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Role of the Mesoamygdaloid Dopamine Projection in Emotional Learning

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Abstract

Rationale: Amygdala dopamine is crucially involved in the acquisition of Pavlovian associations, as measured *via* conditioned approach to the location of the unconditioned stimulus (US). However, learning begins before skeletomotor output, so this study assessed whether amygdala dopamine is also involved in earlier 'emotional' learning. *Objectives:* A variant of the conditioned reinforcement (CR) procedure was validated where training was restricted to curtail the development of selective conditioned approach to the US location, and effects of amygdala dopamine manipulations before training or later CR testing assessed. *Methods:* Experiment 1a presented a light paired (CS+ group) or unpaired (CS- group) with a US. There were 1, 2 or 10 sessions, 4 trials per session. Then the US was removed and two novel levers presented. One lever (CR+) presented the light and leverpressing was recorded. Experiment 1b also included a tone stimulus. Experiment 2 applied intra-amygdala R(+) 7-OH-DPAT (10nmols/1.0µl/side) before two training sessions (Experiment 2a) or a CR session (Experiment 2b). *Results:* For Experiments 1a and 1b, the CS+ group preferred the CR+ lever across all sessions. Conditioned alcove approach during 1 or 2 training sessions, or associated CR tests was low and nonspecific. In Experiment 2a, R(+) 7-OH-DPAT before training greatly diminished leverpressing during a subsequent CR test, preferentially on the CR+ lever. For Experiment 2b, R(+) 7-OH-DPAT infusions before the CR test also reduced leverpressing. *Conclusions:* Manipulations of amygdala dopamine impact the earliest stage of learning in which emotional reactions may be most prevalent.

Keywords: *amygdala, dopamine, learning and memory, emotion, associative learning, behavior, classical conditioning, consolidation, retrograde.*

Introduction

It has been proposed that associative learning may broadly consist of three main stages (Mintz and Wang-Ninio 2001; Lennartz and Weinberger 1992; see also Konorski 1967; Wagner 2008; Wagner and Brandon 1989). The first stage is characterised as the relatively rapid acquisition of a range of physiological measures, *e.g.* galvanic skin responses, heart rate and blood pressure changes (*e.g.* Lennartz and Weinberger 1992), which typically are termed 'emotional' responses. The second stage consists of more readily observable skeletomotor, or behavioural responses, which develop at a relatively slow rate. Finally, a third stage of learning has been described as "efficient motor performance" in the absence of a corresponding emotional component (Mintz and Wang-Ninio 2001), *i.e.* the performance of a habit.

Dissociable components of brain dopamine systems have already been noted to play important roles in the second and third of these stages. For example, the mesoaccumbens dopamine projection may play a significant role in the rate of development of the second, skeletomotor stage of learning. Thus, mesoaccumbens dopamine is thought to govern an activational or gain-amplification component of behaviour (Everitt et al. 1999; Mogenson et al. 1980; Robbins and Everitt 2007), which usually (Robbins and Everitt 2007), though not necessarily (Bardo et al. 1990) is closely linked to conditions of motivational significance. In practise, these conditions may consist of stimuli of predictive significance (*e.g.* Robbins and Everitt 2007; Robinson and Berridge 2003; Wise 2006), or when there is a mismatch between predicted and delivered outcomes (Schultz 2002). A related approach ascribes the degree of "incentive salience" attributed to conditioned stimuli, or the degree to which they are "wanted" also to mesoaccumbens dopaminergic activation (Robinson and Berridge 2008). Third stage, or habitual stimulus-response learning is linked most closely with nigrostriatal dopaminergic innervation of regions of the dorsal striatum (Everitt and Robbins 2005; White and McDonald 2002). A great deal of evidence suggests that different regions of the dorsal striatum govern different categories of habitual behaviour (White and McDonald 2002).

At least three sources of data implicate the mesoamygdaloid dopamine projection in the earliest stage of learning. First, dopamine-dependent effects on

amygdala neuronal responses have been observed after just a very few conditioning trials (Rosenkranz and Grace 2002a; see also Grace and Rosenkranz 2002; Rosenkranz and Grace 2002b). Second, an immunohistochemical technique enabled the simultaneous measurement of dopaminergic activity during the acquisition of a conditioned approach task across a variety of midbrain and forebrain regions (Phillips et al. 2003b). Observations taken soon after the acquisition of an overt behavioural response to a conditioned stimulus confirmed the widespread activation of mesotelencephalic dopamine systems, but found activity to be largely absent following extensive training. Third, although post-session intra-amygdala infusions with dopaminergic agonists have been reported broadly to facilitate recent learning (Hitchcott et al. 1997a; Hitchcott et al. 1997b; Hitchcott and Phillips 1998a), in each case differential drug effects were confined solely to the very earliest stage of development of an overt goal-directed skeletomotor response. Whether mesoamygdaloid dopaminergic involvement is restricted to the initial development of readily observable conditioned responses, or if in fact it extends into the earlier, purely 'emotional' stage of learning is currently unclear.

The current work attempts to pinpoint with more precision the earliest stage at which mesoamygdaloid dopamine begins to exert a significant influence over associative learning. However, the development of a robust conditioned approach response towards the location of the unconditioned stimulus would indicate that the very earliest phase of ‘emotional’ learning no longer predominated. Hence, Experiments 1a and 1b restricted the degree of initial training sufficiently to preclude the development of statistically significant levels of selective conditioned approach to the location of the US, and discovered that a light stimulus nevertheless acquired significant conditioned reinforcing properties. The validity of this methodology was assessed by comparing the ability of positive *vs.* negative light CS-US correlations later to support conditioned reinforcement (*ab origine:* Taylor and Robbins 1984). Subsequently, the dopamine receptor agonist R(+) 7-OH-DPAT was infused directly into the amygdala either immediately prior to just two CS-US training sessions (Experiment 2a), or a subsequent conditioned reinforcement test (Experiment 2b).

Methods

Subjects

A total of 93 male Lister hooded rats took part in these experiments (Charles Rivers, Margate, Kent, UK); 48 in Experiment 1a, 8 in Experiment 1b, 15 in Experiment 2a and 22 in Experiment 2b. Animals were housed in pairs under a 12h:12h light/dark cycle (lights on 08.00h) at a constant temperature of 22°C. Experiments were carried out between 10.00-17.00h. The body weights of animals were reduced to 85% of their free-feeding weight by restricting access to food (Experiments 2a and 2b: following recovery from surgery). Water was available *ad libitum*. All experimental procedures were carried out under the Animals (Scientific Procedures) Act 1986, and were subject to UK Home Office approval (Project Licence PPL 50/01257).

Apparatus

Testing was carried out in eight operant chambers (31 x 24 x 29cm); Med Associates Inc, St Albans, VT, USA). Each chamber was equipped with a dipper (model ENV-202; cup capacity 0.06ml) located within a small recess in the middle of the front wall and was used for the presentation of a 30% w/v sucrose solution which was made up fresh everyday and allowed to reach room temperature before the session. Two retractable levers each 5cm wide, were positioned symmetrically upon this wall 12cm apart and 7cm from the grid floor, either side of the dipper recess (5 x 5 x 3cm). The operant chamber could be illuminated by a white 15W houselight located at the top of wall opposite. Each chamber was also equipped with two white stimulus lights 15W, positioned directly above each retractable lever 18cm above the grid floor, and a 75dB SonAlert sinusoidal tone (2.9kHz) generator. The operant chamber was housed in a sound-attenuating box and external noise was masked further by a ventilating fan mounted on the side of the box.

Each chamber was also fitted with a number of active photobeams for the measurement of activity. Four photobeams recorded horizontal activity, and were positioned 4cm above the grid floor. They were aligned parallel with the wall

containing retractable levers, *i.e.* from front to back at 4cm, 11cm, 19cm and 27cm from the wall with the recess. A fifth photobeam was located in the side walls of the dipper recess and was used to monitor alcove approach behaviour. The apparatus was controlled, and the data collected, by a standard IBM compatible 386 PC with appropriate software platform (Med Associates Inc, St Albans, VT, USA).

Drugs

R(+) 7-OH-DPAT hydrobromide (Semat Technical (UK) Ltd, St. Albans, UK) was dissolved in sterile phosphate buffered saline (PBS), which also served as vehicle. Doses were calculated as that of the base.

Surgery

Surgery was performed under both general and local anaesthesia. In Experiment 2a, rats were first anaesthetised with an i.p. injection of a solution containing 2,2,2-tribromoethanol in sterile PBS (Phillips et al. 1994). In Experiment 2b, rats were first anaesthetised in an induction chamber with 4% halothane and approx 0.8 l/min of N2O and O2 gas (RA Medical Services, Steeton, West Yorkshire, UK). Once anesthetised the rat was moved to a face mask in the stereotaxic frame and delivery of halothane was reduced to 1.5% for maintenance. At the same time, subjects also received 0.1ml amoxycillin i.m. (15mg/ml) to minimise the risk of bacterial infection. Finally, a lignocaine solution (20mg/ml) was applied directly to the exposed areas of skin following the initial incision.

Bilateral stainless-steel guide cannulae (22 gauge single cannulae; Plastics One, Roanoke, VA, USA) were implanted to gain access to the amygdala. The stereotaxic coordinates used were: AP -2.8mm from Bregma, L+/-4.5 from the midline, V -6.6mm from the surface of dura (Paxinos and Watson 1986). Implanted guide cannulae were secured to the skull with a minimum of four stainless steel screws and dental cement. The cannulae were closed by screw-in stainless steel wire obturators (28 gauge dummy cannulae; Plastics One, Roanoke,

VA, USA) and the animals returned to their home cages for a period of recovery of no less than 7 days.

Pre-session Infusions

Intracerebral infusions within the amygdala were made using an infusion pump (Model A, 3.33RPM motor; Razel Scientific Instruments Inc, Stamford, CT, USA). Rats were hand-held while 28 gauge infusion cannulae (Plastics One, Roanoke, VA, USA) were placed into the surgically implanted guide cannulae. The infusion cannulae were attached to the pump microsyringes (Hamilton 801RNE; Scientific Laboratory Supplies Ltd, Hessle, East Yorkshire, UK) by polyethylene tubing filled with sterile PBS. Drug solutions were backloaded within the cannulae and tubing to prevent contamination of the microsyringes. Infusion cannulae projected from guide cannulae by 1mm. The volume infused was 1.0µl over 50 seconds, and infusion cannulae remained in place for a further 1min period. All sessions involving drug infusions were separated by a period of at least 72 hours.

Procedure

US Approach Training

Rats were first trained to consume the sucrose solution from the dipper during 8 sessions in which sucrose was presented 30 times/session according to a variable time 60 second (VT-60sec) schedule (100 possible intervals generated using the progression sequence of Fleshler and Hoffman 1962).

Stimulus Pre-exposure

Rats were presented with stimuli later to be used in Pavlovian training. The single pre-exposure session consisted of four 10sec presentations of each stimulus according to a fixed time of 120 second (FT-120sec) schedule, with the probability of each stimulus type occurring next set at 0.5 (Experiments 1b, 2a and 2b).

Pavlovian CS-US Training

Experiment 1a: Following stimulus pre-exposure, subjects in the positive contingency condition were trained to associate an initially neutral light stimulus, (the conditioned stimulus; CS+) with delivery of sucrose (the unconditioned stimulus; US). Thus, each trial consisted of a 10sec Light stimulus presentation followed immediately by 10sec access to 30% sucrose. A total of 4 trials were presented during each session. Trial frequency was set according to a fixed time 240sec schedule (FT-240sec). Animals in a control, negative contingency condition received an equal number of presentations of the CS+ and the US but these were not paired temporally at any time. Instead, the CS+ and US were presented in alternation on a fixed time 120sec schedule with a total of 4 presentations of each. In both conditions the stimulus was Houselight off, wall-lights on. A total of 1, 2, or 10 training sessions were given (n=8 per training session and contingency conditions). Approach behaviour during the 10sec period immediately preceding the presentation of the stimulus was recorded as a measure of baseline rates of approach behaviour and subtracted from that occurring during presentation of the stimulus itself. Thus, the specificity of the approach response in relation to the CS-US association could be assessed.

Experiments 1b and 2: Following stimulus pre-exposure, each trial consisted either of a 10sec stimulus presentation followed immediately by 10sec access to 30% sucrose solution (CS+ trial), or a 10sec presentation of the control stimulus alone (CS- trial). The probability of each type of trial occurring was 0.5, with the proviso that a total of 4 CS+ and 4 CS- trials were presented during each session. All animals received a total of 8 CS–US pairings across 2 sessions. Trial frequency was set according to a fixed time 240sec schedule (FT-240sec). The stimulus types were Houselight off, wall-lights on for the CS+ as for Experiment 1, with the addition of a SonAlert tone as a control stimulus. In Experiment 1b, the stimulus type-contingency relationship was reversed for half the animals. Approach behaviour during the 10sec period immediately preceding the presentation of the stimuli was recorded as a measure of baseline rates of approach behaviour and subtracted from that occurring during presentation of the stimuli themselves. Thus, the specificity of the approach response in relation to the CS-US association could be assessed. Extraneous behaviour was measured

concurrently and was recorded as horizontal activity within experimental chambers.

Infusions

During Experiment 2, animals received bilateral intra-amygdala infusions of R(+) 7-OH-DPAT (10nmols/1.0µl/side) or an equivalent volume of PBS vehicle immediately before each of two Pavlovian training sessions (Experiment 2a, R(+) 7-OH-DPAT: n=8; Vehicle: n=7) or the conditioned reinforcement test session (Experiment 2b, R(+) 7-OH-DPAT: n=11, Vehicle: n=11). To accustom subjects to the infusion procedure, all subjects received mock infusions before each of the US approach training sessions, and animals in Experiment 2b also received mock infusions before the two Pavlovian training sessions.

Conditioned Reinforcement Test

The ability of the CS+ to act as a conditioned reinforcer (CR) and support a novel lever pressing response was tested 24 hours after the last CS-US training session. During CR testing the US was not presented and the dipper remained inactive, although sucrose was still present in the container. Instead, two novel retractable levers were introduced into the operant chamber. Both levers retracted for 10sec following a response upon either lever during Experiment 1a, but remained extended at all times during the conditioned reinforcement phase subsequent experiments. Depression of one lever (the CR+ lever) resulted in a 10sec presentation of the CS+, while depression of the second lever (the CR- lever) was either without further programmed consequence (Experiment 1a) or resulted in a 10sec presentation of the CS- (Experiments 1b and 2). The position of the CR+ lever was counterbalanced across animals at all times. Sessions began after a response upon either lever, and continued for a total of 30 min. Rates of responding upon both levers and general activity within the chamber were recorded.

Histology

At the conclusion of Experiments 2a and 2b, animals were killed by pentobarbital overdose and the brain removed for histological examination. Brains were blocked and cut at 30μm sections using a vibratome (general scientific, Redhill, UK). The sections were mounted on glass slides and stained with cresyl violet. The accuracy of cannula placements was then assessed (Paxinos and Watson 1986).

Statistical Analysis

Data were analysed initially using parametric analyses of variance, and where appropriate within-factor comparisons were analysed subsequently using simple main effect analyses of variance and appropriate *post hoc* tests (Winer 1971). For Experiment 1a, alcove approach during Pavlovian training (measured as approach during the Pre-CS period subtracted from that during the CS period) was first subjected to a two-way fully independent analysis of variance (**Training Sessions** [1-, 10-Sessions] x **CS-US Contingency** [Positive, Negative]). Training data for the 2-Session group were not included, due to a data recording error during the second session of training. Each condition was then analysed separately using a one-way between subjects analysis of variance (**CS-US Contingency**). US approach was analysed by two-way between-subjects analysis of variance (**Training Sessions** x **CS-US Contingency**). Lever response data during the conditioned reinforcement session were analysed initially for each training session using a two-way repeated measures analysis of variance (**CS-US Contingency** x **Lever** [CR+, CR-]), followed by separate one-way within-subjects analyses of variance (**Lever**) for each Contingency. Approach data from this session were first subjected to a three-way analysis of variance (**Training Sessions** [1-, 2-, 10-Sessions] x **CS-US Contingency** x **Trial Type** [CS+, CS-]) with **CS-US Contingency** as the independent factor, and then further two-way analyses were carried out (**CS-US Contingency** x **Trial Type**) for each training session, with **CS-US Contingency** as the independent factor.

For Experiment 1b, alcove approach during Light or Tone stimulus presentations (measured as approach during the Pre-CS period subtracted from that during the CS period) during the second of two Pavlovian training sessions

was first subjected to a two-way analysis of variance (**Stimulus Modality** [Light CS+, Tone CS+] x **CS-US Contingency** [Positive, Negative]) with **Stimulus Modality** (CS+ Tone, CS+ Light) as the independent factor, then each **Stimulus Modality (**Light CS+, Tone CS+) further analysed with individual one-way within-subjects analyses of variance (**CS-US Contingency** [Positive, Negative]). US approach and locomotor data during the second training session were analysed by one-way between-subjects analysis of variance (**Stimulus Modality [**Light CS+, Tone CS+]). Lever responding data during the conditioned reinforcement test session was initially analysed using a two-way repeated measures analysis of variance (**Stimulus Modality** [Light CS+, Tone CS+] x **Lever** [CR+, CR-]) with **Lever** (CR+, CR-) as the between-subjects variable. Alcove approach data taken from conditioned reinforcement session were subjected to a two-way split-plot analysis of variance (**Stimulus Modality** [Light CS+, Tone CS+] x **CS-US Contingency** [Positive, Negative]) with **CS-US Contingency** [Positive, Negative] as a within-subjects variable. Finally, locomotor activity from the test session was analysed as a one-way between-subjects analysis of variance (**Stimulus Modality** [Light CS+, Tone CS+]).

For Experiment 2, alcove approach data gathered during training was subjected either to a three-way (Experiment 2a) or two-way (Experiment 2a) analysis of variance (**Infusion Group** (Experiment 2a only) [Vehicle, R(+) 7-OH-DPAT] x **CS-US Contingency** x **Session** [Session-1, Session-2]), with **Session** as the sole within-subjects factor. Alcove approach data during the second of the two Pavlovian training sessions were also subjected either to a split-plot two-way (Experiment 2a) or one-way (Experiment 2b) analysis of variance (**Infusion Group** (Experiment 2a only) x **CS-US Contingency**) with **Infusion Group** as the independent factor. US approach and locomotor activity measured during the second training session were analysed by one-way between-subjects analysis of variance (**Infusion Group**).

Leverpresses during conditioned reinforcement were analysed initially using a two-way split-plot analysis of variance (**Infusion Group** x **Lever**) with **Lever** as the within-subjects factor, followed by separate one-way between-subjects analyses of variance for each lever (**Infusion Group**). Alcove approach data from the conditioned reinforcement session were subjected to a two-way split-plot analysis of variance (**Infusion Group** x **CS-US Contingency**) with **CS-**

US Contingency as the within-subjects variable, and for Experiment 2b again as the total number of alcove approaches per leverpress (**Infusion Group**). Locomotor activity from the test sessions were analysed using a between-subjects one-way analysis of variance (**Infusion Group**) and for Experiment 2b again as the total number of beam breaks per leverpress, and finally for beam breaks on the side of the experimental chamber opposite to the levers and alcove.

Results

Experiment 1a: Rate of Development of Conditioned Approach *vs.* Conditioned Reinforcement

CS-US Training

Ten training sessions established a far greater alcove approach response than a single Pavlovian CS-US session (**Training Sessions** $F(1,28)=46.79$, $P<0.001$; Means±SEMs alcove approaches per session: 1-Session, Positive CS-US contingency: -1.75 ±0.98; Negative CS-US contingency: -1.38 ± 0.46; 10-Sessions, Positive CS-US contingency: 11.88 ± 1.47; Negative CS-US contingency: 0.00 ±; 0.38) and this approach response in the 10-Session group was specific to the condition in which the stimulus was positively correlated with the US (**Training Sessions** x **CS-US Contingency** $F(1,28)=51.29$, $P<0.001$; **CS-US Contingency** $F(1,28)=58.44$, $P<0.001$). Separate analysis of the presence of conditioned approach in each Training Session condition confirmed these initial findings: animals in the 10-Session condition demonstrated a clear discriminative approach response to the light stimulus (**CS-US Contingency** $F(1,14)=61.28$, $P<0.001$), whereas animals in the 1-Session condition did not (**CS-US Contingency** $F(1,14)=0.12$, *NS*).

By contrast, alcove approach during US presentations were very comparable across Training Sessions (US-related alcove approach, beam breaks, Means±SEMs: 1-Session, Positive CS-US contingency: 3.75 ±0.16; Negative CS-US contingency: 3.88 ± 0.30; 10-Sessions, Positive CS-US contingency: 4.13 ± 0.30; Negative CS-US contingency: 4.25 ±; 0.25 **Training Sessions** $F(1,28)=2.14$, *NS*; **CS-US Contingency** $F(1,28)=0.24$, *NS*).

Conditioned Reinforcement

Responding on the CR+ lever exceeded responding on the control CR- lever even following a single training session (see Figure 1, Upper Panel; **Lever** $F(1,14)=20.32$, $P<0.001$). However, at this early stage enhanced responding on the

CR+ lever was not specific to the positive CS-US contingency group (**Lever** $F(1,14)=20.32$, $P<0.001$; **CS-US Contingency** $F(1,14)=0.041$, *NS*; **Lever** x **CS-US Contingency** $F(1,14)=1.15$, *NS*). By contrast, a more selective response typical of optimal CR performance was clearly seen following two training sessions. Thus, a preference for responding on the CR+ lever was clearly evident in the positive CS-US contingency condition, yet absent in the negative contingency condition (**Lever** x **CS-US Contingency** $F(1,14)=5.85$, $P<0.05$; **Lever** $F(1,14)=8.02$, $P<0.05$; **CS-US Contingency** $F(1,14)=2.30$, *NS*; Simple Main Effects: Positive CS-US contingency group, **Lever** $F(1,7)=12.33$, $P<0.01$; Negative CS-US contingency group, **Lever** $F(1,7)=0.097$, *NS*). Similarly, ten sessions of positive, but not negative CS-US contingency training also resulted in a robust and preferential rate of leverpressing on the CR+ lever (**Lever** x **CS-US Contingency** $F(1,14)=12.99$, $P<0.01$; **Lever** $F(1,14)=12.99$, $P<0.01$; **CS-US Contingency** $F(1,14)=11.30$, *NS* $P<0.01$; Simple Main Effects: Positive CS-US contingency group, **Lever** $F(1,7)=17.92$, $P<0.01$; Negative CS-US contingency group, **Lever** $F(1,7)=0$, *NS*).

Alcove approach behaviour following each leverpress generally failed to differentiate between CS+ and control conditions (Figure 1, Lower Panel). Consistent with this, discriminative approach in response to the CS+ was not evident at any time in the negative CS-US contingency group (**Training Sessions** x **Trial Type** $F(2,21)=1.46$, NS; 1-Session condition: **Trial Type** $F(1,7)=0.34$, NS; 2-Session condition: **Trial Type** $F(1,7)=1.92$, NS; 10-Session condition: **Trial Type** $F(1,7)=0.10$, NS). This was also true of the positive CS-US contingency group for both the 1-Session and the 2-Session condition (1-Session condition: **Trial Type** $F(1,7)=1.84$, *NS*; 2-Session condition: **Trial Type** $F(1,7)=3.7$, *NS*). Preferential approach behaviour in response to the CS+ did, however, develop in the positive CS-US contingency condition following 10-Sessions of Pavlovian training (10-Session condition: **Trial Type** $F(1,7)=141.75$, $P<0.001$). Thus, approach behaviour recorded during presentations of the CS+ by animals subjected previously to a positive CS-US contingency increased across training sessions, while by contrast approach following CR- leverpresses declined (**Training Sessions** x **Trial Type** interaction ($F(2,21)=24.54$, $P<0.001$; Overall analysis with all groups included: **CS-US Contingency** x **Training Sessions** x **Trial Type** interaction $F(2,42)=8.81$, $P<0.001$; **CS-US Contingency** x **Training**

Sessions interaction F(2,42)=3.79, P<0.05; **CS-US Contingency** x **Trial Type** interaction F(2,42)=15.14, P<0.001; **CS-US Contingency** F(1,42)=7.85, P<0.05; **Training Sessions** F(2,42)=2.46, P=0.09; **Trial Type** F(1,42)=8.09, P<0.05).

Experiment 1b: Stimulus type evaluation

CS-US Training

The light stimulus clearly failed to support a differential approach response during Session 2 of training when paired with the US (Figure 2: Light as CS+, Means±SEMs alcove approaches per session: Positive CS-US contingency: -0.75 ±0.25; Negative CS-US contingency: -0.50 ± 0.29; Light as CS+, **CS-US Contingency** F(1,3)=0.27, *NS*; Overall analysis: **Stimulus Modality** F(1,6)=1.17, *NS*; **CS-US Contingency** F(1,6)=5.17, *NS*), and while the tone stimulus elicited some preferential alcove approach impact (Tone as CS+, Means±SEMs alcove approaches per session: Positive CS-US contingency: 1.00 ±0.41; Negative CS-US contingency: -1.50 ± 0.65), this was also without statistically significant impact (Tone as CS+, **CS-US Contingency** F(1,3)=8.3, *NS*).

Alcove approach in response to the US (alcove beam breaks, Means±SEMs, Tone as CS+: 8.98±0.30; Light as CS+: 8.64±0.27; **Stimulus Modality** F(1,6)=0.70, *NS*), and general locomotor activity within the experimental chamber were unaffected by the two main training conditions of tone or light as the CS+ (locomotor beam breaks, Means±SEMs: Tone as CS+: 343.50 ± 5.55; Light as CS+: 379.50 ± 30.43; **Stimulus Modality** F(1,6)=0.13, *NS*).

Conditioned Reinforcement

Overall rates of response upon the CR+ lever exceeded those on the CR- lever (**Lever** F(1,6)= 29.98, P<0.05), and patterns of lever responding did not differ markedly with CS+ stimulus type (**Stimulus Modality** F(1,6)=0.45, *NS*;

Stimulus Modality x **Lever** F(1,6)=5.26, *NS*). The light stimulus supported a particularly robust pattern of responding (Leverpresses, Means±SEMs: Light stimulus as CS+: 52.5±9.73, ; Light stimulus as CS-: 20.5±9.17; Tone stimulus as CS+: 35.25±6.97; Tone stimulus as CS-: 16.5±3.52).

Levels of alcove approach with the light stimulus as CS+ were low, and clearly did not differ between CS+ and CS- conditions (Alcove approaches per leverpress, Means±SEMs: CS+: 0.15±0.04; CS-: 0.14±0.0.5, **CS-US Contingency** F(1,3)=0.01, NS). Alcove approaches with the tone as CS+ were also generally low, but less consistent across CS+ and CS- conditions (Alcove approaches per leverpress, Means±SEMs: CS+: 0.5±0.05; CS-: 0.03±0.0.2; **CS-US Contingency** F(1,3)=46.14, $p<0.05$; Overall analysis: **Stimulus Modality** F(1,6)=18.84, $p<0.01$; **CS-US Contingency** F(1,3)=17.89, $p< 0.05$; **Stimulus Modality** x **CS-US Contingency interaction:** F(1,6)=16.78, $p<0.05$). Locomotor activity was relatively comparable between groups (locomotor beam breaks, Means±SEMs: Tone as CS+: 431.00 ± 37.66; Light as CS+: 449.00 ± 32.10; **Stimulus Modality** F(1,6)=0.13, *NS*).

Experiment 2a: Conditioned Reinforcement without Conditioned Approach: Effects of Intra-Amygdala R(+) 7-OH DPAT during CS-US Training

Histology

Infusions were located within the amygdala, and were within ± 0.5mm of the intended coordinates in the rostral-caudal plane (see Figure 3). Damage was limited to the immediate area of the infusions.

CS-US Training

Very little approach was observed during presentations of either the CS+ or CS- stimuli during the two Pavlovian training sessions (Figure 4: see also grey bar comparison on Figure showing typical approach rates in 10-Session group

from Experiment 1a), and any approach behaviour that did emerge was not specific to the CS+ stimulus that previously had been paired with the US (**CS-US Contingency** F(1,13)=0.014, *NS*). There was no difference between the approach behaviour of the R(+) 7-OH-DPAT and Vehicle treatment groups (**Infusion Group** F(1,13)=0.32, *NS*; **Infusion Group** x **CS-US Contingency** F(1,13)=0.78, *NS*). Analysis of approach behaviour during the first compared to the second Pavlovian training session also showed no change (**Session** F(1,13)=3.55, *NS*).

Alcove approaches during US presentations in the final Pavlovian training session were very similar between Vehicle and R(+) 7-OH-DPAT groups (alcove beam breaks, Means±SEMs: Vehicle Group: 5.57±1.41; Drug Group: 4.50±0.60; **Infusion Group** F(1,13)=0.54, *NS*), and locomotor activity within the experimental chamber was similarly comparable (locomotor beam breaks, Means±SEMs: Vehicle Group: 481.86±34.22; Drug Group: 389.75±27.00; **Infusion Group** F(1,13)=4.57, *NS*).

Conditioned Reinforcement

A general preference for responding on the CR+ lever over the CR- lever was observed (Figure 5, Upper Panel; **Lever** F(1,13)=22.64, P<0.001). This tendency was, however, greatly attenuated by intra-amygdala R(+) 7-OH-DPAT administration prior to training sessions (**Infusion Group** F(1,13)=5.21, P<0.05), and this reduction in responding was mainly specific to the CR+ lever (**Infusion Group** x **Lever** F(1,13)=6.46, P<0.05). Indeed, R(+) 7-OH-DPAT significantly attenuated responding on the CR+ lever (**Infusion Group** F(1,13)=6.90, P<0.05) but not the CR- lever (**Infusion Group** F(1,13)= 2.01, *NS*).

Alcove approaches during the CR test were relatively uncommon (Figure 5, Lower Panel), and neither Vehicle nor R(+) 7-OH-DPAT groups exhibited any preference for alcove approach during the CS+ stimulus (**CS-US Contingency** F(1,14)=0.37, *NS*; **Infusion Group** F(1,14)=0.18, *NS*; **Infusion Group** x **CS-US Contingency** F(1,14)=0.03, *NS*). Levels of locomotor activity within the experimental chamber were also very comparable across groups (locomotor beam breaks, Means±SEMs: Vehicle Group, 374.25±37.44; Drug Group, 321.25±25.01; **Infusion Group** F(1,13)=2.51, *NS*).

Experiment 2b: Conditioned Reinforcement without Conditioned Approach: Effects of Intra-Amygdala R(+) 7-OH DPAT during the CR Test Phase

Histology

Infusion sites were safely located within the amygdala and approximately 0.5mm of the planned coordinates in the rostral-caudal plane (Figure 6). Limited damage was centred on the infusion site.

CS-US Training

Figure 7 shows alcove approach data during the first and second Pavlovian training sessions. Although there was some small increase in approach behaviour from Session 1 to Session 2 (**Session** $F(1,21)=5.67$, $P<0.05$), this occurred independently of stimulus contingency (**CS-US Contingency** $F(1,21)=3.21$, NS; Session X **CS-US Contingency** $F(1,21)=3.87$, NS; Session 2 only: **CS-US Contingency** $F(1,21)=0.21$, *NS*), and remained at an extremely low level (compare with Figure 1, 10-Sessions of training). Following Pavlovian training, subjects were allocated either to the R(+) 7-OH-DPAT or the Vehicle Group for the conditioned reinforcement test. These two groups were matched on various aspects of performance during the second Pavlovian session (number of approaches during positive contingency stimulus presentations: **Infusion Group** $F(1,20)=0.012$, *NS*; number of approaches during US presentations, **Infusion Group** $F(1,20)=0.31$, *NS*; locomotor activity, **Infusion Group** $F(1,20)=0.69$, *NS*).

Conditioned Reinforcement

Rates of leverpressing on the CR+ lever were higher than on the CR- lever (Figure 8, Upper Panel; **Lever** $F(1,20)=27.88$, $P<0.001$), but were significantly attenuated following intra-amygdala R(+) 7-OH-DPAT infusions (**Infusion Group** $F(1,20)=19.41$, $P<0.001$). Drug-induced impairments in leverpressing rates tended to predominate on the CR+ lever (**Infusion Group** x **Lever** $F(1,20)=4.19$, $P=0.0539$).

Stimulus-related alcove approach was extremely low during the conditioned reinforcement session (Figure 8, Lower Panel), and remained such following administration of R(+) 7-OH-DPAT (**Infusion Group** $F(1,20)=0.58$, NS; **CS-US Contingency** $F(1,20)=0.24$, NS; **Infusion Group** X **CS-US Contingency** $F(1,20)=0.15$, NS). Reduced locomotor activity within the experimental chamber (locomotor beam breaks, Means±SEMs: Vehicle Group: 395.64±20.35; Drug Group: 256.0±20.29; **Infusion Group** $F(1,20)=23.61$, $P<0.001$) occurred to a significantly lesser degree than that for leverpressing (Proportional reductions in behavioural rates following R(+) 7-OH-DPAT administration: Leverpressing -79.19%; Locomotor activity -35.19%). Indeed, it could be argued that locomotor activity actually increased relative to changes in lever-related behaviour (Locomotor beam breaks per leverpress, Means±SEMs: Vehicle Group, 18.68±1.28; Drug Group, 31.16±5.59; **Infusion Group** $F(1,20)=5.1$, $P<0.05$). Consistent with these findings, an analysis of beam breaks on the opposite side of the experimental chamber to the levers and alcove, which would be relatively uncontaminated by drug effects on stimulus-related behaviours unambiguously demonstrated a lack of direct effect of R(+) 7-OH-DPAT on locomotor activity (Beam breaks on left side of chamber, Means±SEMs: Vehicle Group, 127.91±12.83; Drug Group, 101.55±9.24; **Infusion Group** $F(1,20)=2.8$, NS).

Discussion

To further identify the point at which mesoamygdaloid dopamine might first show influence over associative learning, a variant of the conditioned reinforcement procedure was developed which restricted the degree of training so that statistically significant levels of selective conditioned approach to the location of the US were not observed. Experiments 1a and 1b demonstrated that despite the extreme curtailment of initial training, the ability of an initially arbitrary light stimulus subsequently to support a consistent and selective leverpressing response remained entirely dependent on prior stimulus presentations being temporally contiguous with those of a US: an equal number of separate presentations of the light stimulus and the US generally did not support a selective leverpressing response. In Experiment 2a, R(+) 7-OH-DPAT infusions prior to training sessions greatly diminished the ability of the stimulus subsequently to support a selective leverpressing response, and in Experiment 2b, R(+) 7-OH-DPAT infusions immediately prior to the conditioned reinforcement test also resulted in a profound reduction in leverpressing. Taken together, these data perhaps implicate mesoamygdaloid dopamine in the very earliest phase of associative learning, commonly termed 'emotional' learning (*e.g.* Konorski 1967; Lennartz and Weinberger 1992; Wagner 2008; Wagner and Brandon 1989) .

Dopaminergic innervation of the amygdaloid complex demonstrates both neuroanatomical and functional specificity. First, while post-session infusions of dopamine receptor agonists within the central nucleus of amygdala enhanced the acquisition of a Pavlovian conditioned approach response, the same treatment within the basolateral area of the amygdala was without effect (Hitchcott and Phillips 1998a; see also Harmer et al. 1997; Hitchcott et al. 1997a; Hitchcott and Phillips 1998b). By contrast, manipulations of dopamine function within the basolateral area potently impaired the acquisition of a conditioned instrumental response, while the same pharmacological treatment of the central nucleus left such instrumental behaviour relatively intact (Hitchcott and Phillips 1998a; see also Blundell et al. 2001; Blundell et al. 2003; Parkinson et al. 2000). Second, the involvement of the mesoamygdaloid dopamine pathway in the acquisition of appetitive associative learning appears to be functionally quite specific. For example, dopamine overflow increased during trials in which an arbitrary

stimulus (light or tone) was repeatedly paired with an unconditioned stimulus (US), but remained quiescent throughout trials in which the stimulus and US were presented with equal frequency, but separately (Harmer and Phillips 1999). Similarly, immunohistochemical analysis of dopaminergic activity within midbrain and forebrain regions again showed that mere exposure to an arbitrary stimulus and a US was insufficient to lead to a response: temporal contiguity between a potential CS and a US was necessary to activate mesocorticolimbic dopamine (Phillips et al. 2003a; b). Finally, intra-amygdaloid dopaminergic manipulations were effective in enhancing the acquisition of conditioned approach only when the stimulus was presented in close temporal proximity to a US, but otherwise were entirely without effect (*e.g.* Hitchcott et al. 1997a; Hitchcott and Phillips 1998a). In short, activation of mesoamygdaloid dopamine appears to be under a quite exquisite degree of control by current motivational circumstances.

However, dopaminergic activity within the amygdala appears to influence associative learning only indirectly. Thus, while post-session intra-amygdala R(+) 7-OH-DPAT infusions enhanced the acquisition of conditioned approach (Hitchcott et al. 1997a; Hitchcott and Phillips 1998a; b), pre-session infusions robustly blocked the acquisition of the self-same conditioned response (Phillips and Hitchcott 2009). Recent learning is relatively labile (McGaugh 2000), and may be vulnerable to a number of influences, including the introduction of a subsequent learning experience (Müller and Pilzecker 1900). Conversely, otherwise amnestic drugs such as alcohol or benzodiazepines *improve* memory for items presented before the administration of these drugs (Hinrichs et al. 1984; Mueller et al. 1983; Parker et al. 1980). The mesoamygdaloid dopamine projection appears well suited to play a key role in such retroactive modulation of recent learning. For example, dopamine released within the amygdala has a relatively long extracellular half-life (Jones et al. 1995), which may correspond with a notable mismatch between the highest concentrations of amygdala dopamine terminals, and dopamine receptors (Asan 1998; Freedman and Cassell 1994). These findings lend support to a volume transmission view of amygdala dopamine function - a mode of action sometimes lasting minutes rather than milliseconds (Zoli et al. 1998). The high affinity of the D3 dopamine receptor for dopamine (Sokoloff et al. 1992) and its localisation in key regions of the

amygdala (Scibilia et al. 1992) including the central nucleus (Murray et al. 1994), together with behavioural outcomes from pharmacological manipulations linked with the D3 receptor (Hitchcott et al. 1997a; Phillips et al. 2002a; Phillips et al. 2002b) make it an ideal candidate for this form of neural communication. In short, two events, such as a CS and a US, will become associated only to the extent that it is possible concurrently to process their representations during the immediate post-training period (Wagner 1978), and the mesoamygdaloid dopamine system seems particularly well constructed to govern this process.

However, prior findings depended on the observation of a conditioned skeletomotor action; a behaviour far removed from the initial formation of a CS-US association (Mintz and Wang-Ninio 2001). Three lines of evidence suggested possible a relatively early involvement. First, dopaminergic manipulations of the amygdala affect neuronal responsivity following a very few pairings of a stimulus and a US (Rosenkranz and Grace 2002a; see also Grace and Rosenkranz 2002; Greba et al. 2001; Rosenkranz and Grace 2002b). Second, immunohistochemical visualisation of dopamine activity within the central nucleus showed most activity during early and intermediate stages of associative learning (Phillips et al. 2003a; b). Third, Figure 1 of Hitchcott and Phillips (1997b) indicates that post-session intra-amygdala R(+) 7-OH-DPAT enhanced subsequent conditioning during the second session only: later infusions were without differential impact. Comparable effects confined to the very earliest stages of training are evident in a number of related studies (see Figure 2 in each case: Hitchcott et al. 1997a; Hitchcott and Phillips 1998a; Phillips and Morutto 1998).

A novel variant of the CR procedure was therefore developed, in which initial training was restricted so that statistically significant levels of selective conditioned alcove approach (the most typical conditioned response measured) failed to consistently develop. Despite the very limited number of trials (eight in total), Experiments 1a and 1b demonstrated that a preference for the active lever in a subsequent CR test was entirely dependent on the stimulus being paired previously with a US: an equal number of presentations of the stimulus not in conjunction with the US were generally ineffective. These data echo those of Taylor and Robbins (1984), who observed that the ability of an initially arbitrary stimulus subsequently to support an instrumental leverpressing response was entirely dependent upon a prior positive correlation between an initially arbitrary

stimulus and a US, rather than negative or random correlations. Contemporary studies of this phenomenon have invariably employed an initial training phase in which extensive CS-US training is given, and many hundreds of trials might be presented. For example, Hitchcott *et al.* (1997b) provided 300 pairings before moving on to the CR test phase, and Taylor and Robbins (1984) presented over 500 such trials. The implicit rationale would appear to be that extended Pavlovian training maximises the ability of the CS to function as a conditioned reinforcer. For this to follow it is necessary to assume that the form of learning underlying performance of the Pavlovian CR is the same or equivalent to that underlying conditioned reinforcement. However, it is far from clear that this is the case. For example, Pavlovian responses are far more responsive to US devaluation preparations than conditioned instrumental behaviours (Burke et al. 2007; Holland 1998; Parkinson et al. 2005).

The relative sensitivity of the Pavlovian response to devaluation likely denotes reliance upon a detailed representation of the US (Colwill and Motzkin 1994; Holland 1998). By contrast, reported failures of US devaluation procedures to affect responding maintained by conditioned reinforcement suggests that a basic prerequisite for the emergence of this behaviour is the ability of the CS to elicit a more general state of motivational or affective arousal (Burke et al. 2007; Ostlund et al. 2009; Parkinson et al. 2005). The latter is significant inasmuch as previous research has demonstrated that this aspect of learning is relatively rapidly acquired (Lennartz and Weinberger 1992; Powell 1994). Thus, it has long been known that CRs which index a general state of preparedness of the organism, including various autonomic responses and central motive states, appear early in the course of conditioning, often during the first few trials. Specific behavioural CRs which promote adaptation appropriate to the US only appear much later (Konorski 1967).

Thus, minimal initial Pavlovian training in the present study failed to establish reliable conditioned approach behaviour, and yet the same stimulus supported the acquisition of a novel instrumental response. Presumably, a mere eight pairings of stimulus and US were sufficient to engender a state of general affective arousal, elicited initially by the US (30% sucrose solution), and to condition to the CS. Effects of intra-amygdala infusions of R(+) 7-OH-DPAT are at least consistent with this interpretation. Considerable evidence implicates the

amygdala, and in particular the central nucleus, in some forms of affective arousal (*e.g.* Lang and Davis 2006; see also McDannald et al. 2004), and dopaminergic manipulations within the central nucleus, but not basolateral area of the amygdala clearly impact associative learning (Hitchcott and Phillips 1998a). In the present study, infusions of R(+) 7-OH-DPAT prior to Pavlovian training (Experiment 2a) or the CR test (Experiment 2b) reduced the ability of the stimulus to support instrumental responding, presumably by suppressing the ability of the stimulus to acquire or support an affectively arousing, or 'emotional' response..

However, R(+) 7-OH-DPAT infusions immediately prior to the CR test (Experiment 2b) reduced responding to some degree on both levers. While these results resemble those of a motoric character, particularly given that an overall reduction in locomotor activity was also observed, at least four lines of evidence suggest that reduced responding more likely was due to a relative flattening of the arousal response to presentations of the conditioned stimulus. First, a borderline drug x lever interaction in reduced responding was in fact also noted. Second, direct effects of amygdala dopamine manipulations on locomotor activity are not a characteristic feature of this manipulation (*e.g.* Hitchcott and Phillips 1998a; Hitchcott and Phillips 1998b; c; Phillips et al. 2002a; Phillips et al. 2002b). Effects of amygdala dopamine manipulations on even conditioned behaviours have only been noted under conditions of novelty (Hitchcott and Phillips 1998c), or when new learning is required (Phillips and Hitchcott 2009). Third, direct effects on overall locomotor activity were actually less than half those on leverpressing. Fourth, a more specific analysis confined to the side of the chambers opposite to levers showed no significant effects whatever of R(+) 7-OH-DPAT on locomotor activity. Drug effects on CR behaviour were not likely then the outcome of some species of motoric disadvantage, but reflect more consistently an interaction with emotional processes considered above.

To conclude, the present work supported the view that amygdala dopamine may be actively involved in the earliest, 'emotional' phase of learning, using a novel version of the CR procedure in which initial training was restricted to preclude the development of statistically significant levels of selective conditioned approach to the location of the US. The precise roles for other brain dopamine systems in later learning remain to be fully established.

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Figure Legends

Figure 1: Effects of degree of prior training on the subsequent performance of a conditioned reinforcement task (**Upper Panel:** leverpresses; **Lower Panel:** alcove approaches). **S**ubjects previously received 1, 2 or 10 training sessions in which a light stimulus and unconditioned stimulus (sucrose reward) were presented, either together (**Solid lines**: CS+ group) or separately (**Dashed lines**: CS- group). There were 4 trials per session. **Filled circles:** behaviour associated with the instrumental delivery of light stimulus presentations; **Open circles:** behaviour associated with a control lever. The unconditioned stimulus available during training was not available at any time during the conditioned reinforcement session. Values are means per session ± 1SEM. Stars above SEMs or adjacent to circles indicate statistically significant comparisons with the associated CS- condition; other comparisons are as indicated by appropriate lines, **P*<0.05, ***P*<0.01, ****P*<0.001.

Figure 2: Alcove approach behaviour in response to stimulus presentations either paired or unpaired with an unconditioned stimulus (US: sucrose solution in alcove). Data are from Experiment 1b, and shown is the second of two Pavlovian training sessions, 4 trials per session. Conditioned approach behaviour in response to stimulus presentations either paired or unpaired with sucrose reward. Data taken from second of two Pavlovian training sessions, 4 trials per session. **Stimulus Modality: CS+ Tone:** CS+ = tone, CS- = light; **CS+ Light:** CS+ = light, CS- = tone. **Filled bars:** mean approaches per session into an alcove in response to the CS+ stimulus paired with sucrose reward; **Open bars:** mean approaches per session into an alcove in response to the CS- control stimulus unpaired with sucrose reward. **Greyed items:** comparison with conditioned approach behaviour following 10 sessions of training, 4 trials per session (see Experiment 1a). Values are mean approaches into the alcove during the session, ± 1SEM.

Figure 3: Representative locations of infusion sites within the amygdala for Experiment 2a. Plates show coronal sections through the rat brain, and are based upon the atlas of Paxinos and Watson (1986). Numbers adjacent to each section represent distances from Bregma (mm) in the anterior-posterior plane. Infusion sites shown as filled circles.

Figure 4: Alcove approach behaviour in response to stimulus presentations either paired or unpaired with an unconditioned stimulus (US: sucrose solution in alcove). Data are from Experiment 2a, and shown is the second of two Pavlovian training sessions, 4 trials per session. Bilateral infusions of **Vehicle** (1µl/side) or **R(+) 7-OH-DPAT** (10nmols/1µl/side) were made into the amygdala immediately before each training session. **Filled bars:** mean approaches per session into an alcove in response to a stimulus paired with the US; **Open bars:** mean approaches per session into an alcove in response to a stimulus unpaired with the US. **Greyed items:** comparison

with conditioned approach behaviour following 10 sessions of training, 4 trials per session (see Experiment 1a). Values are mean approaches into the alcove during the session, ± 1SEM.

Figure 5: Effects of bilateral R(+) 7-OH-DPAT infusions into amygdala prior to two Pavlovian training sessions on the subsequent performance of a conditioned reinforcement task (**Upper Panel:** leverpresses; **Lower Panel:** alcove approaches per leverpress). **Vehicle Group:** bilateral infusions of vehicle solution (1µl/side); **Drug Group:** bilateral infusions of R(+) 7-OH-DPAT (1nmols/1µl/side). **Filled bars:** behaviour associated with the instrumental delivery of the CS+; **Open bars:** behaviour associated with the instrumental delivery of the CS-. The unconditioned stimulus available during training was not available at any time during the conditioned reinforcement session. Values are means per session, ± 1SEM. Stars indicate statistically significant comparisons with the associated unpaired performance, **P*<0.05, ***P*<0.01.

Figure 6: Representative locations of infusion sites within the amygdala for Experiment 2b. Plates show coronal sections through the rat brain, and are based upon the atlas of Paxinos and Watson (1986). Numbers adjacent to each section represent distances from Bregma (mm) in the anterior-posterior plane. Infusion sites shown as filled circles.

Figure 7: Alcove approach behaviour in response to stimulus presentations either paired or unpaired with an unconditioned stimulus (US: sucrose solution in alcove). Data are from Experiment 2b, and shown are the first and second of two Pavlovian training sessions, 4 trials per session. **Filled bars:** mean approaches per session towards an alcove in response to a stimulus paired with the US; **Open bars:** mean approaches per session towards an alcove in response to a stimulus unpaired with the US. **Greyed items:** comparison with conditioned approach behaviour following 10 sessions of training, 4 trials per session (see Experiment 1a). Values are mean approaches into the alcove during the session, ± 1SEM.

Figure 8: Effects of bilateral R(+) 7-OH-DPAT infusions into amygdala on the performance of a conditioned reinforcement task (**Upper Panel:** leverpresses; **Lower Panel:** alcove approaches per leverpress). Infusions were made immediately prior to the session start. **Vehicle Group:** bilateral infusions of vehicle solution (1µl/side); **Drug Group:** bilateral infusions of R(+) 7-OH-DPAT (1nmols/1µl/side). **Filled bars:** behaviour associated with the instrumental delivery of the CS+; **Open bars:** behaviour associated with the instrumental delivery of the CS-. The unconditioned stimulus available during training was not available at any time during the conditioned reinforcement session. Values are means per session, ± 1SEM. Stars indicate statistically significant comparisons with the associated unpaired performance, ***P*<0.01; and filled circles with associated vehicle performance, • *P*<0.01.