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Interoceptive Pavlovian conditioning with nicotine as the conditional stimulus varies as a function of the number of conditioning trials and unpaired sucrose deliveries

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Interoceptive Pavlovian conditioning with nicotine as the conditional stimulus varies as a function of the number of conditioning trials and unpaired sucrose deliveries

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In rats, the pharmacological (interoceptive) effects of nicotine can serve as a signal (conditional stimulus) in a Pavlovian (classical) conditioning task. In this task, nicotine administration (0.4 mg base/kg, subcutaneous) is typically paired with intermittent access to a liquid sucrose unconditional stimulus; sucrose is withheld on saline sessions. An increase in sucrose receptacle entries (goal tracking) on nicotine sessions indicates conditioning. Given our limited understanding of the functional relationships controlling conditioned responding to a nicotine conditional stimulus, the present research examined nicotine's sensitivity to several manipulations shown to affect the conditioned responding in more widely studied Pavlovian conditioning tasks that use exteroceptive conditional stimuli: number of nicotine conditional stimulus–sucrose unconditional stimulus pairings per session (0, 3, 9, 18, or 36) and the impact of sucrose deliveries in saline sessions. Differential goal tracking developed in fewer sessions and asymptotic conditioned responding magnitude was greater with more nicotine–sucrose pairings. Further, goal tracking was more resistant to extinction (unconditional stimulus withheld) with more conditional–unconditional stimulus pairings during the acquisition phase. The discrimination was not acquired when sucrose presentations (9 or 18) also occurred during saline sessions. Furthermore, expression of the discrimination was disrupted when sucrose was presented in saline sessions; this disruption resulted from goal tracking in saline sessions. These results are consistent with the notion that nicotine-evoked goal tracking results from interoceptive conditioning processes. *Behavioural Pharmacology* **17:161–172 © 2006 Lippincott Williams & Wilkins.**

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All MED-PC programs used in the present article are available on request.

Introduction

A long history of studying the role of interoceptive cues as conditional stimuli (CSs) exists in the Pavlovian conditioning field. Much of the early research was interested in stimulation of the viscera such as the stomach or intestine (Bykov, 1957) or in electrical brain stimulation (Doty, 1961) as the CSs. A particularly relevant example from an appetitive conditioning point of view was reported in Chapter 13 of Bykov's book *The Cerebral Cortex and the Internal Organs*. A dog was surgically prepared so that water could flow in and then out of the stomach (i.e. the interoceptive CS). Importantly, presentation of this CS alone produced very little if any salivation – the primary conditioned response (CR) of interest. This irrigation of the stomach was paired with access to meat powder and bread (i.e. the unconditioned stimulus or US). As described by Bykov (1957), 'After several such combinations we found that if water was allowed to flow into the stomach 20 seconds in advance of the reinforcement, the irrigation alone caused the dog to start licking its lips and turning its head to the food box while there was a copious salivary secretion' (p. 249).

The study of interoceptive cues was later extended to the peripheral administration of ligands. For example, in a conditioned avoidance experiment with dogs, Cook *et al.* (1960) implanted a catheter into the saphenous vein of one hind leg. Infusion of acetylcholine into the catheter served as the interoceptive CS. The US was electric-shock delivered to the opposite (i.e. left) leg. After repeated pairings of the acetylcholine CS with the leg shock US, the dog began to withdraw the left leg within 30 s of CS infusion, but before the US onset. Thus, the right leg infusion of acetylcholine had sufficient stimulus properties to serve as an effective CS that acquired the ability to evoke an avoidance CR in the leg opposite the infusion.

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Of particular interest to us is the extension of this research to the pharmacological effects of abused drugs. This type of research can be categorized into two major classes. One class, drug–drug conditioning, has a drug serve as the CS and the US. A recent example of drug–drug conditioning comes from Shepard Siegel's laboratory. In this research, Siegel and colleagues (e.g. Kim *et al.*, 1999; Sokolowska *et al.*, 2002) investigated the ability of the early pharmacological effects of morphine (early onset cues) to serve as a CS for its later, more profound, analgesic effects in rats [see Greeley *et al.* (1984) for similar research with ethanol]. Other drug–drug conditioning research has used one drug as the CS for the subsequent delivery of a different drug. For instance, Revusky *et al.* (1989) found that a pentobarbital CS paired repeatedly with an amphetamine US in rats came to control an increase in heart rate relative to controls. The second major class, drug–non-drug US conditioning, has a drug that serves as a CS for delivery of a nonpharmacological US. Bormann and Overton (1993) (see also Overton *et al.*, 1993), in a well controlled conditioned suppression experiment with rats, repeatedly paired an intraperitoneal injection of morphine with a foot-shock US. Relative to six other control groups, the morphine CS came to evoke a conditioned fear response as measured by drink suppression. Turner and Altshuler (1976) reported a similar conditioned suppression result in rats using amphetamine as the CS and a decrease in lever pressing as the measure of conditioned fear.

The present research focused on the ability of the pharmacological effects of nicotine to serve as an interoceptive CS for a non-drug appetitive US. We recently developed a Pavlovian appetitive conditioning task to study nicotine as a CS (Besheer *et al.*, 2004; Bevins and Palmatier, 2004). In this task, rats received the nicotine CS (0.4 mg base/kg, subcutaneous) paired with intermittent access to eight liquid sucrose deliveries (i.e. the US) across a 20-min session. Intermixed with these nicotine sessions were saline sessions in which rats were injected with saline, placed into the same conditioning chambers, but with sucrose withheld. Relative to saline (no drug), nicotine came to control a differential approach to the dipper receptacle. This anticipatory food-seeking behavior, hereafter referred to as 'goal tracking' (Boakes, 1977; Farwell and Ayres, 1979), is a widely used measure of Pavlovian conditioning (e.g. Delamater, 1995; Rescorla, 1999; Bouton and Sunsay, 2003). Besheer *et al.* (2004) established that the CS effects of nicotine were blocked by the central and peripheral nicotinic acetylcholine receptor antagonist mecamylamine, but not the peripheral antagonist hexamethonium, suggesting a role of central nervous system receptors. Additionally, the goal-tracking CR decreased with a decrease in the dose of nicotine (CS salience) and with an increase in the injection-to-testing interval up to 100 min. The nicotine-evoked CR also decreased with repeated presentation of the nicotine CS without the sucrose US (i.e. extinction). Finally, Bevins and Palmatier (2004) found, using a fading-dose procedure, that a nicotine dose as low as 0.1 mg/kg could serve as a CS.

As this brief summary demonstrates, knowledge of nicotine's ability to serve as a CS is limited. Accordingly, the goal of the present research was to further our understanding of the CS effects of nicotine by examining its sensitivity to several behavioral manipulations known to affect conditioned responding in more widely studied Pavlovian conditioning tasks with exteroceptive CSs: number of conditioning trials and ratio of CS–US pairings (i.e. unpaired sucrose deliveries). In brief, the magnitude of conditioned responding, within limits, tends to increase with the number of conditioning trials (e.g. Kalish, 1954). Further, an increased number of pairings results in a CR that tends to be more resistant to extinction (i.e. more extinction sessions to reduce CR to control levels; Brabant *et al.*, 2005). To examine these effects, we exposed rats to 36, 18, 9, 3, or 0 sucrose deliveries during each nicotine session. Note that in this experiment sucrose is never delivered in saline sessions. Differential conditioned responding in Pavlovian conditioning tasks, however, is sensitive to the relative number of nontarget CS–US pairings (e.g. Rescorla, 1968; Singh and Banerji, 1986; Murphy and Baker, 2004). Accordingly, we also assessed the importance of this factor by providing different sets of rats with a 36:0, 27:9, or 18:18 ratio of nicotine:saline sucrose pairings at the onset of discrimination training (acquisition) or after acquisition of the discrimination (expression).

Methods

Subjects

Sixty-four male Sprague–Dawley rats obtained from Harlan (Indianapolis, Indiana, USA) were housed individually in clear polycarbonate tubs lined with wood shavings. In the home cage, water was freely available. Food access was restricted such that each rat was maintained at 85% of its normal free-feeding body weight (293 ± 24 g). Each month, the 85% target weight was increased by 2 g. The colony was temperature and humidity controlled and all experimental sessions were conducted during the light portion of a 12-h light:dark cycle. Protocols were approved by the University of Nebraska-Lincoln Institutional Animal Care and Use Committee and followed the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996).

Apparatus

Eight conditioning chambers (ENV-008CT; Med Associates Inc., Georgia, Vermont, USA) were used in these studies. The chambers measured 30.5 × 24.1 × 21 cm (length × width × height), the side walls were aluminum

and the ceiling and front and back walls were made of clear polycarbonate. One aluminum side of each chamber had a recessed liquid dipper well (5.2 × 5.2 × 3.8 cm; length × width × depth). The dipper arm contained a 0.1-ml cup that allowed access to sucrose (26% w/v) in the receptacle when the arm was raised. An infrared emitter/detector unit, 1.2 cm within the receptacle and 3 cm from the floor of the chamber, monitored head entries. Each chamber was enclosed in a sound and light-attenuating polyvinyl chloride cubicle fitted with a fan that provided airflow and masked noise. A personal computer with Med Associates interface and software (Med-PC for Windows, version IV) controlled sucrose deliveries and recorded dipper entries throughout each session.

Procedures: Number of conditional-unconditional stimulus pairings

Discrimination training

For 3 days before the start of discrimination training, all rats were injected subcutaneously with nicotine (0.4 mg/kg) in the home cage to reduce the initial locomotor suppressant effects of nicotine (cf. Bevins and Palmatier, 2003). Rats were randomly assigned to group 0:0, 3:0, 9:0, 18:0, or 36:0 ($n = 8$ per group). The first number refers to the number of sucrose deliveries in each nicotine session; the second number refers to the number of sucrose deliveries in the saline sessions. Discrimination training was conducted from Monday to Friday. Sessions were constructed such that the rats received four nicotine and four saline sessions in random order, with the restriction that no more than two of one session type occurred in a row. On nicotine sessions, rats received a subcutaneous injection of nicotine 5 min before placement in the conditioning chambers for 20 min. Sucrose was available for 4 s, the number of times designated by group assignment (e.g. group 36:0 received 36 sucrose presentations, whereas group 0:0 had no sucrose presentations). To prevent timing of sucrose deliveries, the four computer programs for each group controlling nicotine sessions presented sucrose at different times (see Table 1). On saline sessions, rats were injected subcutaneously with saline 5 min before placement. The 20-min session was identical except that no sucrose was available for any of the groups. Discrimination training lasted for 32 nicotine and 32 saline sessions.

Table 1 Session details for groups in the number of conditional-unconditional stimulus pairings experiment

Group	Mean second to first S*	Range (s)	Mean second between S*	Range (s)
36:0	137	124–152	25	4–80
18:0	126	120–132	58	4–100
9:0	174	132–220	120	12–224
3:0	262	216–340	291	268–676
0:0	No S*; unable to calculate			

Group name denotes the nicotine:saline sucrose ratio during training. S*, sucrose delivery (unconditional stimulus).

Extinction

Extinction began the day following the end of discrimination training. Rats were injected subcutaneously with nicotine 5 min before placement in the conditioning chamber for 20 min; no sucrose was available. A total of 24 sessions were conducted from Monday to Friday.

Procedures: Ratio of conditional-unconditional stimulus pairings (expression)

Retraining discrimination

Following extinction, rats from the number of CS–US pairings experiment just described were retrained using the same intermixed saline and nicotine session discrimination protocol as group 36:0. Given that the goal of this experiment was to determine the impact of sucrose deliveries during saline sessions on discrimination performance, only rats that displayed discrimination performance within 10 nicotine and 10 saline sessions continued to the next phase. Thirty rats had more dipper entries in nicotine sessions than in saline sessions by 20 retraining sessions: five from group 3:0, seven rats from group 9:0, and six rats from each of the remaining groups (i.e. 0:0, 18:0, and 36:0).

Ratio shift

Before the shift in the ratio of nicotine:saline session sucrose deliveries, rats were randomly assigned to group 36:0, 27:9, or 18:18 ($n = 10$) with the restriction that reacquisition did not differ statistically among groups. Rats in group 36:0 continued to receive all sucrose deliveries on nicotine sessions and served as a benchmark for unchanged discrimination training. Rats in group 27:9 received 27 sucrose presentations on nicotine sessions and 9 sucrose presentations on saline sessions. Rats in the 18:18 group received 18 sucrose presentations on nicotine sessions and 18 presentations on saline sessions. Sessions were identical to the training phase except for the number of sucrose deliveries in a session.

Procedures: Ratio of conditional-unconditional stimulus pairings (acquisition)

Discrimination training

For 3 days before the start of discrimination training, naïve rats were injected subcutaneously with nicotine (0.4 mg/kg) in the home cage. Rats were randomly assigned to group 36:0, 27:9, or 18:18 ($n = 8$ per group). Discrimination training commenced with these ratios and was conducted as described in the ratio shift phase of the expression experiment (see previous paragraph).

Dependent measures

The primary dependent measure was the number of dipper entries per second before the first sucrose delivery. A per second measure was necessary because time to first sucrose delivery varied across sessions and groups (see Table 1). Dipper entries before the first sucrose delivery

were used to avoid including dipper entries induced by sucrose in any measure of conditioning. For saline sessions in which no sucrose was delivered, intervals comparable to nicotine sessions for that group were used to equate the time from which dipper entries were derived. Sucrose was never available for the rats in group 0:0. Thus, the eight rats in this group were randomly split into pairs and assigned to have the 'intervals' of one of the other four groups (3:0, 9:0, 18:0, or 36:0). This procedural maneuver ensured that all intervals were represented in group 0:0. For discrimination training/retraining phases of each experiment, we also computed a difference score for each rat on each session. The difference score formula was dipper entry rate before first sucrose delivery on nicotine sessions minus dipper entry rate on a comparable saline session. A value of 0 indicates no discrimination, whereas a positive value indicates development of the Pavlovian drug discrimination. Finally, the use of a difference score provides a measure of conditioning that is adjusted for any shift in goal tracking over time because the saline and nicotine sessions are matched for duration within each group.

Drugs

(–)-Nicotine hydrogen tartrate (Sigma, St Louis, Missouri, USA) was dissolved in 0.9% saline and adjusted to a pH of 7.0 ± 0.2 using a dilute NaOH solution. Nicotine (0.4 mg base/ml) was injected subcutaneously at a volume of 1 ml/kg in all experiments.

Data analyses

For discrimination training/retraining, two-way analyses of variance (ANOVAs) were used to analyze difference scores. A significant group × session interaction on this measure prompted two further sets of analyses. (1) Post-hoc *t*-tests assessed whether a given session was different from a hypothetical 0. Consistent difference scores significantly above 0 indicate acquisition of the Pavlovian drug discrimination. (2) Follow-up two-way ANOVAs on the dipper entry data on saline versus nicotine sessions for each group were also conducted. A significant drug × session interaction prompted pairwise comparisons for each session using Fisher's least significant difference (LSD) tests that control for type I error rate. Other analyses (extinction data, etc.) also used omnibus ANOVAs with post-hoc Fisher's tests prompted by a significant interaction. Statistical significance was declared using a two-tailed rejection region of 0.05 for all tests.

Results

Number of conditional–unconditional stimulus pairings

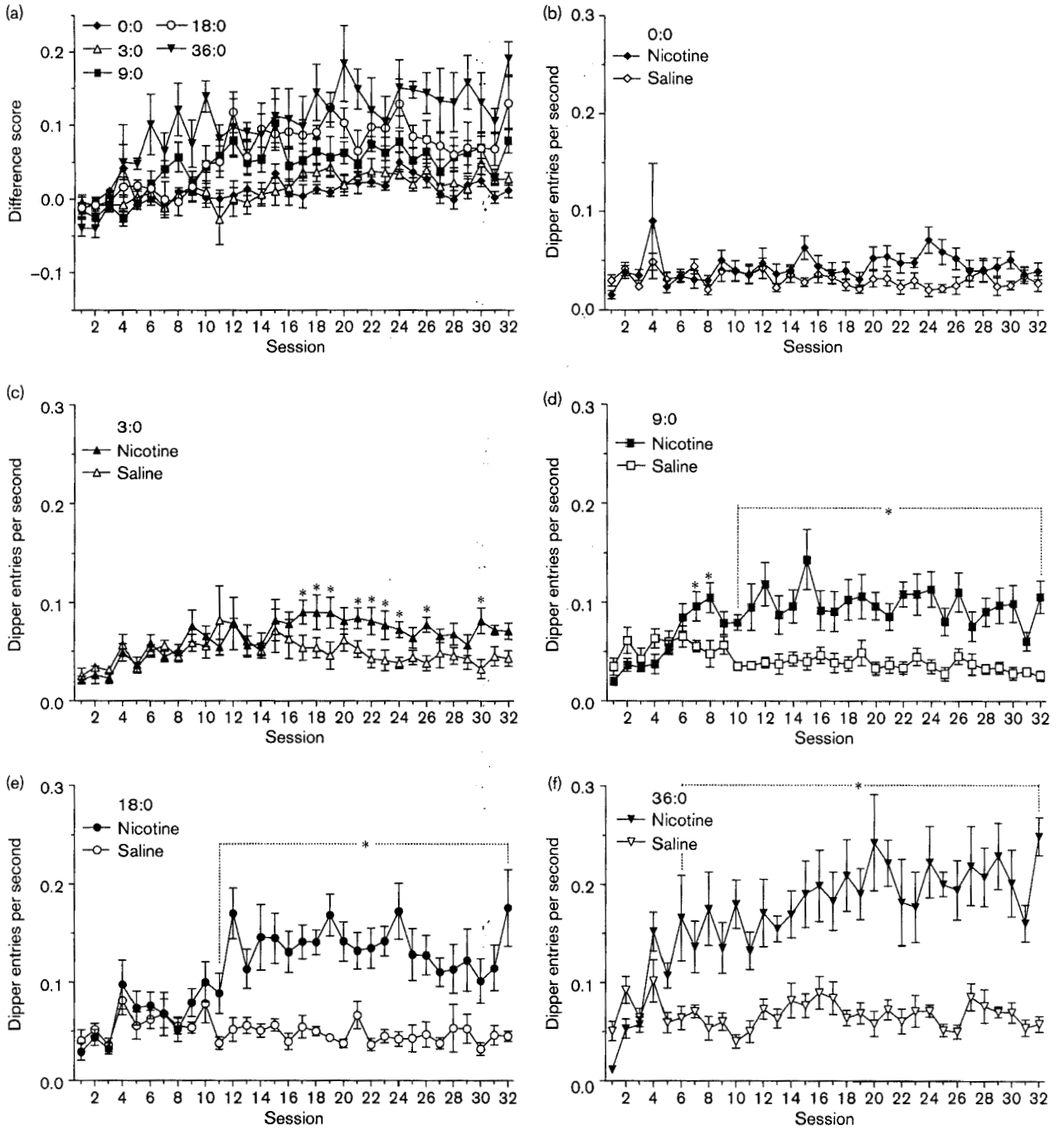
Discrimination training

Figure 1a shows the difference scores for the acquisition phase. The two-way mixed groups ANOVA revealed significant main effects of session [$F(31,1085) = 10.11$, $P < 0.001$] and group [$F(4,35) = 19.20$, $P < 0.001$], and a significant session × group interaction [$F(124,1085) = 1.69$, $P < 0.001$]. The significant interaction suggests that the discrimination was acquired differently across groups. To assess this possibility, further analyses using one-sample *t*-tests compared the difference scores of each session for each group with a hypothetical 0. The following sessions had difference scores that were significantly different from 0: group 0:0 (3, 15, 22–26, and 30) [$t(7) \geq 2.41$, $P \leq 0.05$], group 3:0 (17–19, 21, 24, 26, and 30–32) [$t(7) \geq 2.90$, $P \leq 0.025$], group 9:0 (4, 7, 8, 10–15, 18, 20–26, and 28–32) [$t(7) \geq 2.49$, $P \leq 0.05$], group 18:0 (5, 12–28, and 30–32) [$t(7) \geq 2.37$, $P \leq 0.05$], and group 36:0 (1, 2, 5–8, and 10–32) [$t(7) \geq 2.42$, $P \leq 0.05$].

To explore further the effect of the number of US pairings on acquisition and maintenance of the discrimination, the dipper entry rates for nicotine and saline sessions for each group are shown in Fig. 1b–f. For group 0:0, there was a significant main effect of drug [$F(1,7) = 12.712$, $P < 0.01$], but no significant effect of session or drug × session interaction [$F \leq 1.34$, NS] indicating a tendency for dipper entries to be slightly elevated on nicotine sessions throughout discrimination training. For the remaining groups, there were significant main effects of drug [$F(1,7) \geq 11.373$, $P \leq 0.02$] and session [$F(31,217) \geq 2.654$, $Ps < 0.001$], and a significant drug × session interaction [$F(31,217) \geq 1.89$, $P \leq 0.005$, mean square error (MSE) ≤ 0.03]. To determine when the discrimination was acquired/stabilized, follow-up analyses using Fisher's LSD tests compared dipper entry rates on corresponding nicotine and saline sessions. For group 3:0, dipper entry rates were elevated on nicotine sessions compared with saline on sessions 17–19, 21–24, 26, and 30 ($LSD_{mmd} = 0.031$). For group 9:0, dipper entry rates were higher on nicotine sessions 7, 8, and 10–32 ($LSD_{mmd} = 0.031$). For group 18:0, dipper entry rates were higher on nicotine sessions 11–32 ($LSD_{mmd} = 0.044$). For group 36:0, dipper entry rates were higher on nicotine sessions 6–32 ($LSD_{mmd} = 0.054$).

The previous analyses indicate that the CR was acquired in fewer sessions as the number of pairings of sucrose deliveries per nicotine session increased. It is of interest to determine how the number of nicotine–sucrose pairings affected acquisition of the CR independent of session number. Figure 2a shows the difference scores after every 36 sucrose deliveries for groups 36:0, 18:0, and 9:0 (i.e. groups that displayed reliable discrimination performance). We used 36 because it reflects the lowest number, in which a measure of dipper entries could be derived for all groups, that was not potentially influenced by sucrose deliveries in the session. This maneuver allowed statistical comparison among the three groups for values up to 288 deliveries. Although there was a significant main effect of number [$F(8,168) = 15.97$,

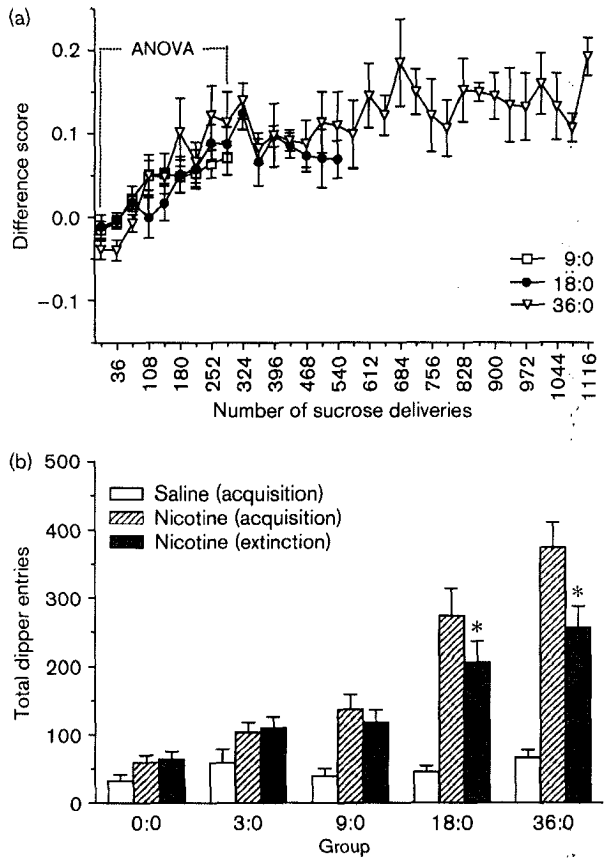
Fig. 1



(a) Mean difference scores (nicotine dipper entry rate minus saline session dipper entry rate) (±1 SEM) of discrimination training for each group in the number of conditional–unconditional stimulus pairings experiment. (b–f) Mean dipper entries per second (±1 SEM) on nicotine and saline sessions during discrimination training for each group; significant difference between dipper entry rates on corresponding nicotine and saline sessions, $*P<0.05$.

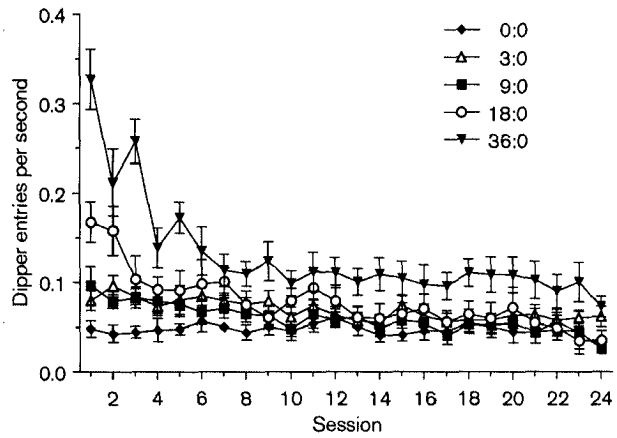
$P < 0.001$], the main effect of group and the number × group interaction were not significant ($F \leq 1.54$, NS), indicating that after equating the number of sucrose deliveries there was no difference in either the rate of acquisition between groups or the CR magnitude after 288 nicotine CS–sucrose US pairings.

Fig. 2



(a) Mean difference score (±1 SEM) of discrimination training for groups 9:0, 18:0, and 36:0 as a function of the number of sucrose presentations. Each data point represents the dipper entry rate after an additional 36 sucrose presentations. (b) Mean total dipper entries (+1 SEM) for each group on the last saline and nicotine session of discrimination training and the first nicotine extinction session; * significant difference between the last nicotine session and the first extinction session, *$P<0.05$.

Fig. 3



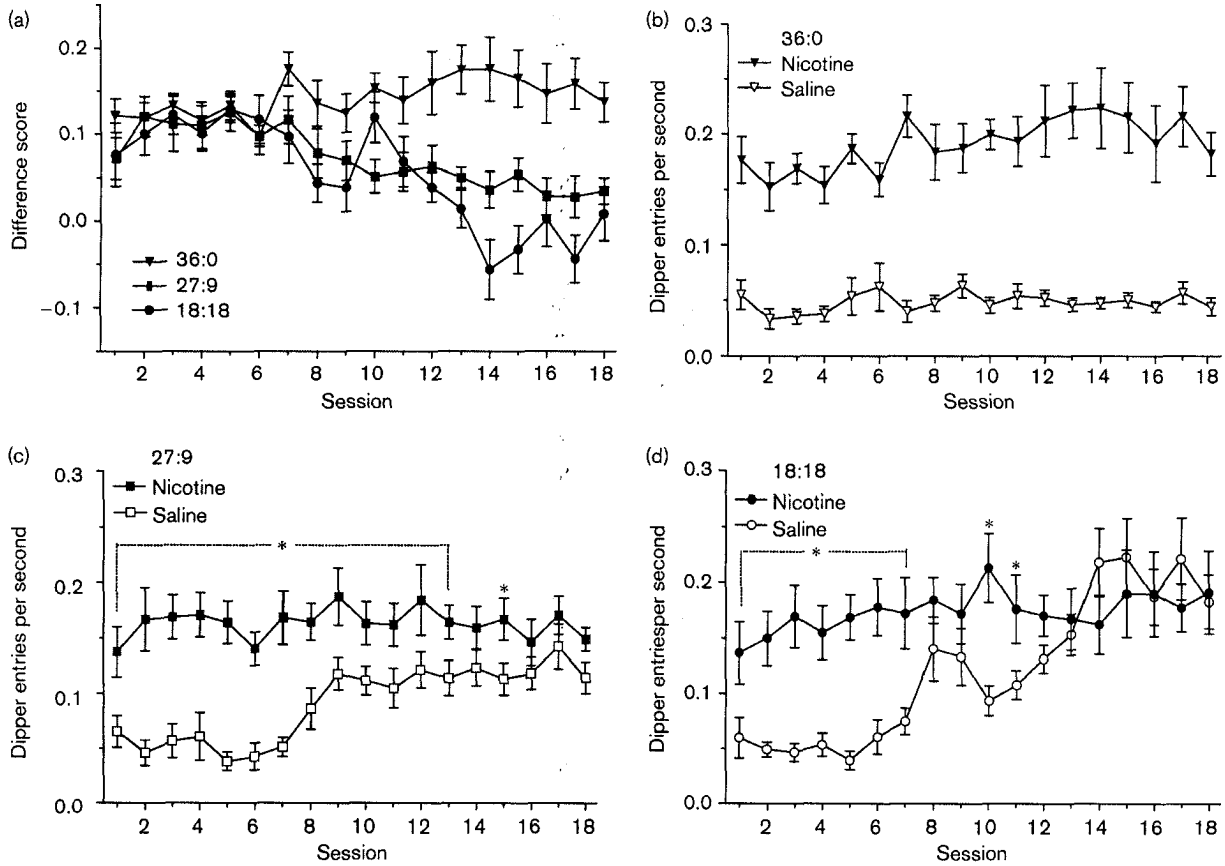
Mean dipper entry rates (±1 SEM) during the extinction phase for each group in the number of conditional–unconditional stimulus pairings experiment.

Extinction

The current design allowed us to examine the ability of nicotine alone to control responding throughout the session. That is, comparing the total dipper entries during the last nicotine training session with the total dipper entries during the first extinction session provides a measure of nicotine-evoked goal tracking in the absence of sucrose. Figure 2b shows the total dipper entries for each group on the last saline and last nicotine session of the acquisition phase, and the total dipper entries for the first nicotine extinction session. Significant main effects of session [$F(2,70) = 130.85$, $P < 0.001$] and group [$F(1,35) = 240.81$, $P < 0.001$], and a significant session × group interaction [$F(8,70) = 18.73$, $P < 0.001$, MSE = 1607.88] were observed. A follow-up one-way ANOVA was conducted on just the saline sessions to determine whether there were differences in total dipper entries between groups on saline sessions. No effect of group ($F < 1$) was observed, indicating no difference in baseline responding by the end of acquisition training. Further, a two-way ANOVA comparing the last nicotine and first extinction sessions found significant main effects of session [$F(1,35) = 54.94$, $P < 0.001$] and group [$F(1,35) = 236.84$, $P < 0.001$], and a significant session × group interaction [$F(4,35) = 20.69$, $P < 0.001$, MSE = 549.48]. Follow-up analyses compared total dipper entries on the last nicotine session and the first extinction session for each group. Groups 18:0 and 36:0 had more total dipper entries on the last nicotine training session than on the extinction session ($LSD_{mmd} = 40.1$), suggesting that the delivery of sucrose added to (or 20 min of extinction decreased) the total level of goal tracking in these groups.

Figure 3 shows the dipper entry rates across extinction sessions for each group. For comparison purposes, this measure is derived from a time period comparable to acquisition. A two-way ANOVA revealed a significant main effect of session [$F(23,805) = 22.00$, $P < 0.001$], indicating that dipper entry rates decreased as nicotine was repeatedly administered without sucrose deliveries. A significant main effect of group [$F(1,35) = 8.93$, $P < 0.001$] and a significant session × group interaction [$F(92,805) = 5.48$, $P < 0.001$, MSE = 0.001] were observed, suggesting that extinction patterns differed depending on the number of nicotine–sucrose pairings that occurred during discrimination training. Follow-up analyses compared the dipper entry rates for each group to group 0:0 on each session ($LSD_{mmd} = 0.03$). Group 3:0 had a higher dipper entry rate on sessions 1–3, 5, 8, 15, and 24. Group 9:0 had higher dipper entry rate on sessions 1–4, 13, and 15. Group 18:0 had higher dipper entry rates on sessions 1–8, 10, and 11. Group 36:0 had

Fig. 4



(a) Mean difference score (±1 SEM) for groups 36:0, 27:9, and 18:18 in the ratio of conditional–unconditional stimulus pairings (expression) experiment when sucrose was delivered in saline sessions. (b–d) Mean dipper entry rate (±1 SEM) on nicotine and saline sessions for each group; significant difference between corresponding nicotine and saline sessions, $^*P<0.05$.

higher dipper entry rates than Group 0:0 across all extinction sessions.

Ratio of conditional–unconditional stimulus pairings (expression)

Retraining discrimination

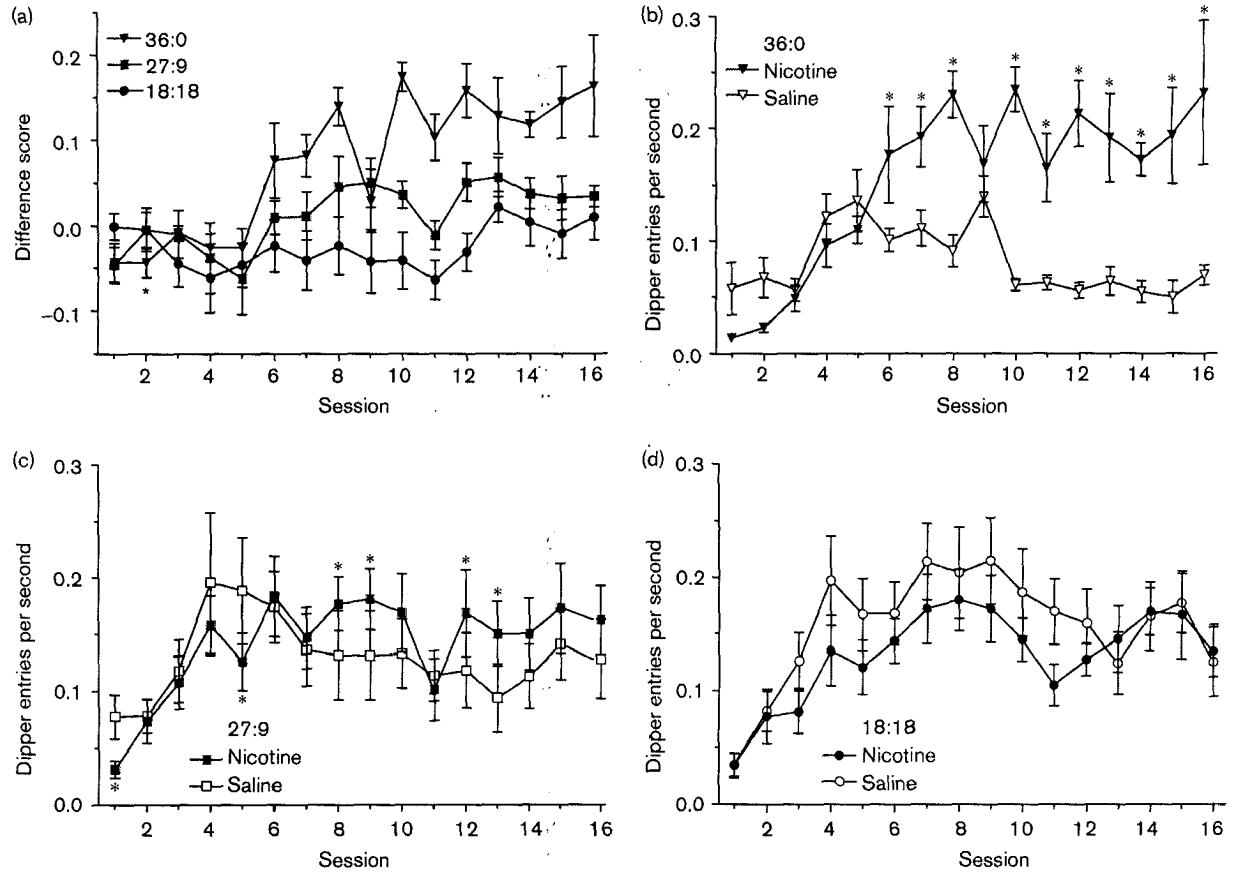
For reacquisition at a ratio of 36:0, only rats that showed reliable discrimination ($n = 30$) were used in analyses (see Methods section). A two-way mixed-groups ANOVA using session as the repeated measure and group as the between-subjects variable was conducted using difference scores (data not shown) to ensure that random assignment did not accidentally result in group (36:0, 27:9, 18:18) differences. A significant main effect of session [$F(9,243) = 2.28$, $P < 0.02$] was noted, indicating that rats reacquired the discrimination. No effect of group and no group × session interaction ($F < 1$) were observed, however, indicating that the groups did not differ during retraining of the discrimination.

Ratio shift

Figure 4a shows the difference scores for groups 36:0, 27:9, and 18:18 after the ratio of sucrose delivery was changed (i.e. sucrose deliveries in saline sessions). Significant main effects of session [$F(17,459) = 4.67$, $P < 0.001$] and group [$F(2,27) = 10.43$, $P < 0.001$], and a significant group × session interaction [$F(34,459) = 3.58$, $P < 0.001$] were found. The interaction suggests that changes in the nicotine:saline sucrose ratio affected expression of the discrimination. The following sessions had difference scores that were significantly different from the hypothetical 0: group 36:0 (1–18) [$t(7) \geq 4.30$, $P \leq 0.002$], group 27:9 (1–13, and 15) [$t(7) \geq 2.59$, $P \leq 0.05$], and group 18:18 (1–7, 10, 11) [$t(7) \geq 2.27$, $P \leq 0.05$].

The dipper entry rates for each group on saline and nicotine sessions are shown in Fig. 4b–d. For group 36:0, there was a significant main effect of drug [$F(1,9) = 61.87$, $P < 0.001$]. No significant effect of

Fig. 5



(a) Mean difference score (±1 SEM) for groups 36:0, 27:9, and 18:18 in the ratio of conditional–unconditional stimulus pairings (acquisition) experiment. (b–d) Mean dipper entry rate (±1 SEM) on nicotine and saline sessions for each group; significant difference between corresponding nicotine and saline sessions, $*P<0.05$.

session or session × drug interaction ($F \leq 1.63$, $P \geq 0.06$), was observed, indicating that the discrimination was maintained throughout the experiment. For groups 27:9 and 18:18, there were main effects of drug [$F(1,9) \geq 11.30$, $P \leq 0.01$] and session [$F(17,153) \geq 3.39$, $P < 0.001$], and a significant drug × session interaction [$F(17,153) \geq 2.40$, $P \leq 0.005$, MSE ≤ 0.003]. Nicotine dipper entry rates were significantly different from the comparable saline dipper entry rates on the following sessions: group 27:9 (1–13 and 15) ($LSD_{mmd} = 0.039$); group 18:18 (1–7, 10, 11, and 14) ($LSD_{mmd} = 0.048$). In summary, the discrimination was disrupted when the nicotine:saline sucrose ratio was changed and the progression of disruption was a function of the ratio.

Ratio of conditional–unconditional stimulus pairings (acquisition)

Discrimination training

Figure 5a shows the difference scores for the naïve rats that received initial discrimination training with the 36:0, 27:9, or 18:18 ratio of nicotine:saline session sucrose deliveries. The two-way ANOVA found a significant main effect of group [$F(2,21) = 12.35$, $P < 0.001$] and session [$F(15,315) = 7.34$, $P < 0.001$], and a significant group × session interaction [$F(30,315) = 2.57$, $P < 0.001$]. The following sessions had difference scores that were significantly different from a hypothetical mean of 0: group 36:0 (2, 7–8, 10–16) [$t(7) \geq 2.53$, $P \leq 0.05$], group 27:9 (13, 16) [$t(7) \geq 2.47$, $P \leq 0.05$], group 18:18 (11) [$t(7) = 2.85$, $P = 0.025$]. Group 36:0 acquired the discrimination; groups 27:9 and 18:18 did not acquire the discrimination.

The dipper entry rates for each group on saline and nicotine sessions are shown in Fig. 5b–d. Separate two-way ANOVAs were conducted on each group. For the 36:0 group, there were significant main effects of drug [$F(1,7) = 20.58$, $P < 0.005$] and session [$F(15,105) = 7.10$, $P < .001$], and a significant drug × session interaction [$F(15,105) = 8.43$, $P < 0.001$, MSE = 0.003]. Follow-up analyses indicated that dipper

entries were elevated for nicotine compared with saline on sessions 6–8 and 10–16 ($LSD_{mmd} = 0.054$). For the 27:9 group, there was no significant main effect of drug ($F < 1$). A significant main effect of session ($F(15,105) = 2.96$, $P < 0.001$) and significant drug × session interaction [$F(15, 105) = 2.57$, $P < 0.005$, MSE = 0.006] were, however, noted. Follow-up analyses indicated less goal tracking on nicotine sessions 1 and 5 ($LSD_{mmd} = 0.044$), and more goal tracking on nicotine sessions 8, 9, 12, and 13. For the 18:18 group, there was a significant main effect of session [$F(15,105) = 5.99$, $P < 0.001$], but no significant main effect of drug [$F(1,7) = 4.09$, $P > 0.08$] or drug × session interaction [$F < 1$].

Discussion

An extant literature demonstrates that stimuli generated within the organism (interoceptive cues) can serve as CSs that come to control responding when reliably paired with another stimulus (i.e. US). Although early Pavlovian conditioning research with interoceptive cues tended to use mechanical stimulation of the viscera (Bykov, 1957; Razran, 1958) or brain stimulation (Loucks, 1933; Doty, 1961), later research has used the pharmacological effects of experimenter-administered ligands (e.g. Cook *et al.*, 1960; Bormann and Overton, 1993; Clements *et al.*, 1996; Kim *et al.*, 1999; Palmatier *et al.*, 2004, 2005). Of particular interest in the present article is the ability of nicotine to serve as a CS in an appetitive Pavlovian conditioning situation. This possibility has not received much empirical attention (Clements *et al.*, 1996; Besheer *et al.*, 2004; Bevins and Palmatier, 2004) despite important implications for tobacco addiction [see Troisi (2003), Bevins and Palmatier, 2004; later Discussion]. The present work extended the sparse research on nicotine as a CS and demonstrated a set of effects that is generally consistent with the notion that nicotine-evoked goal tracking results from interoceptive conditioning processes.

Relative to our previously published research with nicotine as a CS (Besheer *et al.*, 2004; Bevins and Palmatier, 2004), there are several methodological changes that should be highlighted. First, our previous research included dipper training before nicotine/saline discrimination training. That is, rats were trained for several days to access the sucrose within 4 s – nicotine or saline was not administered before any of these sessions. This procedural maneuver can be considered chamber CS–sucrose US pairings. This initial dipper training produced a subsequent pattern of acquisition some have described as 'odd'. That odd pattern included a high level of dipper entries (goal tracking) on early saline sessions reflecting the chamber's control of conditioned responding. As saline sessions continued without sucrose delivery, goal tracking decreased (i.e. extinction of the chamber CS). Further, on early nicotine sessions, goal tracking was the opposite of that in saline sessions (i.e. low) presumably from the locomotor suppressant effect of the 0.4 mg base/kg dose of nicotine (cf. Bevins and Palmatier, 2003). Goal tracking increased with repeated nicotine sessions. The present experiments did not include a dipper training phase. Thus, acquisition looks more like the 'typical' acquisition pattern from a Pavlovian conditioning experiment; conditioned responding starts low with a differential increase occurring to the paired nicotine CS. Another procedural variation from previous research was the 3 days of nicotine treatment before discrimination training. This pretreatment reflects our attempt to decrease the early motor impairing effects of nicotine. Although the current design does not allow us to determine the degree to which this change was successful, the important point is that the Pavlovian drug discrimination was readily acquired despite the procedural changes.

Stable discrimination performance was established with 9, 18, or 36 sucrose deliveries per nicotine session. Rats that received 36 US presentations per session (group 36:0) acquired the discrimination in fewer sessions than groups 9:0 or 18:0. Further, the asymptotic CR magnitude increased with the total number of pairings. If total dipper entries in the first extinction session were used as the measure of conditioning, then groups 36:0 and 18:0 had a more robust CR than group 9:0 (see Fig. 2b). If dipper entries in the early portion of the first extinction session were used as the measure, then the rank order was 36:0 > 18:0 > 9:0 (see session 1 of Fig. 3). As this latter measure tracks dipper entries from an interval in which sucrose had not occurred on previous conditioning sessions, there is less likely to be an influence of extinction on this measure of response magnitude. Regardless of one's preferred measure of asymptotic conditioning, this outcome is consistent with previous Pavlovian conditioning research (Kalish, 1954; Jacobs and Blackburn, 1988; Michel *et al.*, 2003).

For groups 18:0 and 36:0, there were more dipper entries across the last nicotine training (acquisition) session than in the first nicotine extinction session. This result suggests that sucrose deliveries contributed to the overall level of responding and/or that extinction of conditioned responding occurred within the first nicotine-alone session. In contrast, group 9:0 had a similar level of dipper entries from the last nicotine acquisition session to the first nicotine extinction session, suggesting some insensitivity to the initial removal of the US. Despite this early decrease in sensitivity, conditioned responding in group 9:0 extinguished faster than that in groups 18:0 or 36:0. These differences in CR magnitude versus extinction pattern across groups could be used to examine

different empirical questions. For instance, if one is interested in antagonism of the nicotine CS, or substitution of other ligands for the nicotine CS, a higher US density protocol has the advantage of a more robust CR. A more robust CR increases the chances of observing a graded loss of CR control (antagonism) or a graded increase in the evoked CR (substitution) using brief extinction tests. In contrast, a lower density procedure likely has the advantage when manipulation might increase the CR (avoid ceiling effect) or observing an effect might require a longer extinction test session.

Conditioned responding was extinguished faster in group 9:0 than in group 18:0. The goal-tracking CR in group 36:0 also decreased across early nicotine extinction sessions. Dipper entries in this group, however, remained higher than these in group 0:0 even after 24 extinction sessions (i.e. 480 min of nonreinforced nicotine exposure in the chambers). Unpublished research in our laboratory has replicated this effect and suggests that 21 additional nicotine-alone sessions would not be sufficient. Although such resistant appetitive conditioned responding is unusual, it is not unheard of. Krause *et al.* (2003), using a sexual conditioning task with male quail, found that a CS that contained the taxidermic head of a female quail when paired with copulatory opportunity maintained sexual CRs over 126 extinction trials. These authors attributed the persistence of the CR to the relatedness (i.e. ecological relevance) of the CS to the US (see also Domjan *et al.*, 2004). Whether a similar process might explain the remaining conditioned approach CR in the current appetitive conditioning study with nicotine is unclear and, at present, a highly speculative proposition.

The increased resistance to extinction with the increase in the number of CS–US pairings is consistent with research conducted within a Pavlovian conditioning and an experimental analysis (operant conditioning) framework (e.g. Pavlov, 1927; Mikulka and Klein, 1980; Nevin *et al.*, 1990; Shull *et al.*, 2002; Michel *et al.*, 2003; Shahan and Burke, 2004; Brabant *et al.*, 2005). Most of the latter research has been driven by behavioral momentum theory (Nevin, 1992; Nevin and Grace, 2000). The behavioral momentum theory suggests that response strength (i.e. resistance to extinction) is a function of the stimulus–reinforcer (CS–US) relationship. This research tends to use multiple schedules in which different distinct discriminative stimuli are each associated with a schedule of reinforcement. Consistent with the present research, reinforced behavior (e.g. key nose poke in rats) persists longer to a discriminative stimulus that was paired more frequently with food [Nevin *et al.* (1990); Shull *et al.* (2002); but see Nevin and Grace (2005)].

Previous research examining the ability of nicotine to serve as an excitatory CS in a Pavlovian conditioning procedure presented all sucrose US deliveries exclusively during the nicotine state (Besheer *et al.*, 2004; Bevins and Palmatier, 2004). In the present article, we also investigated the effects of degrading this relationship between the nicotine CS and sucrose US by increasing the number of sucrose deliveries in saline sessions. In rats that had already acquired the discrimination (i.e. expression study; Fig. 4), the shift of some sucrose deliveries to saline sessions disrupted the discrimination in a systematic fashion. That is, the Pavlovian drug discrimination was disrupted faster with more saline–sucrose occurrences. Notably, this disruption was not expressed as a loss of goal tracking (i.e. conditioned responding) to the nicotine CS. Rather, dipper entries increased in the saline sessions. This outcome suggests that either the chamber cues and/or the injection cues served as the excitatory CS. One question that remains is whether nicotine still has any control of conditioned responding, or whether the chamber/injection cues serve as the only CS. If sucrose was delivered in saline sessions at the start of acquisition (see Fig. 5), then the discrimination was not acquired – even with a 3:1 ratio of reinforcement in nicotine sessions. It is clear from the increase in dipper entries across sessions that a CR was acquired. Nicotine in the 27:9 and 18:18 groups, however, never systematically evoked more dipper entries again, suggesting that the chamber and/or injection cues served as the CS. This disruptive effect of nontarget CS–US pairings has been observed in a wide variety of conditioning tasks (Singh and Banerji, 1986; Gunther and Miller, 2000; Murphy and Baker, 2004).

Tobacco use and addiction is a major health problem around the world (Mackay and Eriksen, 2002). Nicotine is presumed by most investigators to be the main constituent of tobacco responsible for its chronic use. The factors involved in the acquisition and maintenance of this addiction are complex and obviously vary between individuals. We would argue that learning processes likely play some role in most if not all chronic tobacco users [cf. Bevins and Palmatier (2004); see also Rose and Levin (1991); Pritchard *et al.* (1996); Geier *et al.* (2000)]. Most of the preclinical Pavlovian conditioning research, and hence behavioral and cognitive intervention strategies, have conceptualized nicotine as the US. As a US, the central nervous system effects of nicotine enter into an association with temporally and spatially contiguous environmental cues (e.g. odor, throat irritation, cigarette pack, etc.). Indeed, this framework and research provides the basis for cue-exposure therapy with smokers (cf. Dadds *et al.*, 1997; Niaura *et al.*, 1999).

In contrast, there has been very little attention to the possibility that nicotine might also serve as a CS and

enter into associations with other appetitive stimuli that might occur in a spatially and temporally contiguous manner with its interoceptive cueing effects. One exception to this statement was an article by Clements *et al.* (1996) titled 'Classical conditioning in humans: nicotine as CS and alcohol as US'. In brief, human smokers received an injection of nicotine into the upper arm (0.6 mg) paired repeatedly with 0.5 g/kg of ethanol. Although some physiological measures (heartbeat and electrodermal activity) were suggestive, the authors ultimately concluded that 'the study provided inconclusive evidence for the ability of one drug to act as a CS for the presentation of another in human subjects' (p. 94). Hopefully, the present research, and especially the success of other researchers showing that diazepam (e.g. Alessi *et al.*, 2002) and ethanol (Sitharthan *et al.*, 1997) likely function as CSs in humans, will prompt further empirical and theoretical effort into the potential contribution of the CS effects of nicotine to the tenacity of tobacco addiction.

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