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Dissociable Hippocampal and Amygdalar D1-like receptor contribution to Discriminated Pavlovian conditioned approach learning

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Abstract

Pavlovian conditioning is an elementary form of reward-related behavioral adaptation. The mesolimbic dopamine system is widely considered to mediate critical aspects of reward-related learning. For example, initial acquisition of positively-reinforced operant behavior requires dopamine (DA) D1 receptor (D1R) activation in the basolateral amygdala (BLA), central nucleus of the amygdala (CeA), and the ventral subiculum (vSUB). However, the role of D1R activation in these areas on appetitive, non-drug-related, Pavlovian learning is not currently known. In separate experiments, microinfusions of the D1-like receptor antagonist SCH-23390 (3.0 nmol/0.5 μ L per side) into the amygdala and subiculum preceded discriminated Pavlovian conditioned approach (dPCA) training sessions. D1-like antagonism in all three structures impaired the acquisition of discriminated approach, but had no effect on performance after conditioning was asymptotic. Moreover, dissociable effects of D1-like antagonism in the three structures on components of discriminated responding were obtained. Lastly, the lack of latent inhibition in drug-treated groups may elucidate the role of D1-like in reward-related Pavlovian conditioning. The present data suggest a role for the D1 receptors in the amygdala and hippocampus in learning the significance of conditional stimuli, but not in the expression of conditional responses.

Keywords

Dopamine; D1; Pavlovian conditioned approach; amygdala; subiculum; rats

1. Introduction

When a predictive relationship (i.e. contingency) exists between an environmental stimulus (e.g., a sound) and a biologically-relevant stimulus (e.g., food or pain), the presentation of that environmental event alone can come to elicit a characteristic response (Rescorla, 1988).

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For example, by turning on a metronome (i.e., a conditional stimulus, or “CS”) and then presenting food powder (i.e., an unconditional stimulus, or “US”) to a dog’s tongue, Pavlov demonstrated that the sound of the metronome alone could produce salivation (Pavlov, 1927). Pavlovian, or classical conditioning, is an elementary form of behavioral adaptation that enables an animal to more effectively anticipate important events in a world filled with competitors and predators. However, Pavlovian conditioning can also produce maladaptive responses like the elicitation of withdrawal symptoms following exposure to environmental stimuli (CS) – drug (US) contingencies. This learning can be long-lasting, for exposure to drug-conditioned cues can elicit withdrawal or cravings even after long periods of drug abstinence, thus contributing heavily to the compulsive and seeming intractable nature of addiction (O’Brien, Childress, McLellan, & Ehrman, 1992; Siegel, Baptista, Kim, McDonald, & Weise-Kelly, 2000). Thus, a greater understanding of the neurobiology of appetitive Pavlovian conditioning is likely to yield significant information for addressing problems like drug abuse and relapse.

A crucial role for the mesolimbic dopamine system in appetitive Pavlovian conditioning has been demonstrated in many experiments. Electrophysiological studies in primates have shown that mid-brain dopamine (DA) neurons in the ventral tegmental area (VTA) fire to stimuli that predict upcoming rewards (Schultz, 1998). Microdialysis studies confirm elevated DA in the amygdala following appetitive Pavlovian conditioning (Harmer & Phillips, 1999), a key target of mesolimbic DA system. Pharmacological studies have also begun to determine the post-synaptic mechanisms of DA’s actions in Pavlovian conditioning, implicating a crucial role for DA D1 receptors in Pavlovian conditioned approach (PCA) following systemic blockade (Choi, Morvan, Balsam, & Horvitz, 2009; Eyny & Horvitz, 2003). Local antagonism of D3 receptors in the central nucleus of the amygdala (CeA) have also been shown to produce deficits in PCA. Further, D1-LIKE antagonism in the amygdala during drug-cue conditioning attenuates the ability of that cue to reinstate drug-seeking behavior in a self-administration, re-instatement paradigm (Berglind, Case, Parker, Fuchs, & See, 2005). Another target of the mesolimbic DA system, the ventral subiculum (vSUB), is thought to compete with the amygdala over control of plasticity-related inputs to the Nucleus Accumbens, a site critical for the expression of PCA (Blais & Janak, 2009; Parkinson, Olmstead, Burns, Robbins, & Everitt, 1999). Combined these data strongly suggest a crucial role for dopamine and dopamine receptor activation in appetitive Pavlovian conditioning, in a distributed network.

Dopamine is also hypothesized to serve crucial modulatory functions in neural and synaptic plasticity, which are widely considered to be critical in the cellular/molecular processes instantiating learning. Long-term enhancement of synaptic strength occurs when DA D1R activation is temporally coordinated with NMDAR activation (Jay, 2003; Wickens, Begg, & Arbuthnott, 1996). D1R antagonists block NMDA-dependent LTP in striatal slices (Kerr & Wickens, 2001), while LTP in hippocampal-PFC synapses, *in vivo*, depends on co-activation of D1R and NMDAR (Gurden, Takita, & Jay, 2000; Gurden, Tassin, & Jay, 1999; Jay, Burette, & Laroche, 1995; Jay, Gurden, & Yamaguchi, 1998). More recent data suggests that D1R, via PKA activation, phosphorylates ERK, thereby reconfiguring networks involved in learning and drug use (Kaphzan, O’Riordan, Mangan, Levenson, & Rosenblum, 2006; Valjent et al., 2005). ERK phosphorylates CREB (Das, Grunert, Williams, & Vincent, 1997;

Konradi, Leveque, & Hyman, 1996), a transcription factor thought to be an evolutionarily conserved modulator of memory processes (Nestler, 2001; Silva, Kogan, Frankland, & Kida, 1998). It is presumed that a more thorough understanding of brain reward learning systems will have immense potential for extensively improving outcomes through the development and discovery of new therapeutics to restore reward-related neurobiological pathways gone awry.

However, while substantial data exist on the role of dopamine and dopamine receptors in Pavlovian conditioning, significant omissions also exist. Several of the aforementioned studies used systemic D1R blockade (Choi et al., 2009; Eyny & Horvitz, 2003) or pharmacological agents that also blocked D2 receptors (Di Ciano, Cardinal, Cowell, Little, & Everitt, 2001), which leaves open questions of receptor and region specificity. Lesions of discrete subnuclei of the amygdala produce dissociable effects on learning and performance of Pavlovian conditioned behavior (Blundell, Hall, & Killcross, 2001; Cardinal et al., 2002; Hatfield, Han, Conley, Gallagher, & Holland, 1996; Holland & Gallagher, 2003; Holland, Han, & Winfield, 2002; Holland, Hatfield, & Gallagher, 2001), but they also remain silent on the question of receptor specificity and do not address the possibility that other mechanisms may compensate for or recover function of the lesioned area. In addition, D1-like activation in the amygdala has not been studied in the context of non-drug-related *appetitive* Pavlovian learning, although the amygdala's role in *aversive* conditioning has been well-characterized (Greba & Kokkinidis, 2000; Guarraci, Frohardt, & Kapp, 1999; Lamont & Kokkinidis, 1998; Nader & LeDoux, 1999). D1-LIKE antagonism in both the BLA and CeA blocks appetitive operant conditioning, suggesting a role in appetitive, as well as aversive, learning processes (Andrzejewski, Spencer, & Kelley, 2005). D1-like antagonism in the ventral subiculum (vSUB) also blocks appetitive operant learning (Andrzejewski, Spencer, & Kelley, 2006), although D1-like activation may have been involved in Pavlovian conditioning processes that modulated operant responding. The present experiments, therefore, explored the role of D1-like activation in the BLA, CeA, and vSUB during appetitive Pavlovian conditioning using a discriminated Pavlovian conditioning approach (dPCA) paradigm.

2. Method

2.1 Subjects

Male Sprague-Dawley rats (Harlan, Madison, WI) were housed in pairs in polyethylene cages in colony room with a 12:12 hr light/dark cycle. They were approximately 90 days old at the start of experimentation and weighed approximately 300g each. Each experiment started with 16 rats (8 per group); final n's are presented in the results. All rats were weighed and handled daily and provided with food and water ad libitum prior to surgery. Following recovery from surgery, each rat was reduced to 85% of their ad libitum weight. During food restriction, and prior the start of testing, rats were given approximately 3g of sucrose pellets in their home cages per day; the 85% weight was maintained for the remainder of the experiment. Care of the rats was in accordance with University of Wisconsin-Madison animal care committee guidelines.

2.2 Apparatus

Sessions were conducted in 8 identical commercially constructed conditioning chambers (Coulbourn Instruments, Allentown, PA) enclosed in sound attenuating, ventilated chests. Fans provided some masking noise continuously throughout the session. Two retractable levers, approximately 6 cm apart, were located on the right-side wall (these levers were never projected into the chamber during the present experiments). Spaced equally between the two levers was a feeder trough into which 45 mg Bio-Serv® sucrose pellets could be delivered. The feeder trough was equipped with a photo sensor, such that the number and timing of nose pokes into the tray could be recorded. Above the feeder trough were a row of three stimulus lights (red, yellow, and green; not used in the present experiments) and a 28-V houselight. Experimental events were arranged and recorded via a personal computer in the same room as the chambers, running Graphic State Notation (Coulbourn Instruments, Allentown, PA).

2.3 Surgery

Rats were anesthetized with a ketamine/xylazine mixture (100/10 mg/kg) and placed in a standard stereotaxic surgery device (incisor bar at -3.8 mm; flat skull). Indwelling stainless cannulae (23 gauge) were implanted bilaterally and secured to the skull with stainless steel screws and dental cement. Cannulae were aimed 2.5 mm above the injection targets: the central nucleus of the amygdala (CeA), or the basolateral amygdala (BLA), or the ventral subiculum (vSUB). Stainless steel stylets prevented occlusion of the cannulae. Coordinates (flat skull, in mm) were: CeA, -2.0 AP from bregma, ± 4.0 LM from midline, and -5.7 DV from the skull surface; BLA, -2.8 AP, ± 4.8 LM, and -5.8 DV; vSUB, -6.0 AP, ± 4.3 LM, and -6.2 DV.

2.4 Drugs and Microinfusions

The selective D_1 antagonist, R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SCH-23390), was dissolved in isotonic sterile saline. A dose of $1\mu\text{g}$ (3 nmol) SCH-23390 or vehicle (saline) was administered via bilateral intracerebral microinfusions in a volume of $0.5\mu\text{l}$, each side. After removing the stylets, injectors (30 gauge) were inserted 2.5 mm below the tips of the guide cannulae to the site of the infusion (-8.2 mm DV for CeA, -8.3 mm DV for BLA, and -8.7 mm DV for vSUB). A Harvard Apparatus pump, set at a rate of $0.32\mu\text{l}/\text{min}$, infused drug or vehicle for 1 min 33 s, followed by 1 min of diffusion time. The injectors were removed and the stylets replaced. Rats were immediately placed in the conditioning chambers after microinfusions. The volume of injectate was based on previous studies in our lab (Andrzejewski, et al. (2005)). Quantitative autoradiography studies have demonstrated that injections of $0.33\mu\text{l}$ of SCH-23390 takes at least 20 minutes to diffuse outside of the CeA (Caine, et al. 1995).

2.5 Procedure

The entire experimental timeline and design is represented schematically in Figure 1.

2.5.1 Habituation/Magazine Training—All sessions lasted 20 minutes. Rats were habituated to the chamber and the microinfusion procedure for 3 sessions on consecutive

days immediately before the conditioning phase. Prior to the first two habituation sessions, all rats received a mock infusion. During mock infusions, the rats were lightly restrained, their stylets were removed, and a 10 mm injector was inserted into the guide cannulae, but not into the brain. The pump was turned on for 1 minute and 33 secs, but no solution was infused. One minute later, the injectors were removed and the stylets replaced. The rats were placed immediately into the chambers with the levers retracted. Sucrose pellets were delivered randomly and on average every 60 seconds (RT-60 sec schedule). Prior to the third habituation session, all rats received a saline infusion as described below. The third habituation session also exposed all rats to the RT-60 sec schedule.

2.5.2 Discriminated Approach Training—During the remainder of the experiment, rats were presented the same discriminated Pavlovian conditioned approach procedure (dPCA). All sessions lasted 20 minutes. Rats were placed in a dark conditioning chamber with the levers retracted. Following a variable inter-trial-interval (ITI) of 25 seconds (on average) a stimulus was presented for 5 seconds. If the stimulus was designated as a CS+, stimulus offset coincided with pellet delivery, and a variable ITI ensued. If the stimulus was a CS–, stimulus offset was followed by the variable ITI. The two stimuli were the houselight and flashing houselight (on 0.25 sec, off 0.25 sec, on 0.25 sec, etc.). Stimuli were randomly assigned and counterbalanced across groups such that some rats had the “solid” houselight as CS+ and flashing houselight as CS–, while others has the flashing houselight as CS+ and “solid” houselight as CS–. Each rat received 20 CS+ presentations (followed by a sucrose pellet US delivery) and 20 CS– presentations (followed by nothing) per session. There was no constraint on the number of consecutive CS+ or CS– presentations, nor was there any constraint on the sequence of CS+ and CS– presentations. The type of stimulus presentation was randomly determined. When 20 presentations of one type of stimulus were complete, the remaining trials were the other type of stimulus.

2.6 Experimental Design and Behavioral Testing

After the third habituation session, rats were matched on the basis of the number of nose pokes during session 3, and randomly assigned to one of two groups (vehicle or SCH-23390). Prior to the next 6–7 dPCA sessions (sessions 1–6 or 1–7), rats received an infusion depending on their group assignment and placed immediately in the conditioning chamber (acquisition phase). Sessions and infusions were conducted every other day to minimize the trauma from the injections. On days in which rats were not scheduled to receive infusions, they did not receive dPCA sessions, either, but were weighed, handled and fed. Prior to the next 13–14 sessions (stabilization phase), no infusions were given, but the training conditions remained the same. dPCA sessions during this phase were conducted every day. Prior to the 19th or 20th session, rats were again given an infusion depending on their group assignment (performance test); 1–3 sessions without infusions were conducted after the performance test. Figure 1 shows the experimental design (day to day activities) as well as the two types of sessions (magazine training and dPCA).

2.7 Dependent Variables and Statistical Analyses

Nosepokes during the ITI, CS+, and CS– were recorded for all rats during all sessions and formed the basis of all the dependent measures. A difference score, or the number of nose

pokes during the CS+ minus the number of nose pokes during the CS−, served as the main index of learning. Repeated measures ANOVA or *t*-tests were conducted on the difference scores, CS+, CS−, and ITI responding separately for the acquisition, stabilization and performance phases (sessions 1–7, 8–20, and 21, respectively). *post-hoc* pairwise comparisons were conducted, when indicated, using the Holm-Sidak method of adjusted *t*-tests in SigmaStat 3.5 (Systat Software, Inc.).

2.8 Histological Analysis

After the completion of the experiment, all rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with isotonic saline followed by 10% formalin. Brains were stored in 10% sucrose-10% formalin mixture before sectioning. 60-μm sections were stained with cresyl violet and examined under light microscopy for location of infusion sites. Data from rats with significant damage to the target structure or cannula that missed the target were eliminated. Infusion sites were verified by a blinded observer not affiliated with this research.

3. Results

3.1 Histology

Infusion sites are represented in Figure 2. Two rats were eliminated from the experiment for placements outside the CeA, two for placement outside the BLA, and 1 from the vSUB experiment for significant damage to the hippocampus. One rat from the CeA experiment died during experimentation. Thus, final *n*'s for the 3 studies were: CeA (veh = 6, SCH = 7), BLA (veh = 7, SCH = 7), and vSUB (veh = 8, SCH = 7).

3.2 Discriminated Approach

3.2.1 CeA—As shown in Figure 3, intra-CeA infusions of SCH-23390 impaired the acquisition of discriminated Pavlovian approach behavior. Although ANOVA on difference scores for sessions 1–7 did not result in a significant main effect of drug treatment ($F(1, 11) = 2.70, p = .13$), the effects of session ($F(6, 66) = 5.54, p < .01$), and the drug X session interaction ($F(6, 66) = 3.61, p < .01$) were statistically reliable. *post-hoc* comparisons indicated that the SCH-23390 treated group had statistically lower difference scores during sessions 4 and 7 (p 's $< .05$).

Figure 3 also shows that when infusions were eliminated prior to sessions 8–20, difference scores accelerated for the previously SCH-23390-treated group to comparable control (vehicle-infused) levels. None of the ANOVAs on difference scores for sessions 8–20 were statistically significant (prior Drug treatment: $F(1, 11) = 3.14, p = .10$, Session: $F(12, 132) = 1.67, p = .08$, and Drug X Session interaction: $F(12, 132) = 0.57, p = .86$).

Lastly, an infusion prior to session 21, identical to the treatment received during sessions 1–7, tested the effects of SCH-23390 in performance of a well-learned Pavlovian discrimination. A *t*-test revealed no between-group difference ($t(11) = -0.340, p = 0.74$).

The three panels on the right show nosepoke rates during the three periods (CS+, CS− and ITI) across sessions. For sessions 1–7, ANOVA indicated no main effect of drug on ITI

responding ($F(1, 11) = 0.66, p = .43$), however both the effect of Sessions ($F(6, 66) = 4.54, p < .01$) and the Drug X Session interaction were statistically reliable ($F(6, 66) = 3.62, p < .01$). *post hoc* comparisons indicated lower ITI responding in the drug treated group during sessions 5 and 7. The main effect of drug on CS+ nose-poking rates was nearly significant ($F(1, 11) = 4.16, p = .06$), as was the effect of sessions ($F(6, 66) = 2.20, p = .054$), however the interaction was not so ($F(6, 66) = 0.37, p = .90$). During CS- periods responding was not affected by Drug ($F(1, 11) = 1.29, p = .28$), Sessions ($F(6, 66) = 1.20, p = .32$), or by a Drug $\times$ Sessions interaction ($F(6, 66) = 1.28, p = .28$).

During sessions 8–20, responding during the ITI was not statistically affected by prior Drug treatment ($F(1, 11) = 2.20, p = .17$), nor by a Drug X Session interaction ($F(12, 132) = 1.18, p = .31$), but was influenced by Session ($F(12, 132) = 6.83, p < .01$). There were no statistically significant effects of drug, session, or interaction of drug and session on CS+ or CS- nose-poking rates during sessions 8–20 (All F 's < 1.50).

3.2.2 BLA—As shown in Figure 4, intra-BLA infusions of SCH-23390 also impaired the acquisition of discriminated Pavlovian approach. ANOVA on difference scores for sessions 1–7 revealed a significant main effect of Drug treatment ($F(1, 12) = 11.41, p < .01$) and of Session ($F(6, 71) = 2.82, p < .05$), but no Drug X Session interaction ($F(6, 71) = 0.92, p = .49$).

Figure 4 also shows that when infusions were eliminated prior to sessions 8–20, difference scores quickly accelerated for the previously SCH-23390-treated group to comparable control (vehicle-infused) levels. ANOVA on sessions 8–20 indicated a main effect of Drug treatment ($F(1, 12) = 6.52, p = .03$) and of Session ($F(12, 139) = 3.46, p < .01$), but no Drug X Session interaction ($F(12, 139) = 0.73, p = .72$). Lastly, an infusion prior to session 21, identical to the treatment received during sessions 1–7, tested the effects of SCH-23390 in performance of a well-learned Pavlovian discrimination. Although it appeared that the difference score from session 21 for the vehicle-infused group was lower than the drug-infused group, a *t*-test revealed no between-group difference ($t(12) = 1.68, p = 0.12$). More importantly, no drug-induced decrement in difference scores was apparent.

The three panels on the right show nose-poke rates during the three periods (CS+, CS- and ITI) across sessions. For sessions 1–7, ANOVA with *post hoc* pairwise comparisons (similar to the above) indicated that there were no differences in nose-poke rates during the ITI between drug-treated and vehicle-treated rats (Drug effect: $F(1, 12) = 3.21, p = .10$; Sessions: $F(6, 71) = 2.16, p = .06$; Drug $\times$ Session: $F(6, 71) = 1.34, p = .25$). However, both CS+ and CS- rates were higher in the vehicle-treated group than the SCH-23390-treated rats (For CS+: Drug effect: $F(1, 12) = 33.71, p < .01$; Sessions: $F(6, 71) = 6.60, p < .01$; Drug $\times$ Session: $F(6, 71) = 3.23, p < .01$. For CS-: Drug effect: $F(1, 12) = 7.42, p = .02$; Sessions: $F(6, 71) = 2.14, p = .06$; Drug $\times$ Session: $F(6, 71) = 1.47, p = .20$).

During sessions 8–20, there was no main effect of drug on ITI responding ($F(1, 12) = 0.06, p = .81$), but there was an effect of sessions ($F(12, 140) = 3.60, p < .01$); no Drug $\times$ Sessions interaction was found ($F(12, 140) = 0.71, p = .75$). The effects on CS+ responding during sessions 8–20 were: Drug: $F(1, 12) = 0.91, p = .36$; Sessions: $F(12, 140) = 4.03, p < .01$;

Drug $\times$ Sessions: $F(12, 140) = 2.63, p < .01$. *post-hoc* analyses indicated significantly different CS+ responding between groups during sessions 8, 9 and 11. During the CS– presentations, there was no main effect of Drug on nose-poking ($F(1, 12) = 0.27, p = .61$), but there was a main effect of Sessions ($F(12, 140) = 1.81, p = .05$) and a Drug $\times$ Session interaction ($F(12, 140) = 3.00, p < .01$). Separate *t*-tests comparing nose-poking during session 21 did not reveal any drug effect (ITI: $t(12) = -0.11, p = .91$; CS+: $t(12) = 1.38, p = .19$; and CS–: $t(12) = 0.02, p = .99$).

Also of note, in separate exploratory analyses within each period, there were no statistically significant differences in CS+, CS–, or ITI nose-poking rates within the SCH-23390 group across sessions 1–7. In other words, within the SCH-23390 group, CS+ rates during session 1 were not different than session 2, 3, 4, 5, 6 or 7.

3.2.3 vSUB—Figure 5 shows that intra-vSUB infusions of SCH-23390 also impaired the acquisition of discriminated Pavlovian conditioned approach. ANOVA on difference scores for sessions 1–6 resulted in a significant main effect of drug treatment ($F(1, 13) = 5.405, p = .03$), a main effect of session ($F(5, 65) = 3.872, p < .01$), and a statistically significant drug $\times$ session interaction ($F(5, 65) = 3.338, p < .01$). *post-hoc* comparisons indicated that the SCH-23390 treated group had statistically lower difference scores during sessions 4, 5 and 6. Due to the fact that SCH-23390 infusions were robustly impairing the acquisition of discriminated responding, this phase of infusions was discontinued one session earlier than the previous 2 experiments. While recognizing that this change might limit the potential comparisons to the two previous amygdala targets, it also limited the amount of trauma to the brain produced by multiple injections. Nevertheless, two rats became ill around session 12. They were cage mates and had received different drug infusions; it was unclear what made them ill. They were subsequently eliminated from the experiment, however, their responding during the first phase did not appear to be different from other subjects in their group. Eliminating these two subjects from the first phase did not change the conclusions based on the statistical analysis.

During sessions 7–21, difference scores quickly accelerated for the previously SCH-23390-treated group to comparable control (vehicle-infused) levels. The main effect of prior drug treatment on difference scores for sessions 7–21 was not statistically significant ($F(1, 11) = 2.467, p = .14$). However, there was a significant effect of session: $F(14, 137) = 7.765, p < .01$, but no drug $\times$ session interaction: $F(14, 137) = 1.105, p = .36$.

Lastly, an infusion prior to session 22, identical to the treatment received during sessions 1–6, tested the effects of SCH-23390 in performance of a well-learned Pavlovian discrimination. A *t*-test revealed no between-group difference ($t(10) = -0.926, p = 0.38$).

The three panels on the right show nose-poke rates during the three periods (CS+, CS– and ITI) across sessions. For sessions 1–6, ANOVA indicated that ITI rates were not affected by drug treatment ($F(1, 13) = 0.56, p = .47$), but were influenced by Session ($F(5, 63) = 8.15, p < .01$) and by a Drug $\times$ Session interaction ($F(5, 63) = 2.63, p = .03$). ITI nose-pokes were lower in the SCH-treated group during session 1. CS+ rates were affected by Drug ($F(1, 13) = 4.92, p = .04$), but the effects of Session ($F(5, 63) = 2.05, p = .08$) and the Drug $\times$ Session

interaction were not significant ($F(5, 63) = 1.67, p = .15$). CS– nosepoking was not affected by Drug, Session or Drug $\times$ Session interaction ($F(1, 13) = 2.70, p = .12, F(5, 63) = 0.53, p = .75, F(5, 63) = 0.24, p = .94$, respectively). During sessions 7–21, no main effect of previous drug treatment were found on nose poking rates during the 3 periods, ITI, CS+, or CS– (all F s $< 2.00, p$'s $> .19$). The effect of sessions was significant for CS+ responding ($F(14, 137) = 8.125, p < .01$) but not on CS– or ITI responding. These analyses confirm that CS+ responding continued to increase across sessions 7–21, but CS– and ITI did not change. There were no differences in nosepoking rates between groups during session 21 in the ITI, CS+, or CS– periods.

3.3 Comparison of acquisition during drug-free sessions

In order to fully assess the effects of pre-session SCH-23390 infusions on acquisition of a appetitively-conditioned response and subsequent performance, difference scores during the first 6–7 sessions for the vehicle-treated group were compared to the first 6–7 sessions in which drug was not infused in the SCH-23390 groups. This period in the SCH-23390 treated rats coincides with sessions 8–14 (or 7–12 in the case of vSUB). This analysis essentially shifts the drug-treated group's learning curve to left, treating the first 6–7 sessions with infusions as if they had not occurred. The results of this analysis are shown in Figure 6.

As can be seen in the panels in Figure 6, the former drug-treated groups, when compared to their anatomical controls, acquired the Pavlovian discrimination in a similar fashion. An ANOVA confirmed a learning effect as the main effect of Sessions was highly significant in all three cases (BLA: $F(6, 66) = 4.34, p < .001$, CeA: $F(6, 66) = 8.204, p < .001$, vSUB: $F(5, 49) = 6.531, p < .001$). Neither the main effect of previous Drug treatments (BLA: $F(1, 66) = 1.951, p = .19$, CeA: $F(1, 66) = 0.000, p = .99$, vSUB: $F(1, 49) = 0.015, p = .91$) nor the Drug $\times$ Session interactions (BLA: $F(6, 66) = 0.514, p = .80$, CeA: $F(6, 66) = 1.638, p = .15$, vSUB: $F(5, 49) = 0.277, p = .92$) were statistically significant.

3.4 D1-like receptor antagonism in BLA, CeA, and vSUB has different effects on responding

The effects of D1-like receptor antagonism in the 3 sites studied appears to have differential effects on responding during the learning of a discriminated Pavlovian response, even though the overall measure of learning, difference scores, were affected similarly. Upon a closer review of the data from the last 3 sessions of any experiment (sessions 5–7 or 4–6 in the case of the vSUB experiment), the effects of D1-like receptor antagonism on ITI, CS+ and CS– responding depend on the site of the infusion. Table 1 summarizes those differential effects.

For example, as shown in the table, CS+ and CS– responding was lower during sessions 5–7 in the BLA-drug-infused group relative to vehicle controls. However, ITI responding was not different between the groups. In contrast, ITI and CS+ responding (recall the main effect of Drug on CS+ responding in this experiment was nearly significant at $p = .06$), but not CS– responding, were affected in the CeA-drug-infused group relative to their controls, with ITI responding higher in drug-treated rats. Lastly, only CS+ responding was lower in the vSUB drug-treated group.

4. Discussion and Conclusion

The present experiments demonstrate that D1-like receptor activation in the BLA, CeA, and vSUB are involved in the *acquisition* of appetitive discriminated Pavlovian conditioned approach (PCA). In contrast, D1-like receptor activation in these sites is not required for the *performance* of the behavior after the response was well-learned because infusions of SCH-23390 prior to session 21 or 22 had no statistically reliable effect on behavior.

The present results conform to the results of several other studies demonstrating a role for D1-likes in appetitive Pavlovian conditioning. For example, while systemic D1R antagonism impairs learning in similar PCA procedures (Choi, Balsam, & Horvitz, 2005; Choi et al., 2009; Eyny & Horvitz, 2003), extended training attenuated the effects of SCH-23390 injections (Choi et al., 2005), paralleling a similar shift in D1R-dependency (i.e., learning vs. performance) found in the present experiments. D1R antagonism in the BLA during drug-cue conditioning has been shown to impair that cue's ability to reinstate operant drug-seeking lever-pressing; a putative appetitive CS effect (Berglind et al., 2005). Thus, the present results focus our understanding of the role of D1-like receptors in *appetitive* Pavlovian conditioning by identifying discrete structures involved in this D1R-dependent learning. The present results also suggest that normal reward-related and drug-related processes share some of the same neurocircuitry and neurochemistry, and underscore contemporary theories that propose that the neurobiology of drug use/addiction overlaps heavily with learning/mnemonic processes (Hyman & Malenka, 2001; Nestler, 2001).

In contrast to the effects on learning, it did not appear that D1-like receptor blockade in these limbic targets produced concurrent motivational or motor deficits as evidenced by the following: First, responding during the ITI by drug-treated rats was comparable to rates by vehicle-treated rats. If motor or motivational deficits were produced by drug infusions, ITI nose-poking rates should have decreased in drug-treated rats. Nose-poking rates during the ITI periods were not reduced across the acquisition phase (sessions 1 – 6 or 7; no statistically significant effect of sessions on ITI responding) for SCH-23390 treated rats, nor were those rates lower, within any individual session from vehicle-treated controls (SCH-treated rats following infusions in the CeA did show *elevated* ITI rates during sessions 5 and 7). It is possible that ITI nose-poking rates were significantly reduced by the behavioral contingencies such that a floor effect was reached, thereby obscuring drug-induced deficits, however ITI rates were near 10/min in all 3 drug treated groups. Additionally, it is possible that drug-treatment did constrain nose-poking at that rate, however it is difficult to assess this hypothesis in the current experiment. Second, it did not appear that CS– nose-poking rates were *reduced* by drug treatment, although in the BLA, CS– nose-poking rates were lower in the drug-treated group in comparison to the vehicle-treated group. Nevertheless, nose-poking rates in all 3 periods (ITI, CS–, and CS+) maintained around 10 per minute in the drug treated groups. The principle effect of drug infusion was on the differentiation of nose-poking during those periods. Nonetheless, if global motoric or motivational processes were affected by drug infusions, the prediction would be that CS– nose-poking rates would decrease, which they did not. Third, previous studies from our laboratory demonstrate that D1-like receptor antagonism in these three areas does not reduce spontaneous feeding or locomotion in control experiments (Andrzejewski et al., 2005, 2006), further reducing the likelihood that

D1-like receptor antagonism affects learning secondarily through influences on food motivation or motor behavior. Fourth, there were no statistically significant effects of SCH-23390 infusions after 20 sessions of training, once learning reached asymptotic levels.

4.1 D1-like receptor antagonism differentially influences responding during Pavlovian Conditioned Approach

D1-like receptor activation in different brain structures appears to subserve different processes that produce discriminated PCA. To begin, we assume that the presentation of food (US) following the CS+ produces excitatory conditioning thereby increasing nosepoking during the CS+. The excitatory action of CS+ may also generalize to other stimuli, like the CS–, thereby maintaining or elevating CS– nosepoking, for a short period of time. With training, however, responding to the CS– should extinguish. ITI nosepoking should extinguish as well, for it is never followed by food. However, if responding during the CS– increases over sessions along with increases in CS+, there is evidence that that rat is not learning the discriminative value of the CS+; there is a failure to learn the predictive value of the stimuli from one another. Concurrent increases in CS+ and CS– responding, therefore, may result from general activation or arousal, rather than learning. The difference score measure takes both CS+ and CS– responding into account, but elevated difference scores can result from many combinations of changes in CS+ and CS– responding. Therefore, it is important to consider response rates during the different periods of a session to assess potential differences.

As shown in Table 1, D1-like receptor blockade in the BLA, relative to vehicle controls, affected both responding to the CS+ and CS–, whereas D1-like blockade in the CeA influenced CS+ responding and ITI responding, but not CS–. D1-like receptor blockade in the vSUB affected only CS+ responding. From these data, we can speculate that D1-like receptor blockade in all three structures influences the excitatory processing of the CS+. However, D1-like receptor blockade in the BLA appears to affect the processing of all discrete stimuli, for CS– processing was also affected. The BLA may also be involved in the discrimination of discrete stimuli, filtering relevant and irrelevant events. D1-like receptors in the CeA, while nearly significantly affecting CS+ responding, did increase ITI responding late in training. This may reflect a role for D1-like receptor in the CeA on general arousal, attention to relevant stimuli, or inhibition of activity not predictive of food. Lastly, D1-like receptor activation of the vSUB appears only to affect CS+ processing and thus may be uniquely situated to influence excitatory events.

The differences in responding during the different periods of the dPCA procedure are consistent with some other studies of appetitive Pavlovian conditioning and lesions of these limbic structures. For example, Hatfield et al. (1996) concluded that the BLA was a crucial node of the system responsible for assigning positive incentive value to a CS, whereas the CeA was more involved in attentional processes, a conclusion borne out by substantial data from a number of experiments (Blundell et al., 2001; Blundell, Hall, & Killcross, 2003; Hatfield et al., 1996; Holland & Gallagher, 2003; Holland et al., 2001). We hypothesized that D1-like receptor activity in the vSUB was related to maintaining a “conditioned arousal” process because we found a within-session decline in operant responding by D1-

like receptor blockade during several tests which could not be accounted for by effects on spontaneous (low response cost) feeding or motor behavior (Andrzejewski et al., 2006). It appears from the present results that D1-like receptors in the vSUB play an important role in assigning significance to appetitive stimuli.

4.2 Lesions of the BLA, CeA, and vSUB leave Pavlovian conditioning intact

In apparent contrast to the current results, numerous studies have found that initial appetitive Pavlovian conditioning, using procedures that closely resemble the present experiments, are unaffected by lesions of the BLA, CeA, or vSUB, in some cases (Everitt & Robbins, 1992; Gallagher & Holland, 1992; Hall, Parkinson, Connor, Dickinson, & Everitt, 2001; Hatfield et al., 1996; Parkinson, Robbins, & Everitt, 2000). While the data from BLA-lesion studies appears consistent, two reports (Hall et al., 2001 and Parkinson, et al. 2000) prove equivocal on the question of whether the CeA is important in appetitive Pavlovian conditioning. Parkinson et al. (2000) concluded that the lesions of CeA impair Pavlovian conditioned responding, but Hall et al. (2001) detected no effect following CeA lesions. Another report found that intra-BLA infusions of the NMDA receptor antagonist AP-5 impaired acquisition, but not performance of discriminated Pavlovian approach to an appetitive CS (Burns, Everitt, & Robbins, 1994). Using the same microinfusion methodology reported here, we found that intra-amygdala infusions of SCH-23390 impaired initial operant conditioning (Andrzejewski et al., 2005), in contrast to reports using lesions of the BLA (Balleine, Killcross, & Dickinson, 2003). While certain procedural differences may explain some of these differences (e.g., use of sucrose pellets vs. sucrose solution, auditory vs. visual stimuli, autoshaping vs. conditioned approach), the large and robust effects of amygdala manipulations before, during, and after appetitive Pavlovian conditioning strongly implicate a critical role. We would offer, however, that conclusions from lesion studies are more limited than those from microinfusions for several reasons. First, the microinfusion method allows for certain within-subject manipulations that are not possible with lesions. In the present experiments, rats received microinfusions for several sessions, but then the injections were discontinued. The fact that the formerly-drug-treated rats acquired the discriminated approach rules out the possibility that the two groups differed in some peculiar and/or unmeasured dimension (i.e. akin to a Type I error). Second, compensatory neuroadaptations following lesion-induced damage may obscure the amygdala's fundamental role in Pavlovian learning. In a direct test of lesions versus microinfusions, NMDA receptor blockade with AP-5 in the prefrontal cortex produced deficits in extinction learning, but lesions did not (Lissek & Gunturkun, 2003). Abundant evidence suggests that neuroadaptations in multiple cellular functions occur following lesions, especially DAergic lesions. For example, lesions produce presynaptic increases in synthesis, metabolism, and fractional release of DA in the remaining terminals (Abercrombie, Bonatz, & Zigmond, 1990; Castaneda, Whishaw, & Robinson, 1990; Robinson & Whishaw, 1988; Touchet & Bennett, 1989; Zhang et al., 1988), and post-synaptic DA-receptor supersensitivity (Creese, Burt, & Snyder, 1977; Mishra, Gardner, Katzman, & Makman, 1974; Neve, Kozlowski, & Marshall, 1982). Therefore, while lesion-induced deficits in certain paradigms are well-documented, the *lack* of deficits in some lesion studies makes them difficult to interpret, given the likely recovery of function. Third, the fact that selective pharmacological blockade of NMDA receptors (Andrzejewski, Sadeghian, & Kelley, 2004; Baldwin, Holahan,

Sadeghian, & Kelley, 2000), muscarinic receptors (See, McLaughlin, & Fuchs, 2003) and now D1 receptors, within the amygdala, disrupts appetitive learning indeed demonstrates a role for the amygdala in the plasticity that underlies appetitive learning. Fourth, *in vivo* microdialysis and immunohistochemical studies show that DA levels increase in the amygdala during PCA (Harmer & Phillips, 1999; Phillips, Setzu, Vugler, & Hitchcott, 2003). These robust increases in DA likely serve crucial neural and behavioral functions, and are not merely epiphenomenal.

This is not to claim that there are not limitations of the microinfusion methodology, or with the results of the current study. First of all, D1-like receptor antagonism often produces aberrant activity in an area, that may impact activity elsewhere. While true, manipulations of any area may affect a network and be criticized as “non-specific” in this regard. Yet, the very question of the role of D1-like receptors in a structure requires multiple lines of inquiry, including local, pharmacological ones. Second, the fairly large gap in time between the last “acquisition” infusion and the performance test provided sufficient time for gliosis to occur, thereby limiting the effectiveness of the drug and moderating our conclusions based on the performance test day. Third, the volume of the microinfusion might have produced some spill over into non-targeted areas late in the training sessions, especially in the amygdala, which may temper our claim about site specificity of effects between the CeA and BLA. Lastly, fairly small sample sizes ($n = 6-8$) and a good degree of behavioral variability raise the possibility of false positive results. Yet, robust, statistically significant effects with an appropriate Type 1 error rate were acquired from these samples. While we do not have any data refuting these limitations, the likelihood that dPCA learning results from increases in DA transmission in the amygdala and subiculum mediated through D1-like receptors is strongly supported here.

4.3 Plasticity within the Amygdala and Subiculum or within a broader system?

Synaptic plasticity has been demonstrated in the amygdala and subiculum, using both *in vivo* and *in vitro* models, and could account for the present results (Behr, Wozny, Fidzinski, & Schmitz, 2009; Blair, Schafe, Bauer, Rodrigues, & LeDoux, 2001; Chapman, Kairiss, Keenan, & Brown, 1990; Huang & Kandel, 1998; McKernan & Shinnick-Gallagher, 1997; Pare & Collins, 2000; Rogan & LeDoux, 1995; Watanabe, Ikegaya, Saito, & Abe, 1995). Thus, synaptic plasticity in the amygdala is hypothesized to represent a major mechanism governing learning, and DA is likely to participate in the process for reward-related learning. However, while D1R antagonism could impair synaptic plasticity in the amygdala and subiculum via mechanisms described above, it is important to consider the larger system with which these sub-regions interact and possible changes within the system “as a whole” vis a vis D1R antagonism in the amygdala and subiculum. For example, the nucleus accumbens core (NAcc) appears to be uniquely positioned, as a component of the basal ganglia, to integrate information from cortical and limbic structures and modulate motor output associated with goal-directed behavior (Groenewegen et al., 1991). The NAcc receives projections from the amygdala and subiculum, and D1Rs in the vSUB, through actions on glutamatergic afferents, modulates DA efflux in the NAcc. Likewise, the CeA may also modulate DA transmission in the NAcc by an indirect pathway, via actions on the substantia nigra and VTA (Fudge & Haber, 2000; Krettek & Price, 1978; Price & Amaral,

1981). The CeA, as well as the BLA and vSUB, contain substantial number of D1Rs and, as mentioned above, PCA learning induces DA release into these structures. Data also indicate that DA modulates sensory, thalamic and prefrontal inputs to the BLA (Rosenkranz & Grace, 2001, 2002); ultrastructural analyses have found that that dopaminergic afferents to both the BLA and CeA are in a position to modulate these extrinsic inputs (Asan, 1997).

4.4 Latent Inhibition?

One interesting finding of the current experiments involves the lack of latent inhibition (LI) demonstrated by the drug-treated groups. It has been repeatedly demonstrated that prior to explicit conditioning, repeated presentation of a neutral stimulus delays conditioning to that stimulus, a phenomenon known as “latent inhibition” (Reiss & Wagner, 1972). When infusions were discontinued, drug-treated groups conditioned at a similar rate to the vehicle-infused controls, which suggests that the D1-like receptor-induced deficit involves recognition of the CS+. As shown in Figure 5, the drug-treated groups acquired the discrimination as rapidly as the vehicle-controls after eliminating the first 6–7 sessions of drug injections. However, the drug-treated groups received 20 CS presentations in each of those sessions (120–140 presentations total). There was no evidence that the drug-treated groups did not retrieve all of the pellets (the US). Moreover, all of the rats were trained to retrieve randomly delivered pellets during the first 3 habituation sessions. Thus, there was no evidence for impaired US processing. In contrast, the CS+ was repeatedly presented to the drug-treated rats but no differential conditioning was evident (e.g., difference scores in these groups was near 0). The question of whether this pre-exposure to the CS+ would retard learning when the drug not infused was answered definitively with the lack of a LI effect, as shown in Figure 5, for the previously drug-treated rats conditioned as quickly as the controls. Thus, we may conclude that D1-like receptor blockade affects the representational processing of the CS+. One possible explanation of this effect is that D1-like receptor antagonism has some sort of an amnesic effect on stimulus representation. While an interesting possibility, it is not clear how to test this assertion. Nonetheless, LI has been proposed as a model of the attention deficit seen in schizophrenia. In addition, the basolateral amygdala, subiculum and dopamine have all been implicated in LI (for review, see Gould, 2010), which is entirely consistent with the findings presented here.

Another intriguing possibility is that D1-like receptor disrupted learning about the CS-US relationship. As noted in the introduction, substantial data exist confirming a role for D1-like receptor activation in neural plasticity, in a variety of brain sites and experimental preparations. D1-like receptor activation in the BLA, CeA, and vSUB is required for appetitive operant learning (Andrzejewski et al., 2005, 2006) and the present results suggest that appetitive Pavlovian conditioning is also impaired by D1-like receptor blockade in those sites. Therefore, it appears that these learning processes (i.e. operant and Pavlovian) share some neurobiological mechanisms. There is ample evidence that these two processes are dissociable in *behavioral* terms (Colwill & Rescorla, 1988), *anatomical* features (Corbit, Muir, & Balleine, 2001; Ostlund & Balleine, 2007), *neurochemical* determinants (Dickinson, Smith, & Mirenowicz, 2000), and *gene expression* patterns (Hernandez, Schiltz, & Kelley, 2006; Schiltz, Bremer, Landry, & Kelley, 2007). These observations, when combined, suggest that instrumental and Pavlovian learning are conceptually and

experimentally separable, at least to a certain extent. Regardless, both learning processes engage mechanisms leading to long-term behavioral changes and the present results suggest important neurochemical and anatomical overlap.

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Highlights

- Dopamine D1 receptor (D1R) activation is critically involved in reward learning.
- D1R involvement in three discrete brain sites was interrogated.
- Discriminated Pavlovian approach was impaired following D1R inactivation.
- D1R inactivation produced three differential behavioral profiles, dependent on site.
- Latent inhibition may have been blocked by D1R blockade.

Figure 1.

Section A schematically represents the Experimental Design over the course of consecutive days of experimentation. Section B shows the arrangement of a magazine training session (arrows represent US deliveries, but note that they were randomly programmed). Section C schematically shows the experimental contingencies during discriminated approach sessions; this represents one possibility for CS+, CS-, and US deliveries which were presented differently every session (see Methods for details). Section D illustrates how responses were characterized, in periods; once again, this representation shows only one possibility for the order of presentations of CS+ and CS-, which were random.

Figure 2.

Histological representations of microinjector placements in the central nucleus of the amygdala (CEA), basolateral amygdala (BLA), and ventral subiculum (vSUB). Cresyl violet stained sections were examined under light microscope and the most ventral aspect of infusion sites were estimated. Gray circles represent vehicle infusion locations, while black circles represent SCH-23390 infusions. The numbers on the bottom of each schematic represent millimeters from bregma. Schematic diagrams are reprinted from *The Rat Brain in Stereotaxic Coordinates* (6th ed.) by Paxinos and Watson.

Figure 3.

The effects of SCH-23390 versus vehicle microinfusions in the central nucleus of the amygdala (CeA) prior to discriminated Pavlovian conditioning approach (DPCA) sessions on mean difference scores ($\pm$ SEM, large panel, * $p < .05$, post hoc Tukey, following significant drug $\times$ session interaction). The three panels on the right show nosepoke rate (nosepokes/minute) during the inter-trial interval (ITI), the CS+, and CS– periods, differentiated by condition (SCH-23390 vs. Vehicle). Dashes (–) designate between group differences ($p < .05$) during a particular session following significant drug $\times$ session interaction.

Figure 4.

The effects of SCH-23390 versus vehicle microinfusions in the basolateral amygdala (BLA) prior to DPCA sessions on mean difference scores ($\pm$ SEM, large panel, * $p < .05$, ** $p < .01$, post hoc Tukey, following significant drug $\times$ session interaction). The three panels on the right show nosepoke rate ITI, the CS+, and CS- periods, differentiated by condition (SCH-23390 vs. Vehicle). There were no differences in ITI nosepoking rates. There was a main effect of drug on CS+ and CS- nosepokes, as well as a significant drug $\times$ session interaction on CS+ nosepoking; post hoc significant differences are noted with a dash.

Figure 5.

The effects of SCH-23390 versus vehicle microinfusions in the vSUB on mean difference scores ($\pm$ SEM, large panel, main drug effect, * $p < .05$; post hoc Tukey, following significant drug $\times$ session interaction, * $p < .05$, ** $p < .01$). Nosepoke rates during ITI, the CS+, and CS- periods, differentiated by condition, are shown on the right. There were no main effects on ITI or CS- nosepoking, but there was a significant main drug effect on CS+ nosepoking. A significant drug $\times$ session interaction on ITI nosepoking was found; post hoc Tukey tests revealed a difference during the first session only, again represented with a dash.

Figure 6.

Difference scores (CS+ - CS-) differentiated by drug condition for the first 6 or 7 *drug-free* sessions. For the vehicle groups, data are plotted from the first 6–7 sessions, whereas for the drug-treated groups, data are from sessions 8–14 (or 7–12 for vSUB). There were no significant effects of drug condition; all session main effects were significant ($p < .05$, not shown).

Table 1

Summary of the main effects of SCH-23390 microinfusions in the three targeted regions, versus vehicle, on responding during the three response periods (see Figure 1, section D for schematic of response periods). In all three regions, D1-like receptor antagonism prevented an increase in CS+ responding (* in the CeA represents a significant drug $\times$ session interaction). However, D1-like receptor antagonism affected ITI responding only when infused in the CeA, while D1-like receptor antagonism only affected CS– responding in the BLA. No effects of D1-like receptor antagonism in the vSUB on ITI or CS– responding were found.

	CeA	BLA	vSub
ITI	↑		
CS+	↓ $p = .06$	↓	↓
CS–		↓	