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Abstract

Re-exposure to drug-associated cues causes significant drug craving in recovering addicts, which may precipitate relapse. In animal models of craving, drug-seeking responses for contingent delivery of drug-associated cues sensitizes or "incubates" across drug withdrawal. To date there is limited evidence supporting an incubation effect for behaviors mediated by non-contingent presentation of drug-associated cues. Here we used a model of cue-induced conditioned activity to determine if the conditioned locomotor response to a non-contingent presentation of a drug-associated cue sensitizes across drug withdrawal. In addition, because cue-induced drug-seeking responses are mediated by the rostral basolateral amygdala (rBLA), we investigated whether this structure is critical for the expression of cue-induced conditioned activity. A conditioned association between cocaine (15 mg/kg) and a compound discrete cue (flashing bicycle light + a metronome) was established over 12 conditioning sessions in male Sprague-Dawley rats. In experiment 1, cue-induced conditioned activity was assessed on 3 occasions: 3, 14 and 28 days following the final drug-cue conditioning session. Cocaine-conditioned rats demonstrated reliable cue-induced conditioned activity across all 3 test sessions, however there was no evidence of an incubation effect. To determine whether repeated testing prevented the observation of an incubation effect, rats in experiment 2 were tested either 3-days or 28-days following conditioning; again no incubation effect was observed. In experiment 3, either saline or the $GABA_A$ receptor agonist muscimol was infused prior to testing. Intra-BLA infusions of muscimol prevented the expression of cue-induced conditioned activity. These data support the role of the rBLA in mediating conditioned responses to drug-associated cues. The failure to observe an incubation effect for cue-induced conditioned activity may point to fundamental difference in the manner by which contingent and non-contingent presentations of drug-associated cues influence behavior.

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Key Words

Cocaine, Pavlovian Conditioning, Relapse, Incubation Effect, Basolateral Amygdala, Conditioned Activity

1. Introduction

Drug addiction is a chronically relapsing disorder with re-exposure to drug-associated cues being amongst the most powerful triggers for relapse. Indeed, presentation of drug-associated cues (i.e., videos involving drug-taking, images of drug-related paraphernalia, or hearing a personalized drug-related script) induces profound drug craving, or the desire to re-experience the drug effect, in abstinent cocaine addicts (Volkow et al., 2006; Childress et al., 1999) and is associated with physiological changes that reflect a "drug-like" state (Ehrman et al., 1992). It has been hypothesized that cue-induced drug craving progressively increases over the first several weeks of drug withdrawal (Gawin and Kleber, 1986), which may explain why drug-associated cues are able to induce relapse despite prolonged periods of abstinence. In rodent models of relapse, responding for a cocaine-associated cue is higher after prolonged withdrawal (e.g., one month) than it is after acute withdrawal (e.g., one day), suggesting that the ability of drug-associated cues to influence behavior may sensitize or incubate following drug discontinuation (Lu et al., 2004; Grimm et al., 2001; for review, see Pickens et al., 2011).

In the most prevalent animal model of relapse, including that used to study the incubation effect, drug-associated cues are delivered contingently upon a lever press (Grimm et al., 2001). During training, lever-pressing results in delivery of both the drug and the drug-associated cue (e.g., a light + tone); these cues are thought to gain motivational significance by virtue of being repeatedly paired with the drug effect (See 2005; Berridge, 2004). During tests of reinstatement, rats will lever press for the delivery of drug-associated cues in the absence of the drug, suggesting that drug-associated cues act as secondary reinforcers (e.g., Kantak et al., 2002; Kruzich and See, 2001; Grimm and See, 2000). In contrast, the cues that are presented to abstinent cocaine addicts (i.e., those that cause profound craving) are presented non-contingently (e.g., Childress et al., 1999). This has lead researchers to develop animal models in which drug-associated cues are also delivered non-contingently and their effects on behavior are measured. For example, in a discriminative stimulus task of reinstatement, cocaine-seeking responses are increased in the presence of a cue predictive of cocaine

availability (S+), but not in presence of a cue predictive of non-reward (S-), or in the absence of any cues (Yun and Fields, 2003; Ciccocioppo et al., 2001; Weiss et al., 2000). Similarly, in a cue-induced conditioned activity task, locomotor activity is increased in the presence of a discrete cue previously paired with cocaine, but is unchanged in the absence of that cue (Hotsenpiller et al., 2002; Hotsenpiller et al., 2001; Panlillio and Schindler, 1997).

The basolateral amygdala (BLA; consisting of the lateral, basal and accessory basal nuclei (LeDoux, 2007; Pitkänen et al., 1997)) is required for both contingent and non-contingent presentation of drug-associated cues to influence behavior. Response to contingent presentation of drug-associated cues is associated with increased neuronal activity within the BLA (as indicated by Fos protein expression) (Kufahl et al., 2009). Furthermore, excitotoxic lesions and temporary inactivation of the BLA attenuate responding for response-contingent presentations of drug-associated cues (Gabriele and See, 2010; Kantak et al., 2002; Kruzich and See, 2001; Grimm and See, 2000). Likewise, non-contingent presentation of drug-associated cues increases activation of the amygdala in abstinent cocaine addicts (Bonson et al., 2002; Kilts et al., 2001; Childress et al., 1999). In rodents, re-exposure to a cocaine-associated context or a cocaine-predictive discriminative stimulus increases Fos protein expression in the BLA (Miller and Marshall, 2005; Ciccocioppo et al., 2001), while excitotoxic lesions of the BLA prevent reinstatement of drug-seeking in the presence of a cocaine-predictive cue (Yun and Fields, 2003). However, exposure to a discrete cocaine-paired cue did not increase Fos protein expression in the BLA in the cue-induced conditioned activity task (Hotsenpiller et al., 2002), indicating that the BLA may not be required for the expression of cue-induced conditioned activity.

One goal of the current experiment was to determine the role of the BLA in the expression of cue-induced conditioned activity. First, however we validated a model of cue-induced conditioned activity (Hotsenpiller et al., 2002; Hotsenpiller et al., 2001; Panlilio and Schindler, 1997; Polston and Glick, 2011), and used it to determine if cue-induced conditioned activity “incubated” across drug withdrawal. In the first experiment,

cue-induced conditioned activity was measured 3 times: 3, 14 and 28 days following the final drug-cue pairing. This within-subjects design did not reveal an incubation effect, thus an additional experiment was conducted in which rats were tested either 3 days or 28 days following the final drug-cue pairing. In the third experiment, the BLA was inactivated using the $GABA_A$ receptor agonist muscimol prior to testing. Consistent with previous reports (Hotsenpiller et al., 2002; Hotsenpiller et al., 2001; Panlilio and Schindler, 1997) we observed that activity was increased in the presence of a discrete drug-paired cue, but we did not observe an incubation effect; cue-induced conditioned activity was the same in early withdrawal as it was in late withdrawal. The expression of cue-induced conditioned activity was blocked by inhibition of the BLA.

2. Materials & Methods

2.1 Subjects

Sixty-nine adult male Sprague-Dawley rats bred at Oberlin College were used. Four days before starting behavioral testing (Experiments 1 and 2) or surgery (Experiment 3), rats were individually housed in polypropylene cages (48 cm x 20 cm x 26 cm) and food-restricted diet to approximately 85% of their free feeding weight. Rats used in Experiments 1 and 2 were housed in pairs; rats used in Experiment 3 were housed individually. Rats were fed (LabDiet 5001 rat chow) after daily conditioning sessions. Water was available *ad libitum* while rats were in their home cage. Rats were housed on a 14:10 hr light:dark schedule with lights off at 8 PM in a temperature controlled (22° C) colony room. All experimental procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Academy Press, 1996) and were approved by the Oberlin College IACUC.

2.2 Apparatus

Behavioral procedures occurred in four identical locomotor activity chambers made of clear Plexiglas, each with dimensions of 43.2 cm x 43.2 cm x 30.5 cm (Med-Associates, St. Albans, VT). Each chamber contained three arrays of 16 infrared beams capable of measuring locomotor activity in three dimensions. Locomotor activity chambers were

connected to a PC running Activity Monitor software (version 6.00, Med-Associates) to record activity.

2.3 Drugs

Cocaine hydrochloride (Sigma-Aldrich, St. Louis, MO) was dissolved to a dose of 15 mg/kg in physiological saline (0.9% sodium chloride; Cardinal Health, McGaw Park, IL). Cocaine dose was based upon Hotsenpiller et al. (2002).

Muscimol (Sigma-Aldrich) was dissolved in physiological saline to a final concentration of 50 ng/µl; aliquots were stored at -20°C until use. Muscimol infusion dose was based upon Ishikawa et al. (2008).

2.4 Surgery

Prior to behavioral testing rats used in Experiment 3 (n = 24) were bilaterally implanted with guide cannulae (23-gauge, Plastics One, Roanoke VA) aimed at the basolateral amygdala (BLA). Rats were anesthetized with sodium pentobarbital (65 mg/kg, IP) (Sigma-Aldrich), the skull exposed, burr holes drilled above the BLA, and the cannulae were lowered into place (BLA coordinates relative to bregma: A/P: -2.6 mm, M/L: ± 5.0 mm, D/V: -5.2 mm from dura (Paxinos and Watson, 2009)). Skull screws and dental acrylic secured the guide cannulae in place. Obturators and injector needles (30-gauge) extended 1.5 mm below the guide cannulae.

Rats were given 1 week to recover following surgery before behavioral training started. Throughout the recovery period and training, the obturators were manipulated in order to habituate rats to the handling necessary for infusions and to ensure that obturators remained secure.

2.5 Infusions

Prior to baseline sessions preceding each test session, rats in Experiment 3 (see below) received bilateral infusions of either muscimol (25 ng/0.5 µl/side) or saline (vehicle; 0.5 µl/side). All infusions occurred at a rate of 0.25 µl/min, and injectors were left in place

for an additional two minutes to allow for drug diffusion before being replaced by obturators.

2.6 Cocaine-Cue Conditioning

A timeline for all behavioral training is shown in **Figure 1**. Habituation and training sessions were similar to previously described procedures (Hotsenpiller et al., 2001; Panlilio and Schindler, 1997). Procedures for the habituation and training sessions were exactly the same for Experiments 1, 2 and 3. As detailed below, procedures for the test sessions differed between the three experiments.

2.6.1 Habituation

Prior to training, rats underwent three 60-min habituation sessions. Immediately prior to each session rats were treated with saline (1 ml/kg, IP). Data from these sessions were used to divide rats into cocaine-conditioned and saline-conditioned groups.

2.6.2 Training

Following habituation, rats underwent 12 consecutive training sessions; each training session was divided into a 30-min baseline session and a 30-min conditioning session. During the baseline session, rats were placed in the activity chamber in the absence of any cues. At the completion of the baseline session rats were removed from the chamber, injected with either saline or cocaine and then returned to the chamber for the conditioning session. Conditioning sessions were further divided into cue present (CS+) and cue absent (CS-) sessions. Prior to CS+ sessions, rats in the cocaine-conditioned group were administered cocaine (15 mg/kg, IP) and rats in the saline-conditioned group were administered saline (1 ml/kg, IP) and placed in the activity chamber in the presence of a compound audiovisual cue. The cue consisted of a flashing yellow bicycle light (Ventura LED) and an electronic metronome (Aroma Music Co., China) set to 77 bpm; these were placed directly above the center of each locomotor chamber. Prior to CS- sessions, rats in both the cocaine- and saline-conditioned groups were administered saline (1 ml/kg, IP) and then placed in the activity chamber in the absence of the discrete compound cue.

2.6.3 Testing

2.6.3.1 Experiment 1: Validation of the cocaine-cue conditioning protocol

Rats (n=8 saline; n=8 cocaine) were tested on 3 separate occasions: 3, 14 and 28 days following the completion of conditioning. Each test occurred over 2 days: on one day the rat was tested in the presence of the cue (CS+ session) and on the other day it was tested in the absence of the cue (CS- session). Similar to training, each daily test session began with a 30-min baseline session. At the completion of the baseline session, rats were removed from the chamber, administered saline (1 ml/kg, IP) and then returned to the chamber with either the cue present (CS+ session) or the cue absent (CS- session) for the 30-min test session. The order of CS+ and CS- sessions was counterbalanced across rats.

2.6.3.2 Experiment 2: Determining if non-contingently presented drug-associated cues incubate

In order to determine if repeated testing impeded the observation of an incubation effect separate sets of rats were tested either 3 days (n=6 saline, n=7 cocaine) or 28 days (n=8 saline; n=8 cocaine) following the completion of conditioning. All other procedures were identical to those in Experiment 1.

2.6.3.3 Experiment 3: Effect of BLA inactivation on cue-induced activity

Rats (n=12 saline; n=12 cocaine) were tested 3 days following the completion of conditioning; the test occurred over 4 days. Rats were first infused with either muscimol or vehicle and then placed in the activity chamber for a 30-min baseline session. At the completion of the baseline session, rats were removed from the chamber, administered saline (1 ml/kg, IP) and then placed into the chamber with either the cue present (CS+ session) or the cue absent (CS- session) for the 30-min test session. Rats were tested once under each possible combination: vehicle/CS-, vehicle/CS+, muscimol/CS-, muscimol/CS+; the order of vehicle and muscimol infusions and CS- and CS+ sessions were counterbalanced across rats.

2.7 Statistical Analysis

Data were analyzed with two-way, three-way repeated or four-way repeated measures analyses of variance (ANOVAs) with Condition (cocaine or saline) or Incubation Time (3-day or 28-day) as the between subjects factors. The within subjects factors were Day (or Infusion [vehicle or muscimol]) and Session (CS+ or CS-). Significant main effects and interactions were further analyzed using an estimated marginal means procedure with a Bonferroni correction.

3. Results

3.1 Experiment 1: Validation of the cocaine-cue conditioning protocol

3.1.1 Habituation

Activity of all rats decreased across the habituation sessions ($F(2, 28) = 20.46$, $P < 0.01$; **Figure 2A**); activity was significantly higher in session 1 than it was in sessions 2 and 3 ($P < 0.01$). Neither the main effect of Condition nor the Condition X Day interaction were significant (both $F < 1.0$, $P > 0.05$).

3.1.2 Training

Baseline Sessions: Activity differed across baseline sessions ($F(5, 70) = 4.09$, $P < 0.01$; **Figure 2B**); activity was higher in the first baseline session than it was in the second baseline session ($P < 0.01$). No other main effects or interactions were statistically significant (all $F < 2.12$, all $P > 0.5$).

Conditioning Sessions: Across conditioning sessions there was a significant main effect of Condition ($F(1, 14) = 42.74$, $P < 0.01$), a significant main effect of Session ($F(1, 14) = 52.22$, $P < 0.01$) and a significant Condition X Session interaction ($F(1, 14) = 48.85$, $P < 0.01$; **Figure 2C**). Post-hoc analysis of the interaction revealed that cocaine-conditioned rats exhibited more activity during CS+ sessions than during CS- sessions ($P < 0.01$) and exhibited more activity than saline-conditioned rats during CS+ sessions ($P < 0.01$). No other main effects or interactions were statistically significant (all $F < 1.25$, all $P > 0.05$).

3.1.3 Testing

Baseline Sessions: Activity levels were significantly different across the baseline sessions preceding the three test sessions ($F(2,28) = 15.31$, $P < 0.01$; see **Figure 3A**); activity during the baseline sessions was lower in test 1 than it was in tests 2 and 3 (both $P < 0.01$). No other main effects or interactions were statistically significant (all $F < 4.17$, all $P > 0.05$).

Test Sessions: Across the three tests there was significant main effect of Condition ($F(1, 14) = 4.47$, $P = 0.05$), a significant main effect of Session ($F(1, 14) = 49.63$, $P < 0.01$) and a significant Condition X Session interaction ($F(1,14) = 13.65$, $P < 0.01$; see **Figure 3B**). Post-hoc analysis of the interaction revealed that although both cocaine-conditioned rats and saline-conditioned rats exhibited more activity in the presence of the cue (CS+ session) than in the absence of the cue (CS- session; both $P < 0.05$), cocaine-conditioned rats exhibited more activity than saline-conditioned rats in the presence of the cue (CS+ session, $P < 0.01$). Cocaine-conditioned and saline-conditioned rats exhibited equivalent activity in the absence of the cue (CS- session, $P > 0.05$). These data demonstrate that the cocaine-cue conditioning protocol was effective in establishing an association between the cocaine and the audiovisual cue. No other main effects or interactions were statistically significant (all $F < 1.83$, all $P > 0.05$).

3.2 Experiment 2: ***Determining if non-contingently presented drug-associated cues incubate***

3.2.1 Habituation

There was a significant effect of habituation day ($F(2, 50) = 23.37$, $P < 0.01$; **Figure 4A**); this effect was modulated by Incubation Time (Day X Incubation Time interaction; $F(2,50) = 6.87$, $P < 0.01$). Rats in the 3-day group exhibited more activity than rats in the 28-day group during habituation session 3 ($P < 0.05$). The activity of rats in the 28-day group was lower in sessions 2 and 3 than it was in session 1 (both $P < 0.05$). In contrast, the activity of rats in the 3-day group was lower in session 2 ($P < 0.05$), but not

in session 3, than it was in session 1. No other main effects and interactions were statistically significant (all $F < 1.43$, $P > 0.05$**).**

3.2.2 Training

Baseline Sessions: There were a significant main effects of Day ($F(5,125) = 6.52$, $P < 0.01$; **Figure 4B**) and Session ($F(1, 25) = 5.95$, $P < 0.05$). In addition there was a significant 2-way Day X Session interaction ($F(5, 125) = 2.30$, $P < 0.05$), a significant 3-way Day X Session X Incubation Time interaction ($F(5, 125) = 3.88$, $P < 0.01$) and a significant 4-way Day X Session X Treatment X Incubation Time interaction ($F(5, 125) = 2.56$, $P < 0.05$). Analysis of the 4-way interaction revealed that saline-treated rats in the 3-day group exhibited more activity on CS- baseline sessions 4 and 6 than they did in the corresponding CS+ baseline sessions (both $P < 0.05$). Saline-treated rats in the 28-day group exhibited more activity in CS- baseline session 5 than they did in the corresponding CS+ baseline session ($P < 0.05$). Cocaine-treated rats in the 3-day group exhibited more activity in CS- baseline session 4 than they did in the corresponding CS+ baseline session ($P < 0.05$). Cocaine-treated rats in the 28-day group exhibited more activity on CS- baseline sessions 1 and 5 than they did in the corresponding CS+ baseline sessions (both $P < 0.05$). In addition, saline-treated rats in the 3-day group exhibited less activity than saline-treated rats in the 28-day group on CS+ baseline session 4 and 6 (both $P < 0.05$). Similarly, cocaine-treated rats in the 3-day group exhibited less activity than cocaine-treated rats in the 28-day group on CS+ baseline session 6 ($P < 0.05$). No other main effects and interactions were statistically significant (all $F < 3.08$, all $P > 0.05$).

Conditioning Sessions: During conditioning sessions there was a significant effect of Session ($F(1, 25) = 117.83$, $P < 0.01$; **Figure 4C**), a significant effect of Treatment ($F(1, 25) = 91.26$, $P < 0.01$), a significant Session X Treatment interaction ($F(1, 25) = 109.00$, $P < 0.01$), and a significant Day X Treatment interaction ($F(5, 125) = 2.29$, $P < 0.05$). Analysis of the Day X Treatment interaction revealed that cocaine-treated rats exhibited more activity than saline-treated rats across all days (collapsed across CS+ and CS- sessions; all $P < 0.01$). In addition, cocaine-treated rats exhibited more activity on days

2, 3 and 5 than they did on day 1 (all $P < 0.05$). Analysis of the Session X Treatment interaction revealed that cocaine-treated rats exhibited more activity than saline-treated rats on all CS+ sessions (all $P < 0.01$) and cocaine-treated rats exhibited more activity on CS+ session than on CS- sessions (all $P < 0.05$). No other main effects and interactions were statistically significant (all $F < 2.15$, all $P > 0.05$).

3.2.3 Testing

Baseline Sessions: Rats in the 28-day group exhibited more activity during the 30-min baseline session than rats in the 3-day group ($F(1, 25) = 13.28$, $P < 0.01$; see **Figure 5A**). No other main effects and interactions were significant (all $F < 1.02$, all $P > 0.05$).

Test Sessions: There was a trend for a main effect of Session ($F(1, 25) = 4.06$, $P < 0.10$) and a Session X Treatment interaction ($F(1, 25) = 3.03$, $P < 0.10$). Because we hypothesized *a priori* that cocaine-treated rats would exhibit more activity in the presence of the cue (CS+ session) than in its absence (CS- session); post-hoc analyses were conducted on the Session X Treatment interaction. Consistent with our hypothesis, cocaine-treated rats exhibited more activity in the presence of the cue than in its absence ($P < 0.05$). In addition cocaine-treated rats exhibited more activity than saline-treated rats in the presence, but not the absence, of the cue ($P < 0.05$). No other main effects and interactions were statistically significant (all $F < 2.72$, all $P > 0.05$).

3.3 Experiment 3: Effect of BLA inactivation on cue-induced activity

3.3.1 Histological Analysis

Figure 6 depicts cannulae placements of the rats used in the statistical analyses. Of the 24 rats tested, 5 were excluded from analyses (not shown). Three cocaine-conditioned rats, and one saline-conditioned rat were excluded based on inaccurate cannulae placements, while a third saline-conditioned rat was excluded as an outlier based on multiple sessions of activity greater than 2.5 standard deviations above the group mean. A total of nine cocaine-conditioned rats and ten saline-conditioned rats were included in statistical analyses. Although a few cannulae placements were slightly ventral of the target, these were included in they statistical analyses because the area

of drug diffusion would likely include the BLA (Martin, 1991). Moreover, when rats with ventral placements were excluded from the statistical analysis the same pattern of effects was observed, although a number of these comparisons only resulted in a trend towards significance (i.e., $P < 0.10$) rather than statistical **significance.**

3.3.2 Habituation

Activity of all rats decreased across the habituation sessions ($F(2, 34) = 17.01$, $P < 0.01$; **Figure 7A**); activity was significantly higher on session 1 than it was on session 3 ($P < 0.01$). Neither the main effect of Condition nor the Condition X Day interaction were significant (both $F < 1.0$, $P > 0.05$).

3.3.3 Training

Baseline Sessions: There was a significant Condition X Session X Day interaction for the activity during the 30-min baseline sessions that preceded the conditioning sessions ($F(5, 85) = 3.53$, $P < 0.05$; see **Figure 7B**). During sessions 1 and 4 cocaine-conditioned rats exhibited more activity prior to CS- sessions than they did prior to CS+ sessions (both $P \leq 0.05$). During session 2 saline-conditioned rats exhibited more activity prior to CS+ session than they did prior to CS- sessions ($P < 0.05$). In addition, cocaine-treated rats exhibited more activity than saline-treated rats during baseline CS+ sessions 5 and 6 and baseline CS- sessions 2 and 5 (all $P < 0.05$). No other main effects or interactions were statistically significant (all $F < 3.89$, all $P > 0.05$).

Conditioning Sessions: Across the conditioning sessions there was a significant main effect of Condition ($F(1, 17) = 15.83$, $P < 0.01$), a significant main effect of Session ($F(1, 17) = 18.41$, $P < 0.01$), and a significant Condition X Session interaction ($F(1, 17) = 16.51$, $P < 0.01$; see **Figure 7C**). Cocaine-conditioned rats exhibited more activity than saline-conditioned rats during both CS+ ($P < 0.01$) and CS- ($P < 0.05$) sessions. Cocaine-conditioned rats exhibited more activity during CS+ sessions than CS- sessions ($P < 0.01$). Activity of saline-conditioned rats did not differ across CS+ and CS- sessions ($P > 0.05$). No other main effects or interactions were statistically significant (all $F < 1.00$, all $P > 0.05$).

3.3.4 Testing

Baseline Sessions: In the baseline session preceding the test session, cocaine-conditioned rats were more active than saline-conditioned rats ($F(1,17) = 14.15$, $P < 0.01$; **Figure 8A**). In addition, muscimol infusions significantly decreased activity relative to vehicle infusions ($F(1, 17) = 10.30$, $P < 0.01$). No other main effects or interactions were statistically significant (all $F < 2.86$, all $P > 0.05$).

Test Sessions: In the test session, there were significant main effects of Condition ($F(1,17) = 19.18$, $P < 0.01$), Session ($F(1,17) = 4.95$, $P < 0.01$), and Infusion ($F(1,17) = 14.75$, $P < 0.01$). In addition, there were significant Session X Condition ($F(1,17) = 9.48$, $P < 0.01$), Infusion X Condition ($F(1,17) = 4.38$, $P = 0.05$) and Condition X Session X Infusion interactions ($F(1,17) = 6.08$), $P < 0.05$; **Figure 8B**). Post-hoc analyses on the Condition X Session X Infusion interaction revealed that the conditioning protocol was successful in establishing a cocaine-cue association: following a vehicle infusion, cocaine-conditioned rats exhibited greater activity in the presence of the cue (CS+ session) than in its absence (CS- session) ($P < 0.01$). Inactivation of the BLA blocked the expression of cocaine-cue conditioning: following a muscimol infusion, cocaine-conditioned rats did not exhibit more activity in the presence of the cue (CS+ session) than in its absence (CS- session) ($P > 0.05$). Moreover, the activity of cocaine-conditioned rats in the presence of the cue (CS+ session) was significantly lower following a muscimol infusion than it was following a vehicle infusion ($P < 0.01$). The activity of saline-conditioned rats was not affected by exposure to the cue or by infusions (all $P > 0.05$), and was significantly lower than cocaine-conditioned rats, irrespective of cue or infusion (all $P < 0.05$). The Session X Infusion interaction was not statistically significant ($F < 3.1$, $P > 0.05$).

4. Discussion

Consistent with previous reports, we observed that re-exposure to a discrete compound cue (flashing bicycle light + metronome), previously paired with cocaine administration, induced robust conditioned activity (Hotsenpiller et al., 2002; Hotsenpiller et al., 2001;

Panlilio and Schindler, 1997). Moreover, the conditioned locomotor response lasted for at least 28 days following the last drug-cue pairing and endured despite repeated testing. However, the magnitude of the conditioned locomotor response did not increase across withdrawal suggesting that, unlike cue-induced reinstatement of drug-seeking (reviewed in Pickens et al., 2011; Li et al., 2008; Lu et al., 2004; Grimm et al., 2001), cue-induced conditioned activity does not incubate across drug withdrawal. Finally, the expression of cue-induced conditioned activity requires the BLA; inactivation of the BLA with the $GABA_A$ receptor agonist muscimol prevented cue-induced conditioned activity.

4.1 Cue-induced Conditioned Activity

In experiments 1, 2 and 3, cocaine-conditioned rats exhibited more activity in the presence of the discrete compound cue (flashing bicycle light + metronome) than in the absence of the cue. Furthermore, cocaine-conditioned rats exhibited more activity than saline-conditioned rats in the presence of the cue. Combined these data suggest that the cue gained the incentive motivational significance of cocaine through repeated pairings. In Experiment 1 however, saline-conditioned rats also exhibited more activity in the presence of the cue than in its absence suggesting that the cue itself may increase activity regardless of prior conditioning. Because saline-conditioned rats did not exhibit increased activity in the presence of the cue during training in Experiment 1 or during either training or testing in Experiment 2 and 3, we suggest that this effect may be a false positive. Furthermore, the magnitude of the cue effect was smaller in saline-conditioned rats than it was in cocaine-conditioned rats. Thus, even if the cue itself inconsistently increases locomotor activity, such an increase in activity is not sufficient to account for the cue-induced activity observed in cocaine-conditioned rats.

Drug-associated contexts have also been found to gain incentive-motivational properties of the drugs themselves (reviewed in Crombag et al., 2008). In the current experiment great care was taken to minimize conditioning to the context (i.e., the locomotor activity chambers). First, rats were habituated to the chambers over three 60-min sessions. Second, each training and testing session began with a 30-min

baseline session in which rats were exposed to the environment in the absence of both the cue and the drug. Third, rats were trained using explicit CS-sessions; in these sessions saline administration was paired with the context in the absence of the cue. Previous research and preliminary results from our lab indicated that these measures are necessary in order to minimize conditioning to the context and to maximize conditioning to the discrete compound cue (Wachtel and Paine, 2011; Panlilio and Schindler, 1997). Despite these efforts, there was evidence for contextual conditioning in cocaine-conditioned rats, particularly in Experiment 3. Both during training and during testing the cocaine-conditioned rats exhibited more activity than the saline-conditioned rats during baseline sessions and CS- sessions. Importantly however, this contextual conditioning did not interfere with the ability of the discrete cue to elicit conditioned activity. That is, cocaine-conditioned rats exhibited more activity in the presence of the cue than in its absence.

4.2 The incubation effect

Drug-seeking responses that result in presentation of a drug-associated cue increase across drug withdrawal, a phenomenon termed the “incubation of drug craving” (Pickens et al., 2011). For example, cocaine-seeking responses are greater in late withdrawal (e.g., 1 month) than in early withdrawal (e.g., 1 day), peaking approximately 1 month after discontinuation of cocaine self-administration (reviewed in Pickens et al., 2011; Grimm et al., 2001; Lu et al., 2004). **Moreover, incubation of craving has been observed following self-administration of other drug rewards (e.g., heroin, alcohol and nicotine; reviewed in Pickens et al., 2011) and non-drug rewards (e.g., sucrose; Grimm et al., 2011).** More recently, context-dependent increases in reward-seeking have also been observed using the place-conditioning paradigm (Li et al., 2008). In that experiment, the magnitude of the place preference for a heroin-paired environment was higher in late withdrawal (e.g., 14 days) than it was in early withdrawal (e.g., 1 day) (Li et al., 2008). Based upon these reports, we aimed to determine if non-contingent presentation of a drug-associated cue would also sensitize or incubate across drug withdrawal.

Surprisingly, we did not observe that cue-induced conditioned activity incubated across drug withdrawal—the magnitude of the cue effect was equivalent when tested in either early or late withdrawal. The failure to observe an incubation effect occurred regardless of whether a within subjects or a between subjects design was used. That said, in both Experiment 1 and Experiment 2 activity during the baseline sessions increased as time from training increased. This increase in activity *may* have occluded our ability to observe the incubation effect. However, because the activity during the CS- test sessions was equivalent during the early and late tests, we do not believe that this is the case. Further, the increase in baseline activity was observed in all rats regardless of condition. Thus, we hypothesize that the increased activity during the baseline sessions resulted from dishabituation to the activity chambers—as the time from training increased the rats' memory for the chambers may have diminished.

The failure to observe an incubation effect following non-contingent presentations of drug-associated cues suggests that there maybe something fundamentally different about cues that gain motivational significance through passive administration of drugs compared to cues that gain their motivational significance via self-administration of drugs. Although one report finds that heroin-induced conditioned place preference incubates across drug withdrawal (Li et al., 2008), there are other reports that suggest that both cocaine-induced (Mueller and Stewart, 2000; Brabant et al., 2005) and heroin-induced (Mueller and Stewart, 2002; Lu et al., 2000) conditioned place preference fail to incubate across drug withdrawal. Moreover, there is no clear evidence that responding in the presence of a drug-predictive cue (S+) incubates across drug withdrawal (Ciccopcioppo et al., 2001). In that experiment however, the number of cocaine-seeking responses in the presence of the S+ was greater during protracted withdrawal than it was during early withdrawal, but this comparison was not analyzed statistically (Ciccopcioppo et al., 2001). Moreover, Weiss et al. (2001) demonstrated that the ability of cocaine-predictive cues to reinstate drug-seeking remained stable for up to a month following drug-discontinuation despite repeated testing. These data, combined with the observations of the current

experiment, suggest that non-contingent presentation of drug-associated cues do not result in behavioral effects that incubate across drug withdrawal (but see Li et al., 2008). Rather, the incubation of 'craving' may be relatively restricted to contingent presentations of drug-associated cues.

4.3 Role of the BLA in Cue-Induced Conditioned Activity

Intra-BLA infusions of the $GABA_A$ receptor agonist muscimol blocked the expression of cue-induced conditioned activity. That is, activity in the presence of the cue was significantly lower following an intra-BLA muscimol infusion than it was following a vehicle infusion. Furthermore activity following muscimol infusions was not different in the presence as compare to the absence of the cue. In all rats, intra-BLA muscimol infusions caused a small but significant decrease in activity during the baseline session (i.e., the first 30-min post-infusion). Similar motor impairments following BLA inactivation have been previously observed (Cain et al., 2009; Ishikawa et al., 2008). A generalized motor impairment however, is unlikely to account for the inability of the conditioned cue to increase locomotor activity following intra-BLA muscimol infusions. During the test session, intra-BLA muscimol infusions did not affect activity of cocaine-conditioned rats in the absence of the cue (i.e., comparison of CS- conditions). In addition, muscimol infusions did not affect the activity of saline-conditioned rats during the test session. Thus, although intra-BLA muscimol infusions can decrease locomotor activity, it is unlikely that a general suppression of activity underlies the inability of the cocaine-associated cue to increase locomotor activity. Rather, inactivation of the BLA likely results in a selective decrease in the ability of the conditioned cue to alter behavior.

The data from the current experiment are consistent with previous reports implicating the BLA in the expression of learned associative responses. Because cannulae placements in the current experiment were restricted to the rostral BLA, the current data support the notion that the rostral BLA is important for the expression of drug-cue associations. Previously, it has been observed that the rostral, but not the caudal, BLA is critical for contingent presentations of drug-associated cues to reinstate drug seeking

behavior (Kantak et al., 2002; Mashhoon et al., 2009; Mashhoon et al., 2010). That said, the effects of caudal BLA inactivation on cue-induced conditioned activity were not tested in the current experiment. Thus it remains to be determined if a parallel functional division in the BLA is observed for cue-induced conditioned activity.

The BLA may modulate the locomotor response to the conditioned cue via its interactions with the nucleus accumbens (NAc). The BLA sends glutamatergic afferents to the NAc (LeDoux, 2007; Pitkänen et al., 1997); a connection that is necessary for cue-controlled cocaine seeking under a second order schedule of reinforcement (Ambroggi et al., 2008; Di Ciano and Everitt, 2004). In a paradigm similar to the one used in the current experiment, re-exposure to a drug-associated cue caused an increase in intra-NAc glutamate release and systemic blockade of glutamate AMPA receptors prevented the expression of cue-induced conditioned activity (Hotsenpiller et al., 2001). It is possible that the observed rise in NAc glutamate resulted from increased activity of glutamatergic neurons originating in the BLA. Moreover, it is possible that systemic blockade of glutamate transmission prevented cue-induced conditioned activity by inhibiting neural activity within the BLA, rather than by blocking glutamate receptors in the NAc per se. Future research will determine if cue-induced conditioned activity is mediated by a direct connection from the BLA to the NAc.

Alternatively, it is possible that the BLA maybe interacting with the prelimbic (PrL) prefrontal cortex to modulate the locomotor response to the conditioned cues. The PrL receives a direct connection from the BLA (Hoover and Vertes, 2007). Indeed, asymmetric inactivation of the BLA and PrL decrease reinstatement of drug-seeking behavior under a second order schedule of reinforcement (Mashhoon et al., 2010). Furthermore, inactivation of the PrL (dorsal prefrontal cortex) is sufficient to attenuate cue-induced reinstatement of drug-seeking (McLaughlin and See, 2003). Finally, response contingent presentation of cocaine-associated cues is associated with increased Fos expression in the prefrontal cortex (Kufahl et al., 2009). Thus, it is possible that the PrL plays a similar role in cue-induced conditioned activity as it does in cue-induced reinstatement of drug-seeking behavior.

4.4 Summary and Conclusions

Cue-induced craving is purported to be a major contributing factor to relapse (Pickens et al., 2011; Volkow et al., 2006). In the clinic non-contingent presentations of drug-associated cues results in profound drug craving (Volkow et al., 2006; Childress et al., 1999) and physiological responses resembling a drug-like state (Ehrman et al., 1992). Here we demonstrate that non-contingent presentations of a discrete compound cue can elicit robust conditioned locomotor activity, which persisted for up to one month despite repeated testing. Unlike tests employing contingent presentations of conditioned cues (Pickens et al., 2011), we did not observe an incubation effect whereby the magnitude of the conditioned response sensitized across drug withdrawal. It is unclear whether this is a fundamental difference between contingent and non-contingent presentations of drug-associated cues or whether the testing parameters were such that we could not observe the incubation effect. Finally, this research adds to a growing body of evidence linking the BLA to the expression of learned associations between drug rewards and discrete environmental cues. Using this model, future research may be able to disentangle the neural mechanisms mediating conditioned responses to non-contingent presentations of drug-associated cues; this may lead to more effective relapse prevention strategies.

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Figure Captions

Fig 1. Schematic of the cocaine cue conditioning protocol. Rats underwent three 60-min habituation (H) sessions prior to training. Each training day began with a 30-min baseline session (B) during which rats were placed in the locomotor boxes in the absence of cues; the rats were then removed from the boxes, treated and then returned to the boxes for a 30-min conditioning session. Conditioning sessions were divided into CS+ (cue present) and CS- (cue absent) sessions, which occurred on alternating days. Prior to CS+ sessions rats were administered cocaine (15 mg/kg, IP) or saline (1 ml/kg, IP) and then placed into the activity chamber in the presence of an audiovisual cue (flashing bicycle light + metronome). Prior to CS- sessions all rats were administered saline and then place into the activity chamber without the audiovisual cue. Following training rats were tested on three occasions. Test sessions were similar to training sessions with the exception that all rats were administered saline (1 ml/kg) prior to both CS+ and CS- sessions. Numbers on the bottom indicate experimental day.

Fig 2. Locomotor activity across habituation and training sessions. A) Activity decreased across habituation sessions. B) Activity during the 30-min baseline sessions prior to cue present (CS+) and cue absent (CS-) conditioning sessions. C) Activity during the 30-min conditioning sessions. Cocaine-conditioned rats exhibited more activity during CS+ sessions (following cocaine injection) than CS- sessions (following saline injection). $^{\ddagger\ddagger}P < 0.01$, from session 1; $^{\#\#}P < 0.01$, saline CS+ vs. cocaine CS+; $^{**}P < 0.01$, cocaine CS+ vs. CS-.

Fig 3. Re-exposure to a cocaine-associated cue increases locomotor activity. **Rats were tested 3, 14 and 28 days following conditioning (indicated on the x-axis).** A) Activity during the 30-min baseline session increased across test sessions. B) Although both cocaine and saline-conditioned rats exhibited more activity in the presence of the cue, the activity of cocaine-conditioned rats was greater than that of saline-conditioned rats in the presence of the cue. $^{\ddagger\ddagger}P < 0.01$, different from session 1; $^{\#\#}P < 0.01$, saline CS+ vs. cocaine CS+; $^{**}P < 0.01$, cocaine CS+ vs. CS-; $^{\wedge}P < 0.05$, saline CS+ vs. CS-.

Fig 4. Locomotor activity across habituation and training sessions. A) Activity decreased across habituation sessions. B) Activity during the 30-min baseline sessions prior to cue present (CS+) and cue absent (CS-) conditioning sessions. On several occasions activity was higher during baseline sessions preceding CS- sessions than it was during baseline session preceding CS+ sessions. C) Activity during the 30-min conditioning sessions. Cocaine-conditioned rats exhibited more activity during CS+ sessions (following cocaine injection) than CS- sessions (following saline injection). $^{\ddagger}P < 0.05$, 3-day group from session 1; $^{\dagger}P < 0.05$, 28-day group from session 1; $^{\S}P < 0.05$, 3-day vs. 28-day group; $^{\Psi}P < 0.05$, saline 3-day vs. 28-day; $^{\theta}P < 0.05$, cocaine 3-Day vs. 28-Day; $^{\#\#}P < 0.01$, saline CS+ vs. cocaine CS+; $^{**}P < 0.01$, cocaine CS+ vs. CS-; $^{\partial}P < 0.05$, 3-day cocaine CS+ vs. CS-; $^{}P < 0.05$ 28-day cocaine CS+ vs. CS-; $^{}P < 0.05$, 3-day saline CS+ vs. CS-; $^{\infty}P < 0.05$, 28-day saline CS+ vs. CS-.

Fig 5. Effects of testing either 3-days or 28-days after conditioning on the expression of cue-induced activity. A) Activity during the 30-min baseline session was higher in rats tested 28-days following conditioning than it was in rats tested 3-days following conditioning. B) Regardless of time since conditioning, cocaine-conditioned rats, had greater activity than saline conditioned rats in the presence of the cue (CS+). Furthermore, cocaine-conditioned rats exhibited more activity in the presence of the cue (CS+) compared to in its absence (CS-). $^{\S}P < 0.05$, 3-day vs. 28-day group; $^{\#}P < 0.05$, saline CS+ vs. cocaine CS+; $^{*}P < 0.05$, cocaine CS+ vs. CS-;

Fig 6. Histological representation of BLA cannulae placements. A) Photomicrographs depicting left and right BLA cannula placements. Dotted line shows location of the BLA. B) Schematic showing the location of the cannula tips for saline-conditioned rats (O, n=10) and cocaine-conditioned rats (★, n=9). BLA, basolateral amygdala; CeA, central amygdala. Adapted from Paxinos and Watson (2009).

Fig 7. Locomotor activity across habituation and training sessions. A) Activity decreased across habituation sessions. B) Activity during the 30-min baseline sessions prior to cue present (CS+) and cue absent (CS-) conditioning sessions. C) Activity during the 30-min conditioning session. Cocaine-conditioned rats exhibited more activity during CS+ sessions (following cocaine injection) than CS- sessions (following saline injection) and exhibited more activity than saline-treated rats in both CS+ and CS- sessions. $^{‡‡}P < 0.01$, from session 1; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, saline CS+ vs. cocaine CS+; $*P < 0.05$, $**P < 0.01$, cocaine CS+ vs. CS-; $\wedge P < 0.05$, saline CS+ vs. CS-.

Fig 8. Effect of basolateral amygdala inactivation on the expression of cue-induced locomotor activity. A) Cocaine-conditioned rats exhibited more activity than saline-conditioned rats during the baseline session. Muscimol (MUS) infusions decreased activity. B) Following a vehicle infusion (VEH), cocaine-conditioned rats exhibited increased activity in the presence of the cocaine-associated cue (CS+) compared to in its absence (CS-); an effect that was blocked by a MUS infusion. Cocaine conditioned rats, irrespective of infusion, exhibited more activity than saline-conditioned rats. $^{‡}P < 0.05$, $^{‡‡}P < 0.01$, VEH vs. MUS; $^{\#\#}P < 0.01$, saline vs. cocaine; $**P < 0.01$, cocaine CS+ vs. CS-.

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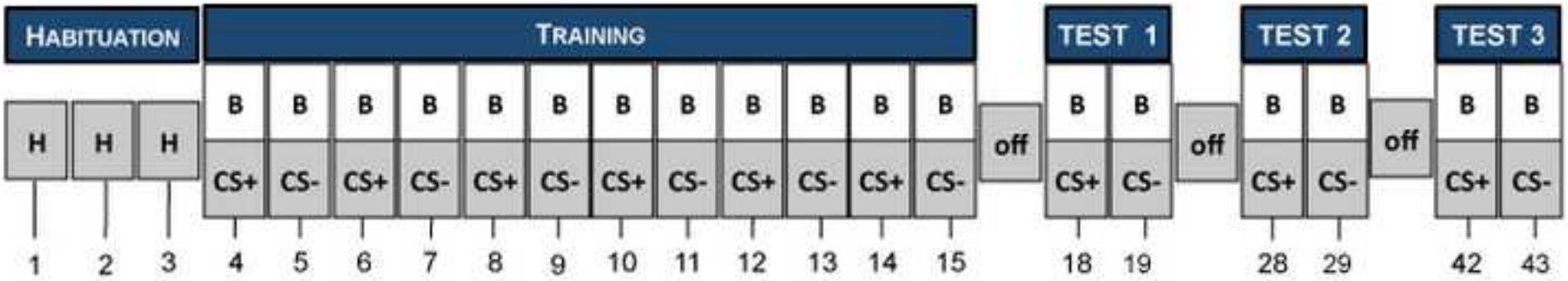

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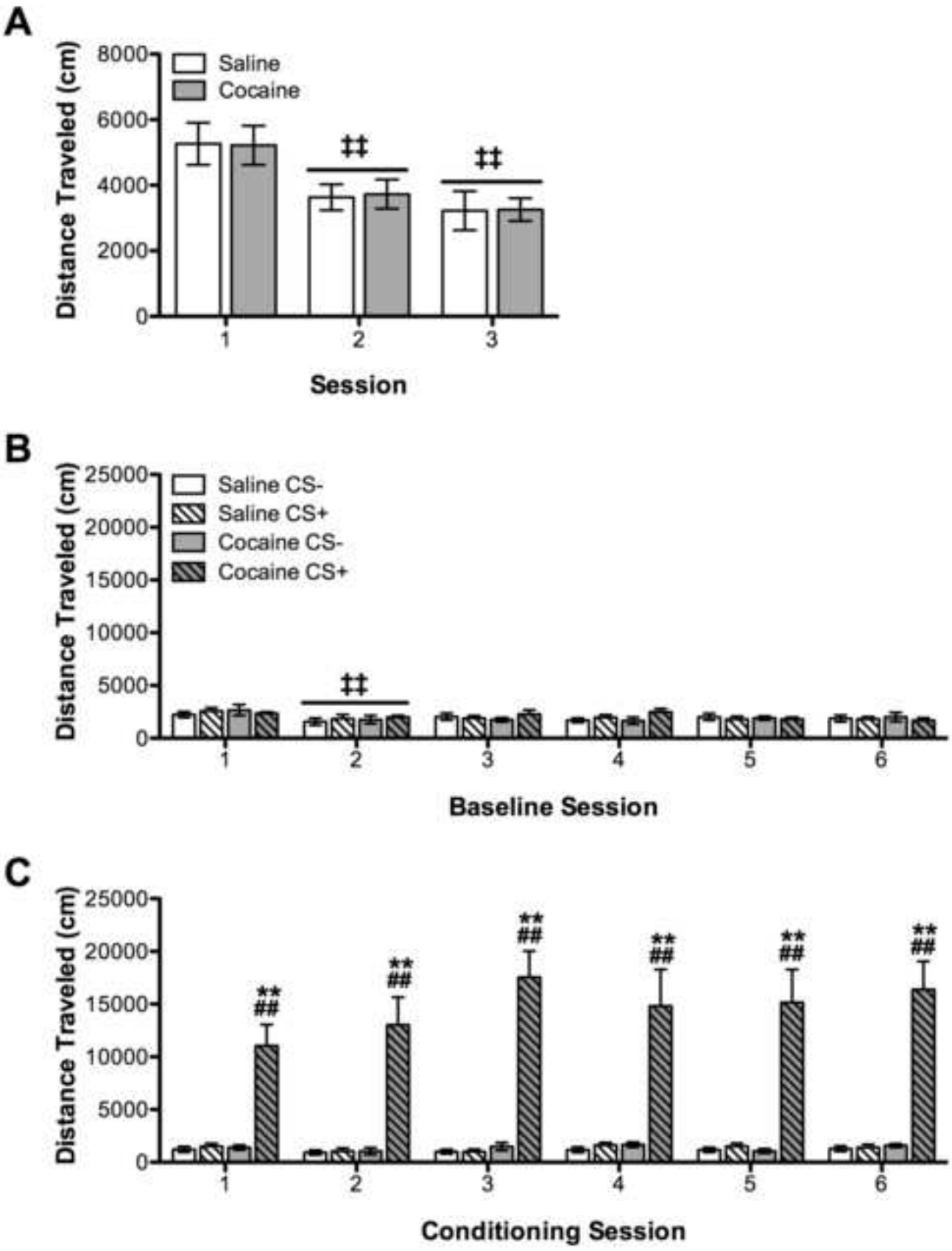

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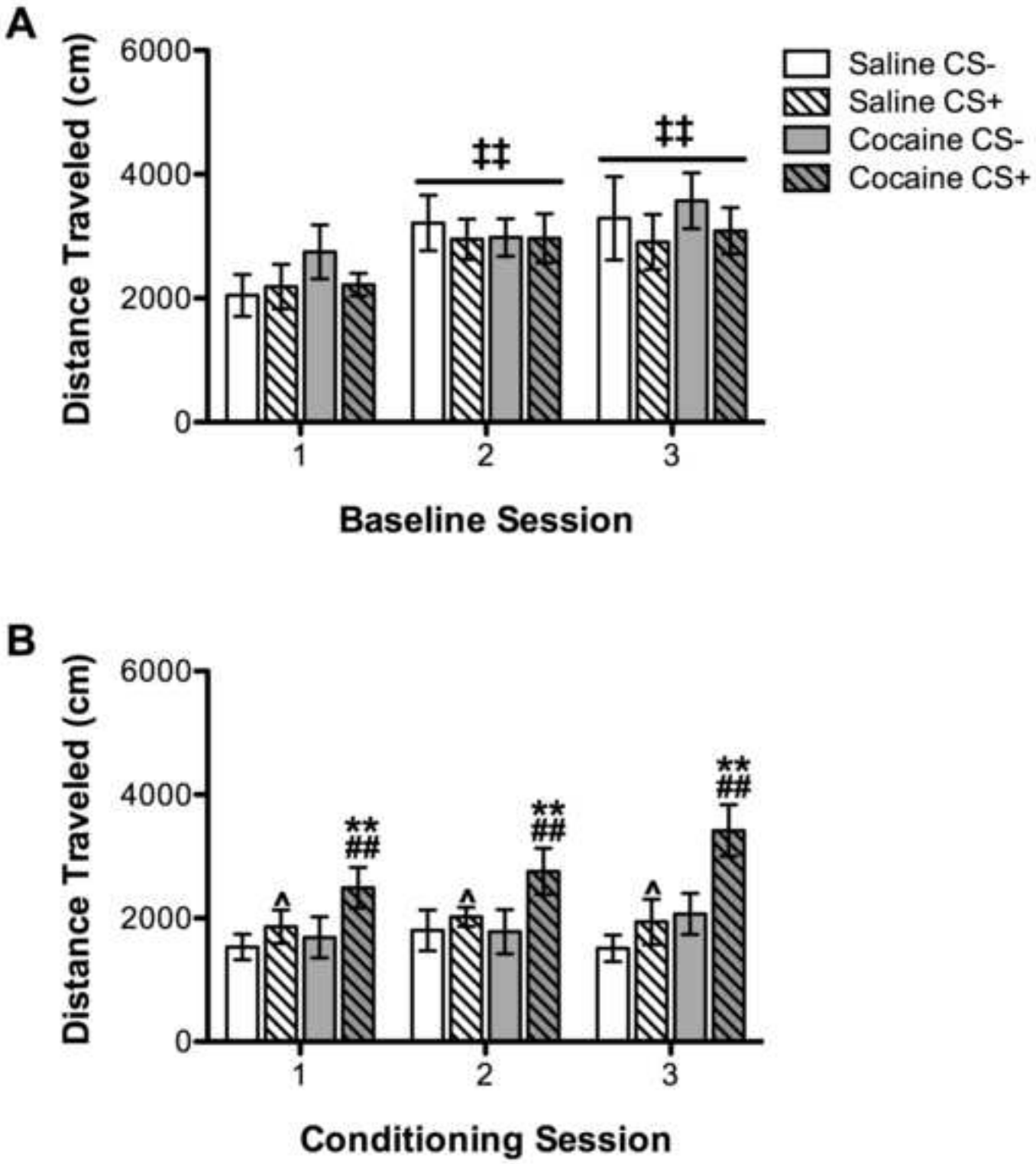

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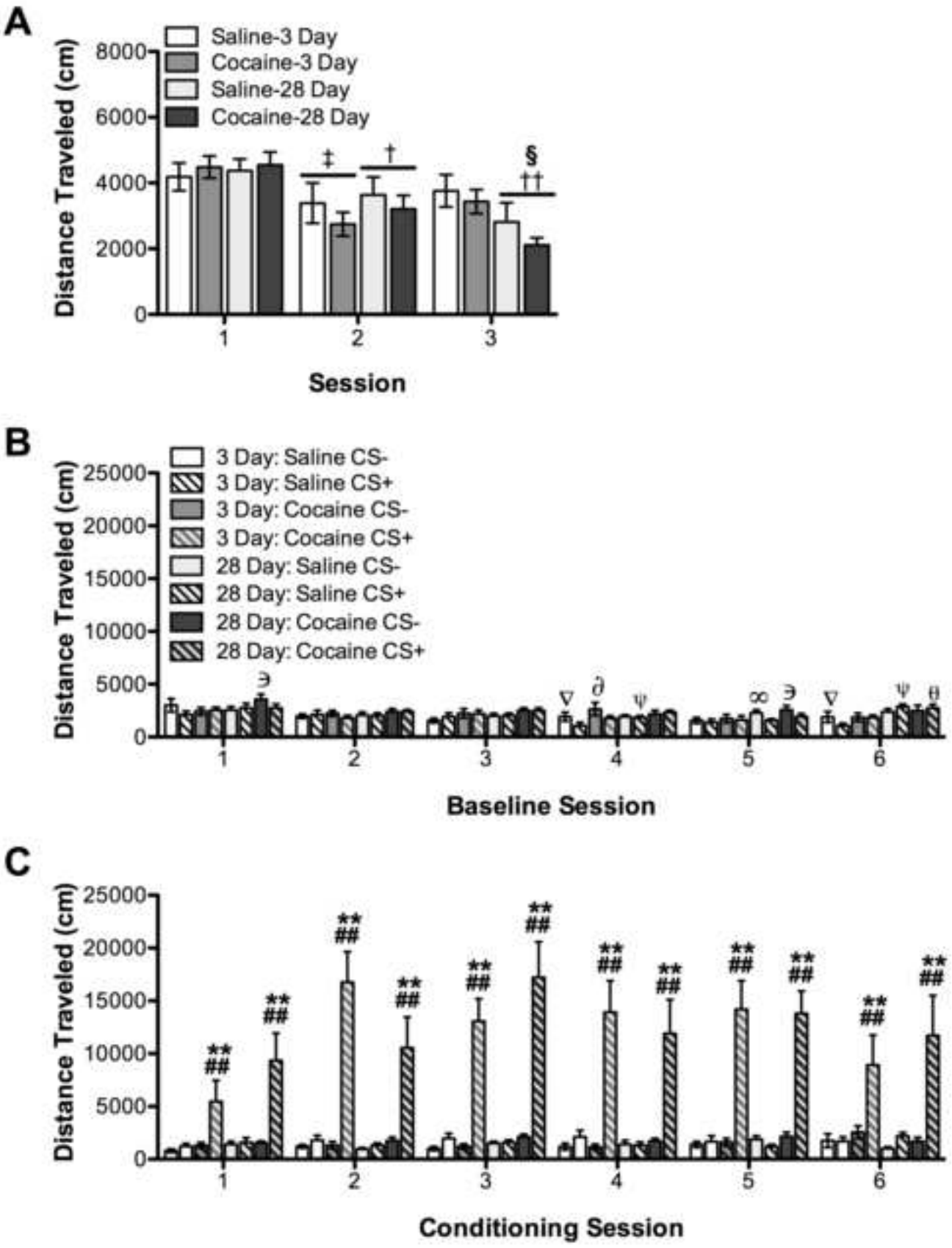

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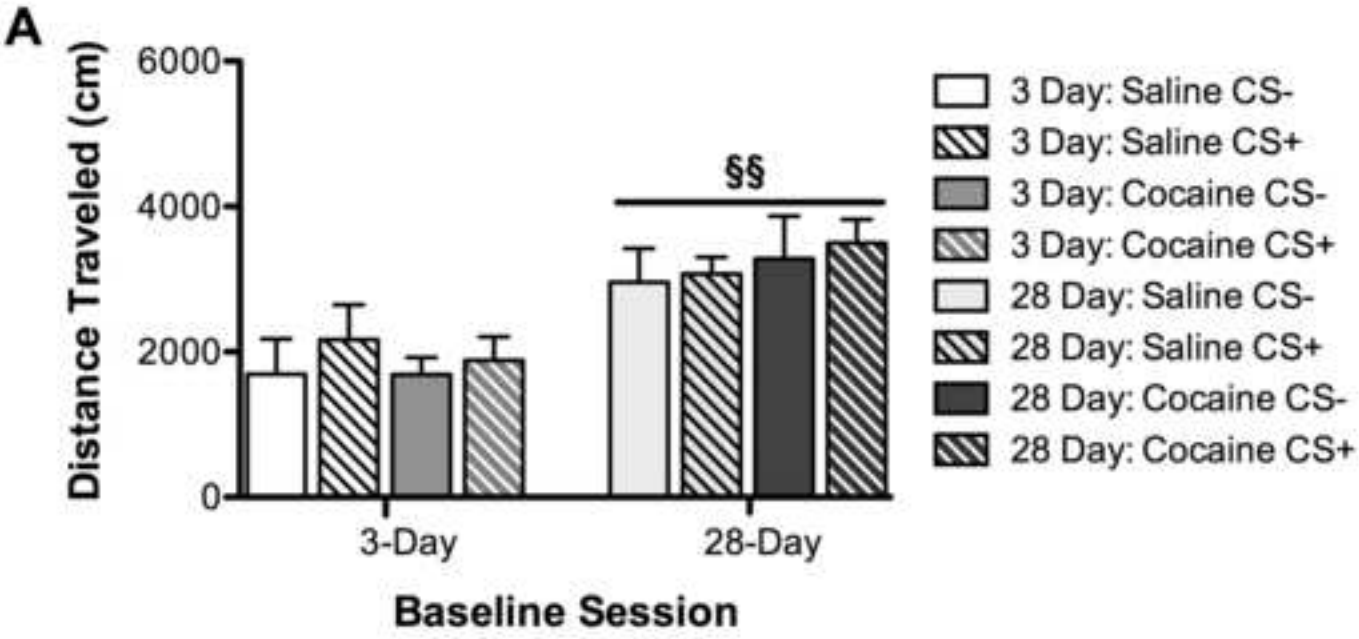

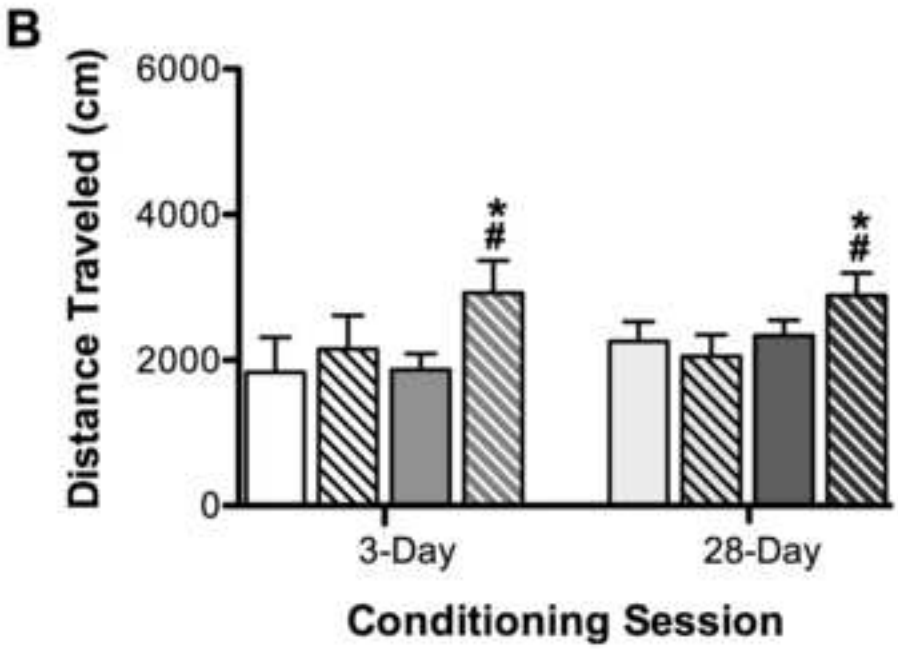

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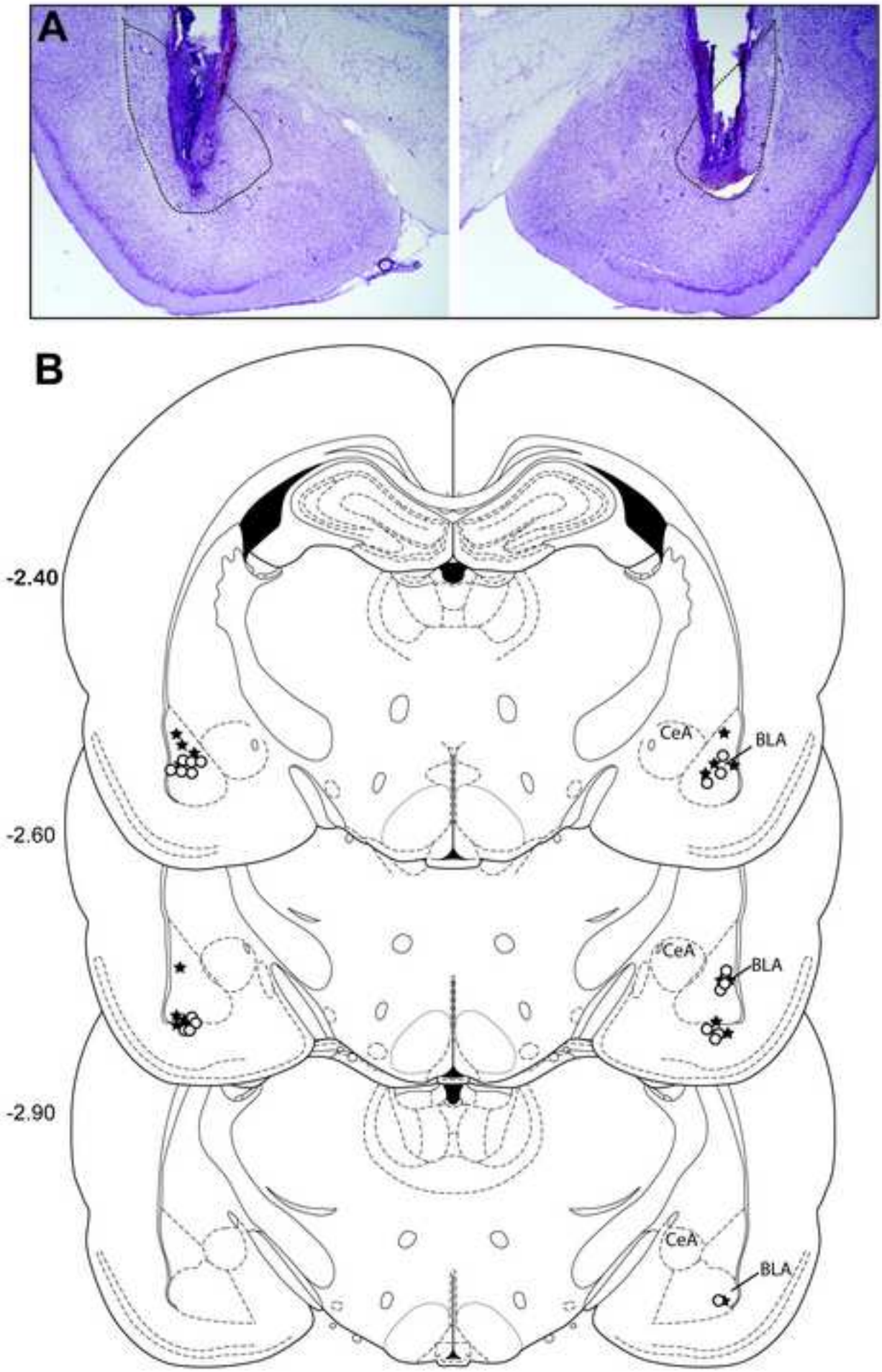

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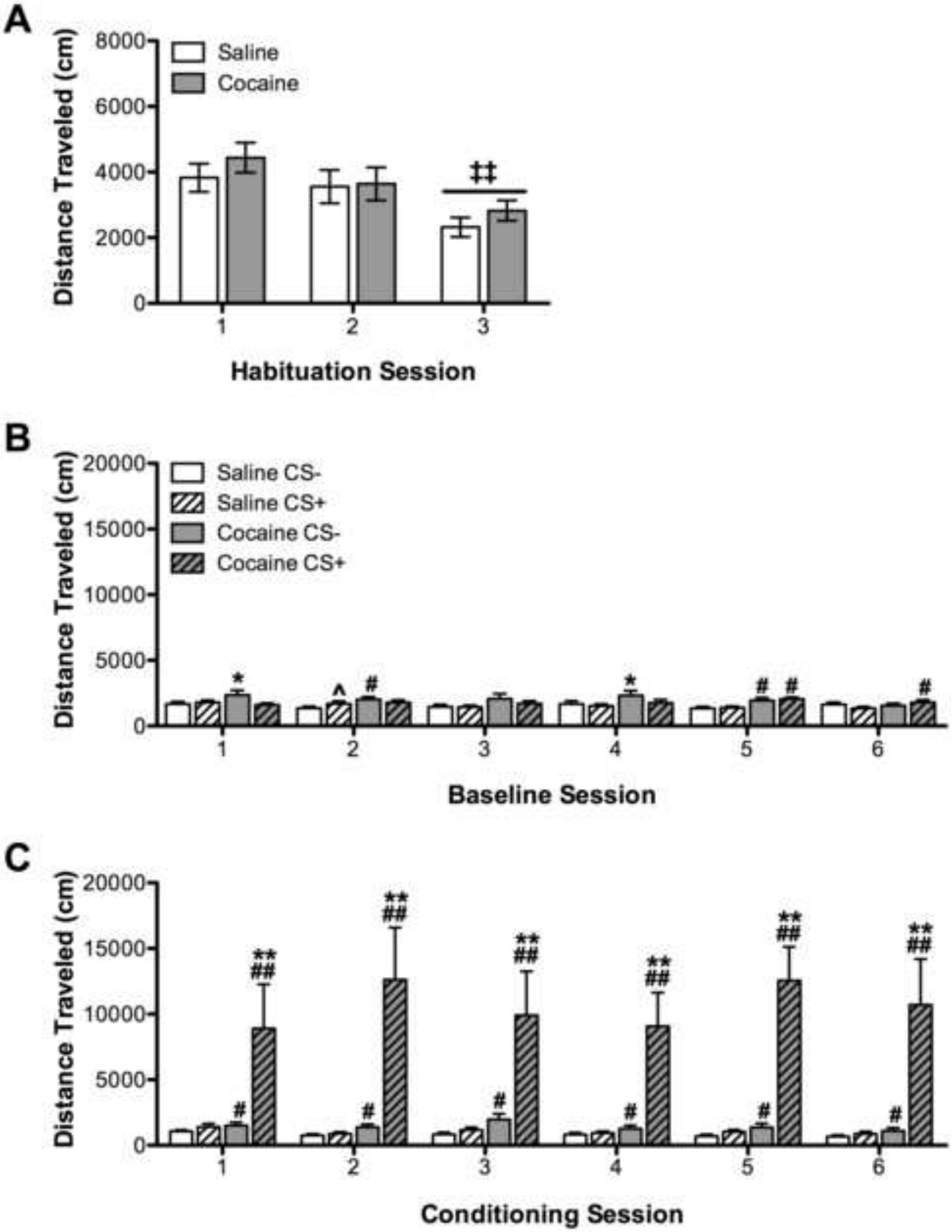

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