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Steven T. Pittenger
University of Nebraska-Lincoln

Rick A. Bevins
University of Nebraska-Lincoln, rbevins1@unl.edu

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Interoceptive conditioning in rats: Effects of using a single training dose or a set of 5 different doses of nicotine

Steven T. Pittenger and **Rick A. Bevins**
University of Nebraska-Lincoln, Department of Psychology, 238 Burnett Hall, Lincoln, NE 68588-0308, USA

Abstract

Interoceptive conditioning contributes to the tenacity of nicotine dependence. Previous research investigating nicotine as an interoceptive stimulus has typically employed administration of a single training dose of nicotine over an extended time. This approach has allowed for careful study of the nicotine stimulus. In humans, the nicotine stimulus is unlikely to be fixed across learning episodes. Thus, from a translational perspective, systematic variation of nicotine dose in training might better approximate interoceptive conditioning in humans. Notably, training with a class or set of discrete exteroceptive stimuli (e.g., different pictures of cars) produces interesting behavioral differences relative to training with a single stimulus. The present study sought to determine whether similar differences would occur if a set of nicotine stimuli were used in place of a single dose. To investigate this question, one group of male Sprague-Dawley rats was trained on a discriminated goal-tracking task with a set of nicotine doses (0.05, 0.125, 0.2, 0.275, and 0.35 mg/kg). A second group received the standard protocol of training with a single nicotine dose (0.2 mg/kg). On each nicotine session, there was intermittent access to liquid sucrose (26%) in a conditioning chamber. On intermixed saline sessions, sucrose was withheld. We examined acquisition, subsequent extinction, transfer of extinction, nicotine generalization, and mecamylamine blockade. Both groups reliably discriminated between nicotine and saline sessions, were sensitive to non-reinforcement, displayed transfer of extinction, demonstrated dose-dependent nicotine generalization, and responding was blocked by mecamylamine. There were no significant differences between the two groups. The unique nature of an interoceptive pharmacological stimulus and the challenges posed for studying the impact of training with a set of interoceptive stimuli are discussed.

Keywords

Pavlovian conditioning; rats; smoking; tobacco; drug discrimination

Introduction

Learning involving interoceptive drug stimuli likely contributes to the tenacity of tobacco dependence (Besheer *et al*, 2004; Bevins and Murray, 2011; Bevins and Palmatier, 2004; Glautier *et al*, 1996; Troisi, 2006). Elucidating the contribution of interoceptive conditioning

Correspondence concerning this article should be addressed to Rick A. Bevins, University of Nebraska-Lincoln, Department of Psychology, 238 Burnett Hall, Lincoln, NE 68588-0308. Phone: 402-472-3721 rbevins1@unl.edu.

to drug addiction is critical to advance our understanding and treatment of addiction (Alessi *et al*, 2002; Bevins *et al*, 2012; Clements *et al*, 1996; Glautier *et al*, 1996; Wise *et al*, 2008). One preclinical model used to study nicotine interoceptive conditioning is the discriminated goal-tracking task with rats (Besheer *et al*, 2004; Charntikov *et al*, 2012; Murray and Bevins, 2007a, b; Polewan *et al*, 2013; Reichel *et al*, 2010; Struthers *et al*, 2009; Wilkinson *et al*, 2006). In this task, systemic nicotine is repeatedly paired with an appetitive event such as intermittent access to sucrose. On intermixed sessions, saline is administered instead of nicotine and sucrose is not available. Following repeated pairings, the nicotine stimulus enters into a conditioned association with the sucrose (Pittenger and Bevins, 2013). This conditioned association is evidenced by greater 'anticipatory' head entries into the dipper receptacle [termed goal-tracking; (Farwell and Ayres, 1979)], following nicotine administration.

The discriminated goal-tracking task has been used for parametric assessment of behavioral factors affecting interoceptive conditioning with a nicotine stimulus. For example, the magnitude of the goal-tracking conditioned response controlled by the nicotine stimulus increases as the concentration of sucrose increases from 4% to 32% (w/v) (Murray *et al*, 2009b). Further, the acquisition of conditioning is accelerated by increasing the number of sucrose presentations per session (Wilkinson *et al*, 2006). Having a higher density of reinforcement in acquisition also produces a goal-tracking response that is more persistent when sucrose deliveries are ceased [i.e., extinction (Wilkinson *et al*, 2006)]. Murray and Bevins (2007b) used the discriminated goal-tracking task to investigate how the interoceptive effects of nicotine vary as a function of training dose. Rats were split into separate groups and trained with an interoceptive stimulus of 0.1, 0.2, or 0.4 mg/kg nicotine. Although the rate of acquisition was similar across doses, conditioned responding controlled by the higher dose of nicotine (0.4 mg/kg) was more resistant to extinction. In addition, when the time between nicotine administration and the assessment of conditioned responding was varied, the 0.4 mg/kg group displayed responding at longer injection-to-placement-intervals, indicating that higher doses continue to control behavior longer than the lower doses. Further, the nicotine dose-effect curves varied with training dose. That is, lower doses of nicotine evoked greater responding in rats trained with the lowest training dose [0.1 mg/kg; (Murray *et al*, 2007b)]. These findings indicate that interoceptive conditioning with the nicotine stimulus tends to follow general principles of learning (Bevins *et al*, 2011) and that salience (dose) of the nicotine stimulus is important (cf. Polewan *et al*, 2013).

The nicotine stimulus can be conceptualized as a complex internal stimulus, with different doses varying in perceptibility, as well as in the neurobiological elements that likely compose the stimulus (Bevins *et al*, 2012; Bevins *et al*, 2011). As briefly summarized in the previous paragraph, differences across doses of the nicotine stimulus manifest themselves in a variety of ways—persistence of responding in extinction, longer lasting control of responding, etc. Studies to date investigating interoceptive conditioning within the discriminated goal-tracking task have used a single unvarying dose of nicotine as the training stimulus. That is, if 0.4 mg/kg was selected as the training dose, then every acquisition and subsequent training session had this 0.4 mg/kg nicotine dose as the stimulus paired with access to sucrose. The present study examined if controlled variation of the nicotine stimulus (dose) that was paired with intermittent access to sucrose would alter expression of interoceptive conditioning relative to a standard condition that receives the same training dose throughout.

Varying the training dose of nicotine across sessions is of considerable interest for at least 2 reasons. First, from a translational perspective, a conditioning history with varying doses of nicotine may more closely resemble that experienced by humans. For instance, an individual

might get interrupted on a work break and only have had a couple puffs on a cigarette. Later in the day, the same person may have several cigarettes on the commute home from work. Whether or not such variation affects expression of interoceptive conditioning is a question of great interest that may help inform researchers on how to increase the translational relevance of current animal models of interoceptive drug conditioning. Second, from an empirical and theoretical perspective, there are data showing that what is learned can vary depending on whether one stimulus, or a class (set) of stimuli, are used in training (Bhatt, 1988; Herrnstein, 1979; Herrnstein and deVillers, 1980b; Herrnstein and Loveland, 1964; Siegel and Honig, 1970; Stemmer, 1980; Wasserman and Bhatt, 1992b; Watanabe *et al*, 1995). Past research in this area, however, has focused on distinct exteroceptive stimuli such as pictures of trees, people, and even paintings. Utilizing a class of interoceptive pharmacological stimuli has not received the same attention [see the Discussion for more on this point and on studies using early onset drug stimuli and drug mixtures; Greeley *et al*, (1984); Kim et al (1999); Mariathasan and Stolerman (1993); Mariathasan *et al*, (1996); Siegel, (2005); Siegel and Ramos, (2002); Stolerman *et al*, (2011); Stolerman and White, (1996); White and Stolerman (1996); Wise *et al*, (2008)]. The present research investigated how administration of a range of nicotine doses (0.05, 0.125, 0.2, 0.275, and 0.35 mg/kg) affected acquisition and altered subsequent extinction, nicotine generalization, and mecamylamine blockade when compared to a group that received a fixed dose (0.2 mg/kg) of nicotine during training.

2. Material and Methods

2.1 Subjects

Sixteen experimentally naive male Sprague-Dawley rats ordered at 275-299 g from Harlan (Indianapolis, IN, USA) were housed individually in clear polycarbonate cages (48.3 × 26.7 × 20.3 cm; length × width × height) lined with wood shavings. Rats had *ad libitum* access to water in home cages. Following acclimation to the colony, rats were handled for a minimum of 2 min per day for 3 consecutive days before access to food (Harlan Teklad Rodent Diet) was restricted to maintain rats at 85% of their free-feeding body weight. The colony room was temperature and humidity controlled. All experimental sessions were conducted during the light portion of a 12 hour light/dark cycle. Protocols were approved by the University of Nebraska-Lincoln Institutional Animal Care and Use Committee.

2.2 Apparatus

Eight conditioning chambers (ENV-008CT; Med Associates, Inc., Georgia, VT, USA) measuring 30.5 × 24.1 × 21.0 (length × width × height) cm were enclosed in sound and light attenuating cubicles fitted with an exhaust fan to provide airflow and mask noise. The front, back, and ceiling of the chambers were clear polycarbonate; side walls were aluminum. A recessed receptacle (5.2 × 5.2 × 3.8 cm; length × width × depth) was on one of the side walls. A dipper arm raised a 0.1-ml cup of sucrose (26% w/v) into the receptacle. To record head entries into the dipper receptacle, each chamber was equipped with an emitter/detector unit placed 1.2 cm into the recessed receptacle and 3 cm above the rod floor of the chamber. A personal computer with Med Associates interface and software (Med-PC for Windows, version IV) controlled sucrose deliveries and recorded dipper entries.

2.2 Drugs

(-)-Nicotine hydrogen tartrate and mecamylamine hydrochloride were purchased from Sigma (St. Louis, MO, USA). Nicotine was dissolved in 0.9% saline and adjusted to a pH of 7.0 ± 0.2 using a dilute NaOH solution. Nicotine doses are reported as the base form. Mecamylamine was dissolved in saline; doses are reported as the salt form. All injections were administered subcutaneous (SC) at a volume of 1 ml/kg.

2.3 Acquisition

To minimize the initial locomotor suppressant effects of nicotine, rats received daily injections of 0.4 mg/kg nicotine in their home cages for the 3 days immediately before the start of the experiment (Bevins *et al*, 2001). At the start of the experiment, rats were divided into 2 groups [stable-dose (StD) or varying-dose (VD)] before the start of acquisition sessions. Discrimination training consisted of 40 daily sessions; 20 nicotine sessions and 20 saline sessions were intermixed. The order of the sessions was pseudo-randomly assigned with the stipulation that rats received no more than 2 consecutive days with the same type of session. Nicotine sessions for the StD group consisted of a 0.2 mg/kg SC nicotine injection 5 min before placement in the chamber for a 20-min session. Nicotine sessions for the VD group consisted of a SC injection with 0.05, 0.125, 0.2, 0.275, or 0.35 mg/kg nicotine. The order of dose was randomly assigned for each rat with the stipulation that each of the 5 doses would be administered before a dose was repeated. Thus, each rat received each dose 4 times over the 20 nicotine sessions. For both groups, there were 36 deliveries of 26% (w/v) sucrose (4 s each) on nicotine sessions. The first sucrose delivery of the session ranged from 124 to 152 s with an average of 137 s; subsequent sucrose deliveries were presented on average every 25 s (range = 4 to 80 s). On saline sessions, rats in both groups received a SC saline injection 5 min before placement in the chamber; sucrose was withheld during saline sessions.

2.4 Extinction

Starting 24 hours after the last acquisition day were 8 daily extinction sessions. For all rats, extinction sessions consisted of a 0.2 mg/kg nicotine injection (SC) 5 min before a 20-min session in which no sucrose was available. The 0.2 mg/kg dose was chosen as it was the training dose for the StD group and the average of all the doses for the VD group. For extinction, one might expect greater persistence of responding in the StD group because they had an appetitive conditioning history consisting of 20 sessions with the 0.2 mg/kg dose (i.e., the extinction dose). In contrast, the VD group received the 0.2 mg/kg dose of nicotine on just 4 of the 20 conditioning sessions. The remaining 16 conditioning sessions may involve different neuropharmacological (stimulus) elements from the 0.2 mg/kg extinction dose (Polewan *et al*, 2013).

2.5 Nicotine Challenge

On the day following the last extinction session, all rats were given nicotine challenge tests. During the challenge, rats were administered 0.35 mg/kg nicotine before a 20-min session in the conditioning chamber with no sucrose available. The 0.35 mg/kg dose was selected because it was the highest dose in the set of nicotine stimuli given to the VD group and it may differ in its neuropharmacological (stimulus) elements from the extinguished 0.2 mg/kg dose. If so, we expect conditioned responding to increase more in group VD when tested with the 0.35 mg/kg dose of nicotine. Challenge sessions occurred daily for 3 consecutive days.

2.6 Reacquisition

Rats were then retrained for 10 reacquisition sessions (5 nicotine and 5 saline). Sessions were identical to those during acquisition. The StD group once again received 0.2 mg/kg before each nicotine session, whereas each rat in the VD group received 0.05, 0.125, 0.2, 0.275, and 0.35 mg/kg nicotine in a random order.

2.7 Nicotine Generalization Tests

Following reacquisition, a nicotine generalization curve was generated. Testing occurred on the 5^{th} day of a 5 day cycle. On days 1 to 4 of the cycle, rats received 2 standard nicotine

and 2 standard saline sessions. The nicotine dose for the StD group remained 0.2 mg/kg, whereas the dose for the VD group continued to receive a dose from the set of doses; order was again randomly assigned. To qualify for testing, rats had to meet the following criteria on days 1-4 of the cycle: dipper entries before the first sucrose delivery on each nicotine session must be 0.01 entries/sec higher than dipper entries during an equivalent amount of time on both saline days (Besheer *et al*, 2004). If a rat did not reach criteria, then it remained in the home cage on the test day. If criteria were met, rats received an injection of nicotine [0.0 (saline), 0.0125, 0.025, 0.05, or 0.1] 5 min before a 4-min test session. Dose order was randomly assigned. No sucrose was available in the chamber during test sessions. When a rat completed its generalization curve, it continued receiving standard conditioning sessions on days 1 to 4 of the cycle, but remained in its home cage on day 5. Testing continued until all rats had been tested with all 5 doses of nicotine.

2.8 Mecamylamine Antagonism of 0.2 mg/kg Nicotine

Following completion of nicotine generalization testing, we assessed how readily the stimulus effects of nicotine could be blocked by mecamylamine, a central and peripheral nicotinic acetylcholine receptor (nAChR) antagonist (Martin *et al*, 1989). The testing cycle was identical to that during nicotine generalization testing, with one exception. On test days, a dose of mecamylamine [0.0 (saline), 0.1, 0.25, 0.5, 0.75, or 1.0 mg/kg] was given 20 min before a 0.2 mg/kg injection of nicotine. Training and testing cycles were again conducted until all rats had been tested with the 5 doses of mecamylamine.

2.9 Mecamylamine Antagonism of 0.1 mg/kg Nicotine

Following completion of the previous phase, rats were tested for mecamylamine blockade of the stimulus effects of nicotine using a lower dose of nicotine; procedures were identical to those during the first mecamylamine antagonism tests except a 0.1 mg/kg dose of nicotine was used on test days.

2.10 Dependent Measures

Head entries into the recessed sucrose receptacle (i.e., dipper entries) were recorded on all sessions. A rate measure of dipper entries per sec before the first sucrose presentation (or an equivalent amount of time for saline sessions) was calculated for acquisition. Using only dipper entries before any access to sucrose avoids any influence of sucrose exposure on this measure of learning. During acquisition, the primary dependent measure was a dipper entries per sec difference score. This measure was calculated by subtracting dipper entries per sec during the saline session from dipper entries per sec during the corresponding nicotine session. The dependent measure for generalization and antagonist tests was total dipper entries during the 4-min test.

2.11 Statistical Analysis

In acquisition and reacquisition, dipper entries difference scores were analyzed with two-way repeated measure analysis of variance (ANOVAs) with Group (StD vs. VD) as a between-subjects factor and Session as the repeated measure. To check for discrimination between nicotine vs. saline sessions in acquisition and reacquisition, dipper entries per sec were analyzed with a two-way within-subjects repeated measure analysis of variance (ANOVA) with Drug (nicotine vs. saline) as a within-subjects factor and Session as the repeated measure for both groups. Total dipper entries during extinction and nicotine challenge (20-min sessions) were again analyzed by two-way repeated measures ANOVAs with Group as the between-subjects factor and Session as the repeated measure. Generalization and antagonism tests were analyzed by two-way repeated measures

ANOVAs with Group as one factor and Dose as the repeated measure. Bonferonni's post-hoc analysis was used to declare statistical significance when appropriate.

3. Results

3.1 Acquisition

Figure 1A shows the mean (± SEM) dipper entries per sec differences scores, as well as individual values across acquisition. Individual data are displayed to reveal the range of values with in each group. Data analysis revealed a significant main effect of Session [$F(19,266)=8.28$, $p<0.001$], but there was no main effect of Group or Group × Session interaction (Fs<1) indicating that acquisition of the discrimination did not differ between groups that were trained with a varying or constant dose of nicotine. Figure 1B shows the dipper entries during saline and nicotine sessions in acquisition for the StD group. There was main effects of Drug [$F(1,19)=44.38$, $p<0.001$] and Session [$F(19,266)=3.62$, $p<0.001$], as well as a significant Drug × Session interaction [$F(19,266)=3.87$, $p<0.001$]. Post-hoc tests revealed a significant increase in responding following administration of nicotine (compared to saline) on sessions 6 to 8 and 10 to 20. Figure 1C shows the dipper entries for the saline and nicotine sessions for the VD group. There was again main effects of Drug [$F(1,19)=64.43$, $p<0.001$] and Session [$F(19,266)=2.52$ $p<0.001$], as well as a significant Drug × Session interaction [$F(19,266)=2.93$, $p<0.001$]. Conditioned responding following nicotine administration was significantly increased on session 8 and 9, 11, 13 to 15 and 17 to 20.

3.2 Extinction

Figure 2A shows total dipper entries across extinction sessions with 0.2 mg/kg nicotine. There was a main effect of Session [$F(7,90)=27.81$, $p<0.001$] indicating a decrease in conditioned responding across sessions. The main effect of Group [$F(1,7)=1.13$, $p=0.30$] and the Group × Session interaction were not significant [F<1]. This data pattern indicates that the persistence of responding in the absence of the sucrose reinforcer was not affected by whether the training dose was constant or variable.

3.3 Nicotine Challenge

Figure 2B shows total dipper entries during the nicotine challenge with 0.35 mg/kg nicotine. There was a main effect of Session [$F(2,28)=10.31$, $p<0.001$], but the main effect of Group and the Group × Session interaction were not significant [Fs<1].

3.4 Reacquisition

Analysis of differences scores for reacquisition (data not shown) revealed a main effect of Session [$F(4,56)=8.09$, $p<0.001$]. There was no main effect of Group [$F(1,4)=2.83$, $p=0.11$] or Group × Session interaction [F<1] indicating comparable reacquisition between the groups. Examination of dipper entries for the StD group showed significant main effects of Drug [$F(1,4)=41.32$, $p<0.001$] and Session [$F(4,56)=3.29$, $p<0.1$], as well as a significant interaction [$F(4,56)=2.59$, $p<0.05$], with nicotine session responding (compared to saline) significantly higher on all reacquisition sessions. Analysis of dipper entries for the VD group also found significant main effects of Drug [$F(1,4)=39.42$, $p<0.001$], Session [$F(4,56)=3.57$, $p<0.01$], and a significant interaction [$F(4,56)=4.04$, $p<0.01$], with nicotine-evoked responding significantly higher on reacquisition sessions 3 to 5.

3.5 Nicotine Generalization

Figure 3A shows total dipper entries during nicotine generalization tests. Analysis revealed a main effect of Dose [$F(4,56)=17.31$, $p<.001$], but no effect of Group and no significant

Group × Dose interaction [Fs<1]. The 0.1 mg/kg nicotine dose evoked significantly more responding than 0 (saline), 0.0125, 0.025, or 0.05 mg/kg nicotine. The 0.05 mg/kg nicotine dose evoked responding significantly higher than the 0 (saline) or 0.0125 mg/kg nicotine.

3.6 Mecamylamine Antagonism of 0.2 mg/kg Nicotine

Figure 3B shows total dipper entries during mecamylamine antagonism of 0.2 mg/kg nicotine. There was a main effect of mecamylamine Dose [$F(5,70)=7.04$, $p<0.001$] indicating a dose-dependent blockade of conditioned responding by mecamylamine. There was no main effect of Group or Group × Dose interaction [Fs<1]. Post-hoc analyses on the main effect of Dose revealed that pretreatment with 0.75 and 1 mg/kg mecamylamine significantly decreased nicotine-evoked responding compared to saline pretreatment. Mecamylamine was not tested alone in this study. However, previous published research from our lab has demonstrated that mecamylamine does not alter general chamber activity, making a motor impairment account for the reduction in goal-tracking here unlikely (Besheer *et al*, 2004; Struthers *et al*, 2009; Wilkinson *et al*, 2010).

3.7 Mecamylamine Antagonism of 0.1 mg/kg Nicotine

Figure 3C shows total dipper entries during mecamylamine antagonism of 0.1 mg/kg nicotine. Again, there was a significant main effect of mecamylamine Dose [$F(5,70)=9.15$, $p<0.001$], but there was no effect of Group and no Group × Dose interaction [Fs<1]. Post-hoc analyses on the main effect of Dose revealed that pretreatment with 0.5, 0.75, and 1 mg/kg mecamylamine significantly decreased nicotine-evoked responding compared to the 0 (saline).

4. Discussion

Elucidating the role of learning involving the interoceptive stimulus effects of drugs is needed for a more comprehensive understanding of addiction (Bevins *et al*, 2012; Bevins *et al*, 2011; Glautier *et al*, 1996; Stolerman *et al*, 2011). The present study investigated how utilizing a class (set) of nicotine stimuli would affect interoceptive conditioning relative to a group that had the same nicotine dose throughout training. To do so, one group of rats with a range of nicotine doses (0.05, 0.125, 0.2, 0.275, and 0.35 mg/kg) and a second group with a fixed dose (0.2 mg/kg) were trained using the nicotine discriminated goal-tracking task. Acquisition of goal-tracking was similar between groups, with both groups discriminating between saline and nicotine. In concordance with previous studies in our laboratory, the nicotine stimulus was sensitive to non-reinforcement, showing attenuated responding across extinction sessions (Besheer *et al*, 2004; Murray *et al*, 2007b; Reichel *et al*, 2010; Struthers *et al*, 2009; Wilkinson *et al*, 2006). This extinction with 0.2 mg/kg transferred to a higher dose of nicotine (0.35 mg/kg). A similar result was found by Polewan et al. (2013) using a 0.4 mg/kg training dose. As in previous studies, stimulus control over conditioned responding was dose-dependent; higher doses of nicotine evoked increased goal-tracking relative to the lower doses (Besheer *et al*, 2004; Murray *et al*, 2007a, b; Murray *et al*, 2009c; Reichel *et al*, 2007; Reichel *et al*, 2010; Struthers *et al*, 2009). Further, mecamylamine dose-dependently blocked goal-tracking evoked by 0.2 and 0.1 mg/kg nicotine; as mecamylamine dose increased, goal-tracking decreased. The 0.1 mg/kg dose of nicotine was blocked by a lower dose of mecamylamine, suggesting that the salience or perceptibility was lower for the 0.1 mg/kg dose than the 0.2 mg/kg dose. This extends previous research that used mecamylamine to block responding evoked by 0.4 mg/kg nicotine (Besheer *et al*, 2004; Murray *et al*, 2007b; Murray and Bevins, 2009a; Struthers *et al*, 2009) and research that blocked goal-tracking by an intravenous nicotine stimulus (Murray *et al*, 2009a). The data pattern across these tests was similar regardless of training condition (i.e., group StD vs. group VD). This similarity suggests that under the current experimental protocol using a

constant versus a varying dose of nicotine, variation of the dose did not produce a difference in interoceptive conditioning or our testing procedures were not sufficiently sensitive to detect existing differences.

Research on the categorization of stimuli in animal models predicts that differences should occur when a set or class of stimuli are used (Bhatt, 1988; Herrnstein, 1979; Herrnstein and deVillers, 1980a; Herrnstein *et al*, 1964; Siegel *et al*, 1970; Stemmer, 1980; Wasserman and Bhatt, 1992a; Watanabe *et al*, 1995). In general, as the number of stimuli in a category increases, acquisition is retarded and generalization during transfer (cf. substitution tests) tests is improved. This effect is highlighted nicely by Bhatt's work (1988). Three groups of pigeons were trained to peck a corresponding key when shown a cat, car, flower, or chair. One group was trained with a single example of each picture. A second group was trained using 4 different exemplars of each category; the third group received 12 different pictures of each category. The group that trained using only a single picture demonstrated the quickest acquisition of correct key choice and was the most accurate. However, this single picture group displayed poor generalization to new pictures of cats, cars, flowers, and chairs (Bhatt, 1988; Wasserman *et al*, 1992a). In contrast, the group trained with 12 examples of each category exhibited significant retardation of acquisition, but there was better generalization when tested with novel pictures (Wasserman *et al*, 1992a).

The brief discussion of research with classes of exteroceptive stimuli in the previous paragraph begs the question of why a similar outcome did not occur here. If the reason lies with the protocol not producing difference in interoceptive conditioning, then the possible parametric manipulations are numerous. Perhaps there were not enough exemplars. Maybe the dose range was too narrow (i.e., go higher than 0.35 mg/kd). Or, the training should have continued much longer before testing. Maybe more non-reinforced stimuli are needed; recall that only saline was used. Or, the nicotine stimuli used were too close together. Perhaps using less optimal training parameters (e.g., fewer sucrose deliveries per session) would reveal differences. If the reason lies with insensitivity of testing procedures, then alternative protocols could also be used in future research. For example, a more selective antagonist could be used such as DhβE (Struthers *et al*, 2009). Rather than antagonist tests, perhaps substitution tests with ligands that share some of the neurobiological stimulus elements with nicotine would reveal differences (Reichel *et al*, 2010).

When reflecting on potential reasons and future experiments, one should keep in mind the qualities of interoceptive drug stimuli that might pose design challenges (Bevins & Murray, 2011; Bevins et al., 2012). A pharmacological stimulus is often quite complex, differing somewhat from more traditional, discrete, exteroceptive cues such as lights or tones. When nicotine is administered, pharmacokinetic processes begin to distribute nicotine throughout the body—central and peripheral receptors are affected. Metabolic processes also start, eventually resulting in excretion of nicotine and its metabolites from the body. Note that the effect of a drug peripherally or centrally will further depend on complex pharmacodynamic processes (Brunzell and Picciotto, 2009; Placzek and Dani, 2009). Combined, these processes are affecting what the perceptible interoceptive nicotine stimulus may be over time. So, the 0.2 mg/kg dose administered to the StD group is experimentally fixed (i.e., the same dose administered each nicotine session), yet the perceptible interoceptive effects of nicotine are, in practice, varying. With this notion in mind, the 0.o5 and 0.125 mg/kg nicotine stimulus of the VD group, albeit not paired with sucrose for entire sessions, would at least be presented to the StD group on every nicotine training session. Thus, the StD group, like the VD group, was presented with a set of stimuli; the set was just not as large as it did not include the higher nicotine doses.

This analysis has empirical support from a series of clever studies by Siegel and his colleagues. In those studies, they established that early interoceptive drug stimuli differ from the latter cues and that these early onset stimuli can function as a signal for later onset of the primary drug effect and evoke a compensatory conditioned response (Kim *et al*, 1999; Siegel, 2005; Siegel *et al*, 2002). The finding, for example, that a low dose of morphine can acquire control over a conditioned analgesic response opposing that of the higher dose (Kim *et al*, 1999) is important for the present discussion. That is, it provides support for the idea that a low dose of a drug has different stimulus effects than a higher dose of the same drug and that these stimuli are discriminably different as evidence by control of different behavior. Similar support for nicotine from the discriminated goal-tracking task was discussed in the Introduction (e.g., Murray *et al*, 2007b; Polewan *et al*, 2013). Additional support from within the drug discrimination literature comes from research with a drug mixture such as midazolam plus nicotine (Mariathasan *et al*, 1993; Stolerman *et al*, 1996; White *et al*, 1996). Under appropriate testing conditions, acquired control over behavior for the "midazolam-like" stimulus element can be separated from those of the "nicotine-like" stimulus elements.

Early on, Pavlov (1927) recognized the import of conditioning involving interoceptive stimuli to diseases and psychopathologies. For addiction, drug effects are an obvious and important source of interoceptive stimuli. Other sources may include interoception related to withdrawal, stress, cognitive deficits, etc. (Koob, 2004). Continued efforts to better understand the role of learning involving interoceptive stimuli are needed for a more comprehensive theory of drug addiction (Bevins *et al*, 2011; Bevins *et al*, 2004; Paulus and Stewart, 2013; Wise *et al*, 2008) and improved intervention and prevention approaches (Alessi *et al*, 2002; Clements *et al*, 1996; Glautier *et al*, 1996).

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Highlights

- Rats had discrimination training with 1 or 5 nicotine doses.
- Both groups learned the discrimination and showed a typical extinction pattern.
- Extinction transfers to a higher nicotine dose and mecamylamine blocked responding.
- No differences in performance were found between the two groups.
- The results may be attributable to the unique qualities of a drug stimulus.

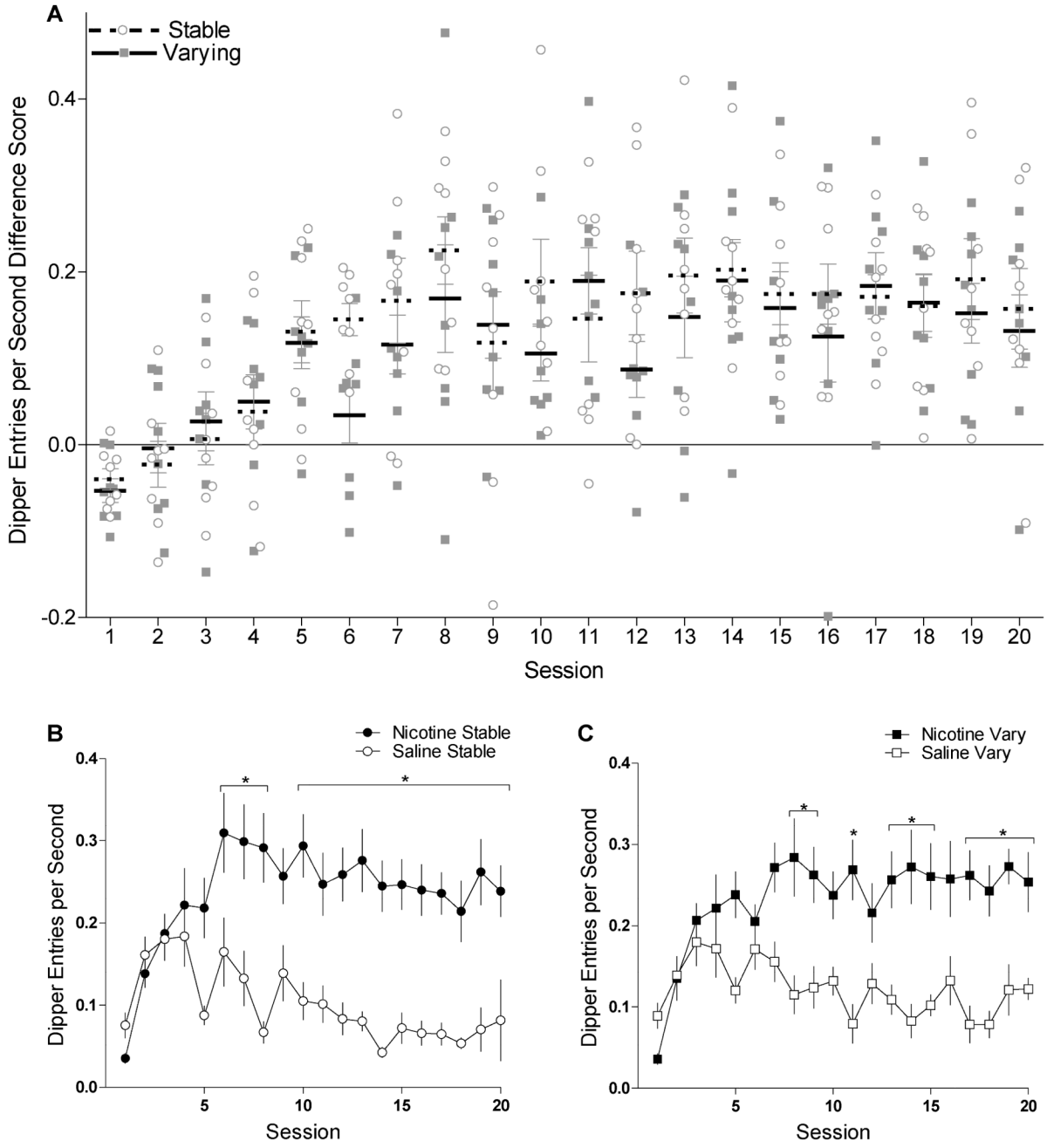

Figure 1.

Panel **A** shows dipper entry rate differences scores between nicotine and saline days during acquisition [individual and mean scores (± SEM) shown]. A score of 0 (solid line) would represent no difference of responding between nicotine and saline days for the session. Panel **B** shows dipper entries per second (±SEM) before the first sucrose delivery (nicotine sessions) or during a comparable time (saline sessions) for the StD group. Panel **C** shows dipper entries per second (±SEM) before the first sucrose delivery (nicotine sessions) or during a comparable time (saline sessions) for the VD group. * denotes significant differences ($p<o.05$) between the nicotine and saline days in a session.

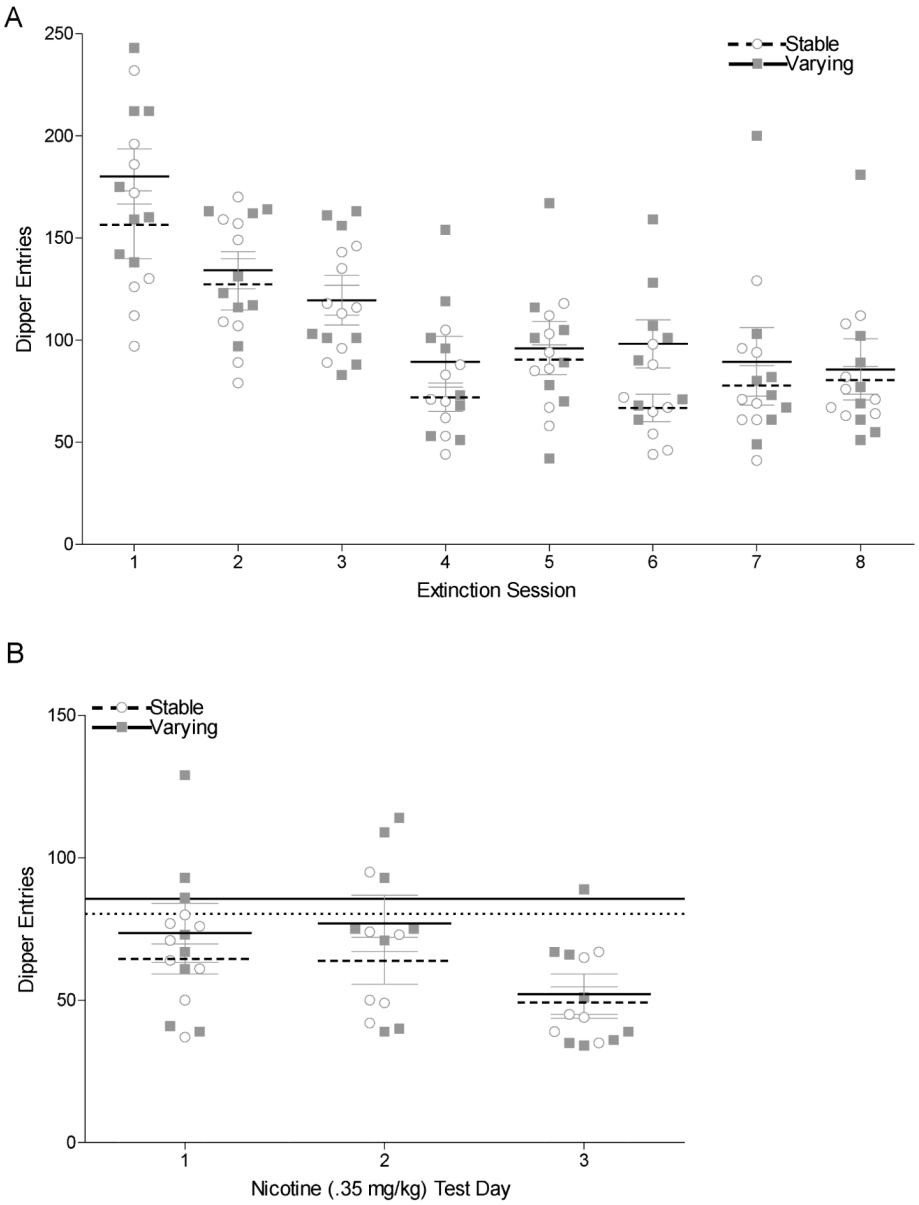

Figure 2.

Panel **A** shows mean (± SEM) and individual total dipper entries during extinction. Panel **B** shows mean (± SEM) and individual total dipper entries during nicotine challenge tests with 0.35 mg/kg nicotine. The solid and dotted line represent responding during the final extinction session for VD and STD group respectively.

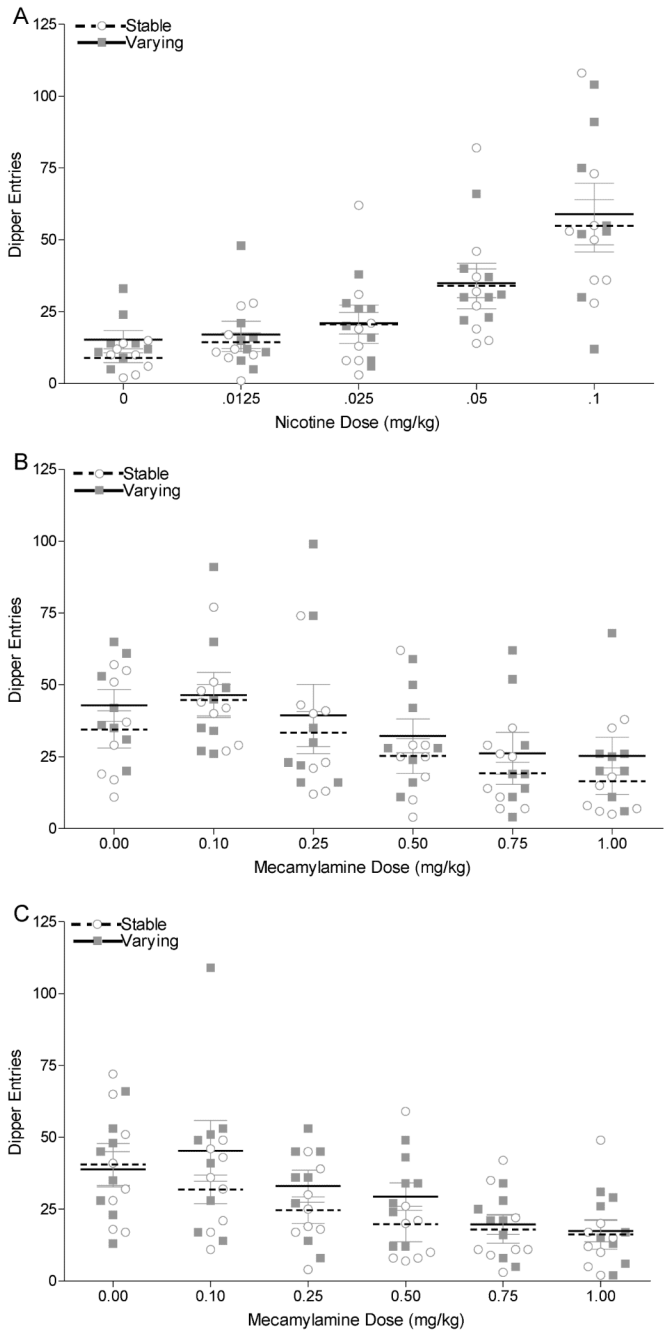

Figure 3.

Panel **A** displays mean (±SEM) and individual dipper entries during nicotine generalization testing. Panel **B** shows mean (±SEM) and individual total dipper entries during nAChR antagonism of a **0.2 mg/kg** dose of nicotine. Panel **C** shows mean (±SEM) and individual total dipper entries during nAChR antagonism of a **0.1 mg/kg** dose of nicotine.