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The Dorsal Agranular Insular Cortex Regulates the Cued Reinstatement of Cocaine-Seeking, but not Food-Seeking, Behavior in Rats

Caitlin V Cosme*[,1], Andrea L Gutman[1] and Ryan T LaLumiere[1,2]

[1]Department of Psychology, University of Iowa, Iowa City, IA, USA; [2]Interdisciplinary Graduate Program in Neuroscience, University of Iowa, Iowa City, IA, USA

Prior studies suggest that the insular cortex (IC), and particularly its posterior region (the PIc), is involved in nicotine craving and relapse in humans and rodents. The present experiments were conducted to determine whether the IC and its different subregions regulate relapse to cocaine-seeking behavior in rats. To address this issue, male Sprague–Dawley rats underwent cocaine self-administration followed by extinction training and reinstatement tests. Before each reinstatement, the PIc or the more anterior dorsal agranular IC (AId) was inactivated to determine their roles in the reinstatement to cocaine seeking. In contrast to the nicotine findings, PIc inactivation had no effect on cue-induced reinstatement for cocaine seeking. However, AId inactivation reduced cued reinstatement while having no effect on cocaine-prime reinstatement. AId inactivation had no effect on reinstatement of food-seeking behavior induced by cues, a food-prime, or cues+food-prime. Based on previous work hypothesizing a role for corticotropin-releasing factor (CRF) in the IC during craving and relapse, a subsequent experiment found that CRF receptor-1 (CRF1) blockade in the AId similarly reduced cued reinstatement. Our results suggest that the AId, along with CRF1 receptors in this region, regulates reinstatement to cocaine seeking, but not food seeking, depending on the type of reinstatement, whereas PIc activity does not influence cue-induced reinstatement.
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Studies examining the reinstatement of cocaine-seeking behavior have found that the medial prefrontal cortex (PFC) is a critical driver of such behavior (LaLumiere *et al*, 2012; McFarland *et al*, 2003), yet considerably less attention has focused on the roles of the lateral PFC in regulating cocaine seeking. However, recent work suggests that the insular cortex (IC), a region in the lateral PFC, may be critically involved in craving and relapse (Naqvi and Bechara, 2010). Human neuroimaging studies have consistently found that drug-associated cues elicit IC activity in participants across multiple types of drug addiction (Brody *et al*, 2002; Kilts *et al*, 2004; Myrick *et al*, 2004). These observations led to a study demonstrating that insula lesions in humans produce significant disruption in nicotine addiction (Naqvi *et al*, 2007), a finding that has been confirmed in subsequent research (Gaznick *et al*, 2014) and has led to increased attention to this region with regard to its role in addiction.

Experiments using rodent models indicate that reversible inactivation of an IC subregion known as the posterior IC (PIc; also known as the granular IC), as well as electrical stimulation of the IC, reduces both nicotine self-administration and reinstatement in rats (Forget *et al*, 2010; Pushparaj *et al*, 2013). In contrast, the more anterior subregions of the IC, including the anterior dorsal agranular IC (AId), appear to drive amphetamine place preference (Contreras *et al*, 2012). Although the role of the IC has not been extensively investigated with regard to cocaine-seeking behavior, prior work has found that cocaine self-administration increases expression levels of the plasticity-associated gene *Arc*, notably, in the AId (Zavala *et al*, 2008). Moreover, the AId innervates the nucleus accumbens (NA) core, a structure known to regulate cocaine seeking in rats, supporting a potential role for the AId in cocaine-seeking behavior (McFarland *et al*, 2003; Voorn *et al*, 2004). Indeed, previous work found that AId inactivation reduces cocaine seeking during a reinstatement test in which a contextual odor stimulus associated with cocaine was presented with a conditioned light cue (Di Pietro *et al*, 2006). In contrast, recent work found that lesions of the anterior portion of the IC, including the AId, potentiated cocaine-seeking behaviors when rats underwent forced abstinence and were then reintroduced to the cocaine-seeking context (Pelloux *et al*, 2013), leaving the role of the IC in the reinstatement of cocaine seeking unclear.

It has been argued that the IC regulates relapse to drug use owing to its role in mediating interoceptive cues (Naqvi *et al*, 2014). A potential key mediator of these interoceptive cues

*Correspondence: CV Cosme, Department of Psychology, University of Iowa, E11 Seashore Hall, Iowa City, Iowa 52242, USA, Tel: +1 319 335 2404, Fax: +1 319 335 0191, E-mail: caitlin-cosme@uiowa.edu
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within the IC is corticotropin-releasing factor (CRF; Naqvi and Bechara, 2009), which is expressed throughout the cortex and at relatively high levels in the IC (Sanchez *et al*, 1999; Van Pett *et al*, 2000). Indeed, evidence suggests that the central CRF system has a critical role in driving drug addiction and relapse (Koob, 2013; Zorrilla *et al*, 2014). Nonetheless, despite the potential significance of this issue, the role of the IC, including its different subregions and CRF1 (CRF receptor-1) receptors, has not been extensively examined in the reinstatement of cocaine-seeking behavior. Therefore, the present study investigated whether these two subregions of the IC, the AId and PIc, regulate cue-induced reinstatement, as well as whether blocking CRF1 receptors in the AId influences cocaine-seeking behavior during reinstatement.

MATERIALS AND METHODS

Subjects

Male Sprague–Dawley rats (250–275 g at time of arrival; Charles River Laboratories; $n = 82$) were single-housed on a 12-h reverse light cycle (and kept at a constant temperature) with food and water *ad libitum* in an AAALAC-approved vivarium. All animals were given at least 5 days of acclimation before undergoing surgery. All procedures were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by University of Iowa Institutional Animal Care and Use Committee.

Surgery

Rats were anesthetized with ketamine (100 mg/kg, i.m.) and xylazine (6 mg/kg, i.m.). Ketorolac (3 mg/kg) was given as an analgesic on the day of surgery and the day immediately following surgery. For catheter implantation for cocaine self-administration, a 13-cm piece of Silastic tubing was threaded under the skin from the back to the ventral side of the rat and inserted into the right jugular vein. A silicone ball affixed 4 cm from the end of the catheter served as a stopping point for insertion. Catheters were secured using silk sutures. The opposite end of the catheter was externalized through a small hole in the skin between the animal's shoulder blades. The externalized end was connected to a 22-gauge guide cannula that was secured in the middle of a harness that was looped around the rat's forelimbs.

For both cocaine and food self-administration experiments, the rats were then placed in a small animal stereotaxic instrument (Kopf Instruments, Tujunga, CA). Jewelers screws were affixed to the skull surface. Bilateral cannulae (Plastics One, Roanoke, VA) were implanted and secured with dental cement, aimed at the PIc and AId with coordinates as follows: AId: 3.1 mm anterior to and 4.2 mm lateral from Bregma and 4.4 mm ventral from the skull surface (cannula aimed 2 mm above the AId); PIc: 0.5 mm posterior to and 6.0 mm lateral from Bregma and 3.9 mm ventral from skull surface (cannula aimed 3 mm above the PIc). The coordinates were developed based on prior work (Contreras *et al*, 2012; Di Pietro *et al*, 2006) and refined in our laboratory. Following surgery, all animals received 3 ml sterile saline subcutaneously and a topical application of the local anesthetic bupivacaine to both the animal's head and chest. Obdurators were placed in all cannulae and maintained throughout reinstatement testing. Rats were then returned to their home cages and permitted to recover for 5–7 days. During this time, catheters were flushed daily with 0.1 ml of heparinized saline (100 USP) to ensure catheter patency and 0.1 ml of cefazolin (100 mg/ml) to reduce the opportunity for infection.

Cocaine Self-Administration and Extinction

All self-administration experiments occurred in standard operant chambers (Med Associates, Fairfield, VT) that contained two retractable levers, a house light, a cue light, and a tone generator (4500 Hz). Rats were food deprived 24 h before a 15-h overnight food-training session, during which each active lever press resulted in a single food pellet (45 mg) on a fixed ratio 1 (FR1) schedule. After food training, rats were given ~20 g of food daily, which was maintained throughout all training and testing. Before the start of self-administration, all animals had their catheters checked for patency using 0.1 ml of sodium brevital (1 mg/ml).

One day after food training, self-administration began. Rats underwent 2-h self-administration sessions where presses on the active lever produced a single infusion of cocaine (50 μl infusion of 200 μg cocaine dissolved in sterile saline, given over 2.18 s; cocaine kindly provided by NIDA) and a 5-s light and tone cue on an FR1 schedule. A 20-s timeout period followed each infusion. Inactive lever presses had no consequence. Rats underwent daily self-administration 6 days per week for a minimum of 12 days. In order to move into extinction, rats were required to take at least 10 infusions of cocaine per day for at least 10 days, including the last 3 days of self-administration, and demonstrate discrimination between the active and inactive lever. During extinction, active lever presses did not produce cocaine infusions or the light and tone cues. Rats' lever pressing was extinguished for a minimum of 7 days and rats only began reinstatement testing if they had 28 or fewer active lever presses for at least two consecutive days immediately before the reinstatement session. The final 2 days of extinction training before each reinstatement session served as the extinction baseline.

Food Self-Administration and Extinction

Following surgery and recovery, rats were given 20 g rat chow per day following each self-administration, extinction, and reinstatement session. The food-seeking experiments followed previous protocols established by McFarland and Kalivas, 2001. Initially, active lever presses produced a single food pellet (45 mg; BioServ) on an FR1 schedule along with the same light/tone cues used in the cocaine self-administration studies. As training progressed, the reinforcement schedule, including both the food pellet and the cues, increased to FR3 and then FR5. This schedule of reinforcement was used to help ensure robust reinstatement, as established by McFarland and Kalivas, 2001. Rats were required to receive 100 pellets per day for at least 3 days before moving onto the next schedule. Extinction procedures began when these criteria were achieved on the FR5

schedule. Rats' active lever pressing was considered extinguished once active lever presses reached $<10\%$ of the active lever presses achieved on the final day of self-administration for two continuous days, with a minimum of 7 days of extinction training.

Microinjections

Intra-AId or intra-PIc microinjections were given before each reinstatement test. Microinjectors (with 2 and 3 mm projections for the AId and PIc, respectively) were connected to PE20 tubing, which was attached to 10-µl Hamilton syringes controlled by an infusion pump. The microinjections were 0.2 µl/side, given at a rate of 0.3 µl/min. Following each microinjection, injectors were left in position for 1 min to allow for diffusion. Immediately following the microinjection, rats were placed into the operant chamber for their appropriate reinstatement test. Microinjected drugs consisted of the $GABA_{B/A}$ receptor agonists baclofen and muscimol (BM; given as a cocktail at 1 and 0.1 mM, respectively), dissolved in artificial cerebrospinal fluid (aCSF) as the vehicle, or the CRF1 antagonist antalarmin (6.0 mM), dissolved in a 70% DMSO/30% aCSF solution as the vehicle. Doses of drugs were chosen based on previous studies (Blacktop *et al*, 2011; LaLumiere *et al*, 2012).

Reinstatement Testing

Each reinstatement test lasted 2 h and, during the reinstatement session, active lever presses never produced a cocaine infusion. Between reinstatement tests, lever pressing was re-extinguished to baseline for a minimum of 3 days using the same criteria described above. For all reinstatement tests, microinjections occurred immediately before testing. For cued reinstatement for either cocaine seeking or food seeking, active lever presses produced the light and tone cues that were previously paired with the drug infusion or delivery of food pellet during self-administration. The cued reinstatement of food seeking was performed on the same FR1 schedule that the rats engaged in cued reinstatement of cocaine seeking used. The cocaine-prime reinstatement used in Experiment 1 consisted of an injection of cocaine (10 mg/kg, i.p.) immediately before the reinstatement session. For food-prime reinstatement, two pellets were placed in the food hopper before the start of the session, and for the first 30 min of the session a single pellet was non-contingently dispensed into the hopper every 2 min, following previously published procedures (McFarland and Kalivas, 2001). The remaining 90 min of the food-prime reinstatement session was a standard extinction session. During the duration of the food-prime reinstatement session, active lever presses had no consequence. The cue+food-prime reinstatement combined both sets of procedures described above.

Experiment 1. In the first experiment, the AId was inactivated before the cued reinstatement testing described above via BM microinjections. To determine whether AId inactivation had any effect alone, a subset of rats that underwent the cued reinstatement also underwent an inactivation-alone test in which the AId was inactivated before a standard extinction session. A separate group of rats received BM microinjections into the AId before a cocaine-prime reinstatement.

Experiment 2. As previous work has identified the PIc as a critical subregion of the IC for mediating nicotine craving and relapse (Forget *et al*, 2010; Pushparaj *et al*, 2013), the second experiment examined whether the cued reinstatement findings from Experiment 1 extended to the PIc. Before the reinstatement testing, rats received BM microinjections into the PIc to inactivate the region.

Experiment 3. As prior work has suggested that CRF within the IC may be involved in addiction processes (Naqvi and Bechara, 2009), Experiment 3 examined whether CRF1 receptor blockade in the AId alters the reinstatement of cocaine-seeking behavior. Therefore, rats received intra-AId microinjections of either the CRF1 receptor antagonist antalarmin or vehicle immediately before undergoing cued reinstatement.

Experiment 4. In order to determine whether the AId has a similar role in the reinstatement of food-seeking behavior, the AId was inactivated via BM microinjections immediately before cued, food-prime, or cue+food-prime reinstatement. In this case, all rats underwent all three reinstatement tests in the described order.

Histological Analysis

Rats were overdosed with sodium pentobarbital (100 mg/kg, i.p.) and intracardially perfused using phosphate-buffered saline. All brains were placed in 3.7% formaldehyde for a minimum of 24 h. Coronal slices (75 µm thick) were taken and mounted onto gelatin-coated slides. Sections were stained with Cresyl violet and each animal was analyzed for accurate placement of microinjector termination points. Data from any rat whose injection tracts terminated outside the borders of the AId or PIc were excluded from analysis.

Data Analysis

Reinstatement lever pressing data were analyzed using two-way analyses of variance (ANOVA) with both comparisons as repeated measures (extinction *vs* reinstatement; aCSF *vs* drug). *Post hoc* analysis was completed using Holm–Sidak's multiple comparison tests. *P*-values <0.05 were considered significant. All measures were expressed as mean $\pm$ SEM. Each group's *n* is indicated in the figure.

RESULTS

Out of a total of 82 rats used in the present experiments, 44 rats were included in the final data. Rats were excluded due to misplaced (15 rats) or unverifiable microinjection termination locations (11 rats), failure to acquire self-administration (1 rat), clogged cannula (3 rats), and illness or death of rat before completion of reinstatement testing (8 rats). When determining the termination of microinjector tips, conservative criteria were used and any rats in which one or both injector tracts were not clearly visible were excluded, which resulted in the relatively high number of rats excluded due to misplaced or unverifiable microinjection locations. Figure 1a shows the number of active and inactive lever presses and cocaine infusions over the final 12 days of cocaine self-administration. Figure 1b shows the number of active and inactive lever presses and food pellets received over the final 12 days of food self-administration. Figure 1c and d

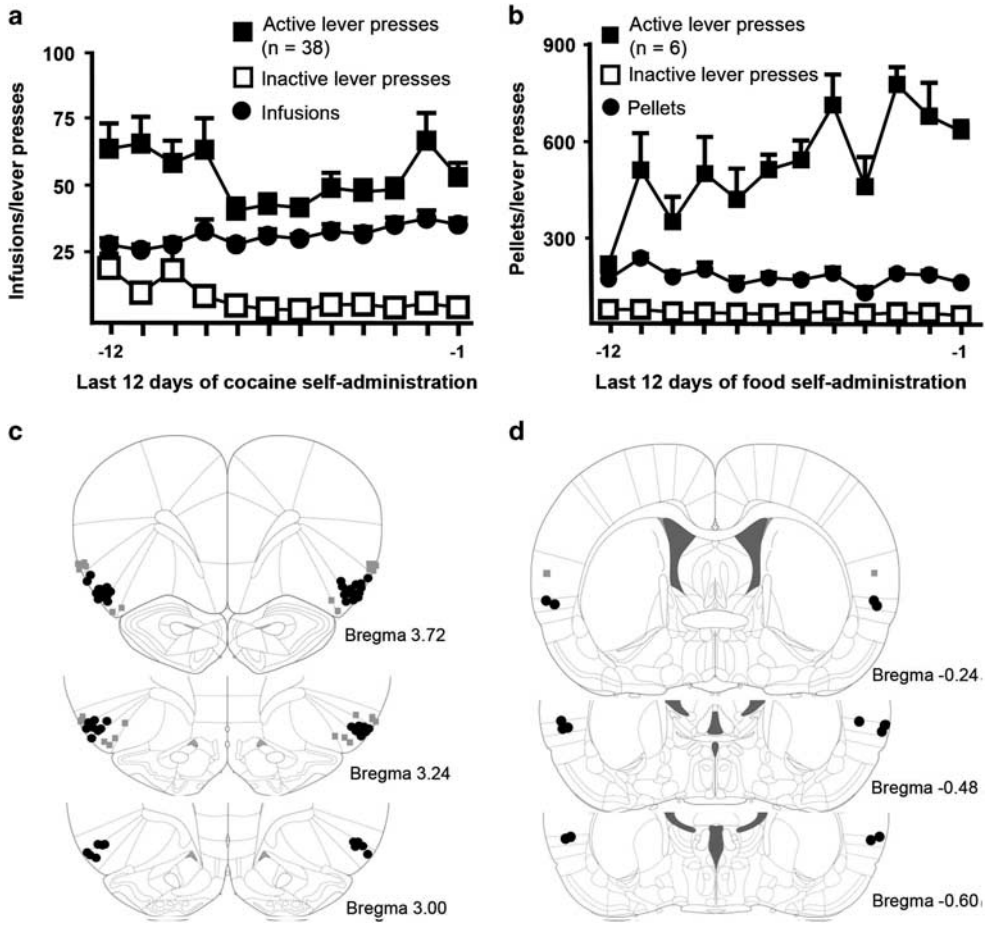

Figure 1 (a) Number of active and inactive lever presses and cocaine infusions for the last 12 days of cocaine self-administration for all rats included in the final analysis. (b) Number of active and inactive lever presses and food pellets for the last 12 days of food self-administration for all rats included in the final analysis. (c, d) Diagrams showing the termination of needle tracks for microinjections aimed at the AId and PIc, respectively. Black circles indicate correct placements. Gray squares indicate incorrect placements. Figures are adapted from Paxinos and Watson (2007), and A/P coordinates (in mm) are given relative to Bregma.

shows the location of the microinjector tips, both correctly and incorrectly placed, in the AId and PIc, respectively.

Experiment 1. AId Inactivation Reduces Cued Reinstatement of Cocaine-Seeking Behavior

Figure 2 shows the active and inactive lever presses (Figure 2a–c and Figure 2d–f, respectively) across the different reinstatements examined in Experiment 1 in which the AId was inactivated before the reinstatement tests. AId inactivation significantly reduced active lever presses for cued reinstatement (Figure 2a). Because two rats had cued reinstatement data that were more than two standard deviations beyond the mean (one for aCSF and one for BM), they were excluded from the cued reinstatement analysis. A two-way repeated measures ANOVA of active lever presses indicated a significant effect of reinstatement ($F_{(1,9)} = 15.91$, $P < 0.01$), a significant effect of microinjection ($F_{(1,9)} = 10.40$, $P < 0.05$), and a significant interaction ($F_{(1,9)} = 12.57$, $P < 0.01$). *Post hoc* tests revealed that although both treatment groups showed increased active lever pressing during reinstatement compared with the extinction baselines ($P < 0.05$), the BM-treated group had significantly fewer active lever presses compared with the aCSF-treated group ($P < 0.05$). AId inactivation alone (Figure 2b), when given before an extinction session, had no effect on active lever pressing. A two-way repeated measures ANOVA of active lever presses indicated no effect of reinstatement ($F_{(1,5)} < 1$, $P > 0.05$), no effect of microinjection ($F_{(1,5)} = 2.304$, $P > 0.05$), and no interaction ($F_{(1,5)} < 1$, $P > 0.05$). Figure 2c shows the active and inactive lever presses from the rats that underwent the cocaine-prime reinstatement. A two-way repeated measures ANOVA of active lever presses indicated a significant effect of reinstatement ($F_{(1,12)} = 18.65$, $P < 0.01$), no effect of microinjection ($F_{(1,12)} < 1$, $P > 0.05$), and no interaction ($F_{(1,12)} < 1$, $P > 0.05$). Both BM- and vehicle-treated rats had significant reinstatement compared with their extinction baseline ($P < 0.01$). There were no significant differences in inactive lever pressing across any of the reinstatements.

Experiment 2. PIc Inactivation has no Effect on Cued Reinstatement of Cocaine-Seeking Behavior

Figure 3 shows the active and inactive lever presses (panels Figure 3a and b, respectively) for rats with PIc inactivation during cue-induced reinstatement. Rats treated with vehicle

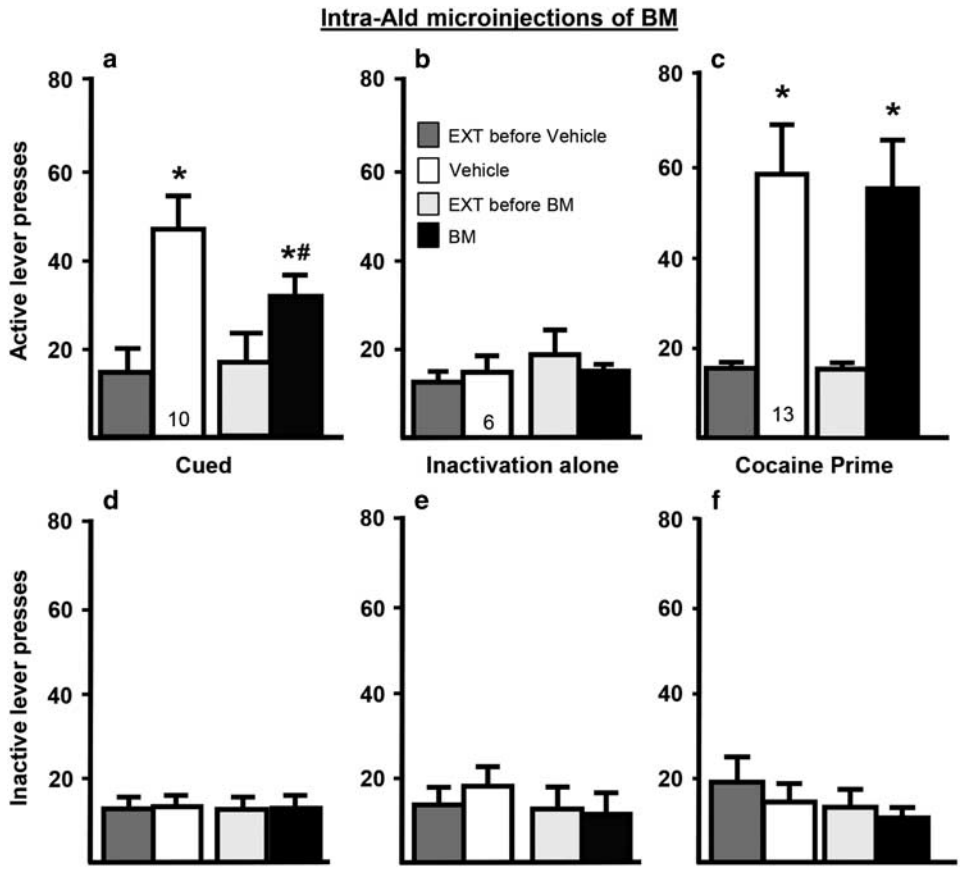

Figure 2 AId inactivation, via baclofen/muscimol (BM; 0.1 and 1.0 mM, respectively) microinjections, regulates cued reinstatement to cocaine-seeking behavior. (a–c) The active lever presses and (d–f) the inactive lever presses across the different tests for Experiment 1 are shown. (a) Intra-AId microinjections of BM significantly reduced active lever pressing during cued reinstatement compared with vehicle–controls. (b) Intra-AId microinjections of BM had no effect on active lever presses when given alone before a standard extinction session in a subset of rats used in a. (c) Intra-AId microinjections of BM had no effect on active lever presses when given before a cocaine-prime reinstatement test. (d–f) There were no effects of reinstatement or microinjections on inactive lever presses across any of the tests. $^{*}P<0.05$ compared with extinction baseline. $^{\#}P<0.05$ compared with vehicle–control group. EXT, extinction baseline.

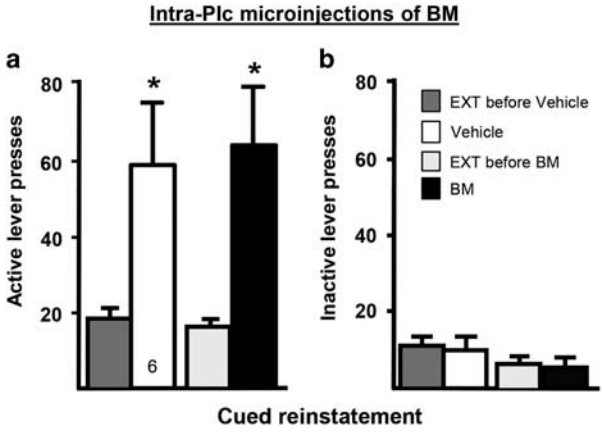

Figure 3 PIc inactivation, via baclofen/muscimol (BM; 0.1 and 1.0 mM, respectively) microinjections, has no effect on cued reinstatement of cocaine-seeking behavior. (a, b) Active and inactive lever presses, respectively, for Experiment 2. Intra-PIc microinjections of BM had no effect on active lever presses during cued reinstatement compared with vehicle–control injections. There were no effects of reinstatement or microinjections on inactive lever presses. $^{*}P<0.05$ compared with extinction baseline. EXT, extinction baseline.

or BM showed equivalent levels of active lever presses (Figure 3a). A two-way repeated measures ANOVA of active lever presses indicated a significant effect of reinstatement ($F_{(1,5)}=8.209$, $P<0.05$), no effect of microinjection ($F_{(1,5)}<1$, $P>0.05$), and no interaction ($F_{(1,5)}=1.192$, $P>0.05$). *Post hoc* tests found significant differences between the extinction baseline and both the vehicle- and BM-treated rats ($P<0.05$). Active lever pressing for the BM group was not significantly different from the vehicle group. There were no significant differences in inactive lever presses for any of the reinstatement tests.

Experiment 3. Blockade of Intra-AId CRF1 Receptors Reduces Cued Reinstatement of Cocaine-Seeking Behavior

Because the results of Experiments 1 and 2 indicated that the AId, but not the PIc, regulated reinstatement of cocaine seeking, Experiment 3 examined the role of the CRF1 receptors in the AId only. Figure 4 shows the active and inactive lever presses (Figure 4a and b, respectively) during cue-induced reinstatement, in which intra-AId microinjections of the CRF1 receptor antagonist antalarmin were given

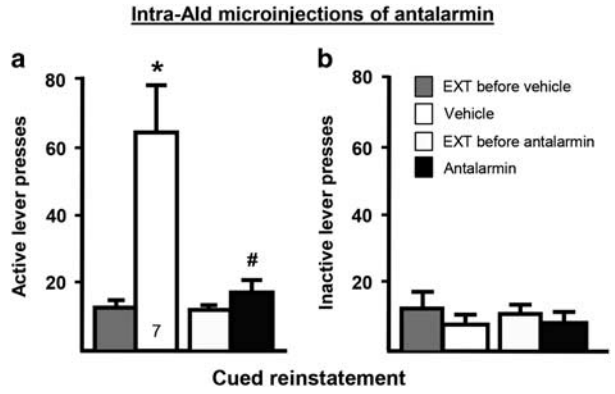

Figure 4 Blockade of CRF1 receptors in the AId reduces cued reinstatement to cocaine-seeking behavior. (a, b) Active and inactive lever presses, respectively, for Experiment 3. Intra-AId microinjections of the CRF1 receptor antagonist antalarmin (6.0 mM) reduced active lever presses during cued reinstatement compared with vehicle–control injections. There were no effects of reinstatement or microinjections on inactive lever presses. $^{*}P<0.05$ compared with extinction baseline. $^{\#}P<0.05$ compared with vehicle–control group. EXT, extinction baseline.

before the reinstatement test. The results were similar to those of Experiment 1 with AId inactivation. Blockade of CRF1 receptors in the AId significantly reduced active lever presses for cued reinstatement (Figure 4a). A two-way repeated measures ANOVA of active lever presses indicated a significant effect of reinstatement ($F_{(1,6)}=18.40$, $P<0.01$), a significant effect of microinjection ($F_{(1,6)}=8.384$, $P<0.05$), and a significant interaction ($F_{(1,6)}=8.138$, $P<0.05$). *Post hoc* tests revealed that aCSF-treated rats showed a significant increase in active lever pressing compared with their extinction baseline and the antalarmin-treated group ($P<0.05$), whereas the antalarmin-treated group did not significantly differ in active lever presses compared with its extinction baseline ($P>0.05$). There was no significant difference in inactive lever presses for the cued reinstatement test ($P>0.05$).

Experiment 4. AId Inactivation has no Effect on the Reinstatement of Food-Seeking Behavior

Figure 5 shows the active and inactive lever presses (Figure 5a–c and Figure 5d–f, respectively) for the reinstatement of food-seeking behavior from Experiment 4. Overall, the results indicated no effect of AId inactivation on food-seeking behavior. A two-way repeated measures ANOVA of active lever presses during cued reinstatement indicated a significant effect of reinstatement ($F_{(1,5)}=49.04$, $P<0.001$), no effect of microinjection ($F_{(1,5)}<1$, $P>0.05$), and no interaction ($F_{(1,5)}<1$, $P>0.05$). Both BM- and vehicle-treated rats had trends toward a significant cued reinstatement compared with their extinction baselines ($P<0.09$). A two-way repeated measures ANOVA of active lever presses during food-prime reinstatement indicated a significant effect of reinstatement ($F_{(1,5)}=20.84$, $P<0.01$), no effect of microinjection ($F_{(1,5)}<1$, $P>0.05$), and no interaction ($F_{(1,5)}<1$, $P>0.05$). Both BM- and vehicle-treated rats had significant food-prime reinstatement compared with their extinction baselines ($P<0.05$). A two-way repeated measures ANOVA of active lever presses during cue+food-prime reinstatement indicated a significant effect of reinstatement ($F_{(1,5)}=17.00$, $P<0.01$), no effect of microinjection ($F_{(1,5)}<1$, $P>0.05$), and no interaction ($F_{(1,5)}<1$, $P>0.05$). Both BM- and vehicle-treated rats had trends toward a significant cue+food-prime reinstatement compared with their extinction baselines ($P<0.07$). Visual inspection of the data in all cases did not suggest that AId inactivation had any effect on food-seeking reinstatement. There were no significant differences in inactive lever pressing in all cases ($P>0.05$).

Analysis of Data for Rats With Misplaced Cannula

Supplementary Figure 1 shows the active lever presses for rats with misplaced microinjection sites from Experiment 1 (Supplementary Figure 1A and B), Experiment 2 (Supplementary Figure 1C), Experiment 3 (Supplementary Figure 1D), and Experiment 4 (Supplementary Figure 1E–G). Although the low *n*'s for misplaced microinjections for most of the experiments preclude drawing any conclusions, the data for the misplaced microinjections from Experiment 1 (Supplementary Figure 1A) indicate that BM microinjections outside the AId did not reduce cued reinstatement of cocaine seeking.

DISCUSSION

The present findings indicate that AId activity and the CRF1 receptors within the AId regulate cocaine-seeking behavior. AId inactivation decreased cocaine seeking for cued reinstatement but had no effect on cocaine-prime reinstatement, suggesting a differential role for the AId depending on the type of reinstatement. In contrast, PIc inactivation had no effect on cued reinstatement. Similar to the findings with AId inactivation, blockade of the CRF1 receptors in the AId reduced cued reinstatement. Additional experiments found that AId inactivation had no effect on reinstatement of food-seeking behavior, suggesting a selective role for the AId in drug seeking.

The IC has been increasingly implicated as a critical component for relapse, as imaging studies have demonstrated that drug-associated cues elicit IC activity across multiple drugs of abuse (Brody *et al*, 2002; Kilts *et al*, 2004; Myrick *et al*, 2004). Moreover, IC damage in humans produces a profound loss of nicotine craving and relapse (Naqvi *et al*, 2007). Similarly, rodent studies have found that PIc inactivation and electrical stimulation reduce nicotine self-administration and reinstatement induced by nicotine-associated cues or a nicotine prime (Forget *et al*, 2010; Pushparaj *et al*, 2013). The present results, however, indicate that PIc inactivation had no effect on cued reinstatement to cocaine seeking. In contrast, AId inactivation as well as intra-AId administration of a CRF1 receptor antagonist altered cocaine-seeking behavior produced by cued reinstatement. A role for the AId in regulating such behavior is consistent with previous work, suggesting that the AId is involved in amphetamine- and cocaine-related behavior, including relapse (Contreras *et al*, 2012; Di Pietro *et al*, 2006; Pelloux *et al*, 2013).

Although studies investigating the role of the anterior IC in addiction are limited, previous work using coordinates for

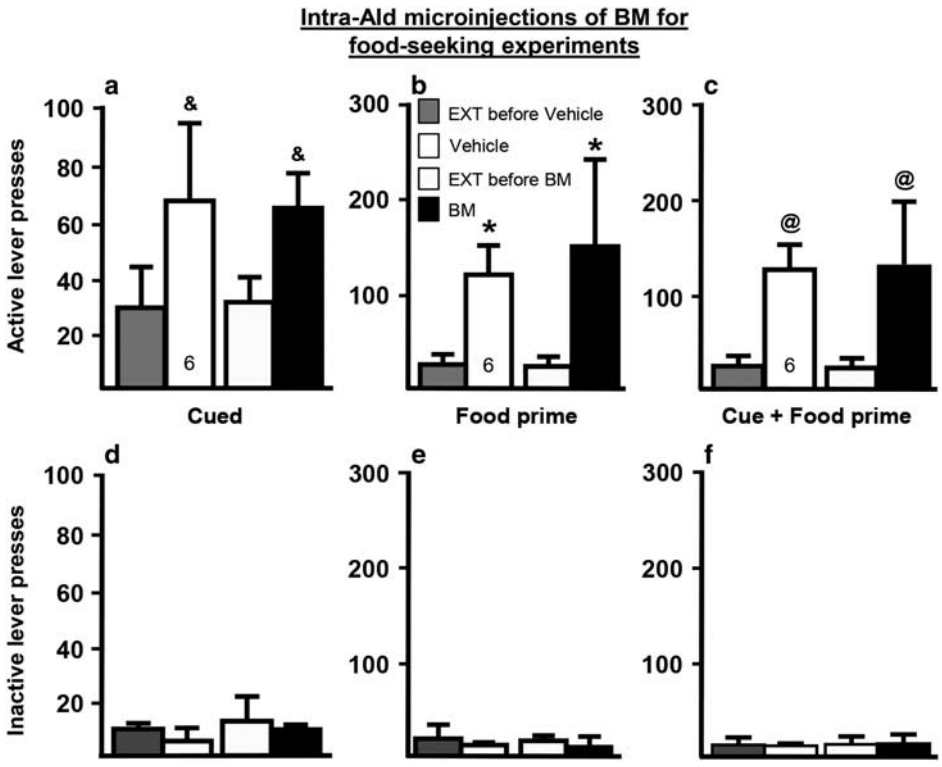

Figure 5 AId inactivation, via baclofen/muscimol (BM; 0.1 and 1.0 mM, respectively) microinjections, has no effect on reinstatement of food-seeking behavior. (a–c) The active lever presses and (d–f) the inactive lever presses across the different reinstatement tests are shown. (a–c) Intra-AId microinjections of BM had no effect on active lever pressing during cued, food-prime, and cue+food-prime reinstatement. (d–f) There were no effects of reinstatement or microinjections on inactive lever presses across any of the reinstatement tests. $^{*}P<0.05$ compared with extinction baseline; $^{@}P<0.07$ compared with extinction baseline; $^{\&}P<0.09$ compared with extinction baseline. EXT, extinction baseline.

AId microinjections akin to the ones used in the present study found that AId inactivation via lidocaine reduced odor context-dependent cue-induced reinstatement of cocaine seeking, but had no effect on sound context-dependent cue-induced reinstatement (Di Pietro *et al*, 2006). Although the present experiments found that AId inactivation attenuated lever pressing when the AId was inactivated before the cued reinstatement, the current methods were different from those of the Di Pietro *et al*, (2006), as the prior study used a contextual cue (sound) and a discrete contingent cue (light) in contrast to the contingent tone+light cues used in the current study. A potentiation of cocaine seeking following IC manipulation has previously been observed by Pelloux *et al*, (2013) in which lesions of the anterior IC (including the AId) were found to increase cocaine-seeking behavior when animals were reintroduced to the drug-taking context following an abstinence period (Pelloux *et al*, 2013). The procedures used in the present experiments, however, were significantly different from those used by Pelloux *et al*, (2013). Nonetheless, taken together, these results suggest that the role of the AId in cocaine-seeking behavior may be rather complex.

The IC is a critical region for the mediation of interoceptive cues (Goldstein *et al*, 2009; Paulus and Stewart, 2014), and these interoceptive cues appear to be critical to addiction and relapse. In the presence of external cues, it has been argued that the IC receives information regarding these cues, and that this drives the recall of drug-specific interoceptive cues, which produce subjective craving and relapse behaviors (Naqvi and Bechara, 2009; Naqvi *et al*, 2014). In the present study, however, the cues are delivered in a response-contingent manner and are thus conditioned reinforcers, although they may also act as antecedents to future lever pressing during the session. Therefore, whether the cues used in the present study induce the recall of interoceptive cues in rodents is difficult to ascertain, though the present results are consistent with the hypothesis that the AId is involved in behavior related to drug-associated cues. As each drug of abuse produces its own unique set of interoceptive cues (Naqvi *et al*, 2014), it is possible that the discrepancy between the nicotine findings and the present cocaine-seeking findings with regard to the PIc and AId are due to such differences. That the present results indicate that AId inactivation did not alter the reinstatement of food seeking suggest that the AId is not generally involved in the reinstatement of reward-related behavior. Given that the AId maintains a population of CRF1 receptors (Potter *et al*, 1994; Sanchez *et al*, 1999) and that it has been suggested that CRF in the AId may be involved in the mediation of these interoceptive cues (Naqvi and Bechara, 2009), the present work also examined the role of CRF1 receptors in the AId in regulating reinstatement and found that CRF1 receptor blockade reduced cued reinstatement. To our knowledge, these are the first findings demonstrating a role for CRF in the AId in drug-seeking behavior.

The differences in results between the present work and the nicotine studies may also involve differences in anatomical connections and/or differential activation of structures during reinstatement. The present study targeted the granular cortex in the PIc, which projects to the agranular regions but otherwise appears to maintain relatively few connections with other forebrain regions likely involved in drug addiction (Shi and Cassell, 1998). In contrast, the AId region targeted in the current experiments projects to the NA core and most regions of the amygdala, maintains reciprocal connections with the prelimbic and infralimbic cortices, and also receives input from the medial orbital cortex (Hoover and Vertes, 2007, 2011; Shi and Cassell, 1998; Vertes, 2004; Voorn *et al*, 2004). The anatomical connections suggest that the PIc may act upstream of the AId anatomically and at least functionally for nicotine seeking, but the PIc appears to have no role in cue-induced cocaine seeking, suggesting a distinct circuit for such reinstatement. Indeed, Naqvi and Bechara (2009) have hypothesized that drug-associated cues activate the IC via the ventromedial PFC and the amygdala both of which connect with the AId directly, thereby providing a circuit whereby cued reinstatement can bypass the PIc, although it is not clear why this is not the case for nicotine seeking. As the AId projects to the NA core (Reynolds and Zahm, 2005) and inactivation of the core or blockade of glutamate receptors in the core prevents cued and cocaine-prime reinstatement (Backstrom and Hyytia, 2007; Cornish and Kalivas, 2000; Fuchs *et al*, 2004a; McFarland and Kalivas, 2001), activity in this pathway may be responsible for the present cued reinstatement results. As AId inactivation alone had no effect on lever pressing, it appears that the AId does not act similarly to the infralimbic cortex in suppressing cocaine-seeking behavior (eg, Peters *et al*, 2008).

Studies have examined other regions of the lateral PFC, including the nearby lateral orbitofrontal cortex (lOFC), in cocaine-seeking behavior. Intriguingly, lOFC lesions made before self-administration potentiate contextual and cocaine-prime reinstatement and have no effect on cued reinstatement, whereas lesions made after self-administration have no effect on contextual reinstatement (Fuchs *et al*, 2004b; Lasseter *et al*, 2009). However, lOFC inactivation, via BM microinjections given before the test trial akin to the design of the present study, was found to impair cued and contextual reinstatement and have no effect on cocaine-prime reinstatement, similar to the present results. Based on examination of the histology results from these previous studies, the lOFC microinjections appear to be ~0.5 mm anterior to the present microinjections and 1–2 mm medial to the present microinjections, making it unlikely that the 0.2-μl microinjections used in the current study spread to the lOFC. Nonetheless, the present findings, together with previous studies, suggest that lPFC subregions are differentially involved in the reinstatement of drug seeking and deserve increased attention, particularly considering the apparent heterogeneity of function among these subregions.

Given that there must be CRF release for antalarmin to have any effects and that general inactivation is not activity dependent, it is surprising that the inactivation appeared to be less robust at impairing cued reinstatement than the CRF1 receptor blockade. However, comparing two different drugs is difficult, as it is possible that the relative doses produced smaller or larger areas of physiological effects. The antalarmin dose that was used, therefore, may have resulted in greater spread of physiologically effective concentrations of antalarmin compared with the spread of the physiologically effective concentrations of the GABA receptor agonists. If this is the case, higher doses of BM may be required to produce the same results that were seen with antalarmin. Given that different doses of lidocaine produced different results in the study by Di Pietro *et al*, (2006) and given the relatively large size of the AId, especially in the rostral–caudal dimension, it is possible that increased doses of BM would produce more robust attenuation of cued reinstatement.

CONCLUSIONS

The present results indicate a critical role for the AId and specifically CRF1 receptors in the AId in the cued reinstatement of cocaine-seeking behavior. Of interest, PIc inactivation had no effect on the reinstatement of cocaine seeking, suggesting subregional specificity for these effects. Moreover, AId inactivation had no effect on the reinstatement of natural reward (food) seeking, suggesting that the present results are not due to general effects on the reinstatement of operant behavior. The present findings also indicate that AId activity and the effects of CRF1 receptor activation in the AId differentially influence such behavior depending on the type of reinstatement. These findings provide significant evidence of the critical nature of the AId in the circuitry underlying cued reinstatement of cocaine-seeking behavior.

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