5-HT2CRs in the CeA attenuate cocaine-seeking behavior and support the idea that 5-HT2CR agonists have potential as treatments for cocaine addiction.

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Statement of Interest

None.

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