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Retrieval-induced NMDA receptor-dependent Arc expression in two models of cocaine-cue memory

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Abstract

The association of environmental cues with drugs of abuse results in persistent drug-cue memories. These memories contribute significantly to relapse among addicts. While conditioned place preference (CPP) is a well-established paradigm frequently used to examine the modulation of drug-cue memories, very few studies have used the non-preference-based model conditioned activity (CA) for this purpose. Here, we used both experimental approaches to investigate the neural substrates of cocaine-cue memories. First, we directly compared, in a consistent setting, the involvement of cortical and subcortical brain regions in cocaine-cue memory retrieval by quantifying activity-regulated cytoskeletal associated gene (Arc) protein expression in both the CPP and CA models. Second, because NMDA receptor activation is required for Arc expression, we investigated the NMDA receptor dependency of memory persistence using the CA model. In both the CPP and CA models, drug-paired animals showed significant increases in Arc immunoreactivity in regions of the frontal cortex and amygdala compared to unpaired controls. Additionally, administration of a NMDA receptor antagonist (MK-801 or memantine) immediately after cocaine-CA memory reactivation impaired the subsequent conditioned locomotion associated with the cocaine-paired environment. The enhanced Arc expression evident in a subset of corticolimbic regions after retrieval of a cocaine-context memory, observed in both the CPP and CA paradigms, likely signifies that these regions: (i) are activated during retrieval of these memories irrespective of preference-based decisions, and (ii) undergo neuroplasticity in order to update information about cues previously associated with cocaine. This study also establishes the involvement of NMDA receptors in maintaining memories established using the CA model, a characteristic previously demonstrated using CPP. Overall, these results demonstrate the utility of the CA model for studies of cocaine-context memory and suggest the involvement of an NMDA receptor-dependent Arc induction pathway in drug-cue memory interference.

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Keywords

Arc; Conditioned activity; Conditioned place preference; NMDA receptor; Reconsolidation

1 Introduction

Discrete stimuli or contexts previously associated with the rewarding properties of drugs are a major cause of relapse among addicts. When repeatedly paired with drug intake, initially neutral stimuli acquire incentive motivational value and encounters with these drug-associated cues induce intense physiological cravings in addicts (Childress et al., 1988). These conditioned responses to drug-paired cues are a major cause of relapse. Indeed, degree of craving measured during treatment for drug dependence is predictive of drug use resumption (Hartz et al., 2001). Because drug-paired cues evoke memories that influence drug-seeking and relapse, a better understanding of the neurobiological basis for this phenomenon will aid in the development of novel, biologically-based therapies for drug addicts.

To date, drug-cue memory interference studies have relied on measures of drug-seeking, such as instrumental responding for drug access (self-administration) or choosing a previously drug-paired environment [conditioned place preference (CPP)]. The study of drug-cue memories could be expanded to include behavioral measures distinct from drug-seeking. The conditioned activity (CA) model is a Pavlovian paradigm in which repeated, non-contingent pairings of a psychostimulant with an environment produces enhanced locomotion upon drug-free exposure to this environment (Pickens and Crowder, 1967; Beninger and Hahn, 1983; Beninger and Herz, 1986; Mazurski and Beninger, 1991). Like self-administration and CPP, CA relies on an association between environmental cues and drug. Unlike self-administration and CPP, CA is not based on preference; instead, it allows for memory strength assessment by measuring conditioned locomotion in the drug-paired context. Because the CA model employs a nonoperant response, it can be used to study the neurochemical basis of drug-conditioned behaviors without the confounding influences of goal-directedness or response-reward expectancy (Olmstead et al., 2001).

The activity-regulated cytoskeletal-associated gene (*Arc/Arg3.1*) is an effector immediate-early gene (IEG) widely implicated in experience-dependent synaptic plasticity and memory consolidation (Steward et al., 1998; Guzowski et al., 2000; Holloway and McIntyre, 2011). Several groups have used Arc as a marker of neuronal activity to investigate corticolimbic circuits underlying drug-cue memories (Schiltz et al., 2005; Zavala et al., 2008), but direct comparisons of brain regions involved using two separate models of drug-context memories are lacking. In the present study, we examined Arc expression in brain regions activated during drug-cue memory retrieval in both the CPP and CA models, training and testing subjects in a common setting, with the behavioral task as the only variable. We sampled areas previously implicated in cue-elicited drug seeking behaviors (e.g., amygdalar, corticolimbic, hippocampal, and accumbens regions). Additionally, we measured Arc expression in regions we had no *a priori* reason to believe would be involved in drug-related memories (e.g., motor and somatosensory cortices). Because CPP and CA models both

result in the formation of drug-cue associative memories, we expected substantial overlap in brain regions showing enhanced levels of Arc expression. However, because CPP consists of a choice-based component, but CA does not, task-specific regional involvement was also anticipated.

Previous studies have shown that N-methyl-D-aspartate (NMDA) glutamate receptors are required for both the induction of *Arc* transcript (Lyford et al, 1995) and for targeting the newly synthesized transcript to active synapses (Wallace et al., 1998; Steward and Worley, 2001). Using CPP, we have previously demonstrated that NMDA receptor antagonist (MK-801 or memantine) administration immediately following memory retrieval diminishes cocaine-paired compartment preference in subsequent tests (Alaghband and Marshall, 2013). Although the CA model has been used to investigate the role of NMDA receptors in acquisition (Stewart and Druhan, 1993; Cervo and Samanin, 1996) and expression (Bespalov and Zvartau, 1996; Bespalov et al., 2000) of drug-cue memories, no studies to date have used CA to interrogate drug memory maintenance and persistence. Here, we used CA to explore the role of NMDA receptors in post-retrieval modification of a cocaine-context association. Specifically, these experiments compared the effects of systemic administrations of two NMDA receptor antagonists, MK-801 and memantine, on cocaine-CA memory maintenance.

2 Materials and methods

2.1. Subjects

Male Sprague-Dawley rats (Charles River Laboratories; Hollister, CA) weighing 200-225 g upon arrival were individually housed in a temperature-controlled (21 ± 2°C) colony room with *ad libitum* access to food and water. Lights were on from 06:00 to 18:00, and all training and testing procedures were conducted between 08:00 and 14:00 each day. All experiments were conducted in accordance with the National Institutes of Health guidelines for animal care and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

2.2. Drugs

Cocaine-HCl, (+)-MK-801 hydrogen maleate, and memantine-HCl were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and dissolved in saline (0.9% NaCl). For training, cocaine-HCl was dissolved to a final concentration of 12 mg/ml (of the salt) and administered in a volume of 1 ml/kg body weight. MK-801 and memantine (0.2 and 10 mg/kg, respectively; doses as free-base) and 0.9% saline were given at 2 ml/kg body weight. All drug treatments and saline were administered intraperitoneally (i.p.). The two NMDA receptor antagonists were used to ensure that any effects were due to common actions on this receptor system. Both MK-801 and memantine function as open-channel antagonists that block NMDA receptors only when these channels are activated (Chen et al. 1992). However, MK-801 and memantine interact with the NMDA receptor complex in distinct ways. Memantine is a low-affinity blocker with rapid blocking kinetics (Ribeiro Do Couto et al. 2004; Tzschentke and Schmidt 1999), while MK-801 is a high-affinity blocker with slower kinetics (Parsons et al. 1999). Additionally, these compounds differ in their binding

site. MK-801 binds to the PCP site inside the pore of the receptor (Moring et al. 1994; Sakurada et al. 1993), whereas memantine is believed to bind at or near the Mg2+ binding site (Chen and Lipton 1997; Chen et al. 1992).

2.3. Apparatus

2.3.1. Place preference apparatus—Conditioning took place in a three-chamber apparatus (Med Associates, Inc.) consisting of two larger compartments (28 × 21 cm) separated by a smaller compartment (12 × 21 cm). The two larger compartments differed in visual, olfactory, and tactile cues. One compartment had white walls and a wire mesh floor above pine shavings, while the other compartment had black and white checkered walls and a metal-bar floor above cedar shavings. The middle compartment had two gray walls and a solid gray polyvinyl floor above corncob bedding. Additionally, this middle compartment had a white wall and a checkered wall leading into the correspondingly patterned adjacent compartments. Guillotine doors, patterned to match the outer compartments, separated the three compartments and were lowered on training days and raised on test days. Photobeams located in the walls were used to quantify time spent and distance traveled in each compartment.

As described below, some animals were placed in a control environment consisting of a clear plastic box (40 × 40 cm), distinct from the home cage, located in a room separate from both the CPP apparatus and holding rooms.

2.3.2. Activity chamber apparatus—Conditioning took place in activity monitors (Med Associates, Inc.) consisting of open-field boxes (43.2 × 43.2 × 30.5 cm) with transparent Plexiglas walls and an opaque plastic floor. Boxes were equipped with 16 photocell beams (2.5 cm off the floor) for measuring horizontal activity.

2.4. Arc protein analysis

2.4.1. Arc protein expression in the cocaine-CPP model—All animals were taken into an anteroom distinct from the colony holding room 1 h prior to the start of behavioral procedures. An unbiased, counterbalanced design was used for all conditioning trials. Baseline preference of each animal was assessed during an initial test, in which animals were allowed free access to all compartments of the CPP apparatus for 15 min, and the time spent in each of the large outer compartments was recorded. Animals were assigned to one of three treatment groups such that each group had similar mean time spent in each compartment, as determined by a Wilcoxon signed ranks test. Paired (n=11) and Paired-Unexposed Control (n = 11) groups underwent identical training procedures for CPP, but were exposed to different environments on day of sacrifice. An Unpaired group (n = 11) served as a control for drug treatment.

Conditioning began one day after the initial test and took place over 9 consecutive days (Figure 1A). On days 3, 6, and 9, the Paired and Paired-Unexposed Control animals received saline injections (1 ml/kg, i.p.) before placement in the control environment. A counterbalanced design was used such that on days 1, 4, and 7 half of the Paired and Paired-Unexposed Control animals received injections of cocaine immediately before placement in

the checkered compartment. The remaining Paired and Paired-Unexposed Control animals received saline before placement in the white compartment. On days 2, 5, and 8, the treatment and side were alternated. The Unpaired animals received saline injections before placement in CPP compartments on days 1, 2, 4, 5, 7, and 8. On days 3, 6, and 9, the Unpaired animals received cocaine injections before placement in the control environment. After treatment administration, all animals remained in their respective environments for 30 min. Forty-eight hours after the last conditioning session, the Paired and Unpaired groups underwent a 3 min re-exposure to the cocaine-paired side of the CPP apparatus. They were sacrificed 45 min after the start of the exposure to maximize Arc expression (Ramírez-Amaya et al., 2005). By contrast, the Paired-Unexposed Control group underwent a drug-free preference test forty-eight hours after the last conditioning session using the same procedure as that for the initial test. Starting the day after the final preference test, the Paired-Unexposed Control animals were handled for three consecutive days by a researcher other than the one who trained the animals on cocaine-CPP. On the fourth day after the final preference test, the animals were taken from their home cage and euthanized by this experimenter.

2.4.2. Arc protein expression in the cocaine-CA model—In this experiment, all animals were taken directly from the colony holding room into the activity chamber room, where they underwent behavioral procedures. Baseline horizontal activity (cm traveled) was measured by placing each animal in the activity chamber for a 30 min initial test. Animals were assigned to Paired (n = 12), Activity-Yoked Paired (n = 12), and Unpaired (n=12) groups so that the initial test horizontal activity of the three groups did not differ. Training began one day after the initial test and lasted 7 days. Paired animals received cocaine, while Unpaired animals received saline prior to placement in the activity chamber for 30 min (Figure 1B). Two hours after removal from the activity chamber on each day, Paired animals were given saline and Unpaired animals were given cocaine in the colony holding room (home cage). Two days after training, the Paired and Unpaired groups were re-exposed for 6 min to the activity chamber in a drug-free state, during which horizontal activity counts were measured. A 6-min session was chosen because it was 1/5 of the initial test session duration (as for the CPP experiment). The Activity-Yoked Paired group was designed to control for the potential contribution of locomotion to Arc expression on the final test day. These animals underwent the same training as the Paired group, but, on the final day, they were allowed to locomote in the activity chamber only until they matched the average distance traveled by the Unpaired group during the 6-min session, at which time they were removed. All animals were euthanized 45 min after the start of exposure to the activity boxes.

2.5. Tissue processing and Arc immunohistochemistry

Rats were given an overdose (375 mg/kg) of sodium pentobarbital (i.p.) and decapitated 2.5 min later. Brains were rapidly extracted and frozen in isopentane (~−80 °C), then stored at -20 °C until sectioned on a cryostat. Twenty μm-thick sections were mounted onto slides, such that all experimental groups were represented on each slide. Also, the positions of the experimental groups' sections were counterbalanced on each slide. These procedures controlled for slide-to-slide and within-slide variations in fluorescent signal associated with the staining procedure. The sections were post-fixed for 5 min in 4% paraformaldehyde

dissolved in phosphate buffered saline (0.1 M sodium phosphate with 0.9 % NaCl pH 7.4; PBS) and were then washed (3 × 5 min) with Tris buffered saline (0.1 M Tris with 0.9 % NaCl pH 7.4; TBS). Endogenous peroxidase activity was inhibited by incubation for 15 min in 2% H_2O_2 in TBS. Sections were washed (3 × 5 min) in TBS and nonspecific antibody binding was blocked with TSA blocking buffer (Perkin Elmer) for 10 min. Sections were incubated for 1 h in anti-Arc primary antibody made in rabbit (1:2000 diluted in TSA blocker; Synaptic Systems catalog #156002). Sections were then washed in TBS with 0.05% Tween (TBS-T) and incubated for 1 h in HRP-conjugated anti-rabbit secondary antibody (1:500 diluted in TSA blocking buffer; Jackson ImmunoResearch). Sections were washed in TBS-T and Arc-positive cells were fluorescently labeled with TSA-CY3 (1:50; 15 min; light-protected). After TBS washes (3 × 5 min), slides were coverslipped with Vectashield.

2.6. Image acquisition and analysis

Figure 2 presents representative photomicrographs showing Arc protein expression of the Paired, Unpaired, and Control groups in selected brain regions. Figure 3E-G illustrate the specific brain regions analyzed using the atlas planes from Paxinos and Watson (2007). Sections taken +2.76 mm from bregma contained the cingulate cortex (Cg1), prelimbic cortex (PrL), infralimbic cortex (IL), orbitofrontal cortex and claustrum (OF&Cl), and primary somatosensory cortex (S1). Sections taken +2.04 mm from bregma contained the dorsal caudate putamen (dCPu), dorsal endopiriform nucleus (DEn), nucleus accumbens core (AcbC), and nucleus accumbens shell (AcbSh). Sections taken -2.76 mm from bregma contained the primary and secondary motor cortices (M1 & M2), dentate gyrus (DG), CA3 and CA1 subfields of the hippocampus, basolateral amygdala (BLA), lateral amygdala (LA), central amygdala (CeA). Images of immunofluorescent Arc-positive cells were obtained using a Leica DM-2000 microscope equipped with a DC-500 cooled camera. The number of Arc-positive cells in defined regions of each brain section was determined with a computer-assisted image analysis program (MCID; InterFocus Imaging; Linton, Cambridge, England). Analysis of Arc immunoflourescence was done at a magnification of 10X for all brain regions except for dCPu, AcbC, and AcbSh, which were done at 20X. Area and density criteria for defining Arc-positive cells were determined by a preliminary survey of each sampled brain region. Arc levels were expressed as a count density, i.e. the number of labeled cells sampled per μm^2. Because of the difficulty distinguishing individual cells from the high neuropil staining in CA1 and CA3 regions, the intensity of Arc labeling in the cellular layers of these hippocampal regions was measured as integrated optical densities, expressed as gray levels. These values were corrected for background antibody labeling by subtracting gray level readings taken in adjacent areas of motor cortex (for CA1) or dorsal thalamus (for CA3). All readings were taken from both hemispheres of each section. Because analysis by paired-samples t-tests showed no significant hemispheric asymmetries in Arc activation, values for each region are reported as an average of readings from both hemispheres.

2.7. Behavioral procedures using the CA model to investigate post-retrieval NMDA receptor antagonism

The CA training procedures were conducted as described in section 2.4.2. A test measuring horizontal activity was administered two days after the last training day. This test (test 1)

was conducted using animals in a drug-free state with the same procedures as for the initial test. Results of test 1 were used to assign the Paired and Unpaired animals to experimental (NMDA receptor antagonist) and control (saline) groups, ensuring that that the Paired and Unpaired group had mean horizontal activities that did not differ. To determine whether MK-801 treatment blunts the ability of the cocaine-paired context to facilitate locomotor activity using the CA paradigm, one day after test 1, all animals were re-exposed to the activity chamber for 6 min (retrieval trial), then given either 0.2 mg/kg MK-801 or saline immediately afterward, followed the next day by another activity test (test 2). This two-day paradigm of re-exposure followed by treatment and locomotor activity test was repeated four additional times with two days rest intervening. To further test the potential role of NMDA receptors in the maintenance of a previously learned cocaine-CA memory, we examined whether memantine would interfere with cocaine-CA maintenance in a different set of animals. The experimental paradigm used was identical in all respects to the experiment above, except that memantine (10 mg/kg) was administered post-retrieval and the two-day paradigm of re-exposure followed by treatment and locomotor activity count test was repeated twice rather than four times (Figure 5).

2.8. Statistical analysis

CPP Arc expression was analyzed using two-way repeated measures ANOVA (SPSS), with brain regions as within-subjects variables, treatment (Paired, Unpaired, Paired-Unexposed Control) as between-subjects factors, and test as the repeated measure. Significant results were further analyzed with one-way ANOVA *post hoc* tests. Paired-Unexposed Control animals underwent the same training and testing procedures as the other two groups, but were not exposed to the test apparatus on the day of euthanasia and thus showed basal Arc expression that was significantly lower than that of the Paired and Unpaired groups. Because the comparisons of interest were between Paired and Unpaired animals, the post hoc ANOVAs did not include the Paired-Unexposed control group. For the CPP experiment behavioral data, Wilcoxon signed-ranks tests were conducted to compare Preference Scores (PS, the time spent in the cocaine-paired compartment minus the time spent in the saline-paired compartment) on the final versus initial tests. For the CPP experiments, results were considered significant at $p \leq 0.05$.

The identical approach was taken for the CA Arc expression analyses, except that treatment (Paired, Unpaired, Activity-Yoked Paired) was the between-subjects factor. Because multiple *post hoc* comparisons were performed (Paired vs. Unpaired and Paired vs. Activity-Yoked Paired), the Bonferroni correction was used. For the behavioral data of the CA experiment, one-way ANOVA was used to compare distance traveled (cm) between the Paired and Unpaired groups during the 6 min retrieval session.

For the NMDA receptor antagonism studies, initial analyses were done with two-way repeated measures ANOVA, with horizontal activity count as the within-subjects variable, treatment (NMDA receptor antagonist vs. saline) as the between-subjects factor, and test as the repeated measure. Significant effects were further analyzed using one-way ANOVA *post hoc* tests. For the CA experiments, results were significant at $p \leq 0.05$, unless Bonferroni corrections indicated lower p values were necessary.

3 Results

3.1 Retrieval of cocaine-CPP memory results in increased Arc expression in the PrL, IL, Cg1, and BLA

Brief re-exposure of trained rats to the cocaine-paired side of the CPP apparatus caused a significant increase in Arc immunofluorescence in the PrL, IL, Cg1, and BLA in Paired as compared to Unpaired animals (Figures 2 [top row] and 3A-D). ANOVAs comparing the density of Arc-positive cells (counts/μm^2) of each brain region across all three groups revealed a significant main effect of group ($F_{(2,30)} = 77.96$, $p < 0.0001$) and a significant group-by-brain region interaction ($F_{(26,390)} = 18.06$, $p < 0.0001$). When only the Paired and Unpaired groups were included, ANOVAs comparing the Arc density of each brain region revealed a significant main effect of group ($F_{(1,20)} = 6.11$, $p = 0.02$) as well as a significant group-by-brain region interaction ($F_{(13,260)} = 2.59$, $p < 0.01$). Compared to the Unpaired group, the Paired group showed significantly greater Arc expression in the PrL ($F_{(1,20)} = 20.56$, $p < 0.001$) , IL ($F_{(1,20)} = 6.21$, $p = 0.02$) , Cg1 ($F_{(1,20)} = 9.57$, $p < 0.01$) , and BLA ($F_{(1,20)} = 6.73$, $p = 0.02$) , and a trend towards increased Arc in the OF&Cl ($F_{(1,20)} = 3.49$, $p = 0.08$). It is noteworthy that Arc immunoreactivity in the accumbens regions, hippocampal regions, dCPu, and the other amygdala regions did not differ between Paired and Unpaired groups ($F_{(1,20)} = 0.0078 - 2.7$, $0.1 < p$'s < 0.9). As expected, the control regions S1, M1 & M2, and DEn also showed no differences in Arc between Paired and Unpaired groups ($F_{(1,20)} = 0.14 - 1.2$, $0.3 < p$'s < 0.7).

The Paired-Unexposed Control group was used to verify that conditioning procedures in this cohort of animals resulted in a significant preference. These animals did not express a preference for either side of the apparatus on initial test (time spent in prospective saline-paired compartment: 350.23±35.66 s vs. time spent in prospective cocaine-paired compartment: 344.64±21.48 s; $z = -0.267$, $p = 0.8$), but showed a significant preference for the cocaine-paired side on final test (time spent in saline-paired compartment: 256.61±28.80 s vs. time spent in cocaine-paired compartment: 442.69±26.94 s; $z = -2.58$, $p = 0.01$). Notably, the Unpaired group showed significantly higher levels of Arc in brain regions analyzed compared to the Paired-Unexposed Control group in the CPP model (Figure 3A-D). This group difference is likely due to the animals' experience on the day of sacrifice. Whereas the Unpaired animals waited in the anteroom, where they had been placed prior to cocaine-CPP training, and were exposed to the experimenter who administered cocaine on training days, the Paired-Unexposed Control animals were taken directly from their home cage to be euthanized by a familiar experimenter who had not previously trained or tested the animals. The generally greater Arc expression of the Unpaired compared to the Paired-Unexposed group may be due to arousal induced by the testing procedures just prior to euthanasia.

3.2. Retrieval of cocaine-CA memory results in increased Arc expression in the PrL, IL, AcbSh, and BLA

The CA Paired group showed a significantly greater level of locomotion than the Unpaired group during the 6 min retrieval test (Paired: 2304.92±162.58 cm, Unpaired: 1680.99±188.54 cm; $F_{(1, 22)} = 6.28$, $p = 0.02$). The average distance the Activity-Yoked

group was permitted to travel was within 1% of that traveled by the Unpaired group during the retrieval session (Activity-Yoked Paired: 1671.96±12.83 cm). Brief re-exposure to the CA activity box resulted in significant increases of Arc protein in the PrL, IL, AcbSh, and BLA of the Paired and Activity-Yoked Paired groups, compared to the Unpaired control animals (Figures 2 [middle row] and 3H-K).

ANOVAs comparing Arc-positive cell densities of each brain region across all three treatment groups revealed a significant group-by-brain region interaction ($F_{(26, 429)} = 1.99$, $p < 0.01$) and a trend towards a main effect of group ($F_{(2, 33)} = 3.03$, $p = 0.06$). Paired and Activity-Yoked Paired groups did not differ in Arc levels across brain regions ($F_{(1, 22)}$ = 0.012 - 2.66, 0.1 < p's < 0.9). By contrast, the Paired group exhibited greater Arc expression in the PrL ($F_{(1,22)} = 10.58$, $p < 0.01$), IL ($F_{(1,22)} = 16.80$, $p < 0.001$), AcbSh ($F_{(1,22)} = 7.18$, $p = 0.014$), and BLA ($F_{(1,22)} = 8.33$, $p < 0.01$) compared to the Unpaired group. Thus, elevated Arc expression in these brain regions depends on the association between the activity chamber and cocaine administration, but not on the amount of locomotion. Notably, the Arc-positive cell densities within the DG, CA3, CA1, Cg1, dCPu, and the other amygdala regions did not differ between the Paired and Unpaired groups (Figures 2 [bottom row] and 3H-K; $F_{(1,22)}$ = 0.04-2.90, 0.1 < p's < 0.9). Additionally, control regions S1, M1 & M2, and DEn showed no differences in Arc immunoreactivity between the Paired and Unpaired groups ($F_{(1,22)}$ = 0.14 - 0.87, 0.3 < p's < 0.7).

Additional analyses demonstrated that the Paired and Activity-Yoked Paired groups did not differ in Arc density across brain regions and that the Paired groups exhibited greater Arc immunoreactivity in regions of the Acb, amygdala, and frontal cortex compared to the Unpaired control group. Thus, elevated Arc expression reflects the association between the activity chamber and cocaine administration, and not locomotor activity *per se*. Further, comparisons between Activity-Yoked Paired and Unpaired animals were not performed because these groups differed in both the amount of time they were exposed to the cocaine-paired environment as well as the associations with the compartment in which they are placed on the day of euthanasia. Consequently, the interpretation of observed differences between these groups would be less certain than comparisons between the Paired and Unpaired groups.

3.3 Post-retrieval NMDA receptor antagonist MK-801 administration attenuates conditioned locomotion as compared to saline-treated animals.

Brief (6 min) re-exposure to the CA activity chamber followed immediately by 0.2 mg/kg MK-801 (i.p) blunted the subsequent ability of the cocaine-paired context to facilitate locomotor activity, as evidenced by less conditioned locomotion in this group compared to saline-paired animals (Figure 4). This was supported by the results of ANOVAs comparing horizontal activity counts from tests 1 through 6 of the Paired groups, which revealed a significant treatment-by-test interaction ($F_{(5, 140)} = 2.68$, $p = 0.02$) and a significant main effect of treatment ($F_{(1, 28)} = 5.39$, $p = 0.03$). After four rounds of exposure to the activity chamber, each followed by 0.2 mg/kg MK-801 treatment, the MK-801-Paired group showed significantly lower activity than the corresponding Saline-Paired group on tests 5 and 6 ($F_{(1,28)} = 7.62$, $p \leq 0.01$; $F_{(1,28)} = 8.82$, $p < 0.01$, respectively). By contrast, ANOVAs

comparing activity from tests 1 through 6 of the Unpaired groups revealed neither a significant treatment-by-test interaction ($F_{(5, 135)} = 0.55$, $p = 0.7$), nor a main effect of treatment ($F_{(1, 27)} = 0.18$, $p = 0.7$).

3.4 Post-retrieval NMDA receptor antagonist memantine administration attenuates conditioned locomotion as compared to saline-treated animals

A second NMDA receptor antagonist, memantine, was used to further test the role of NMDA receptors in maintaining a previously established cocaine-CA memory. As shown in Figure 5, after repeated re-exposure to the test chambers, each of which was followed by 10 mg/kg memantine administrations, the Memantine-Paired group's activity was significantly reduced compared to the Saline-Paired group. When ANOVAs were used to compare activity across tests 1 through 4 of the Paired groups, a significant treatment-by-test interaction was found ($F_{(3,48)} = 4.79$, $p < 0.01$), and a trend towards a significant main effect of treatment ($F_{(1, 16)} = 3.86$, $0.05 < p < 0.1$) was observed. Further, after three rounds of cue re-exposure followed by memantine treatment, the Memantine-Paired group's activity was significantly lower than the corresponding Saline-Paired group on test 4 ($F_{(1,16)} = 7.52$, $p = 0.014$). ANOVAs comparing horizontal activity from tests 1 through 4 in the Unpaired groups revealed no main effect of treatment ($F_{(1, 16)} = 0.41$, $p = 0.5$).

4 Discussion

By directly comparing corticolimbic Arc expression arising from context re-exposure in the CPP and CA models, the present experiments show substantial overlap in specific forebrain regions activated in these two paradigms. In both CPP and CA, we show significant increases in Arc protein levels in the PrL, IL, and BLA of drug-paired animals briefly re-exposed to the environment in which they received drug compared to unpaired controls (Figures 2 and 3). Although others have investigated corticolimbic circuits activated by drug-cue memories using either CPP or CA, this is the first study to examine brain region involvement in each model within a common setting, using internally consistent methodology. Overall, the present findings agree with intracerebral infusion studies implicating these same brain regions in drug-cue memory retrieval. For example, NMDA receptor antagonist D(-)-2-amino-5-phosphonopentanoic acid (D-APV) infusion into the BLA before memory reactivation disrupted the drug associated memory and abolished subsequent instrumental responding for conditioned reinforcement (Milton et al., 2008). A large body of evidence suggests that the BLA participates in Pavlovian conditioning (Dunn and Everitt, 1988; See, 2002) and is specifically implicated in the reconsolidation of drug-cue memories (Lee et al., 2005, 2006). That evidence is consistent with our observation of post-retrieval conditional increase in Arc positive cells within this structure in both CPP and CA.

Other studies suggest that projections from the PrL to the AcbC promote cocaine-seeking, while projections from the IL to the AcbSh mediate the expression of extinction of cocaine-seeking (Peters et al., 2008, 2009). Notably, we found elevated levels of Arc in the PrL *and* IL of Paired animals using both paradigms. Much research indicates that memory retrieval initiates reconsolidation and extinction, two processes with distinct temporal signatures.

Brief exposures to conditioned cues result in memory destabilization followed by restabilization, thus preferentially leading to reconsolidation, while more prolonged and repeated exposures favor extinction (Eisenberg et al., 2003; Pedreira and Maldonado, 2003; Suzuki et al., 2004; Power et al., 2006; Mamiya et al., 2009; Inda et al., 2011). It can be argued that the brief exposures (3 min for CPP; 6 min for CA) to the cocaine-paired environment in the present studies may have lead to both memory reconsolidation and initiation of extinction of drug-seeking behavior. In this regard, it is notable that the magnitude of IL (but not PrL) Arc expression attributable to the pairing of cocaine with the context was greater in the CA (compared to the CPP) paradigm, consistent with the longer exposure to drug-paired context of the CA favoring extinction. In line with this view, we also observed a significantly enhanced level of Arc protein expressed within the AcbSh in the Paired group of the CA but not the CPP study. Thus, our data are consistent with projections from the IL to the AcbSh mediating extinction of cocaine-seeking (Peters et al., 2008, 2009).

Although we found increased cue-induced Arc expression in many of the expected corticolimbic regions, there are notable exceptions. The Paired and Unpaired groups showed no difference in levels of Arc protein in the AcbC in either the CPP or CA experiments. Previous studies from this laboratory found elevated levels of c-fos in the AcbC (Miller and Marshall, 2004) and activation of ERK in the D1 cell population of the AcbC (Fricks-Gleason and Marshall, 2011) in paired versus unpaired animals. Post-retrieval infusions of MEK inhibitor into AcbC interfered with local ERK phosphorylation and disrupted cocaine-CPP memory (Miller and Marshall, 2005a). Thus, while much evidence points to a role for AcbC plasticity in cocaine-context memories, activation of *Arc* in the AcbC may not contribute to this plasticity.

Additionally, the dorsal hippocampus has been suggested to play a role in the expression of drug context-induced cocaine-seeking (Fuchs et al., 2005, 2007), and its functional integrity is required for post-reactivation reconsolidation of memories that facilitate context-induced cocaine seeking (Ramirez et al., 2009). Also, Hearing et al. (2010) reported that re-exposure of animals to a cocaine-associated context in a self-administration model increased *Arc* mRNA in the DG, CA1 and CA3 hippocampal fields. By contrast, we did not observe retrieval-induced enhanced Arc protein expression within the dorsal hippocampus. The distinction between *Arc* mRNA and protein may explain this discrepancy because McIntyre et al (2005) reported that brain manipulations that enhance or diminish memory consolidation have corresponding influences on dorsal hippocampal Arc protein, but not mRNA. In addition, Ramirez et al (2009) present evidence that, despite its involvement in a circuit facilitating reconsolidation of cocaine-context memories, the dorsal hippocampus is not the site of anisomycin-sensitive memory restabilization.

Although the CPP and CA models both result in the formation of drug-environment memories, the CPP model is preference-based whereas the CA model is not. Although animals in neither the CPP nor CA experiments were asked to make a choice on day of sacrifice, animals in the CPP model learn during their initial test that the two large compartments of the apparatus are connected and that they can select between them. This decision-making feature may help explain the increased Arc protein observed in the Cg1 of

the Paired versus Unpaired groups in the CPP, but not the CA, model. The Cg1 is reciprocally connected to the PrL, and both structures send projections to the BLA (Conde et al., 1995). These projections may be responsible for coordinated interaction between prefrontal regions and the amygdala, which are thought to be necessary in planning and implementing appropriate motor responses (Salinas and McGaugh, 1996; McDonald, 1998). The Cg1 plays a significant role in evaluative processes and choice behavior (Hillman and Bilkey, 2010). Human imaging studies show activation of this region during voluntary decision-making (Walton et al., 2004; Yoshida and Ishii, 2006; Croxson et al., 2009). Also, Cg1 lesions in primates are associated with suboptimal choice behavior; these animals fail to use positive or negative feedback to optimize behavior during choice tasks (Amiez et al., 2006; Kennerley et al., 2006). Thus, Arc induction in the Cg1 may be specific to retrieval of memories that involve a behavioral choice.

The lack of significant *Arc* activation in various control regions further supports the conclusion that the enhanced Arc levels in specific corticolimbic regions are involved in retrieval of cocaine-cue memories formed in both the CPP and CA models. The S1 and M1 & M2 showed no group differences, suggesting that Arc protein levels in these regions are not related to the underlying drug-associated memory or locomotor activity levels. The absence of increased Arc expression in the dCPu is in agreement with other studies (Schiltz et al., 2005; Zavala et al., 2008), suggesting that this region does not play a strong role in cue-induced drug-seeking (however, see Hearing et al., 2008). Zavala et al. (2008) found an increase in *Arc* mRNA in the OF of animals presented with cues previously paired with cocaine administration compared to controls, but we did not see such a group difference in either model when we analyzed the OF & Cl together. However, Zavala et al. (2008) used a reinstatement paradigm in extinguished animals, so the discrepancy in findings may arise because the OF contributes specifically to reinstatement of an extinguished behavior.

Since NMDA receptor-activation is required for the expression of Arc, we also investigated the post-retrieval NMDA receptor-dependency of memories formed using the CA model. Post-retrieval administration of either MK-801 or memantine blunted the ability of subsequent exposure to the cocaine-paired context to facilitate locomotor activity, evidenced by a significant reduction in conditioned locomotion as compared to saline-treated animals. NMDA receptor antagonist-treated animals in the unpaired group showed levels of locomotion similar to the unpaired saline-treated animals. If these antagonists' influences on locomotion had been attributable to unconditioned pharmacological effects, we would have expected a similar reduction in locomotion in the MK-801/memantine-unpaired animals and the MK-801/memantine-paired group. Furthermore, the present design of administering NMDA receptor antagonists *after*, but not before, memory reactivation affords significant benefits because it eliminates potential confounding influences of these drugs on attention, arousal, or other processes during memory reactivation (Gold and Van Buskirk, 1976; Introini-Collison et al., 1992; McGaugh 1992).

The results presented here agree with our previous findings that NMDA receptor antagonists have parallel effects on impeding the consolidation and reconsolidation of cocaine-CPP (Alaghband and Marshall, 2013), supporting a role for these receptors in the strengthening of both recently acquired and retrieved associations. The finding that NMDA receptor

antagonists disrupted cocaine-conditioned locomotion suggests that NMDA receptor antagonism does not simply interfere with the underlying motivation to seek cocaine or cocaine-associated *cues*. Instead, post-retrieval NMDA receptor antagonism disrupts the association formed between the effects of cocaine and the context in which cocaine is administered, irrespective of the particular conditioned response.

The memory impairments arising from post-retrieval administration of the NMDA antagonists suggest that NMDA receptor activation is necessary after stimulus presentation during both memory consolidation and reconsolidation. During memory reconsolidation, the original memory is placed in a labile state, making it subject to the effects of inhibition of cellular and molecular processes downstream of NMDA receptors. We hypothesize that signal transduction pathways activated by Ca^{2+} influx through the NMDA receptor might mediate memory reconsolidation in ways similar to their involvement in acquisition and consolidation. Burgos-Robles et al. (2007) found that consolidation of extinction requires post-training activation of NMDA receptors within the ventromedial prefrontal cortex (vmPFC). Interestingly, the NMDA receptor antagonist CPP selectively reduced burst firing in the vmPFC, and rats that showed impaired burst firing in the IL subregion of the vmPFC were unable to consolidate extinction learning. In fact, the degree of bursting in the IL neurons after extinction training predicted subsequent recall of extinction.

Moreover, it has recently been shown that motor training increases Arc expression in a subset of frontal cortical excitatory neurons and these neurons show post-training persistent firing, and the persistent firing was diminished in animals carrying a genetic deletion of *Arc* (Ren et al., 2014). This implies that *Arc* is a key molecular link between prior experience and the establishment of persistent firing patterns in select frontal cortical neurons. In the present experiments, enhanced Arc expression in several corticolimbic regions after retrieval of a cocaine-context associated memory suggests that neurons undergoing neuroplasticity contribute to updating information about cues previously associated with cocaine.

Drug-cue memory retrieval may place brain circuits involving the PrL, IL, and the BLA into a labile state, thus making them susceptible to reconsolidation interference. Because Arc activation depends on NMDA receptors, these brain regions are promising sites for the contribution of NMDA receptors to these forms of memory plasticity and may represent loci at which systemic administration of NMDA receptor antagonists exerted their mnemonic influences.

In summary, our findings suggest that drug-cue memory retrieval may place brain circuits involving the PrL, IL, and the BLA into a labile state, raising the possibility of therapeutically manipulating NMDA receptor-dependent Arc expression during memory reconsolidation. This offers a promising potential target for development of novel therapeutics that can disrupt drug-cue memories and diminish relapse incidence among drug abusers.

Acknowledgements

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Abbreviations

CPP	conditioned place preference
CA	conditioned activity
Arc/Arg3.1	activity regulated cytoskeletal-associated gene
IEG	immediate early-gene
NMDA	N-methyl-D-aspartate
Cg1	cingulate cortex
PrL	prelimbic cortex
IL	infralimbic cortex
OF&Cl	orbitofrontal cortex and claustrum
S1	primary somatosensory cortex
dCPu	dorsal caudate putamen
DEn	dorsal endopiriform nucleus
AcbC	nucleus accumbens core
AcbSh	nucleus accumbens shell
M1 & M2	primary and secondary motor cortices
DG	dentate gyrus
CA3 and CA1	subfields of the hippocampus
BLA	basolateral amygdala
LA	lateral amygdala
CeA	central amygdala
D-APV	D(-)-2-amino-5-phosphonopentanoic acid
vmPFC	ventromedial prefrontal cortex

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Highlights

► Retrieval causes enhanced Arc protein in the frontal cortex and amygdala

► NMDA receptor antagonists blunted locomotion facilitation by cocaine-paired context.

► Retrieval-induced lability allows for involvement of NMDA receptors.

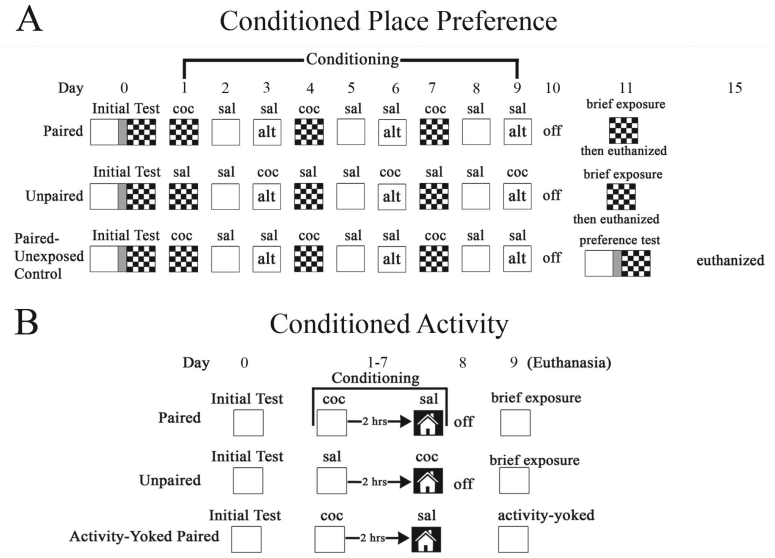

Figure 1. Schematic representation of experimental timeline for CPP and CA Arc protein expression experiments. (A) Schematic representation of CPP paradigm

All animals were given free access to the CPP apparatus and tested for initial preference on day 0 of the experiment. Drug treatment and order of environment in which animals were confined are represented by the individual squares. On day 11, Paired and Unpaired animals underwent a 3 min re-exposure in the cocaine-paired environment and were euthanized for Arc immunohistochemistry 45 min after start of exposure. The Paired-Unexposed Control group underwent a Preference Test on day 11, and four days later they were taken from their home cage and euthanized for Arc immunohistochemistry. **(B) Schematic representation of CA paradigm.** Baseline horizontal activity (distance traveled in cm) was measured by placing each animal in the activity chamber for a 30 min initial test on day 0 of the experiment. Training began one day after the initial test and lasted 7 days. Paired animals received cocaine while Unpaired animals received saline prior to placement in the activity chamber for 30 min. Two hours after removal from the activity chamber, Paired animals were given saline and Unpaired animals were given cocaine in the colony holding room (home cage). On day 9, Paired and Unpaired animals were re-exposed for 6 min to the activity chamber. Rats in the Activity-Yoked Paired group were allowed to locomote in the activity chamber only until they matched the average distance traveled by the Unpaired group during the 6-min session, at which time they were removed from the activity chamber. All animals were euthanized 45 min after the start of exposure to the activity boxes.

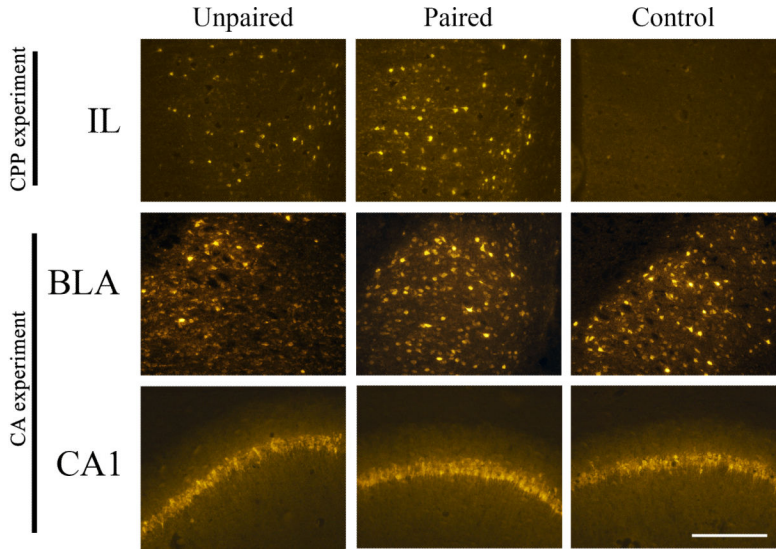

Figure 2. Representative photomicrographs show Arc protein expression of the Paired, Unpaired, and Control groups in selected brain regions

For the IL, Arc protein expression is depicted for the CPP experiment. For the BLA and CA1, Arc protein expression is depicted for the CA experiment. Control groups for the CPP and CA experiments differed. Control column denotes image from the Paired-Unexposed Control group and the Activity-Yoked group for the CPP and CA experiments, respectively. Scale bar, 80 μm for regions shown.

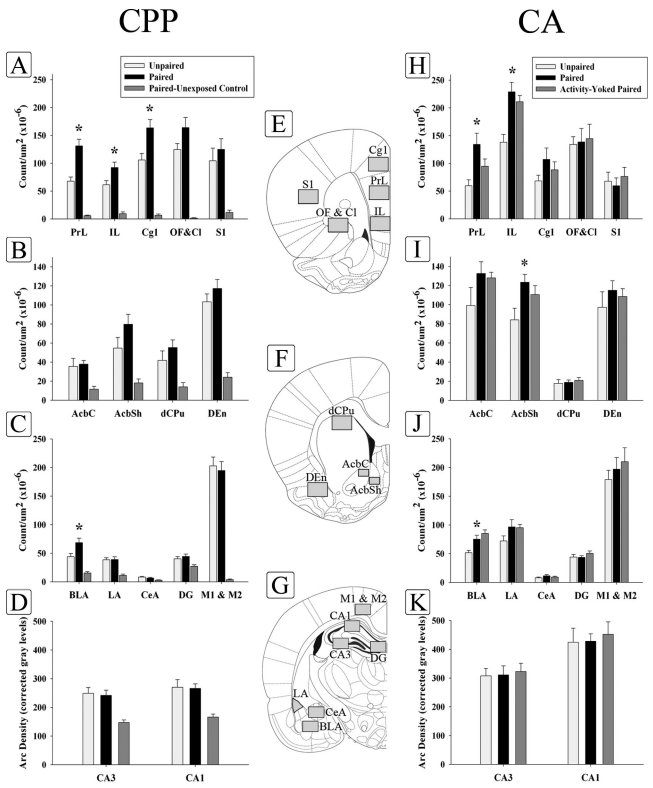

Figure 3. Paired animals show greater levels of Arc expression in the PrL, IL, and BLA in both the CPP and CA experiments compared to their respective controls

(A-C) Quantification of Arc protein expression in the Paired (n = 11), Unpaired (n = 11), and Paired-Unexposed Control (n = 11) groups for brain regions analyzed at 2.76, 2.04, or -2.76 mm from bregma, respectively. **(D)** Arc expression in the CA 3 and CA1 regions analyzed at -2.76 mm from bregma. Overall statistical analyses of the CPP data for all brain regions yielded a significant group-by-brain region interaction and main effect of group (two-way ANOVA, $p < 0.05$). * Indicates significant differences from Unpaired groups (one-way ANOVA, $p < 0.05$). **(E-G)** Brain regions analyzed at 2.76, 2.04, or -2.76 mm from bregma, respectively. **(H-J)** Quantification of Arc protein expression in the Paired (n = 12), Activity-Yoked Paired (n = 12), and Unpaired (n = 12) groups for brain regions analyzed at 2.76, 2.04, or -2.76 mm from bregma, respectively. **(K)** Arc density in the CA 3 and CA1 regions analyzed at -2.76 mm from bregma. Overall statistical analyses of the CA data for all brain regions yielded a significant group-by-brain region interaction (two-way ANOVA, $p < 0.05$). * Indicates significant group difference from the Unpaired groups (one-way ANOVAs, $p < 0.025$).

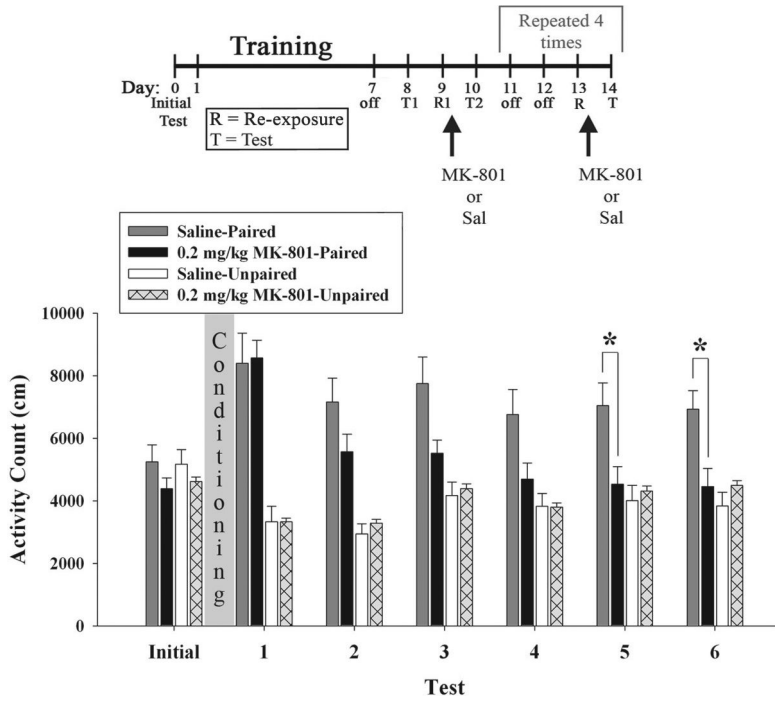

Figure 4. Post-retrieval MK-801 administration interferes with cocaine-CA memory maintenance

Top Schematic representation of the experimental timeline. *Bottom* Locomotion (distance traveled in cm) in MK-801-treated and saline control animals when tested in the activity chamber. Values represent mean ± SEM. Saline group (n = 15); 0.2 mg/kg MK-801 group (n = 15); Saline-Unpaired group (n = 15); MK-801-Unpaired group (n = 14). * Indicates significant group differences between the Saline-Paired and MK-801 Paired groups (one-way ANOVAs, $p \leq 0.01$). *R*, 6 min re-exposure to the activity chamber; *Sal*, saline; *T*, Test.

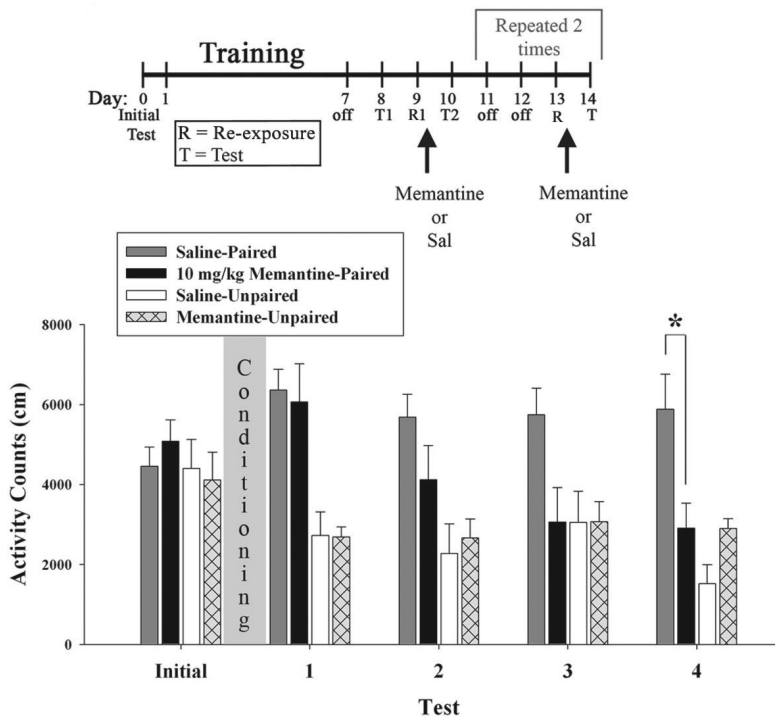

Figure 5. Post-retrieval memantine administration interferes with cocaine-CA memory maintenance

Top Schematic representation of the experimental timeline. *Bottom* Locomotion (distance traveled in cm) in memantine-treated and saline control animals when tested in the activity chamber. Values represent mean ± SEM. Saline group (n = 9); 10 mg/kg Memantine group (n = 9); Saline-Unpaired group (n = 9); MK-801-Unpaired (n = 9). * Indicates significant group differences between the Saline-Paired and Memantine-Paired groups (one-way ANOVAs, $p < 0.017$). *R*, 6 min re-exposure to the activity chamber; *Sal*, saline; *T*, Test.