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Interoceptive conditioning with nicotine using extinction and re-extinction to assess stimulus similarity with bupropion

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Abstract

Bupropion is an atypical antidepressant that increases long-term quit rates of tobacco smokers. A better understanding of the relation between nicotine and this first-line medication may provide insight into improving treatment. For all experiments, rats first had nicotine (0.4 mg base/kg) and saline session intermixed; intermittent access to sucrose only occurred on nicotine session. Nicotine in this protocol comes to differentially control “anticipatory” dipper entries. To more closely examine the overlap in the interoceptive stimulus effects of nicotine and bupropion, we assessed whether subsequent prolonged and repeated non-reinforced (extinction) sessions with the bupropion stimulus could weaken responding to nicotine (i.e., transfer of extinction). We also examined whether retraining the discrimination after initial extinction and then conducting extinction again (i.e., re-extinction) with bupropion would affect responding. We found that bupropion (20 and 30 mg/kg) fully substituted for the nicotine stimulus in repeated 20-min extinction sessions. The extent of substitution in extinction did not necessarily predict performance in the transfer test (e.g., nicotine responding unchanged after extinction with 20 mg/kg bupropion). Generalization of extinction back to nicotine was not seen with 20 mg/kg bupropion even after increasing the number of extinction session from 6 to 24. Finally, there was evidence that learning in the initial extinction phase was retained in the re-extinction phase for nicotine and bupropion. These findings indicate that learning involving the nicotine stimuli are complex and that assessment approach for stimulus similarity changes conclusions regarding substitution by bupropion. Further research will be needed to identify whether such differences may be related to different facets of nicotine dependence and/or its treatment.

INTRODUCTION

In the United States, tobacco consumption is responsible for approximately one fifth of all deaths [ca. 443,000 deaths per year (CDC, 2008)]. The United States currently loses more

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DISCLOSURE

The authors declare no conflict of interest.

than \$193 billion on healthcare and loss of productivity associated with the use of tobacco products (CDC, 2008). Although 70% of smokers express a desire to quit, only 40% of all smokers make a serious quit attempt each year. Without treatment, of those that quit, only about 5% will remain abstinent for a year (CDC, 2008; Lemmens *et al.*, 2008). Behavioral and/or pharmacological interventions increase this success rate 3 to 6 fold depending on the study (Lemmens *et al.*, 2008). Although these interventions improve outcomes, relapse rates remain high. If more successful treatment strategies are to be designed, then we need to better understand the basic mechanisms underlying this tenacious habit.

When nicotine, the primary addictive constituent of tobacco, is ingested, it produces a complex internal stimulus composed of many neurobiological elements. This drug stimulus can acquire control over behavior (Murray and Bevins, 2011). In the laboratory, human participants will learn to discriminate nicotine from saline and place poker chips in a particular pile when under the influence of nicotine (Perkins *et al.*, 1999). Similarly, rats responding, such as pressing a left lever 25 times to receive a food pellet, will come under the control of the nicotine stimulus (Colpaert, 1999; Solinas *et al.*, 2006; Wooters *et al.*, 2009). These stimulus effects, and the acquired appetitive properties conferred by the learning history, likely contribute to the development of nicotine dependence and the tenacity of habitual tobacco use (Bevins and Palmatier, 2004; Bevins and Murray, 2011).

We have been studying interoceptive conditioning involving nicotine using a discriminated goal-tracking (DGT) task. In the DGT task, rats have the nicotine stimulus reliably paired with access to a non-drug appetitive stimulus (e.g., liquid sucrose); in the absence of nicotine (i.e., saline), the reinforcer is not available. After a brief period of training, a goal-tracking response comes to be differentially evoked by the nicotine stimulus. Goal-tracking refers to approach behavior to a location where the reinforcer has occurred in the past (Boakes, 1977; Farwell and Ayres, 1979). Previous research has shown that this acquired appetitive behavior controlled by the nicotine stimulus does not reflect state-dependent learning (Bevins *et al.*, 2007; Murray and Bevins, 2011), cannot be attributed to a non-associative or stimulant effect of nicotine (Besheer *et al.*, 2004; Murray *et al.*, 2009; Reichel *et al.*, 2007; Wilkinson *et al.*, 2006), and follows many postulates of Pavlovian conditioning (Bevins and Murray, 2011; Murray *et al.*, 2009).

One such postulate, extinction, refers to the non-reinforced presentation of a stimulus that had been previously paired with a reinforcer; responding evoked by the stimulus tends to decrease over non-reinforced presentations (Pavlov, 1927; Wasserman and Miller, 1997). Some forms of extinction likely occur as a natural consequence of quitting smoking. That is, during abstinence, some stimuli previously associated with smoking (e.g., sight of a cigarette or package, smell of coffee, etc.) occur in the absence of nicotine. Of course, exposure to smoking-related stimuli can facilitate relapse and, in principle, this effect should be alleviated to some extent with cue-exposure (extinction) therapies (Conklin, 2006; Lee *et al.*, 2007).

In part, the difficulty in quitting that tobacco users often experience may result from the appetitive learning history with the nicotine stimulus. This learning history with the nicotine stimulus remains intact when one quits without the aid of a pharmacotherapy and may

contribute to how quickly a lapse in smoking cessation leads to return of pre-cessation smoking patterns (Bevins and Palmatier, 2004; CDC, 2008). Pharmacotherapies such as bupropion or nicotine replacement may not only serve to alleviate withdrawal symptoms, such as depressed mood and cognitive deficits, they may also facilitate decoupling of the reinforcers in the environment from the stimulus effects of nicotine (i.e., extinction). In the DGT task, non-reinforced presentations of the nicotine stimulus produce a systematic decrease in the goal-tracking conditioned response (Besheer et al., 2004; Murray et al., 2009; Wilkinson et al., 2006). This effect occurs even when the dose of nicotine in extinction is lower than the training dose (Polewan et al., 2013).

Of primary interest in the present report is the finding that the acquired appetitive response controlled by the nicotine stimulus can be blunted by non-reinforced presentations of other ligands that share stimulus effects with nicotine (Reichel et al., 2010). More specifically, rats were first trained with nicotine as the conditioned stimulus in the DGT task. Then, in the subsequent extinction phase, a ligand that shares stimulus effects with nicotine [e.g., nornicotine or varenicline (Chantix®)] was used as the stimulus for extinction. The effectiveness of this extinction history was assessed in a challenge test where all rats were tested with the training dose of nicotine. Reichel et al. (2010) observed that the goal-tracking response in rats that had received either nornicotine or varenicline in extinction was diminished relative to controls; albeit less effective than receiving nicotine in extinction. This diminished responding, referred to as “transfer of extinction learning”, reflects generalization of the non-reinforcement history with the “nicotine-like” ligand back to the original excitatory nicotine stimulus.

Although varenicline will partially substitute for nicotine in extinction, we have not examined the other United States FDA approved non-nicotine pharmacotherapy for smoking cessation—bupropion (Zyban®). Accordingly, one goal of the present study was to determine whether extinction learning involving the bupropion stimulus would transfer back to the nicotine stimulus as evidenced by weaker responding relative to controls. Experiment 1 tested whether extinction (6 sessions) with bupropion as a substitution ligand would transfer back to the nicotine stimulus. The low dose bupropion (20 mg/kg) in this experiment did not substitute for the nicotine stimulus in the transfer test. Therefore, Experiment 2 tested whether prolonged extinction (up to 24 daily sessions) with 20 mg/kg bupropion would deepen extinction and hence enhance transfer of extinction back to nicotine. Another potential approach to deepening extinction is re-extinction. Re-extinction refers to a second extinction attempt after the previously extinguished stimulus has been retrained (see later for more detail). Experiment 3 examined re-extinction learning with the bupropion stimulus.

MATERIALS AND METHODS

Animals

There were 187 young adult male Sprague-Dawley rats (275–299 g upon arrival from Harlan; Indianapolis, IN, USA) used in the present experiments. Rats were housed individually in clear polycarbonate cages (48.3 × 26.7 × 20.3 cm) lined with wood shavings. The temperature- and humidity-controlled colony room was kept on a 12-h light/dark

schedule. All experiments were conducted during the light cycle. Water was freely available in the home cage and access to chow (Harlan Teklad Rodent Diet; Harlan, USA) was restricted to maintain rats at 85% of their free-feeding body weight. Four weeks into the study, this 85% target weight was increased by 2 g. All experimental protocols were approved by the University of Nebraska-Lincoln Animal Care and Use Committee.

Apparatus

All experiments were conducted in commercially available conditioning chambers (ENV-008CT; Med Associates, Inc.; St. Albans, VT, USA) measuring 30.5 × 24.1 × 21 cm (l × w × h) enclosed in a sound and light attenuating cubicle equipped with an exhaust fan. Each chamber had aluminum sidewalls, metal rod floors with polycarbonate front, back, and ceiling. A recessed receptacle (5.2 × 5.2 × 3.8 cm; l × w × d) was centered on one of the sidewalls. A dipper arm when raised provided access to 0.1 ml of 26% (w/v) sucrose in the receptacle. Access to the dipper was monitored by an infrared beam mounted 1.2 cm into the receptacle and 3 cm above the rod floor. Beam breaks for dipper entries were recorded using Med Associates interface and software (Med-PC for Windows, version IV).

Drugs

Bupropion hydrochloride (Toronto Research Chemicals; Toronto, ON, Canada) and (–)-nicotine hydrogen tartrate (Sigma; St. Louis, MO, USA) were dissolved in 0.9% saline. The pH of nicotine was adjusted to 7.0 ± 0.2 with a dilute NaOH solution. All drugs were administered at 1 ml/kg. Bupropion was administered intraperitoneal (IP); nicotine was injected subcutaneous (SC). Injection routes, drug doses, and time between injection and onset of behavioral assessment were based on previous reports from our laboratory (Besheer et al., 1999; Bevins et al., 2001; Murray and Bevins, 2007; Wilkinson et al., 2010). Nicotine dose is reported as base, whereas bupropion doses are reported as salt.

Behavioral Procedures

Experiment 1: 6 Days of Extinction with Bupropion

Preliminaries: Rats (n=48) were acclimated to the colony for 5 days and then handled for a minimum of 2 min on each of three consecutive days before the start of the experiment. To attenuate initial locomotor suppressing effects of nicotine (Besheer et al., 2004; Bevins et al., 2001), all rats received nicotine (0.4 mg/kg; SC) on each of the three days before the start of the training phase.

Discrimination Training: For each of the 32 daily training sessions, rats were injected SC with either nicotine (0.4 mg/kg) or saline 5 min before placement in the chamber for a 20-min session. Each rat received a unique order of 16 nicotine and 16 saline sessions with the restriction that no more than two of the same session type (nicotine or saline) occurred in a row. Sucrose was available only during nicotine sessions. Access to sucrose was initiated between 124 to 152 s from the start of the session. There were 36 separate 4-sec deliveries of sucrose per nicotine session. Time between sucrose deliveries ranged from 4 to 80 s (mean = 25 s). On intermixed saline sessions, sucrose was in the dipper well, but the dipper cup was never raised (i.e., no sucrose access). Discrimination training, as well as the

remaining phases of the experiment, was conducted in a dark chamber and there were no other programmed cues of any kind during the session.

Extinction: Following this training, rats were assigned to one of four groups (n=12 per group) with the condition that groups responding throughout the training did not differ statistically, as confirmed by two-way (Group \times Session) mixed factor analysis of variance (ANOVA). For the 6 daily extinction sessions, rats received saline, 0.4 mg/kg nicotine, 20 mg/kg bupropion, or 30 mg/kg bupropion before placement in the chamber for a 20-min session without any sucrose deliveries. Bupropion was administered 15 min before the session; nicotine was administered 5 min before the session. To control for any possible time and/or route of administration effects, all rats received counterbalanced saline injections. For example, rats that had nicotine as the extinction ligand received an IP saline injection 15 min before the session onset. In contrast, rats that had bupropion as the extinction ligand received saline (SC) 5 min before the session.

Transfer of Extinction Tests: Following extinction, all rats were tested for transfer of extinction learning back to the nicotine training stimulus. On 4 separate test days, rats in all groups were injected with 0.4 mg/kg nicotine and placed in the chamber 5 min later for a 20-min test session. A saline injection was also 15 min before chamber placement so that time of injection did not serve as signal for the test ligand.

Experiment 2: Extensive Extinction with 20 mg/kg Bupropion—Following discrimination training, which was identical to Experiment 1, rats (n=60) were assigned to one of five groups with the condition that group performance did not differ statistically in the training phase. Depending on group assignment (n=12 per group), rats were injected with 20 mg/kg bupropion for 24, 12, 6, 3, or 0 (saline control) consecutive days before the transfer of extinction tests. Rats that received less than 24 extinction sessions with bupropion had the remainder of the 24 sessions as non-reinforced saline sessions. For example, rats assigned to receive 6 extinction sessions with bupropion were first given 18 consecutive saline sessions. This protocol controls for handling, number of injections, and chamber exposure. Thus, only the number of extinction sessions with the bupropion stimulus varied between groups. The transfer tests followed the extinction phase by 24 h and were identical to those in Experiment 1. That is, all rats were challenged with the training dose of nicotine before each 20-min test.

Experiment 3: Re-extinction with Bupropion—Unless otherwise noted, the procedural details were identical to Experiment 1. Rats (n=79) first had excitatory conditioning with the nicotine stimulus. Following this training, rats were assigned to receive saline, nicotine (0.4 mg/kg), 20 mg/kg bupropion, or 30 mg/kg bupropion for 6 consecutive extinction sessions (see Figure 1A for timeline). Following 24 h after the last day of extinction, rats had one transfer of extinction test with the training dose of nicotine. All rats then had 8 daily sessions (4 nicotine and 4 saline) of retraining the discrimination. In our experience, 8 retraining sessions is more than sufficient to reestablish stable conditioned responding to the nicotine stimulus. Following retraining, rats were assigned to one of 7 groups (n=10 to 12). Figure 1B depicts group assignment. The name of each group refers to

the ligand used in each extinction phase. For example, rats in the NIC/NIC group were extinguished and re-extinguished with nicotine. Rats in the SAL/30BUP group had saline in the first extinction phase and 30 mg/kg bupropion in the second extinction phase. The re-extinction phase was procedurally the same as extinction. Rats were injected with their assigned ligand at the prescribed injection-to-placement interval for each of the 6 sessions. Following 24 h after the last re-extinction session, there were 4 daily transfer of extinction tests with the 0.4 mg/kg training dose of nicotine.

Dependent Measures

For discrimination training, the primary dependent measure was the rate of dipper entries per second before the first sucrose delivery or an equivalent time for saline sessions. A rate measure was required as the time to the initial sucrose delivery varies across nicotine sessions. Further, using dipper entries before the initial sucrose delivery kept the measure of conditioning from being contaminated by sucrose access. Because sucrose was not available in the extinction, the re-extinction, or the transfer test phase, we used total dipper entries in the 20-min session as the dependent measure.

Statistical Analyses

An omnibus analysis of variance (ANOVA) preceded planned comparisons. Higher-order interactions were further analyzed by two-way ANOVAs and followed, if necessary, by limited Tukey's HSD post-hoc tests ($p < 0.05$). For discrimination training, a Drug (nicotine vs, saline) \times Session within-subjects repeated measures ANOVA was performed. For Experiments 1 and 3, the effect of ligand on extinction used 4×6 (Drug \times Session) repeated measures ANOVA. The effect of re-extinction drug on re-extinction was analyzed using this analytical approach. To examine the effect of extinction history on transfer of extinction tests, a one-way ANOVA on the first transfer test of Experiment 3, or two-way repeated measures ANOVAs (Drug \times Session) were performed for Experiment 1 and second transfer test of Experiment 3. Given the nature of the design of Experiment 2, an omnibus Group \times Session repeated measures ANOVA in the extinction phase was not employed. Rather, corresponding session data from the extinction phase of Experiment 2 were analyzed using planned LSD mean pairwise comparisons restricted to contrasting each test value with corresponding vehicle control session.

RESULTS

Experiment 1: 6 Days of Extinction with Bupropion

Discrimination Training—For the training phase, an omnibus ANOVA revealed a significant main effect of Drug [i.e., nicotine vs. saline; $F(1,94)=1282.83$, $p < 0.001$] and Session [1–16; $F(15,1410)=21.12$, $p < 0.001$], as well as a significant Drug \times Session interaction [$F(15,1410)=35.88$, $p < 0.001$]. Responding on the first nicotine session was lower than saline; this switched, with nicotine-evoked responding higher than saline for sessions 4 to 16 (Figure 2A).

Extinction—In extinction, there was a main effect of Drug [i.e., saline, nicotine, 20 or 30 mg/kg bupropion; $F(3,263)=10.97$, $p < 0.001$]; the main effect of Session [$F(5,263)=2.00$,

$p=0.07$] and Drug \times Session interaction ($F<1$) were not significant. For the main effect Drug, the saline group had less dipper entries than nicotine or either bupropion group (Figure 2B). Notably, there were no significant differences in the magnitude of the conditioned response during extinction between the bupropion and nicotine groups. This data pattern suggests that bupropion across repeated 20-min extinction sessions fully substituted for the interoceptive stimulus effects of nicotine.

Transfer of Extinction Tests—There was a significant main effect of Drug [$F(3,175)=11.07$, $p<0.001$] and Session [$F(3,175)=5.14$, $p<0.01$], but no Drug \times Session interaction ($F<1$). For the main effect of Drug, rats previously treated with nicotine or 30 mg/kg bupropion in the extinction phase had lower levels of responding than rats that had saline or 20 mg/kg bupropion (Figure 2C). This outcome indicates that extinction learning with 30 mg/kg bupropion was comparable to extinction with the nicotine stimulus in the transfer test. Notably, extinction learning with 20 mg/kg did not generalize back to the nicotine stimulus.

Experiment 2: More Extensive Extinction with 20 mg/kg Bupropion

Discrimination Training—As in Experiment 1, there was a significant main effect of Drug [$F(1,118)=202.88$, $p<0.001$], a main effect of Session [$F(15,1770)=20.99$, $p<0.001$], and a significant Drug \times Session interaction [$F(15,1770)=33.97$, $p<0.001$]. Dipper entries on nicotine sessions 1 and 2 were lower than saline; this pattern reversed (i.e., elevated goaltracking on nicotine sessions) from session 4 to 16 (data not shown).

Extinction—Rats that had 24 extinction sessions with 20 mg/kg bupropion had greater goal-tracking than saline controls on extinction sessions 1 to 3, 5, and 10; responding was comparable to saline thereafter (see Figure 3A and 3B for all extinction data). Rats that had 12 extinction sessions with bupropion had higher responding than saline controls on extinction sessions 1 to 3, 5, 6, 9, 10, and 12. Rats that had 6 days of bupropion treatment differed from saline controls on sessions 1 to 4, and 6 of extinction. Rats that had 3 days of extinction with bupropion had higher responding than controls on all three sessions.

Transfer of Extinction Tests—For the nicotine transfer test, there was a main effect of Session [$F(3,165)=14.5$, $p<0.001$] indicating decreased responding across tests. However, there was no main effect of Group or Group \times Session interaction [$Fs<1$; Figure 3C]. Although there is a mean tendency for less responding after 24 extinction sessions, the results of this analysis indicate that even extended extinction did not facilitate transfer of extinction learning with 20 mg/kg bupropion.

Experiment 3: Re-extinction with Bupropion

Discrimination Training—There was a main effect of Drug [$F(1,2506)=1591.92$, $p<0.001$], a main effect of Session [$F(15,2506)=26.03$, $p<0.001$], and a significant Drug \times Session interaction [$F(15,2506)=47.38$, $p<0.001$]. Dipper entries on nicotine sessions were higher than saline on sessions 4 to 16 (data not shown).

Initial Extinction—There was a main effect of Group [$F(3,448)=100.86$, $p<0.001$] and of Session [$F(5,448)=5.46$, $p<0.001$], as well as a Group \times Session interaction [$F(15,448)=3.09$, $p<0.001$]. Overall, dipper entries were higher in the nicotine and bupropion (20 and 30 mg/kg) groups than in saline controls (Figure 4). The group treated with 30 mg/kg bupropion had lower responding relative to nicotine and 20 mg/kg bupropion only on the first extinction session. Saline controls were lower than nicotine and 20 mg/kg bupropion across all the sessions, and from 30 mg/kg on sessions 2 to 6. Thus, bupropion appears to substitute fully for the nicotine stimulus across repeated extinction sessions.

Transfer Test 1—Following initial 6 days of extinction, we tested the generalization of the non-reinforcement history with bupropion to the nicotine training stimulus (0.4 mg/kg nicotine). On this transfer test, there was a main effect of Group [$F(3,121)=7.89$, $p<0.001$]. Saline controls had higher dipper entries than rats that had nicotine in the initial extinction phase (Figure 4). The tendency for conditioned responding to be reduced in the groups that had bupropion in the extinction phase did not reach criterion for statistical significance.

Retraining of Discrimination—The differences in initial extinction histories did not affect reacquisition (data not shown). There was no main effect of Group ($F<1$) and interactions that included Group were not significant ($F_s < 1.5$). Responding on all nicotine retraining sessions was higher than saline sessions.

Re-extinction—Table 1 shows all significant main effect group comparisons for re-extinction. Overall, there was a main effect of Group [$F(6,360)=41.45$, $p<0.001$] and Session [$F(5,360)=4.59$, $p<0.001$], as well as a Group \times Session interaction [$F(30,360)=1.83$, $p<0.01$]. Using separate planned comparisons, we first asked whether re-extinction differed from initial extinction. Overall, responding in the re-extinction phase for group NIC/NIC was lower than group SAL/NIC that had extinction with nicotine the first time [main effect of Group; $F(1,105)=9.70$, $p<0.01$; Figure 5A]. Similarly, responding in re-extinction for group 20BUP/20BUP was lower than group SAL/20BUP [main effect of Group; $F(1,100)=41.39$, $p<0.001$; Figure 5B]. This data pattern indicates that extinction learning in the initial phase was not completely lost by retraining with nicotine. A similar effect was not seen between group 30BUP/30BUP and group SAL/30BUP (Figure 5C).

The next planned analyses examined differences between groups that had an extinction history with a ligand only once (i.e., SAL/20BUP, SAL/30BUP, SAL/NIC); group SAL/SAL serves as a benchmark and was included in the analyses. There was a significant main effect of Group [$F(3,195)=55.59$, $p<0.001$] and of Session [$F(5,195)=2.50$, $p<0.05$]; the interaction was also significant [$F(15,195)=1.85$, $p<0.05$; Figure 5D]. Overall, responding in SAL/NIC group was higher than SAL/SAL. Both SAL/20BUP and SAL/30BUP displayed responding comparable to SAL/NIC; responding in these groups differed from SAL/SAL. This substitution pattern replicated the full substitution between bupropion and nicotine seen in Experiment 1. The interaction was driven by differences in responding observed on sessions 1 to 4. Specifically, responding in group SAL/NIC differed from the SAL/SAL on sessions 1 to 3, while responding in SAL/20BUP group was higher than SAL/SAL on sessions 1 to 4. Further, on session 1, responding in SAL/20BUP group was higher than SAL/30BUP.

Planned analyses of extinction curves among groups that had re-extinguished with the same ligand (i.e., 20BUP/20BUP, 30BUP/30BUP, or NIC/NIC) revealed a main effect of Group [$F(3,215)=61.87$, $p<0.001$] and a Group \times Session interaction [$F(15,215)=2.82$, $p<0.001$; Figure 5E]. The main effect of Session was not significant [$F(5,215)=1.55$, $p=0.17$]. Overall, responding in NIC/NIC group was significantly higher than SAL/SAL, 20BUP/20BUP, and 30BUP/30BUP. The pattern of responding in groups 20BUP/20BUP and 30BUP/30BUP now suggests only partial substitution for the nicotine stimulus. That is, overall levels of goal-tracking in those groups were higher than SAL/SAL but lower than NIC/NIC. This partial substitution is in contrast to the full substitution seen with single extinction in this study (Figure 5D) and Experiment 1 (Figure 2B). As for the significant Group \times Session interaction, responding in NIC/NIC group was higher than SAL/SAL on sessions 1 to 6. Responding in 20BUP/20BUP was higher than SAL/SAL on sessions 1 and 3. Responding in 30BUP/30BUP was higher than SAL/SAL on sessions 2, 3, 4, 6 and lower than NIC/NIC on session 1.

Transfer Test 2—These planned comparisons were designed to elucidate to what degree the extinction or re-extinction with bupropion generalizes back to the nicotine stimulus. All significant main effect group comparisons for the second transfer test are listed in the Table 1. An omnibus ANOVA revealed a main effect of Group [$F(6,216)=12.23$, $p<0.001$] and of Session [$F(3,216)=3.48$, $p<0.05$], as well as a Group \times Session interaction [$F(18,216)=2.12$, $p<0.01$]. With separate planned comparisons, we first asked whether or not rats with single extinction histories differ from those with re-extinction histories in their responding evoked by the nicotine stimulus. Overall, responding in group SAL/NIC was higher than NIC/NIC [main effect of Group; $F(1,63)=14.60$, $p<0.001$; Figure 6A]. Similarly, responding in SAL/20BUP was higher than 20BUP/20BUP [$F(1,59)=13.29$, $p<0.001$; Figure 6B]. Transfer test performance of groups NIC/NIC and 20BUP/20BUP suggests that a history of retraining and re-extinction enhances generalization of extinction learning across the ligands. A similar outcome did not occur with 30 mg/kg bupropion. Overall responding in SAL/30BUP group was lower than 30BUP/30BUP group [$F(1,63)=10.53$, $p<0.01$; Figure 6C]. The Group \times Session interactions were not significant in these planned comparisons.

A separate analysis of groups with single extinction histories (SAL/20BUP, SAL/30BUP, SAL/NIC, and SAL/SAL control) revealed main effects of Group [$F(3,117)=7.45$, $p<0.001$] and Session [$F(3,117)=2.92$, $p<0.05$], and a significant interactions [$F(9,117)=2.10$, $p<0.05$]. Overall (see Figure 6D for all comparisons), responding in SAL/SAL group was higher than NIC/NIC. Response pattern in SAL/30BUP group indicates generalization of extinction to the nicotine stimulus; goal-tracking differed from group SAL/SAL, but not from SAL/NIC. Similar transfer of extinction was not seen in group SAL/20BUP with goal-tracking in that group lower than SAL/NIC and not different from the SAL/SAL control. This pattern of substitution replicates the findings from the transfer tests in Experiment 1 (recall Figure 2C). The interaction was driven by the differences on session 1 where responding in group SAL/30BUP and group SAL/NIC was lower than group SAL/SAL.

Analysis of transfer test 2 data, with groups that had extinction and re-extinction with the same ligand (20BUP/20BUP, 20BUP/30BUP, NIC/NIC, and SAL/SAL), revealed significant main effects of Group [$F(3,129)=16.91$, $p<0.001$] and Session [$F(3,129)=3.00$,

$p < 0.05$], and a significant interaction [$F(9,129) = 3.34$, $p < 0.01$]. Overall responding (the main effect) in NIC/NIC group was lower than in SAL/SAL, 20BUP/20BUP, and 30BUP/30BUP groups (see Figure 6E). The overall pattern of responding suggests that reextinction with 20 mg/kg bupropion enhanced generalization of extinction learning with responding evoked by the nicotine stimulus partially weakened; goal-tracking in 20BUP/20BUP group was higher than in NIC/NIC and lower than in SAL/SAL groups. Recall that transfer of extinction with 20 mg/kg bupropion did not occur without this reextinction (Figure 4). Group 30BUP/30BUP did not show substitution for nicotine as responding in this group was higher than NIC/NIC and did not differ from SAL/SAL; this is in contrast to a full substitution of SAL/30BUP for SAL/NIC seen in re-extinction transfer tests including groups with single extinction histories (Figure 6D). The Group \times Session interaction was driven by the differences observed on session 1 and 2. Specifically, responding in groups 20BUP/20BUP and 30BUP/30BUP was similar to NIC/NIC on the session 1, yet differed from SAL/SAL; full substitution for nicotine stimulus. Further, responding in SAL/SAL group on sessions 1–2, and responding in 20BUP/20BUP and 30BUP/30BUP on session 2, was higher than responding in NIC/NIC group.

DISCUSSION

Internal or interoceptive stimuli can be powerful modulators of behavior. Their control of behavior can be modified by experiences (i.e., learning processes). This learning regarding interoceptive stimuli is involved in such important health issues as pain, aging and cognition, eating disorders (Craig, 2003; Kanoski, 2012), and drug addiction (Bevins and Palmatier, 2004; Bevins et al., 2012). We have been using the discriminated goal-tracking task to study the behavioral and neural processes of interoceptive conditioning involving the nicotine stimulus (Bevins and Murray, 2011; Bevins et al., 2012; Charntikov et al., 2012). The research in the present report extends previous studies showing bupropion substitution for the interoceptive stimulus effects of nicotine (Besheer et al., 2004; Wiley et al., 2002; Wilkinson et al., 2010; Young and Glennon, 2002). These earlier studies use testing protocols designed to minimize learning, such as very brief tests (e.g., 4 min) without the reinforcer [see Bevins et al. (2012) for more on this issue]. An alternative approach to studying the similarity of interoceptive stimuli is to test whether the ligand of interest will evoke substitution-like responding in prolonged (i.e., 20 min) and repeated extinction sessions. This approach essentially asks the extent to which the test ligand will continue to evoke the acquired behavior even though the reinforcer is withheld (see Reichel et al., 2010; Wilkinson et al., 2010). The current experiment further extends past research on bupropion substitution for nicotine by examining whether a learning history of non-reinforcement with bupropion would generalize back to the nicotine stimulus. Across the three experiments in this report, we found that (i) bupropion (both 20 and 30 mg/kg doses) fully substitutes for the interoceptive effect of nicotine during repeated 20-min extinction sessions, (ii) the extent of substitution in the repeated extinction sessions did not necessarily predict performance in the transfer test, (iii) transfer of extinction was not seen with 20 mg/kg bupropion even after increasing the number of extinction session from 6 to 24, (iv) there was evidence that what was learned in the initial extinction phase was retained in the re-extinction phase for nicotine

and bupropion, and (v) re-extinction with bupropion dose dependently affected performance to nicotine stimulus in the subsequent transfer tests.

Using prolonged and repeated extinction testing, that allows for the opportunity to learn about non-reinforcement, we found that 20 and 30 mg/kg bupropion evoked goal-tracking that was comparable to nicotine. This full substitution of bupropion for the nicotine stimulus differs somewhat from previous findings from our laboratory using brief substitution tests. For example, Besheer et al. (2004) found partial substitution with 20 mg/kg bupropion in a 2-min test. Wilkinson et al. (2010), however, used 4-min tests and found full substitution by bupropion at 20 mg/kg, but 30 mg/kg dose evoked only partial substitution. This comparison suggests that using repeated and more prolonged extinction test sessions within the discriminated goal-tracking task can provide different answers regarding stimulus substitution. This suggestion is consistent with previous research using this approach (Polewan et al., 2013; Reichel et al., 2010). For example, using the brief 4-min testing protocol, the $\alpha_4\beta_2$ nicotinic acetylcholine receptor agonist ABT-418 (0.6 mg/kg) evoked goal-tracking comparable to nicotine (Reichel et al., 2010). However, in repeated extinction sessions, like those used here, ABT-418 only evoked a partial conditioned response in the first session, and no substitution in the subsequent 5 extinction session.

As detailed earlier, another way of assessing stimulus similarity among ligands is to assess whether extinction learning generalizes to the training stimulus. Interestingly, in the present report, the extent of substitution in the repeated extinction sessions did not necessarily predict performance in the transfer test. For example, 20 and 30 mg/kg bupropion evoked goal-tracking comparable to nicotine across repeated extinction sessions; a result that suggests full substitution for the nicotine stimulus. In Experiment 1, full substitution in the extinction phase by bupropion did not translate into full substitution in the transfer test. During the transfer test, responding in rats that had 20 mg/kg bupropion in extinction had slightly attenuated goal-tracking, but did not differ from controls that did not receive extinction. In contrast, the extinction learning with 30 mg/kg fully generalized back to the nicotine stimulus in this initial study. However, in Experiment 3, and in our unpublished studies, transfer of extinction learning with this higher dose of bupropion appears to be more like the 20 mg/kg dose; a mean tendency toward decreased responding when challenged with nicotine, but not meeting cutoff for significance. We believe further investigation of these dissociations between brief substitution tests, repeated extinction tests, and transfer tests will provide a better understanding of the drugs under investigation (cf. Bevins et al., 2012). For example, what other factors besides stimulus similarity produce the responding evoked by bupropion in the extinction phase? Do these other factors play a role in the effectiveness of bupropion as a smoking cessation drug? Are there ways to enhance extinction learning to bupropion in a manner that would reliably attenuate conditioned responding controlled by the nicotine stimulus in the transfer test? Re-extinction seems to be one answer to this latter question, but increased number of extinction trials does not seem to work, at least with the 20 mg/kg dose of bupropion.

Extinction involves new inhibitory learning that competes with previous excitatory stimulus-reward association (Bouton, 1988, 2000; Rescorla, 2001) -- nicotine-sucrose in our case. One of the ways to improve or deepen extinction learning is to increase the number of

opportunities to learn about non-reinforcement in the extinction phase. (Bouton and Sunsay, 2003; Denniston et al., 2003; Mackintosh, 1975; Pearce and Hall, 1980; Rescorla and Wagner, 1972). Thus, we expected that increasing the number of extinction sessions with 20 mg/kg bupropion would deepen the inhibitory association with the bupropion stimulus and non-reinforcement. If there is sufficient overlap between the bupropion stimulus and the nicotine stimulus, then strengthening this association should translate into a blunted response when challenged with nicotine in the transfer test. This approach did not work with the 20 mg/kg bupropion stimulus. In Experiment 2, rats extinguished with 20 mg/kg bupropion, up to 24 separate occasions (versus 6 in Experiment 1), still did not differ from controls that did not receive any extinction history with bupropion. Future research could try alternative approaches to initial extinction that might deepen extinction. Perhaps extinction that was more spaced such as a session every other day may be more effective. Some published studies with more typical exteroceptive stimuli presented repeatedly within a single session show that temporal spacing does affect extinction learning (e.g., Moody et al., 2006). What if extinction was conducted more like a discrimination procedure with intervening saline days? Whether or not access to sucrose was provided on these intermixed saline sessions would also be of interest.

There is a very limited literature on re-extinction, even with exteroceptive stimuli (cf. Langton and Richardson, 2009; Leslie and Norwood, 2013; Quirk, 2002). The paucity of research is surprising given the interest in extinction and cue-exposure approaches to so many health issues including drug addiction (Conklin and Tiffany, 2002; Conklin, 2006; Ferguson and Shiffman, 2009; O'Brien et al., 1990). Past research with the discriminated goal-tracking task has examined reacquisition following extinction with a nicotine stimulus (Besheer et al., 2004; Murray and Bevins, 2007). That research has never examined reextinction with the nicotine stimulus, or any other ligand for that matter. We found that the pattern of conditioned responding in extinction changes in re-extinction with the very same stimulus. For example, re-extinction with 20 mg/kg bupropion evoked significantly lower responding relative to rats receiving extinction with this same dose for the first time (see Figure 5B). This outcome suggests that something learned in the initial extinction phase remained, at least partly, intact following the brief retraining of the discrimination. Notably, a similar effect was observed in rats re-extinguished with nicotine (Figure 5A), but not with 30 mg/kg bupropion (Figure 5C). The literature with exteroceptive stimuli examining retention of extinction learning following retraining is mixed. For example, Quirk (2002) found that re-extinction of fear conditioning controlled by a tone in rats was faster in comparison to initial extinction. Others in the fear conditioning field have not shown this effect (Langton and Richardson, 2009; Leslie and Norwood, 2013).

Across the three experiments, extinction with 20 mg/kg bupropion in the initial extinction phase did not affect subsequent responding to the nicotine stimulus. In contrast, re-extinction with 20 mg/kg bupropion resulted in significantly lower conditioned responding evoked by the nicotine stimulus (see Figure 6B). Re-extinction reflects 6 additional sessions in which the reinforcer is withheld while the rat is on bupropion. However, this doubling of the extinction sessions seems an unlikely account for diminished responding to the nicotine stimulus following re-extinction. Namely, neither 12 nor 24 consecutive extinction sessions with 20 mg/kg bupropion were sufficient to decrease the subsequent responding evoked by

the nicotine stimulus (Experiment 2; Figure 3C). It seems that this reacquisition and re-extinction experience impacted how learning regarding non-reinforcement with 20 mg/kg bupropion generalized back to the nicotine stimulus.

The suggestion that re-extinction can engage different processes than initial extinction is consistent with research elucidating neural processes underlying re-extinction using a Pavlovian fear conditioning task. For example, Langton and Richardson (2008) first paired illumination of a light with a foot shock in rats. In a subsequent extinction phase, the light was presented without a foot shock. In this initial extinction phase, administration of the partial NMDA agonist D-cycloserine accelerated extinction of fear evoked by the light. These same rats were then retrained with the light–shock pairings and then subjected again to extinction. In this re-extinction phase, the rate at which conditioned responding to light (i.e., freezing) decreased was not affected by the D-cycloserine. This finding suggests that learning in the initial extinction phase is NMDA-dependent, whereas learning in the reextinction is NMDA-independent; this conclusion has been supported further in a series of follow-up experiments (Langton and Richardson, 2010, 2009).

These studies by Langton and Richardson (2008, 2009, 2010), combined with the re-extinction study in the present report, support the notion that re-extinction engages distinct neural networks from extinction that may be of import to how conditioned responding is expressed and what factors may diminish control of this responding. Of interest in future work with the nicotine stimulus will be to identify the behavioral and neural processes that may differ between extinction and re-extinction of interoceptive conditioning. For example, what is the role of NMDA in the extinction and re-extinction with nicotine as an interoceptive stimulus? Are there distinct neural networks that are differentially involved in the extinction and re-extinction with nicotine as an interoceptive stimulus? Answers to these questions may provide a better understanding of relapse in chronic human smokers who on average initiate 7 lifetime quit attempts with about 40% of smokers making a serious attempt to quit each year (CDC, 2005; Colby *et al*, 2011). These quit attempts, sometime done cold turkey and sometimes with the aid of a pharmacotherapy or cognitive/behavioral intervention, reflect potential learning opportunities. Because nicotine is a complex stimulus able to function as a primary reinforcer and an interoceptive stimulus susceptible to modification by learning, it is critical to take these effects into consideration and design therapies that address, and periodically reassess, nicotine’s multifaceted control of patient behavior.

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Highlights

- We examined bupropion substitution for the nicotine stimulus in extinction
- Bupropion substituted for nicotine during repeated extinction
- Substitution in extinction did not predict performance in the transfer test
- Pattern of substitution differed in extinction and re-extinction

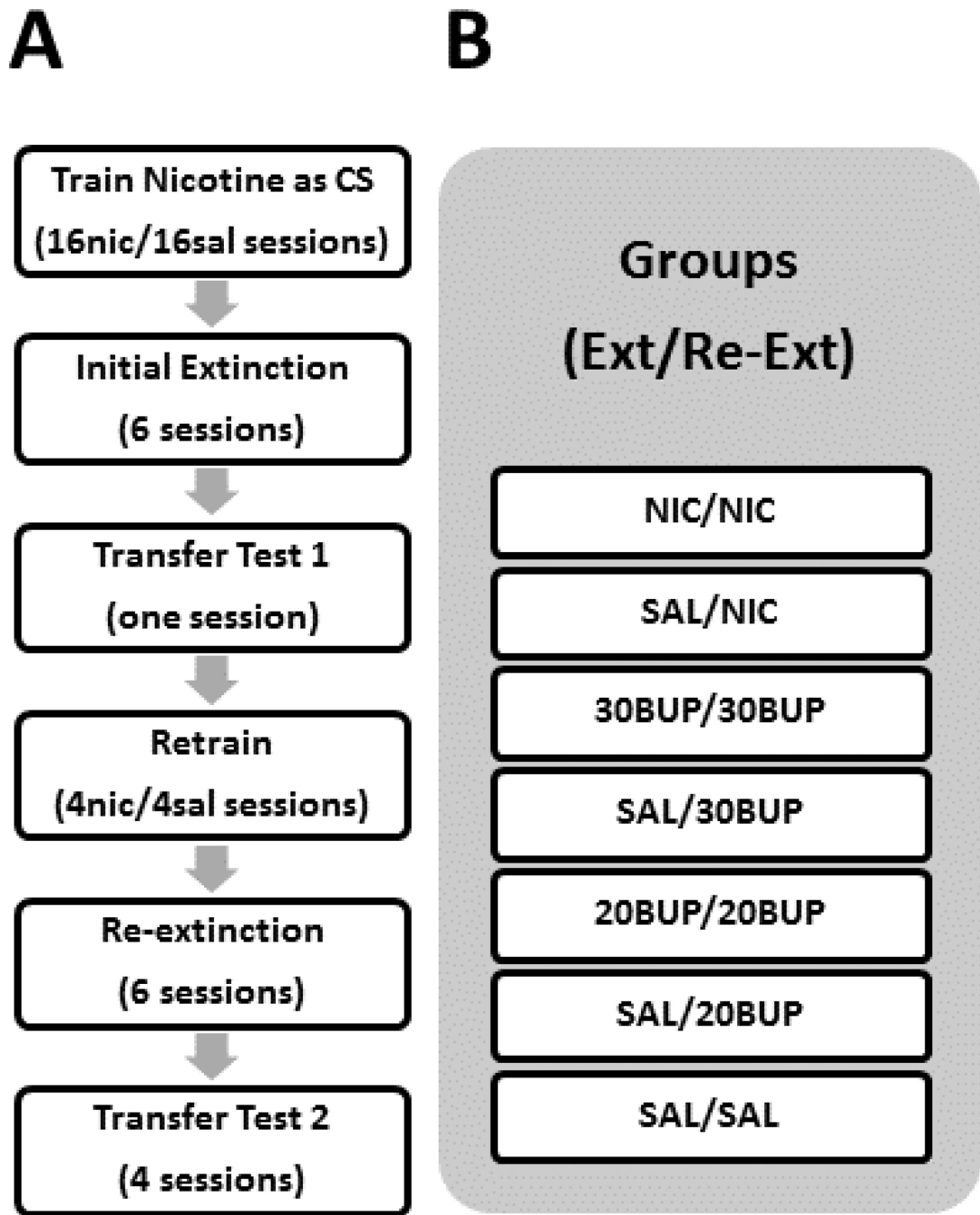


Fig. 1. Graphical representation of procedures employed in Experiment 3 (A) Experimental time line and (B) Group, extinction, and re-extinction ligand assignments.

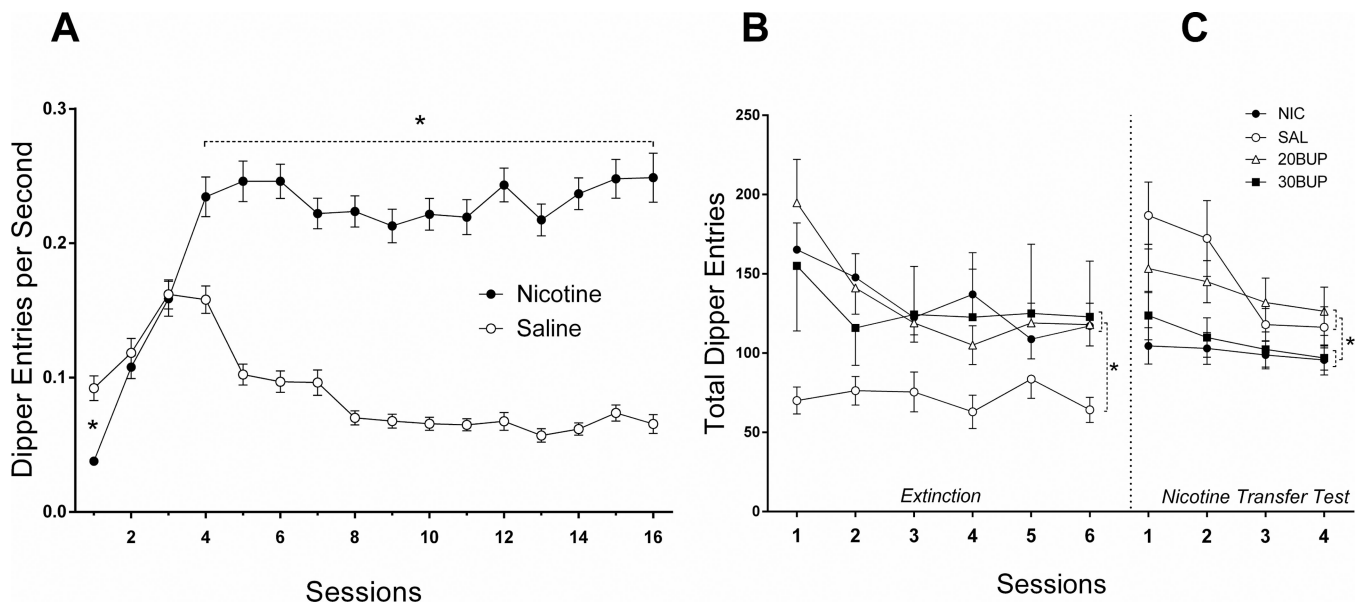


Fig. 2. Data from all three phases of Experiment 1. (A) A mean (\pm SEM) number of dipper entries per second during 2 min prior to initial sucrose or equivalent in timing no reward presentations. *Denotes sessions with significant differences between saline and nicotine evoked responding. (B) A mean (\pm SEM) number of total dipper entries during the six extinction sessions. *Denotes overall group differences. (C) A mean (\pm SEM) number of total dipper entries during four nicotine transfer tests. *Denotes overall group differences.

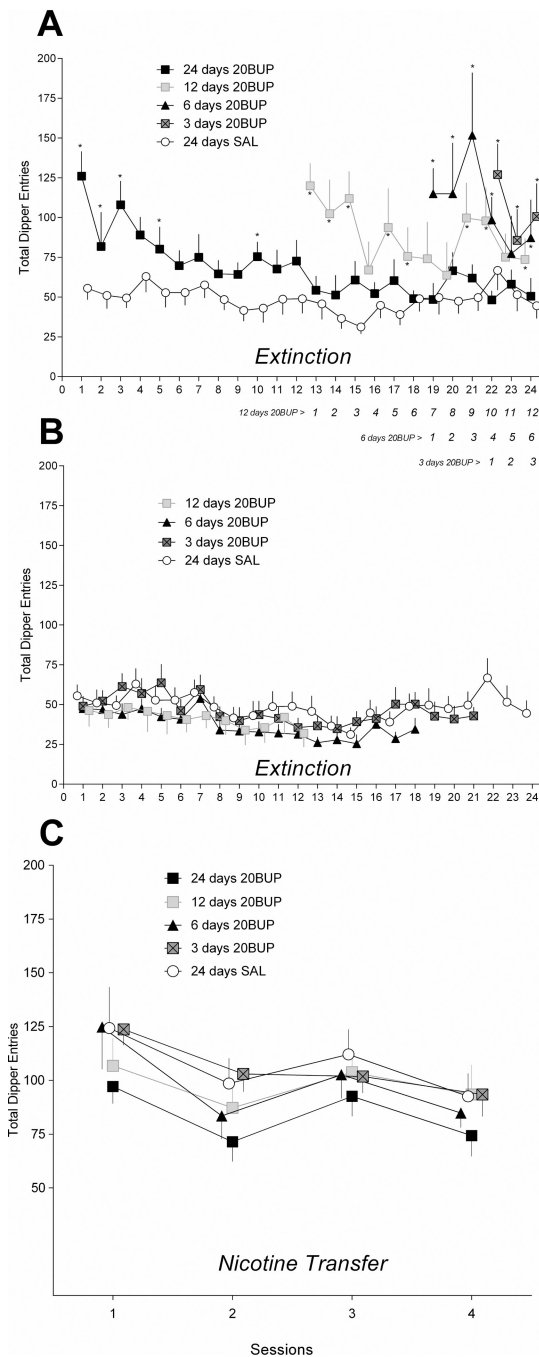


Fig. 3. Data from extinction and nicotine transfer phases of Experiment 2. (A) A mean (\pm SEM) number of total dipper entries during extinction sessions with bupropion and saline control. *Denotes group means significantly different from a corresponding session of saline controls. (B) A mean (\pm SEM) number of total dipper entries during extinction sessions with saline prior to extinction with bupropion; includes saline control. (C) A mean (\pm SEM) number of total dipper entries during four nicotine transfer tests.

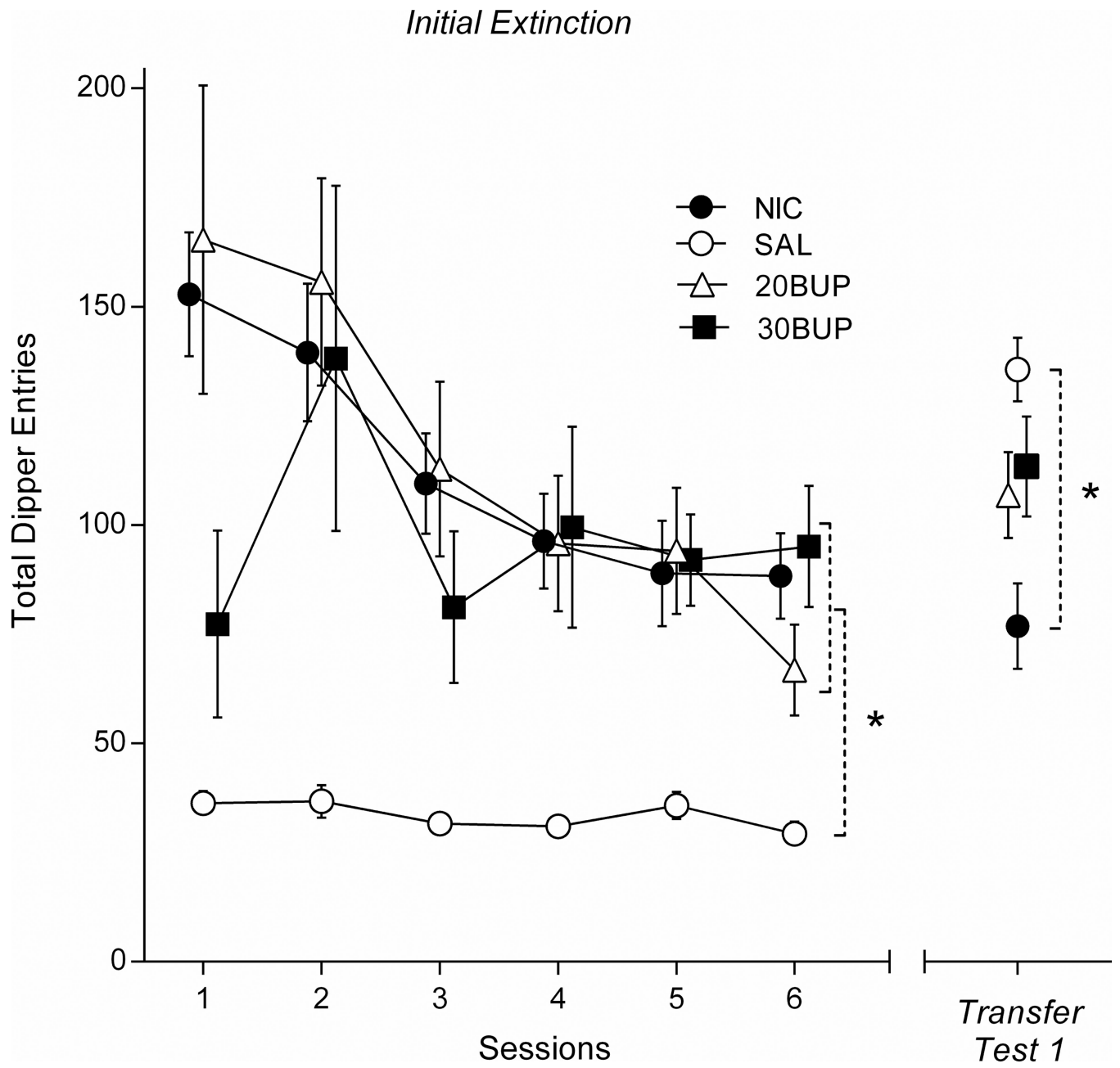


Fig. 4. A mean (\pm SEM) number of total dipper entries during extinction and transfer test sessions of Experiment 3. *Denotes overall group differences during extinction and transfer test 1.

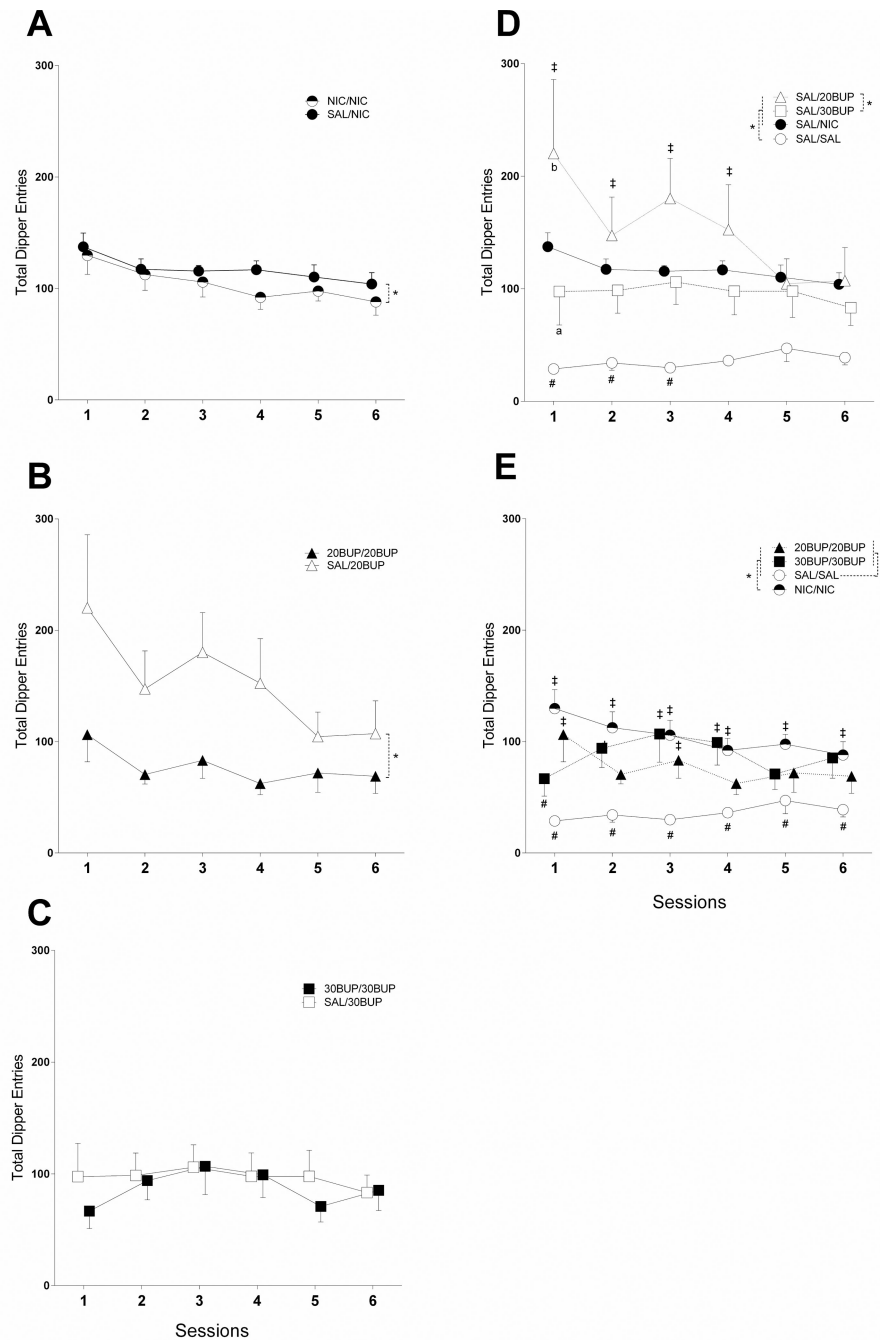


Fig. 5. A mean (\pm SEM) number of total dipper entries during re-extinction sessions of Experiment 3. Each panel represents data from each of separate planned analyses: (A–C) comparison of groups receiving extinction ligand in either one or both of extinction phases, (D) comparison of groups with single extinction histories, (E) comparison of groups receiving extinction ligand in both extinction and re-extinction phases of the experiment. *Denotes overall group differences. ‡Denotes differences from a corresponding SAL/SAL session. #Denotes difference from a corresponding SAL/NIC session. ^{ab}Indicates where^a is different from^b.

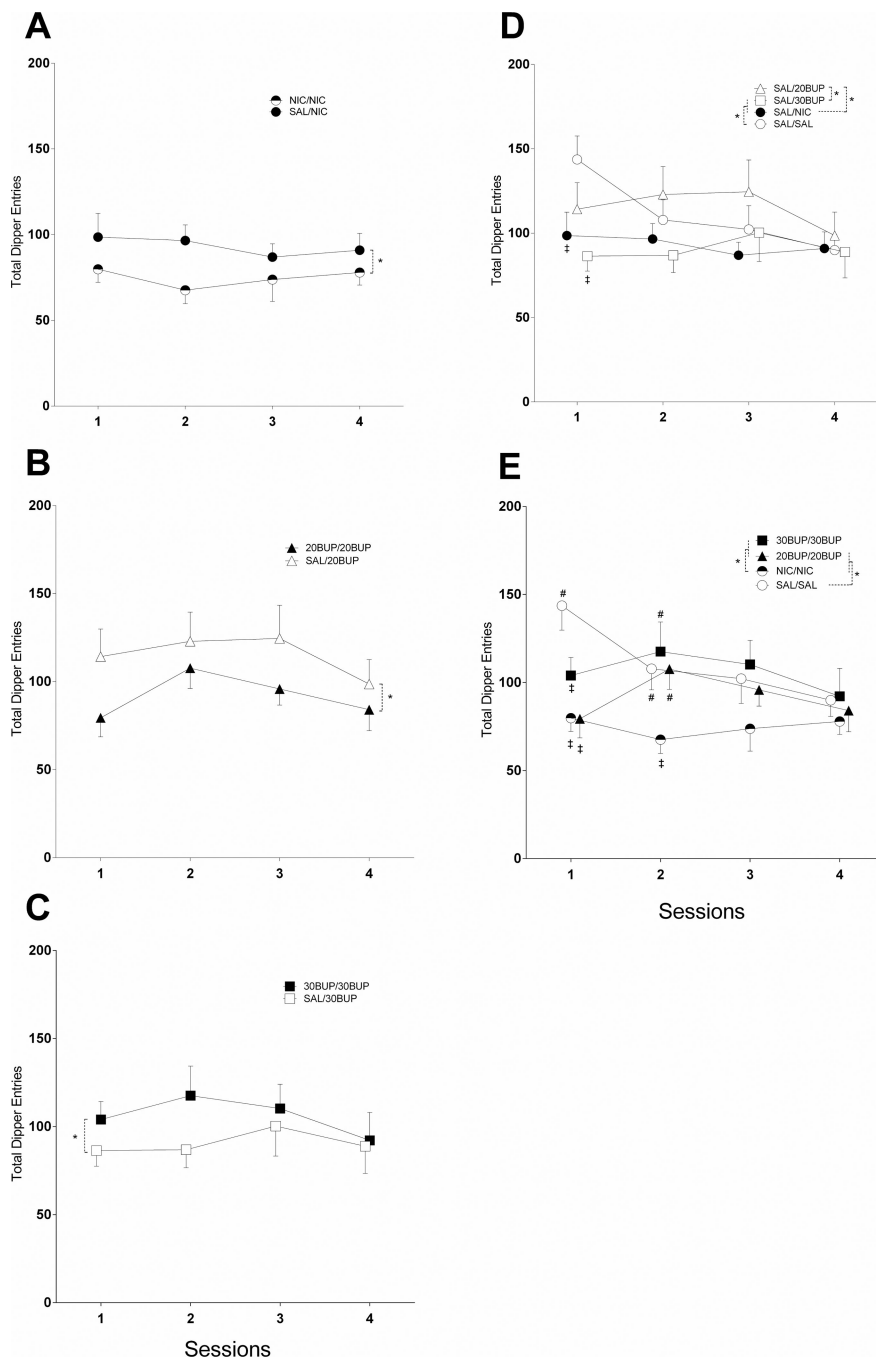


Fig. 6. A mean (\pm SEM) number of total dipper entries during transfer test 2 of Experiment 3. Each panel represents data from each of separate planned analyses: (A–C) comparison of groups receiving extinction ligand in either one or both of extinction phases, (D) comparison of groups with single extinction histories, (E) comparison of groups receiving extinction ligand in both extinction and re-extinction phases of the experiment. *Denotes overall group

differences. ‡Denotes differences from a corresponding SAL/SAL session. #Denotes difference from a corresponding SAL/NIC or a NIC/NIC sessions.

TABLE 1
Overall group means comparisons during Re-Extinction and Second Transfer Test

Phase	Group	NIC/NIC	SAL/NIC	SAL/SAL	20BUP/20BUP	SAL/20BUP	30BUP/30BUP	SAL/30BUP
Re-Extinction	NIC/NIC			***	**	***		
	SAL/NIC			***	***	***	**	
	SAL/SAL	***	***		***	***	***	***
	20BUP/20BUP	**	***	***		***		
	SAL/20BUP	***	***	***	***		***	***
	30BUP/30BUP	***	**	***	***	***		***
Second Transfer Test	NIC/NIC	**	***	***	*	***	***	
	SAL/NIC	**	*			***		
	SAL/SAL	***	*		*			**
	20BUP/20BUP	*						
	SAL/20BUP	***	***	***	**			***
	30BUP/30BUP	***						***
	SAL/30BUP		**	***		***		

Significance:

* p<0.05,

** p<0.01,

*** p<0.001