

Amphetamine exposure selectively enhances hippocampus-dependent spatial learning and attenuates amygdala-dependent cue learning

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Abstract

Behaviorally sensitizing regimen of amphetamine exposure has diverse effects on learning, memory and cognition that is likely to be a consequence of long term neural adaptations occurring in the cortico- limbic-striatal circuitry. In particular, altered dopamine signaling in the nucleus accumbens and medial prefrontal cortex has been implicated to underlie amphetamine-induced changes in behavior. The present study sought to test the hypothesis that repeated amphetamine exposure disrupts the regulation of limbic information processing and the balance of competing limbic control over appetitive behavior. Mice received 7 intra-peritoneal injections of d-amphetamine (2.5mg/kg or 5mg/kg) or vehicle solution (saline) and were trained in a 1) simultaneous conditioned cue and place preference task using a 6 arm radial maze, found to depend upon the integrity of the hippocampus (HPC), and basolateral amygdala (BLA) respectively and a 2) conditional BLA-dependent cue-, and HPC-dependent place learning task using an elevated T-maze. In both tasks, the vehicle pre-treatment group acquired cue learning first, followed by the emergence of significant place/spatial learning. In contrast, pre-treatment with repeated amphetamine caused marked deviations from normal acquisition patterns of place and cue conditioning, significantly facilitating HPC-dependent place conditioning in the first task, while attenuating BLA-dependent cue conditioning in both tasks. These findings provide the first demonstration of aberrant regulation of HPC and BLA-dependent learning as a result of amphetamine exposure, highlighting the importance of the meso-corticolimbic dopamine system in maintaining the balance of limbic control over appetitive behavior.

Key words: conditioned place preference, learning and memory systems, nucleus accumbens, dopamine, schizophrenia, addiction

Introduction

Repeated exposure to amphetamine (AMPH) induces enduring behavioral and neural sensitization which is **revealed by** the exaggeration of behavioral and neurochemical responses to subsequent acute AMPH challenge. Used widely as an animal model of escalating drug craving in human addiction (Robinson and Becker, 1986; Robinson and Berridge, 1993, 2001), much research has implicated long-term neural adaptations involving augmented dopaminergic neurotransmission in the striatum (Paulson and Robinson, 1995; Pierce and Kalivas, 1995, 1997) in underlying the expression of AMPH sensitization (see Vanderschuren and Kalivas, 2000 for review).

However, AMPH exposure has far wider consequences on aspects of learning, memory and cognition. Acute or repeated exposure to AMPH in animals facilitates many forms of appetitive learning including the acquisition of pavlovian approach to, and instrumental responding for a reward-associated cue (Harmer and Phillips, 1998; Taylor and Jentsch, 2001), and habit learning (Nelson and Killcross, 2006; Nordquist et al., 2007). Repeated infusions of AMPH directly into the nucleus accumbens (NAc) enhance the acquisition of a novel instrumental responding for response-contingent conditioned reinforcers (Taylor and Robbins, 1984; Taylor and Horger, 1999), and response *non-contingent* food cues (Wyvell and Berridge, 2000), highlighting the importance of elevated mesolimbic dopamine in mediating these effects.

In contrast, a sensitizing regimen of AMPH treatment can cause cognitive disturbances reminiscent of symptoms of schizophrenia (Castner and Goldman-Rakic, 2003). Impaired performance in spatial working memory with a concomitant decrease in DA turnover in the dorsolateral prefrontal cortex and caudate putamen has been reported in primates pre-treated with AMPH (Castner et al., 2005). Escalating doses of AMPH in rats also impair visual attentional task performance; a deficit that was reversible with the application of a D1 receptor agonist into the medial prefrontal cortex (mPFC;

Fletcher et al., 2007). Indeed, AMPH withdrawal is often associated with a decreased dopaminergic neurotransmission in the prefrontal cortex (Paulson and Robinson, 1995; Pierce and Kalivas, 1997; Castner et al., 2005).

The NAc and mPFC are crucial components of a wider cortico-limbic-striatal circuitry which regulates the control of adaptive behavior by behaviorally significant stimuli (Cardinal et al., 2002). Both regions receive dopaminergic inputs from the ventral tegmental area which overlap with converging glutamatergic inputs from key limbic structures, namely the hippocampus and amygdala (Pennartz et al., 1994; Mulder et al., 1998; French and Totterdell, 2003), implicated in spatial/contextual learning and the formation of associations between discrete cues and behaviorally salient events respectively (O'Keefe and Nadel, 1978; Morris et al., 1982; Phillips and LeDoux, 1992; Burns et al., 1993; Ito et al., 2006). Much evidence suggests that these limbic inputs compete for control over appetitive behavior (White and McDonald, 1993; Ito et al., 2006). The objective of the present study was thus to test the hypothesis that repeated AMPH exposure causes dysregulation in the balance of control over appetitive behavior by the two competing learning and memory systems, using two tasks; 1) a novel concurrent cue and spatial conditioning task in which spatial cues and a non-spatial elemental cue were in direct competition with one another for associative strength, and the rate of acquisition monitored by conditioned preference tests; 2) parallel conditional cue and spatial conditioning task in which the presence or absence of specific floor inserts in the start arm acted as a conditional cue in determining which of the two goal arms would be rewarded. We present novel findings that AMPH sensitization alters the balance of limbic information processing, preferentially enhancing hippocampal control under circumstances in which HPC-dependent and BLA-dependent information are directly in competition with one another for control over appetitive behavior, while suppressing BLA control over appetitive behavior.

Methods

Subjects were 80 female C57BL/6 mice (Harlan, UK) weighing between 18-22g at the time of surgery or drug treatment. They were group housed (6) in a room held at a temperature of 21°C under a 12hr light/dark cycle (lights on at 0700hrs). Water was available *ad libitum*, but prior to the start of behavioral testing, food (laboratory chow, Purina) was restricted to 2g lab chow/day, sufficient to maintain pre-operative/treatment body weight and growth. All experiments were conducted during the light phase, in accordance with the United Kingdom 1986 Animals (Scientific Procedures) Act Project License No30/2561.

Surgery

In all surgical procedures, animals were anaesthetised with isoflurane (Abbott Lab Ltd, Kent, UK), and placed in a stereotaxic frame (Kopf, USA) with the incisor bar set at -1.0mm below the inter-aural line. A 5µl Hamilton syringe adapted with a 34 gauge stainless steel needle was then lowered into the hippocampus or basolateral amygdala for bilateral lesions. For hippocampus lesions, four injections of 0.07M NMDA (Sigma-Aldrich, Dorset, UK) in 0.1M sterile phosphate buffer (sterile PB) were made according to established methodology (Deacon et al., 2002), using the following coordinates (in mm from bregma); 0.1µl each at AP=+1.8, +1.3, +0.7, L=±1.5, ±1.8, ±2.4, V=-1.8, -2.0, -2.0; 0.15µl at AP=+0.7, L=±3.1, V=-3.5. The infusion cannula was left in place for a further 3min after each infusion to allow complete diffusion of the toxin from the tip. For basolateral amygdala lesions, one infusion of 0.07M NMDA buffered to pH7.4 using 0.1M sterile PB, was made at the following co-ordinates: 0.1µl at AP=+2.5, L=±3.25, DV=-4.8 (SS) followed by 2min diffusion time. Sham control groups were treated identically to the lesion groups, except that they did not receive any form of infusions. Following surgery, mice were allowed a recovery period of at least 7 days prior to behavioral testing, with food available *ad libitum*.

Amphetamine drug injections

40 C57BL/6 mice were assigned to 3 groups of 13 or 14 mice: Amphetamine 5mg/kg (AMPH 5; n=13); Amphetamine 2.5mg/kg (AMPH2.5; n=13) and Vehicle (VEH; n=14). AMPH5 and AMPH2.5 mice were given 7 daily injections (I.P) of d-amphetamine sulphate (at dose of 2.5mg/kg or 5mg/kg). Mice in the VEH group received 7 daily injections (I.P) of saline. **All injections took place in a separate room to their housing and behavior testing environment.** All mice were then given a minimum of 7 days of drug-free period, before any form of behavioral testing began.

Conditioned cue and place preference task:

Radial arm maze apparatus

Behavioral testing took place in a six arm radial maze made of wood and painted grey, elevated 80cm from the floor. The maze consisted of 6 open arms with a 1cm raised edge (60cm x 7cm) emanating from a central compartment which was surrounded by a transparent Perspex cylinder (18cm diameter, 30cm high) with 6 manually operated doors allowing access to each of the arms. Stainless steel wells in which mice could be rewarded with 0.1ml of sweetened condensed milk were located at the end of each arm. The maze itself was placed in a large room with various extra-maze cues (stools, posters, sink) which remained in the same position for the duration of the experiment. The floor of the maze was wiped down with ethanol solution after each session to eliminate any odour traces and the maze was rotated to varying degrees (60°, 120° or 180°) at the end of the testing day to minimize conditioning to intra-maze cues.

Experimental procedure (Figure 1)

Habituation: All mice were given one 15min habituation session in which they were free to explore the whole maze.

Conditioning (Sessions 1-3, 5-7, 9-11): Mice were given 3 rounds of 3 daily sessions of concurrent cue and place conditioning, such that they received a total of 9 reward-cue and reward-place pairings (see Figure 1). For each daily session, mice were confined in each of the 6 arms for 3 minutes, with the order of presentation of the arms randomized across sessions for each animal. **They received 3 aliquots of 0.1ml condensed milk within the 3 minute confinement period in an arm** which contained a continuous striped floor insert stimulus (cue conditioning), and an arm which was in a particular spatial location (place conditioning), but did not receive any reward in the rest of the 4 arms. The floor insert was moved randomly between different arms (all arms except the place arm) across each session, and the maze was rotated after each session to minimize conditioning to the arms themselves.

Conditioned Cue and Place Preference Test (Session 4, 8, 12): Following each round of 3 conditioning sessions, mice were given 5min to explore the entire apparatus in the absence of any reward. Thus, a total of 3 preference tests were conducted for each animal. The time spent in each arm location was recorded.

Conditional cue and spatial learning task

Subjects

Subgroups of animals from Experiment 1 (BLA group n=6; BLA sham, n=6; Saline, n=6; AMPH 2.5 n=7; AMPH 5 n=7) were subsequently tested on the conditional cue and spatial learning task in a new room with novel spatial cues and floor inserts.

T maze apparatus (Figure 2)

Behavioral testing was conducted in an elevated wooden, grey-painted T-maze which consisted of a start arm (35 x 10cm) and two goal arms (35x 10cm). Specially adapted stainless steel food wells

were placed at the end of both arms, comprising a small well embedded in another well half filled with condensed milk (but inaccessible to the animal) to ensure that the animals did not solve the task on the basis of odour cues. The maze was surrounded by a 10cm raised wall, and was placed in a different room to the radial maze, with a number of distinct extra-maze cues. White perspex and wire mesh floor inserts extending the whole length of the start arm were used as conditional cues, and sand paper and green felt floor inserts were used in the goal arms for the conditional cue trials.

Experimental procedure

Habituation: Mice were given 4 days of 2min free exploration of the T maze apparatus and given the opportunity to collect rewards from the ends of the two goal arms.

Conditioning: Mice were trained to learn three floor insert-reward contingencies (Figure 2); 1) white perspex floor insert in the start arm signaled the availability of reward (0.1ml condensed milk) in the left goal arm; 2) wire mesh insert – reward in right goal arm, and 3) no floor insert – reward in sand paper goal arm (but not in green felt goal arm). The pairing of the start-arm floor insert and rewarded goal arm and the assignment of reward contingencies to goal-arm floor inserts were counterbalanced across animals, and remained constant for each mouse across sessions. The first two associations assessed spatial learning, and their successful acquisition has previously been shown to depend upon the integrity of the hippocampus (Schmitt et al., 2004). The last association tested cue learning, and the position of the goal arm floor inserts (green felt or sand paper) was changed (right or left goal arm) from trial to trial. The mice received a total of 28 sessions of 12 trials with an inter-trial interval of around 5min. Each session consisted of 4 cue learning trials, and 8 spatial learning trials (4 with each floor insert) presented in a pseudorandom sequence, with no more than 3 successive trials with the same contingencies.

Locomotor sensitization test in amphetamine-pretreated mice

AMPH- or saline-pretreated mice were placed in individual transparent plastic cages (26x15x17) equipped with 2 sets of infrared photobeams located 14cm apart and 1.5cm above the floor, and their locomotor activity was measured in 5min time bins. 2 hours after the beginning of the session, all mice were given 1 injection of AMPH (2.5mg/kg, i.p.) and placed back into the activity cages. Locomotor activity was then monitored for another 2 hours.

Histological procedure

All BLA and HPC and sham-lesioned mice were anaesthetised with sodium pentobarbitone (0.25ml/animal, 200mg/ml Euthatal, Rhone Merieux, UK) and perfused intracardially via the ascending aorta with 0.01M phosphate-buffered saline (PBS), followed by 10% formalin saline. Brains were then removed, stored in 10% formalin and transferred to a 30% sucrose cryoprotectant solution on the day before sectioning. Coronal sections (50µm) of the brain were cut using a freezing microtome and then stained with cresyl violet.

Data Analysis

All data were analysed using the SPSS statistical package version 15.0 (SPSS, Chicago, IL). Data generated for each test session in the conditioned preference task consisted of the absolute time spent in each of the 6 arms of the radial maze within 5min (**see Table 1**). These times were subsequently converted into 'discrimination ratios' which were calculated as, time spent in each arm/total time spent in all the arms **to control for differences in the degree of exploration between mice within, as well as between groups**. Repeated measures analysis of variance (ANOVA) **was thus applied to the discrimination ratios** with lesion or treatment group as the between-subjects factor and test (3 levels) and/or arms (3 levels; CUE, PLACE, NR) as the within-subjects factor(s). Further ANOVAs, and *post-hoc* tests including

Dunnett's test (for comparisons of treatment/lesion group performance with sham group performance) and one sample t-tests (to evaluate performance above chance level), were conducted upon confirmation of significant interactions. **However, where there were no significant differences in the total time spent exploring the arms in conditioned preference tests between treatment/lesion groups, selective ANOVAs were also conducted on the absolute time spent in the cue and place arms with lesion or treatment group as the between subject factor, and arms (CUE VS. PLACE) as the within subject factor.** For locomotor sensitization tests, repeated measures ANOVA were conducted with bins of 5min and pre- post-AMPH challenge as within-subject factors, and treatment group as the between-subjects factor. Furthermore, a Spearman's correlation analysis between the magnitude of locomotor activity change following an AMPH challenge (calculated as (mean of 3 bins of baseline activity) - (mean of 3 bins of peak activity level after AMPH) for each animal), and the performance in the first test (cue and place preference discrimination ratio) was conducted. **Data from the conditional cue and spatial learning task were expressed as mean percent correct responses in blocks of 8 trials for the conditional cue learning, and in blocks of 16 trials for the conditional spatial task, and were subjected to repeated measures ANOVA with block as the within-subject factor, and treatment or lesion groups as a between-subject factor.**

Results

Lesion assessment

The extents of the excitotoxic lesions of the HPC and BLA are shown schematically in Figure 3, based on Paxinos and Franklin's stereotaxic atlas of the mouse brain (2001). Excitotoxic lesions of the HPC induced by NMDA infusions consistently extended rostrally from -0.94mm to -3.80mm posterior to the bregma, encompassing all the hippocampal subfields and dentate gyrus of the dorsal and ventral HPC. All HPC-lesioned mice (n=12) were therefore included in the data analyses.

Excitotoxic lesions of the BLA induced by NMDA resulted in significant neuronal damage to the basal amygdaloid nucleus and the lateral nucleus of amygdala, typically extending from -0.94mm to -2.06mm posterior to bregma, while sparing the central nucleus and medial nucleus of the amygdala. Three mice were excluded from data analysis on the basis of bilateral damage extending into the central nucleus of the amygdala, such that the final group numbers were 9 BLA-lesioned mice.

Effects of BLA and HPC lesions on rates of cue and place learning

Acquisition of cue and place learning in mice with BLA, HPC and sham lesions is shown in Figure 4. For increased clarity of viewing differential rates of cue and place learning, the acquisition is expressed as the mean performance above (or below) chance level discrimination ratio (1/6) for the cue-arm, place-arm and non-rewarded arms (mean of performance in **4 non rewarded** arms – following confirmation of no significant differences in the mean discrimination ratios of each of the 4 arms) for each of the 3 tests.

Sham-lesioned mice showed significant preference for the cue-arm over the place-arm and non-rewarding arms in the first test ($F(2,24)=24.87$, $p<0.0001$), showing that 3 conditioning sessions were sufficient to establish the cue-reward association (significantly above chance level: $t=6.10$, $p<0.0001$). In contrast, conditioned preference for the place-arm took longer to emerge, reaching a significant level of

preference above chance only in Test 3 (after 9 conditioning sessions; $t=4.90$, $p<0.001$: Test 2, $t=1.97$, $p=0.07$). This pattern of acquisition of place learning in the sham group was confirmed by a significant effect of test using repeated measures one way ANOVA ($F(2,24)=4.92$, $p<0.016$).

Overall three way-ANOVA conducted on discrimination ratio data across the 3 test sessions for all lesion groups revealed a significant Arm x Lesion group interaction ($F(4,62)=9.48$, $p<0.0001$), and a near significant lesion effect ($F(2,31)=2.89$, $p=0.07$), as well as significant main effects of Arm ($F(2,62)=74.75$, $p<0.0001$) and Test ($F(2,62)=11.65$, $p<0.0001$), and significant Arm x Test interaction ($F(4,124)=5.45$, $p<0.001$). Separate ANOVAs on the performance of the BLA and HPC groups for cue learning and place learning revealed that the BLA-lesioned mice showed significantly reduced preference for the cue-arm compared to the overall level of preference shown by sham-operated mice (lesion effect, $F(1,20)=11.12$, $p<0.003$), but a level of place preference that was not different from sham level of performance (no lesion effect; $F(1,20)=1.50$, ns). Post-hoc analyses revealed that the level of preference for the cue-arm shown by BLA-lesioned mice was significantly lower than the sham-group in Test 1 ($p<0.001$), and this was supported by the fact that neither of the discrimination ratios for the cue-arm in Tests 1 and 2 were significantly different from chance level in the BLA group (Test 1; $t=1.46$, ns: Test 2; $t=1.82$, ns). The BLA-lesioned mice did, however, reach a level of cue preference above chance after 9 sessions of conditioning (Test 3, $t=4.215$, $p<0.003$).

There was also a trend towards significantly increased levels of place-arm preference in the first test in the BLA group compared to that in the sham group (one way ANOVA on all 3 groups for place in test 1, $F(2,33)=3.73$, $p<0.035$, *post hoc* analysis between BLA and sham groups; $p=0.08$), however, the actual level of place-arm preference shown by the BLA group did not reach a level significantly above chance till Test 3 (Test 1; $t=2.13$, $p=0.066$; Test 2; $t=2.23$, $p=0.056$, $t=4.25$, $p<0.0003$), as with the sham group performance.

HPC-lesioned mice showed the opposite pattern of results, with a significantly reduced overall level of preference for the place-arm compared to the sham-operated mice across all tests (lesion effect; $F(1,23)=12.76$, $p<0.002$), but not for the cue-arm (no lesion effect; $F(1,23)=1.16$, ns). Post-hoc analyses revealed that the level of preference for the place-arm in the HPC group was significantly lower than in the Sham group in Test 3 ($p<0.003$). A significant difference between place preference performance between the HPC and sham group in Test 2 was also confirmed by post-hoc analysis ($p<0.038$). As with the sham-lesioned group, HPC-lesioned mice showed significant preference for the cue-arm in Test 1 ($t=9.62$, $p<0.00001$), and this level of performance significantly above chance was maintained in tests 2 and 3 (Test 2; $t=5.90$, $p<0.0001$; Test 3; $t=7.98$, $p<0.00001$).

In summary, BLA-lesioned mice were significantly slower to acquire cue learning, only achieving a level of conditioned cue preference above chance level after 9 conditioning sessions, compared to the 3 sessions required for the sham-lesioned mice and HPC-lesioned mice. In contrast, HPC-lesioned mice were significantly impaired in acquiring place learning, but not cue learning, with no evidence of place preference even after 9 conditioning sessions.

Effect of repeated AMPH pre-treatment on rates of cue and place learning

Acquisition of cue and place learning in mice pre-treated with saline (VEH) or AMPH (2.5mg/kg or 5mg/kg) is shown in Figure 5. Saline-pretreated mice exhibited cue-arm preference over the place-arm and non-rewarding arms ($F(2,24)=13.48$, $p<0.0001$) that was also significantly above chance level in Test 1 ($t=5.67$, $p<0.0001$), and which remained significantly above chance in subsequent tests (test 2: $t=6.25$, $p<0.0001$; test 3; $t=5.18$, $p<0.0002$). Significant preference for the place-arm in the saline treatment group was apparent in Test 2 ($t=6.44$, $p<0.0001$) and Test 3 ($t=5.68$, $p<0.0001$).

Overall three way-ANOVA conducted on discrimination ratio data across the 3 test sessions for all treatment groups revealed a significant Arm x Treatment group interaction ($F(4,72)=7.28$, $p<0.0001$),

as well as significant main effects of Arm ($F(2,72)=197.63, p<0.0001$) and Test ($F(2,72)=11.34, p<0.0001$), and significant Arm x Test interaction ($F(4,144)=8.03, p<0.0001$). Separate ANOVAs on the performance of the AMPH treatment groups compared to saline treatment groups for cue learning revealed that both the AMPH 2.5 and AMPH 5 treatment groups showed significantly reduced preference for the cue-arm compared to the overall level of preference shown by saline-treated mice across all tests (AMPH 2.5 treatment effect; $F(1,25)=15.51, p<0.001$, AMPH 5 Treatment effect; $F(1,25)=18.39, p<0.0001$). Dunnett's tests revealed the level of cue-preference to be significantly different (lower) to the saline-treated level of performance in Test 1 ($p<0.005$) and 2 ($p<0.05$) for the AMPH 5 group, and for Test 3 ($p<0.05$) for the Amph 2.5 group. **However, ANOVAs conducted on the absolute times spent in the cue and place arms in TEST 1, upon confirmation of no significant difference in the overall exploratory time between treatment groups ($F(2,39)=0.004, p=1.0$), revealed that there was a significant Arm x Treatment interaction for both AMPH groups when compared to the Saline group (AMPH 2.5: $F(1,25)=15.5, p<0.001$; AMPH 5: $F(1,25)=15.67, p<0.001$). Subsequent Dunnett's tests conducted on the absolute times spent in the cue arm in Test 1 confirmed that mice in both the AMPH2.5 and AMPH 5 groups spent significantly less time in the cue arm than the mice in the Saline group in Test 1 ($p<0.05$), indicating attenuated cue learning even in Test 1 in the AMPH 2.5 group.**

ANOVA comparing place-arm preference between the treatment groups across 3 tests revealed that the strength of place preference increased across the three tests in all groups ($F(2,74)=13.66, p<0.0001$), and that there was a significant effect of AMPH treatment in the degree of place preference that was expressed ($F(2,37)=3.67, p<0.035$). Both AMPH groups showed place preference significantly above chance level in Test 1 (AMPH 2.5: $t=6.25, p<0.0001$; AMPH 5: $t=3.84, p<0.002$), which was also significantly different from the level of place preference seen in saline-treated mice in the AMPH 2.5 group (Dunnett's $p<0.05$).

In summary, mice that had received repeated AMPH showed significantly reduced conditioned cue preference across the three tests. In contrast, both AMPH-pre-treatment groups showed facilitation of place learning, with significant establishment of place preference after 3 conditioning sessions, compared to the 6 sessions required by the saline-treated group.

Effect of basolateral amygdala lesions on conditional cue and spatial learning

Acquisition curves of conditional cue and spatial learning in BLA-lesioned, and sham-operated mice are shown in Figure 6. All mice were successful in acquiring conditional associations between the absence of a floor insert in the start arm with the availability of reward in the goal arm with a specific cue (block effect: $F(13,130)=7.64$, $p<0.0001$). However, BLA-lesioned mice showed a significantly lower level of correct performance throughout training, compared to sham-group performance (lesion effect: $F(1,10)=13$, $p<0.005$). In contrast, there were no differences in the performance of BLA-lesioned mice and sham-operated controls (no lesion effect: $F(1,10)=0.58$, $p=0.46$), in the ability to use start arm floor inserts as a conditional cue in solving the spatial version of the task (block effect; $F(15,150)=5.39$, $p<0.0001$).

Effect of AMPH exposure on conditional cue and spatial learning

Acquisition curves of conditional cue and spatial learning in saline- and AMPH-pretreated mice are shown in Figure 7. While all mice were able to acquire the cue version of the conditional task ($F(13,221)=13.22$, $p<0.0001$), the overall levels of correct responses in both the AMPH2.5 and AMPH 5 groups were significantly lower than those achieved by the sham group (treatment effect with all groups; $F(2,17)=22.88$, $p<0.0001$; Amph 2.5 vs. Saline: $F(1,11)=62.05$, $p<0.0001$; Amph 5 vs. Saline: $F(1,11)=15.50$, $p<0.01$). All saline- and AMPH-pretreated groups were able to solve the spatial version of the conditional learning task (block effect; $F(13,221)=8.21$, $P<0.0001$; Saline: $F(13,65)=5.22$, $p<0.0001$; AMPH 2.5: $F(13,78)=2.63$, $p<0.01$; AMPH 5; $F(2,45)=2.45$, $p<0.01$) and the performance

between saline- and AMPH-pre-treated groups did not differ (no treatment effect: AMPH 2.5 vs. Saline; $F(1,11)=0.31$, $p=0.59$; AMPH 5 vs. Saline, $F(1,11)=2.34$, $p=0.15$).

In summary, both BLA-lesioned and AMPH-pretreated mice were significantly attenuated in the acquisition of conditional cue learning, but not in the acquisition of a spatial version of the same task.

Locomotor Response to AMPH Challenge

Overall 3 way ANOVA conducted on the changes in the mean number of beam breaks broken before, and following a single AMPH challenge (see Figure 8) revealed a significant effect of the challenge on locomotor activity in all groups ($F(1,35)=134.5$, $p<0.0001$) and amph challenge x treatment group interaction ($F(2,35)=10.48$, $p<0.0001$), as well as a significant difference in the overall level of locomotor activity between the groups ($F(2,35)=4.78$, $p<0.01$). Selective analyses on the baseline locomotor activity (bins 1-24) showed that there was a significant difference in the activity levels between the groups ($F(2,36)=4.11$, $p<0.025$) which was due to the AMPH 5 group showing much lower overall activity levels compared to those of saline-treated rats in this period ($p<0.01$). All groups did, nevertheless, show reduced levels of activity prior to the AMPH challenge ($F(23,828)=105.0$, $p<0.00001$). Selective analyses on the locomotor activity in the period following the AMPH challenge (bins 25-48) revealed a significant difference in activity levels between the treatment groups ($F(2,35)=7.32$, $p<0.002$) and a significant group x bin interaction ($F(46.828)=2.08$, $p<0.00001$). Post-hoc analyses attributed these effects to significantly enhanced increase in locomotor activity (locomotor sensitization) in both AMPH 2.5 and AMPH5 groups compared to saline-treated mice (AMPH 2.5; $p<0.017$, AMPH 5; $p<0.001$). Analyses comparing activity levels between the AMPH 2.5 and AMPH5 groups showed that there was no significant difference between the overall levels in increased activity between the groups after an AMPH challenge ($F(1,23)=0.71$, ns), but a significant bin x treatment interaction ($F(23,529)=1.71$, $p<0.02$), reflecting the fact that mice in the AMPH 5 group showed sustained increase in locomotor activity at the

end of the session, by which time locomotor activity in the AMPH2.5 group returned to levels similar to saline-treated mice.

Correlative analyses between performance in Test 1 and degree of locomotor sensitization

Pearson's correlation analyses between the degree of locomotor sensitization shown by each AMPH-pretreated mouse, and their cue and place preference performance in Test 1 (the test most sensitive to differential rates of conditioning between cue and place learning- see Figure 9) showed that there was a significant positive correlation between the magnitude of locomotor sensitization and the degree of place preference ($r=0.42$, $p<0.038$), but not cue preference ($r=-0.17$, $p=0.40$). The same analyses in saline-pretreated mice did not yield any significant correlations (place vs locomotor activity increase; $r=0.11$, $p=0.70$; cue vs locomotor activity increase; $r=0.008$, $p=0.98$).

Discussion

The present study provides novel and important findings that a sensitizing regimen of AMPH has selective modulatory effects on limbic information processing; enhancing place conditioning which is dependent on the integrity of the hippocampus (HPC), while attenuating cue conditioning that is dependent on the integrity of the basolateral amygdala (BLA), specifically under conditions in which the two forms of associative information are competing for control over appetitive behavior. However, when HPC-dependent and BLA-dependent learning occur in parallel, AMPH exposure causes attenuation of cue learning, in the absence of facilitation of spatial learning. The facilitation of place conditioning showed a significant positive correlation with the degree of locomotor sensitization shown by each animal, indicating the commonality between the neural substrates of the two phenomena. These findings highlight the possible significance of the dopamine system in regulating the balance of information processing between learning and memory systems, and how disruption to this regulation could lead to manifestations of the behavioral abnormalities associated with schizophrenia and addiction.

Acquisition of cue and spatial learning is BLA and HPC-dependent

Data from control lesion and saline pre-treated mice revealed that under conditions in which elemental cues are explicitly **competing for associative control over appetitive behavior**, elemental cues have predominant control early in training. Spatial cues then begin to have equal control over preference behavior after further conditioning sessions. Selective excitotoxic lesions of the mouse HPC caused a marked deviation from this acquisition pattern, abolishing the contribution of conditioned spatial cues in guiding preference behavior, consistent with its well established role in spatial learning in rats and mice alike (O'Keefe and Nadel, 1978; Morris et al., 1982; Cho et al., 1999; Deacon et al., 2002), and conditioned place preference in rats (Ferbinteanu and McDonald, 2001; Ito et al., 2006). In contrast,

selective excitotoxic lesions of the mouse BLA retarded the ability of a conditioned discrete cue to guide preference behavior, consistent with previous studies showing BLA lesion-induced deficits in conditioned cue preference (Everitt et al., 1991; Ito et al., 2006) and similar paradigms involving the acquisition of conditioned discriminative approach behavior towards a CS signalling availability of sucrose in rats (Burns et al., 1993). The selective sensitivity of the conditioned cue and place preference tests to HPC and BLA lesions respectively make it highly likely that the expression of preference for the cue-arm or place-arm is a result of acquired appetitive learning, rather than approach behavior elicited by inherent activational properties of the stimuli.

Successful performance of the conditional T maze task depends upon making correct behavioral choices on the basis of trial-specific information (occasion setting stimulus) presented in the start arm, which in turn depends on the retrieval of appropriate discrete cue-reward association (in cue trials), and place-reward association (in spatial trials). Vehicle mice and sham-operated controls showed a similar incremental acquisition pattern to that of the conditioned cue and place preference task, acquiring the cue version at a markedly faster rate than the spatial version of the task. **Excitotoxic lesions of the HPC in mice have been previously shown to impair the spatial version of this task (Schmitt et al., 2004),** which was attributed to an impairment in a spatial-temporal tagging or encoding mechanism. Excitotoxic lesions of the BLA in the present study attenuated the cue version of the task, but not the spatial version of the task, again supporting the idea that the BLA is selectively involved in the processing of elemental cue information, as opposed to spatial information.

The nature of the effects of BLA lesions on both conditioned cue preference and conditional cue learning here was transient, with the effects being most debilitating in the early part of acquisition. Similarly, a slowed, but not abolished acquisition of discrete cue conditioning has been previously reported with BLA-lesions (Burns et al., 1993; Ito et al., 2006). These findings raise the

possibility that other associative mechanisms mediated by neural substrates outside of the BLA can also subserve this form of learning, the most likely candidate being the dorsal striatal-mediated stimulus-response mechanism (Packard & Knowlton, 2002). The involvement of the same stimulus-response mechanism in the successful performance of the conditional spatial learning cannot be ruled out completely. Nevertheless, the fact that HPC-lesioned mice show chance level (50%) performance in conditional spatial learning which persists throughout the course of training (Schmitt et al., 2004) undoubtedly indicates an strong HPC contribution to the acquisition of the task.

Effect of repeated AMPH on acquisition of cue learning

Repeated AMPH exposure led to a marked change in the acquisition patterns of conditioned cue and place preference with a significant attenuation of conditioned cue preference that resembled the effects of BLA lesions, and a concomitant enhancement of HPC-dependent place preference. BLA-dependent cue learning in the conditional cue and spatial learning task was also attenuated in AMPH-pre-treated mice, although in this task, there was no concomitant facilitation of conditional spatial learning. The clear attenuation of cue learning in the present study is somewhat surprising given previous findings showing that a history of repeated AMPH *facilitates* the acquisition of pavlovian discriminative approach behavior (Taylor and Jentsch, 2001) which is to some extent dependent on the integrity of the BLA (Burns et al., 1993) and the meso-amygdaloid dopamine system (albeit an effect of post-training DA manipulations; Hitchcott et al., 1997). **However, what is apparent is that repeated drug exposure leads to substantial alterations in BLA function, and the nature or direction of drug-induced changes in BLA-mediated behaviors may depend on the extent to which the functions of other brain regions that are closely connected with the BLA are affected. Thus, a recent study found that repeated cocaine pre-treatment caused BLA neurons to persistently respond to outdated associative cue information during reversal learning of odour cue- go, no go discriminations,**

previously shown to be dependent on the integrity of the orbito-frontal cortex (Stalnaker et al., 2007). Such inflexibility in associative encoding in the BLA was observed in the absence of an actual deficit in reversal learning following lesions of the BLA indicating that while the BLA itself is not necessary for this process, its altered function following repeated cocaine exposure can still have debilitating effects on processes that depend on other brain structures with which it's connected.

Neural mechanism of attenuated BLA control over appetitive behavior

There is much evidence pointing to the idea that the attenuation of BLA control over appetitive behaviour in the present study is a consequence of altered meso-amygdaloid DA transmission following repeated AMPH exposure, which in turn affects meso-accumbens DA transmission. Thus, following repeated AMPH treatment, DA response in the amygdala to a subsequent AMPH challenge is augmented (Harmer et al., 1997), and the disruption of DA terminals in the amygdala prevents the development of behavioral sensitization induced by both systemic and intra-VTA AMPH pretreatment (Bjijou et al., 2002). It has been suggested that there is an inverse relationship between DA in the BLA and NAc (Jackson & Moghaddam, 2001; Louilot & Beeson 2000) with BLA DA exerting an inhibitory influence upon NAc DA under normal circumstances (Louilot et al., 1985). Repeated exposure to an escalating dose of AMPH has been shown to disrupt the balance of DA neurotransmission in the NAc core and shell, and lead to exaggerated conditioned freezing responses to a tone previously paired with shock (Pezze et al., 2002), a form of conditioning well established to depend on the integrity of the BLA (Phillips and LeDoux, 1992). It is possible that such imbalances in NAc core and shell DA could also lead to attenuation of BLA control over appetitive behaviour.

There is also evidence to suggest that the function of the BLA may be compromised following repeated AMPH exposure due to decreased DA tone in the mPFC (Paulson and Robinson, 1995; Pierce

and Kalivas, 1997; Hedou et al., 2001). In the event of a decrease in DA levels in the mPFC, there is a concomitant increase in responsiveness to glutamate, and increased activity of mPFC pyramidal cells (Peterson et al., 2000; Hedou et al., 2001). While this may have an excitatory influence upon some sub-cortical structures such as the ventral tegmental area (VTA) and NAc, neurophysiological evidence suggests that it can have a suppressive effect on the BLA through recruitment of inhibitory interneurons (Rosenkranz and Grace, 2001). A dysfunctional BLA account here would be consistent a considerable number of reports implicating decreased activation in the amygdala in schizophrenic patients (Schneider et al., 1998; Calder et al., 2001; Gur et al., 2002; Takahashi et al., 2004), and further validates the use of AMPH sensitization in modelling certain aspects of schizophrenia in animals.

Effect of repeated AMPH on acquisition of spatial learning

The present findings suggest that under conditions in which HPC-dependent and BLA-dependent information are in direct competition over the control of appetitive behavior, AMPH exposure causes HPC-mediated input to dominate behavior. It is noteworthy, however, that AMPH exposure did not facilitate the acquisition of conditional spatial learning here, which is consistent with previous studies showing the absence of facilitation upon other measures of HPC-dependent spatial learning following AMPH exposure. Thus, Russig et al., (2003) failed to show any effects of a sensitizing regimen of repeated AMPH administration on the acquisition rate of water maze performance. There has even been a demonstration of impairment in non-associative spatial learning in mice following repeated AMPH administration (Mandillo et al., 2003). Such failures to observe repeated AMPH-induced facilitation in spatial learning may indicate that the neural adaptations caused by repeated AMPH exposure serve to enhance HPC control over behavioral output at the level of expression, as opposed to enhancing HPC-dependent associative learning at the level of acquisition. This may explain why Gelowitz et al., (1994) observed enhanced acquisition of spatial learning in the

Morris water maze in rats that have not only been pre-treated with AMPH, but also given *acute* doses of AMPH prior to each session of training throughout the course of acquisition.

Neural mechanism of enhanced HPC control over appetitive behavior

There are a number of putative mechanisms by which HPC-dependent information may gain predominant control over behavior following AMPH exposure. The presence of a positive correlation between the degree of locomotor sensitization exhibited by each animal, and the degree of conditioned place preference exhibited by the same animal in the present study strongly points to the fact that the neural and neurochemical alterations that underlie locomotor sensitization also contribute to the facilitation of place learning. It is well established that sensitized animals have enhanced activity of the meso-accumbens DA system (Kalivas and Stewart, 1991; Henry and White, 1992), that is dependent on the extent of behavioral sensitization exhibited by the animal (Brady et al., 2003) and specifically related to behaviorally significant information (Wan and Peoples, 2008). This DA pathway has also been implicated in attributing incentive salience to reward-associated cues, thereby making cues powerful elicitors of pavlovian approach responses (Robinson and Berridge, 1993, 2001). Thus, in line with this incentive salience hypothesis, AMPH exposure may have led to excessive attribution of salience to HPC inputs. This aberrant salience attribution could arise as a direct result of a state of HPC overdrive in AMPH withdrawal, in light of recent electrophysiological evidence showing that repeated AMPH administration augments ventral hippocampal output, which in turn increases baseline/tonic DA activity in the nucleus accumbens (Lodge and Grace, 2008).

Neural mechanism of AMPH-induced alteration in the balance of limbic control over appetitive behavior

The present findings suggest that repeated AMPH exposure disrupts the *balance* of competing limbic control over appetitive behavior. The aberrant regulation of HPC and BLA control over

conditioned preference behavior is likely to be a consequence of altered interactions between converging dopaminergic inputs and limbic glutamatergic inputs at the level of the NAc and mPFC. It is well established that repeated psychostimulant exposure enhances meso-accumbens DA activity (Vanderschuren and Kalivas, 2000). Electrophysiological evidence lends support to the idea that NAc DA regulates the balance of limbic information processing by strengthening the most behaviorally relevant and salient inputs, whilst inhibiting other competing inputs. Most pertinent is the demonstration by Floresco et al., (2001) that co-activation of NMDA receptor-mediated glutamatergic HPC-NAc pathway and post-synaptic D1 receptors in the NAc has the net effect of enhancing HPC-evoked activity, and facilitating meso-accumbens DA release. Importantly, this not only ensures that there is a preferential enhancement of HPC inputs, but also serves to inhibit the effect of subsequent BLA input to the same neuron. Thus, as discussed previously, it is conceivable that repeated AMPH induces an abnormal state of hyperdopaminergic activity in the NAc as a consequence of HPC overactivity, leading to HPC dominance over behavioral output and a concomitant suppression of BLA control over behaviour, as seen in the present study.

Furthermore, substantial evidence indicates that repeated AMPH exposure leads to marked deficits in cognitive and executive functions that are accompanied by reduced function of the mesocortical DA system. Thus, a sensitizing regimen of AMPH administration impairs spatial working memory in primates, an effect accompanied by a reduced turnover of DA in the prefrontal cortex (Castner et al., 2005). Visual attentional performance is also impaired in AMPH sensitized rats, with evidence of decreased function of D1 receptors in the mPFC (Fletcher et al., 2009). An opposing input selection mechanism similar to that between HPC and BLA inputs seems to operate between HPC and mPFC inputs in the NAc. Thus, when HPC activation occurs, LTP is induced at HPC inputs in the NAc due to D1 receptor and NMDAR activation, and LTD is induced at mPFC inputs via D2 receptor activation, to shift processing in the NAc towards limbic information. Conversely, when mPFC activity

is dominant, LTD is induced at HPC inputs, and LTP at mPFC inputs due to decreased D2R stimulation (decreased tonic DA transmission in NAc; Goto and Grace 2005b), providing a neural mechanism for set shifting of behavioral strategies. Cocaine sensitization has been shown to disrupt the induction of synaptic plasticity in the NAc that correlated with an increase in perseverative errors when a switch in response strategy was required, indicative of reduced PFC influence upon NAc activity (Goto and Grace, 2005b).

In summary, the present study provides the first functional demonstration of altered limbic information processing and consequent control over appetitive behavior following a sensitizing regimen of repeated AMPH administration in mice. The manifestation of an aberrant limbic control over conditioned preference behavior is likely to be driven by altered interactions between converging dopaminergic inputs and limbic glutamatergic inputs within the cortico-striatal circuitry, coupled with an overactive HPC and an underactive BLA. These findings, together with previous demonstrations of competitive interactions between HPC and BLA-mediated learning (McDonald and White, 1993; Chai and White, 2004; Ito et al., 2006) indicate that the balance of HPC-NAc and BLA-NAc throughput is normally kept under tight regulation. Dysregulation in these systems, as seen here as a result of repeated AMPH treatment, can lead to manifestations of abnormal patterns of behavior, which may include psychotic symptoms in schizophrenia (see Castner and Goldman-Rakic, 2003) and increased contextual control over drug-craving and relapse in drug addicts due to salience misattribution.

Disclosure/Conflict of interest

There is no conflict of interests to report.

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	Arm	HPC	BLA	Sham	Saline	Amph (2.5mg/kg)	Amph (5mg/kg)
TEST 1	<i>Total Time</i>	235.4±5.2**	131.4±16.6	160.2±14.8	138.8±8.3	137.9±10.9	138.9±7.4
	<i>Cue</i>	78.8±4.6	26.3±4.6**	53.9±6.1	46.6±4.8	34.3±3.9*	30.9±3.0**
	<i>Place</i>	34.2±5.0	35±6.2	30.6±5.0	25.1±2.6	39.3±5.5*	35.2±4.7
	<i>NR arms</i>	30.6±4.3	18.5±3.7	18.9±3.5	17.1±2.1	16.1±2.0	19.6±2.1
TEST 2	<i>Total Time</i>	197.1±11.3	161.7±14.4	183.4±7.5	144.0±8.5	174.4±8.73**	156.8±8.3
	<i>Cue</i>	60.3±4.9*	37.3±6.4*	50.9±3.9	43.8±3.4	39.5±3.5	32.2±3.3*
	<i>Place</i>	32.8±3.7*	47.8±6.2	51.3±6.0	41.6±4.2	56.8±4.3	57.6±5.2**
	<i>NR arms</i>	26.0±4.6	19.1±3.6	21.2±3.0	15.7±2.0	20.5±3.3	20.8±2.5
TEST3	<i>Total Time</i>	200.2±10.9	121±9.7**	187.5±11.8	165.3±9.0	197.7±13.1*	168.7±8.7
	<i>Cue</i>	75.2±5.5	34.4±3.8	63.6±6.9	47.0±3.4	39.9±5.3	40.5±3.9
	<i>Place</i>	32.1±4.8*	36.9±6.0	47.1±5.6	56.5±7.1	83.4±14.3	48.7±6.0
	<i>NR arms</i>	23.3±3.9	12.4±2.6	18.7±2.8	14.0±2.3	18.9±3.1	17.8±2.7

Table 1 Time spent (mean ± SEM) in cue, place and non-rewarded (NR)arms, and total time spent (mean ± SEM) exploring all the arms during the 3 conditioned cue and place preference tests in mice with hippocampus (HPC), basolateral amygdala (BLA) , or sham lesions, and in mice pre-treated with saline or 2 doses of amphetamine (AMPH). Note that the time shown for the NR arms represents the pooled mean of the time spent in all 4 non rewarding arms for each animal, and thus may not be truly representative of the commonly observed unequal distribution of the actual time spent in the 4 arms. Asterisks denote significant differences in the times spent (**p<0.01; *P<0.05) from the control groups (sham or saline). Statistical tests on the time spent in the cue/place arms were conducted only for test sessions in which the total time spent in the apparatus was not significantly different between groups.

Titles and legends to figures

Figure 1. Schematic representation of NMDA lesions of the hippocampus (left) basolateral amygdala in mice (right). Areas shaded in grey and black represent the largest and smallest extent of neuronal damage in a single animal, respectively.

Figure 2. **Concurrent cue and place conditioning task** (Conditioning Sessions 1-3 shown): mice were confined in each of the 6 arms of the radial maze for 2min on each day of conditioning (+ depicts the rewarding arms, the rest of the arms were non-rewarding). The apparatus was rotated by varying degrees, and the stripy floor insert (cue) was moved to a different arm between sessions to minimise conditioning to intra-maze cues. A conditioned cue and place preference test was conducted on the day after the third session (Session 4). This cycle of 3 conditioning sessions followed by a conditioned preference test was repeated a further 2 times.

Figure 3. **Parallel conditional cue and spatial learning task**. Mice were trained to learn 3 floor insert-outcome contingencies based on spatial or non-spatial cues in the goal arms. + depicts the location of the reward.

Figure 4. **Conditioned cue and place preference tests** (5min) for sham-operated controls, HPC and BLA lesion groups expressed as the mean \pm SEM performance above or below chance discrimination ratio level (0.16666) for the cue-arm, place-arm and non-rewarding (NR) arms.

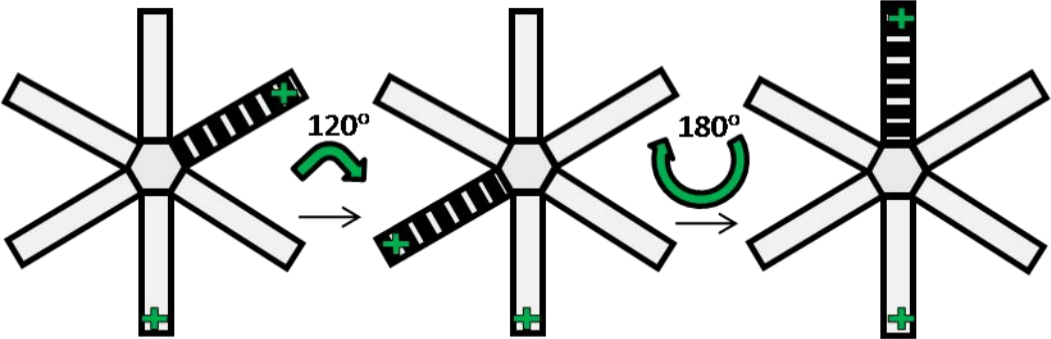
Figure 5. **Conditioned cue and place preference tests** (5min) for saline pre-treated controls, AMPH2.5 and AMPH5 pre-treated mice expressed as the mean \pm SEM performance above or below chance discrimination ratio level (0.16666) for the cue-arm, place-arm and non-rewarding (NR) arms.

Figure 6. **Performance of conditional cue (A) and spatial learning (B)** in BLA-lesioned and sham-operated control mice expressed as the mean \pm SEM percent correct trials in blocks of 8 trials in conditional cue learning, and in blocks of 16 trials in conditional spatial learning.

Figure 7. **Performance of conditional cue (A) and spatial learning (B)** in saline- and amphetamine-pretreated (AMPH 2.5mg/kg and AMPH 5mg/kg groups) mice, expressed as the mean \pm SEM percent correct trials in blocks of 8 trials in conditional cue learning, and in blocks of 16 trials in conditional spatial learning.

Figure 8. **Locomotor sensitization test**, showing mean \pm SEM locomotor beambreaks in 5minute bins before, and after an AMPH challenge (2.5mg/kg) for saline pre-treated controls, AMPH2.5 and AMPH5 pre-treated groups.

Figure 9. Correlation between performance in the first conditioned cue and place preference test (discrimination ratios) and degree of locomotor sensitization shown by each animal in the AMPH groups.



Session 1

Session 2

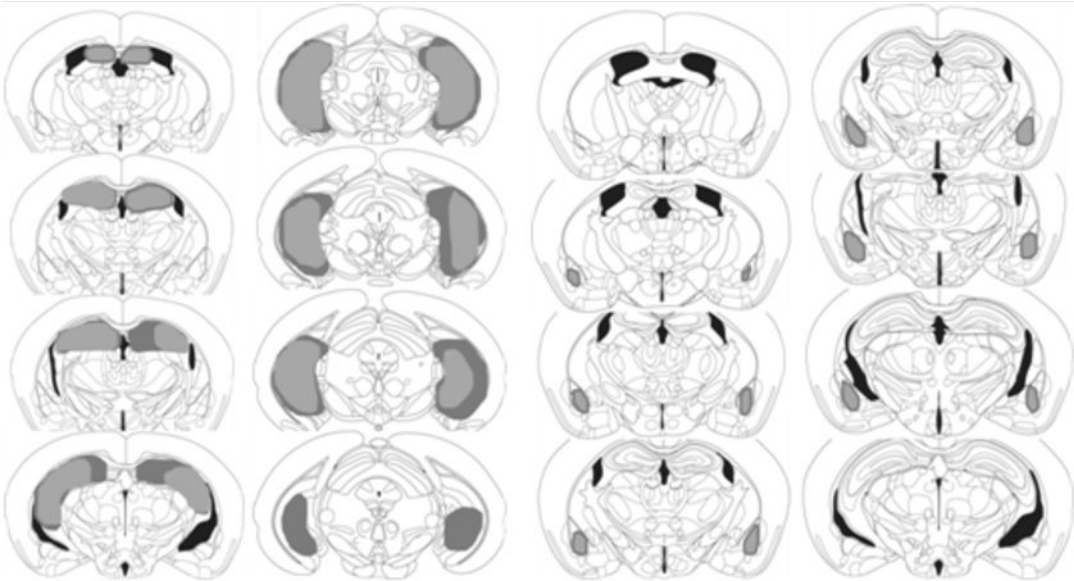
Session 3

Spatial - Left

Non-spatial Cue

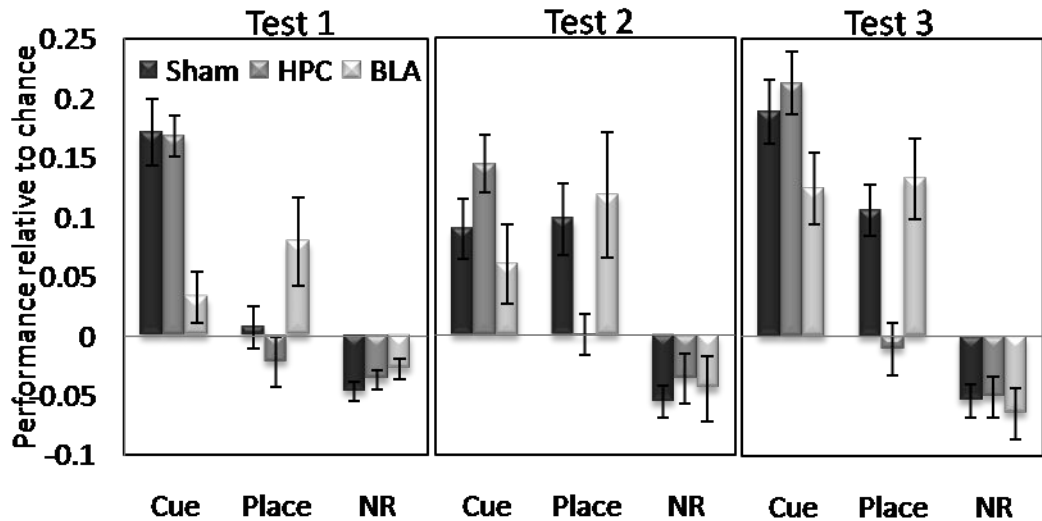
Spatial - Right

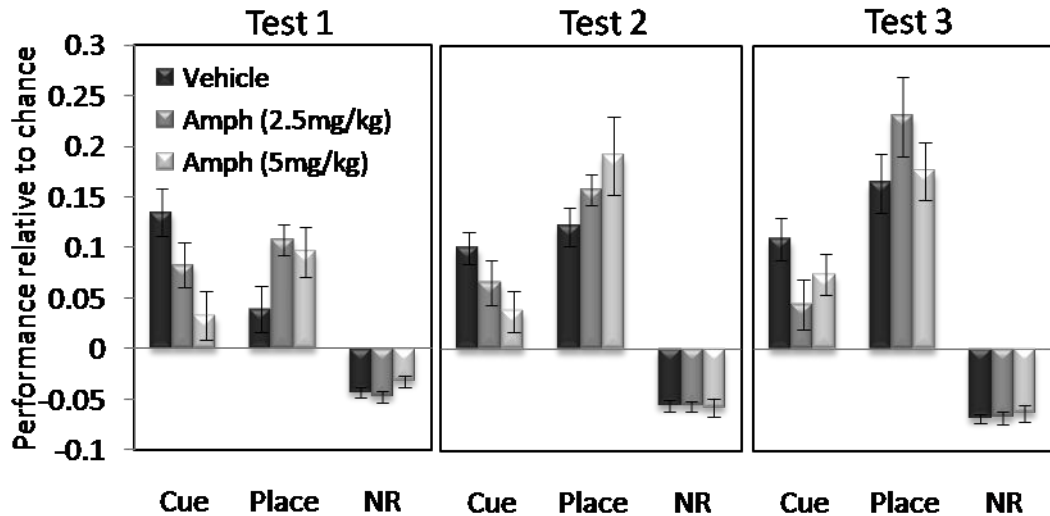


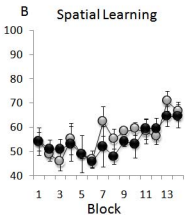
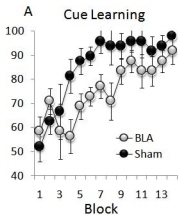


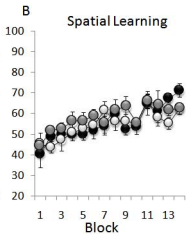
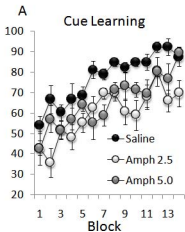
HPC

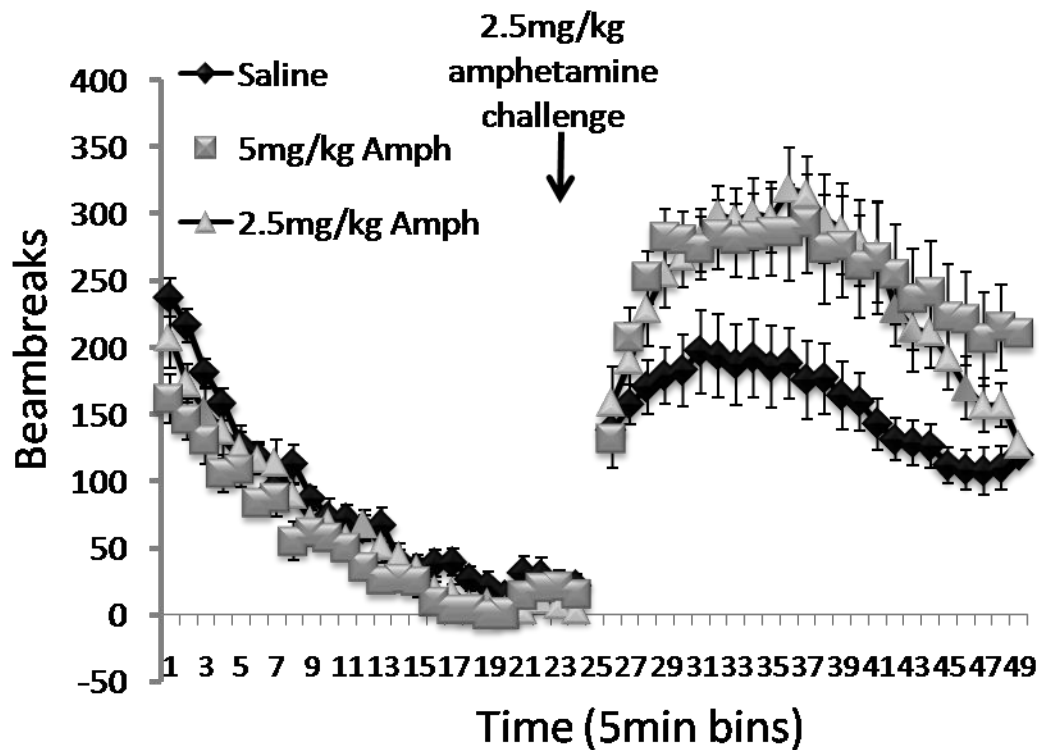
BLA



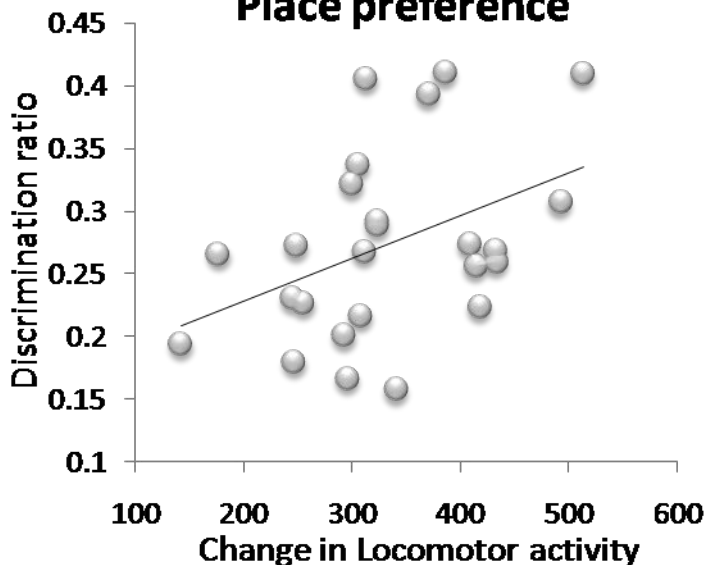








Place preference



Cue preference

