Interoceptive conditioning with the nicotine stimulus: extinction learning as a method for assessing stimulus similarity across doses

Robert J. Polewan, Stephanie A. Savala and Rick A. Bevins

Interoceptive conditioning involving the nicotine stimulus likely contributes to chronic tobacco use. To better understand the nature of this interoceptive conditioning, we compared generalization during repeated extinction with generalization in a 'transfer of extinction' test using a wide range of test doses. Rats were first trained in the discriminated goal-tracking task in which nicotine (0.2 or 0.4 mg/kg), but not saline, was paired with repeated intermittent access to sucrose. Across sessions, nicotine acquired control of approach behavior directed at the location of previous sucrose deliveries. Extinction followed with eight 20-min sessions without sucrose access; extinction doses of nicotine ranged from 0.05 to 0.6 mg/kg. In rats trained with 0.4 mg/kg, the 0.1, 0.2, and 0.6 mg/kg doses evoked comparable responding across extinction sessions; substitution was only partial at 0.05 and 0.075 mg/kg (i.e. above saline controls, but less than the training dose). With the 0.2 mg/kg training dose, complete generalization was seen only at the 0.1 and 0.4 mg/kg doses. After extinction, rats were given a transfer test with their training dose. Rats trained with 0.4 mg/kg showed full transfer of extinction learning with 0.1, 0.2, and 0.6 mg/kg (i.e. responding comparable with extinction with the training dose). Partial transfer was observed at 0.075 mg/kg. With the 0.2 mg/kg nicotine dose, only 0.4 mg/kg fully generalized; 0.075, 0.1, and 0.6 mg/kg showed partial transfer. Extinction with 0.05 mg/kg dose did not show transfer to either training dose. These findings indicated that conclusions regarding stimulus similarity across nicotine doses can vary with testing protocol. ***Behavioural Pharmacology*** **24:45–54 © 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Smoking is the leading cause of preventable death in the USA with over 443 000 people dying per year (Center for Disease Control, 2009). Approximately 20.6% or 46 million US adults over the age of 18 smoke cigarettes. The health and economic impact of chronic tobacco use and nicotine dependence has been well documented (Center for Disease Control, 2011). Although behavioral and/or pharmacological approaches have improved long-term smoking session rates, a majority of individuals that receive treatment still relapse within a year (Garret *et al.*, 2001; Schröter *et al.*, 2006; Rose, 2009). Associative learning processes involving nicotine, the primary addictive constituent of tobacco products, likely contributes to the tenacity of the addiction and the high relapse rates (Perkins *et al.*, 1999; Caggiula *et al.*, 2001; Bevins and Palmatier, 2004; Benowitz, 2008; Perkins, 2009). Further understanding of the conditioning and learning processes involving nicotine will likely reveal improved or new strategies for treatment.

Along these lines, the physiological effects of nicotine serve as a conditioned stimulus (CS) for when an appetitive event or unconditioned stimulus (US) will co-occur (e.g. Besheer *et al.*, 2004; Bevins and Palmatier, 2004; Wilkinson *et al.*, 2006; Murray and Bevins, 2007b; Bevins, 2009; Murray and Bevins, 2009; Reichel *et al.*, 2010). More specifically, in a discriminated goal-tracking (DGT) task, rats receive intermixed sessions in which they are injected subcutaneously with either nicotine or saline. During nicotine sessions, rats receive intermittent access to liquid sucrose regardless of their behavior; sucrose is not available on saline sessions. The interoceptive stimulus effects of nicotine come to control an 'anticipatory' increase in head entries into the dipper receptacle relative to saline. This behavior directed at the location of previous sucrose deliveries is referred to as 'goal-tracking' (Boakes, 1977; Fawell and Ayres, 1979) and is widely used as a measure of conditioning (Delamater, 1995; Bouton and Sunsay, 2003; Rescorla, 2006; Costa and Boakes, 2009; Danna and Elmer, 2010). Like conditioning with exteroceptive CSs, subsequent repeated nonreinforced presentations of the nicotine CS (i.e. extinction) produces a progressive decrease in the conditioned goal-tracking response (Besheer *et al.*, 2004; Wilkinson *et al.*,

All Med-PC programs used in this research are available upon request in an updated form. Those requests, as well as requests for reprints, should be sent to Rick A. Bevins at rbevins1@unl.edu.

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2006; Murray and Bevins, 2007b; Murray and Bevins, 2009). Behavioral and neurobiological research has provided copious evidence that extinction is a new form of learning that counteracts or interferes with the expression of the previously learned conditioned response (CR) (Pavlov, 1927; Konorski, 1948; Bouton, 1991; Bouton, 1993; Rescorla, 1997; Bevins *et al.*, 1999; Rauhut *et al.*, 2001; Rescorla, 2001; Bouton, 2002; Davis and Myers, 2002; Delamater, 2004; Quirk and Mueller, 2008).

Reichel *et al.* (2010) took this notion that attenuated responding in extinction is new competing learning and assessed whether expression of interoceptive conditioning with nicotine could be weakened if extinction was conducted with a ligand that shared stimulus effects with nicotine (see also Bevins *et al.*, 2012). To this end, Reichel *et al.* (2010) used ABT-418, nornicotine, and varenicline as the ligands to replace nicotine in extinction. These ligands were selected because they prompt conditioned responding comparable with the nicotine CS in standard 4-min generalization (substitution) tests that presumably minimize learning across repeated testing of different doses. These findings parallel the operant drug discrimination literature with ABT-418 (Damaj *et al.*, 1995), nornicotine (Goldberg *et al.*, 1989), and varenicline (Paterson *et al.*, 2010; Jutkiewicz *et al.*, 2011) substituting fully for the discriminative stimulus effects of nicotine (see Smith *et al.*, 2007; LeSage *et al.*, 2009 for partial substitution with varenicline).

Interestingly, when these ligands (i.e. ABT-418, varenicline, and nornicotine) supplanted nicotine in an extinction phase, conclusions regarding how similar they were to nicotine changed relative to 4-min substitution tests. Briefly, across six 20-min extinction sessions (1/day), varenicline and nornicotine evoked only a partial CR; ABT-418 did not evoke any nicotine-like responding after about 10 min into the first extinction session. Following 24 h after the last extinction session was a challenge test in which rats were tested with the training dose of nicotine (0.4 mg/kg). Exposure to repeated extinction sessions following administration of varenicline or nornicotine produced diminished responding to nicotine; administration of ABT-418 had no effect on responding. We have come to refer to this generalization of extinction effect as 'transfer of extinction learning' (Reichel *et al.*, 2010; Bevins *et al.*, 2012).

Most closely related to the present research, Reichel *et al.* (2010) also examined whether a low dose of nicotine (0.05 mg/kg) would be effective at attenuating responding to a 0.4 mg/kg training dose in this transfer of extinction task. The 0.05 mg/kg dose was selected for that experiment because it prompted partial substitution for the nicotine CS in a wide range of studies. Further, this dose is on the lower end of the range of median effective doses (ED_{50}) reported when the 0.4 mg/kg nicotine served as the CS (range = 0.033–0.099 mg/kg; mean = 0.072 mg/kg; see Murray *et al.*, 2007a, 2007b; Struthers *et al.*, 2009; Murray *et al.*, 2010; Reichel *et al.*, 2010; Wilkinson *et al.*, 2010; Dion *et al.*, 2012). The 0.05 mg/kg dose of nicotine evoked a partial goal-tracking CR in the first five of the six 20-min extinction sessions. Despite the partial CR throughout most of the extinction phase, conditioned responding evoked by the 0.4 mg/kg training dose was not significantly attenuated in the transfer test (see fig. 2 of Reichel *et al.*, 2010). The goal of the present research was to extend the research of Reichel *et al.* (2010) by using this transfer of extinction approach to provide a detailed and parametric assessment of the similarity of the interoceptive stimulus effect of a broad range of nicotine doses. To this end, rats were trained with either 0.2 or 0.4 mg/kg nicotine as the CS. They then received saline or nicotine (0.05, 0.075, 0.1, 0.2, 0.4, or 0.6 mg/kg) repeatedly without sucrose. Following this extinction, rats were tested with either 0.2 or 0.4 mg/kg nicotine, without sucrose, to ascertain whether such a history of repeated extinction affected responding controlled by the training dose (stimulus).

Methods

Subjects

Male Sprague–Dawley rats (*Rattus norvegicus*), 70–90 days old (275–290 g upon arrival), were obtained from Harlan (Indianapolis, Indiana, USA). Rats were individually housed in clear 48.3 × 26.7 × 20.3 cm ($l \times w \times h$) polycarbonate cages lined with aspen shavings. All rats received free access to water; access to food (Harlan Tekland Rodent Diet) was restricted to maintain individual rats at 85% of their free-feeding weight. The colony room was temperature-controlled and humidity-controlled and on a 12-h light : dark cycle (lights on at 06 : 00). All sessions occurred during the light cycle. Experimental 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) measuring 30.5 × 24.1 × 21.0 cm ($l \times w \times h$) were used in this study. The chamber side walls were aluminum; the front and back walls and the ceiling were clear polycarbonate. Chamber floors consisted of 19 stainless steel rods. Each chamber was equipped with a recessed receptacle (5.2 × 5.2 × 3.8 cm; $l \times w \times d$) on the right side wall. A dipper arm raised a 0.1 ml cup of 26% sucrose (w/v) into the receptacle. An infrared emitter/detector unit, 1.2 cm into the receptacle and 3 cm above the chamber floor, monitored head movement into the receptacle. Chambers were individually enclosed in a light-attenuating and sound-attenuating cubicle fitted with a fan to provide airflow and mask noise. A personal computer with Med Associates interface and software (Med-PC for

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Windows, version IV; Med Associates Inc.) controlled sucrose deliveries and recorded dipper entries.

Experiment 1: transfer of extinction after training with 0.4 mg/kg nicotine

Preliminaries

Rats ($n = 112$) were handled for at least 2 min a day for 3 consecutive days. On the last day of handling, rats were weighed to establish a free-feeding weight for each rat. Access to food was then restricted to gradually decrease and then maintain each rat at 85% of its free-feeding weight. Twenty-four hours after the last day of handling, rats were injected subcutaneously with 0.4 mg/kg of nicotine in the home cage for 3 consecutive days to attenuate the initial locomotor suppressant effects of nicotine (cf. Bevins and Palmatier, 2003). Discrimination training began the following day.

Discrimination training

Discrimination training occurred for 32 consecutive days. All rats received intermixed nicotine and saline sessions. On nicotine sessions, rats were injected subcutaneously with nicotine (0.4 mg/kg) 5 min before placement in the conditioning chambers. Each session lasted for 20 min and there were 36 presentations of the sucrose US, 4 s each. The temporal pattern of sucrose presentation varied across sessions so that the average interval between deliveries was 25 s (range 4–80 s); the average interval before the first sucrose delivery was 137 s (range 124–152 s). On saline sessions, rats were injected subcutaneously with 0.9% saline 5 min before the 20-min session. There was no access to sucrose on saline sessions. Sucrose was in the trough even on saline sessions. Sessions were arranged quasi-randomly with the restriction that no more than two of a certain session type (nicotine or saline) occurred in a row.

Extinction training

Extinction began 24 h after the last discrimination training session. Before the first extinction session, rats ($n = 16$/group) were assigned to one of seven doses of nicotine [0.0 (saline), 0.05, 0.075, 0.1, 0.2, 0.4, or 0.6 mg/kg], with the restriction that performance in discrimination training did not differ across groups. Extinction occurred across 8 consecutive days. Rats were injected subcutaneously with their assigned dose of nicotine 5 min before placement in the conditioning chamber for 20 min. Sucrose was not available in these sessions.

Transfer testing

The transfer of extinction learning phase followed 24 h after the last extinction session. All rats were administered the 0.4 mg/kg training dose of nicotine used in discrimination training 5 min before a 20-min test session; no sucrose was available.

Experiment 2: transfer of extinction after training with 0.2 mg/kg nicotine

Male rats ($n = 112$; 16/group) were handled, trained, and tested as described in Experiment 1. The only difference was that the training dose of nicotine was 0.2 mg/kg.

Dependent measures

The dependent measure for discrimination training was the rate of dipper entries before the first sucrose delivery on nicotine sessions. This approach avoids any influence of US access on the measure of conditioning. For saline sessions, we derived a measure of dipper entries per second from the start of the session that was equated to the nicotine sessions. For the extinction and transfer test phase, the total number of dipper entries across the 20-min session was used as the dependent measure.

Drugs

(–)-Nicotine hydrogen tartrate (Sigma, St Louis, Missouri, USA) was dissolved in 0.9% saline and brought to a pH of 7.0 ± 0.1 with a dilute NaOH solution. Nicotine was injected subcutaneously at a volume of 1 ml/kg. All nicotine doses are reported as the base form.

Data analyses

The last four nicotine and four saline sessions for discrimination training were analyzed with mixed-model repeated-measures analysis of covariance (ANOVA) with two within-subjects factors (session and drug) and a between-subjects factor (group). This analysis provided an assessment that the discrimination had been acquired and that there were no significant differences between the groups before the start of extinction. Extinction data were analyzed with a mixed-model repeated-measure ANOVA, with session as a within-subjects factor and groups as a between-subjects factor. For the transfer test, dipper entries were divided into five 4-min bins and then analyzed with mixed-model repeated-measure ANOVA. To delineate the source of a significant Group × Bin interaction, a follow-up one-way ANOVA on each bin was used. Fisher's least significant difference [$LSD_{\text{minimum mean difference (mmd)}}$] tests were used for subsequent pairwise comparisons. On the transfer test, in which all rats received the training dose of nicotine, full transfer of extinction learning was declared when the total number of dipper entries differed significantly from the group that received saline during extinction training, but did not differ from the group that received the training dose during extinction training. Partial transfer of extinction learning was declared when the total number of dipper entries was significantly higher than the group that received saline, yet responding was significantly lower than the group that received the training dose during extinction training. Median effective doses (ED_{50} values) were calculated from the ascending portion of nicotine generalization curves. Statistical significance was set at P value less 0.05 for all tests.

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Results

Experiment 1: transfer of extinction after training with 0.4 mg/kg nicotine

Discrimination training

The three-way ANOVA at the end of training revealed only a significant main effect of Condition (nicotine vs. saline; $F_{1,105} = 629.75$, $P < 0.001$). The mean rates of dipper entries per second on the last four nicotine sessions [Mean (±SEM); 0.23 (±0.008), 0.22 (±0.008), 0.23 (±0.009), 0.23 (±0.008)] were higher than corresponding saline sessions [0.07 (±0.004), 0.06 (±0.004), 0.06 (±0.004), 0.06 (±0.003)].

Extinction training

The results of this phase are split into two figures for ease of visual presentation. Figure 1a shows extinction with 0.05, 0.075, and 0.1 mg/kg nicotine, whereas Fig. 1b shows 0.2 and 0.6 mg/kg nicotine; saline and the 0.4 mg/kg training dose of nicotine are displayed on both graphs to facilitate comparison. There were significant main effects of Session ($F_{7,735} = 23.45$, $P < 0.001$) and Group ($F_{6,105} = 4.59$, $P < 0.001$), as well as a significant Group × Session interaction ($F_{42,735} = 94.86$, $P < 0.001$). Relative to the group that received the 0.4 mg/kg training dose of nicotine in extinction, responding evoked by saline, 0.05, and 0.075 mg/kg were lower across the first four extinction sessions, as was also the case for 0.1 mg/kg in session 2 ($LSD_{mmd} = 32.87$). By the last four sessions, responding at all doses of nicotine was at the level of the 0.4 mg/kg training dose except for 0.6 mg/kg, where responding was lower on the sixth extinction session. The saline group remained lower than the 0.4 mg/kg group.

Transfer testing

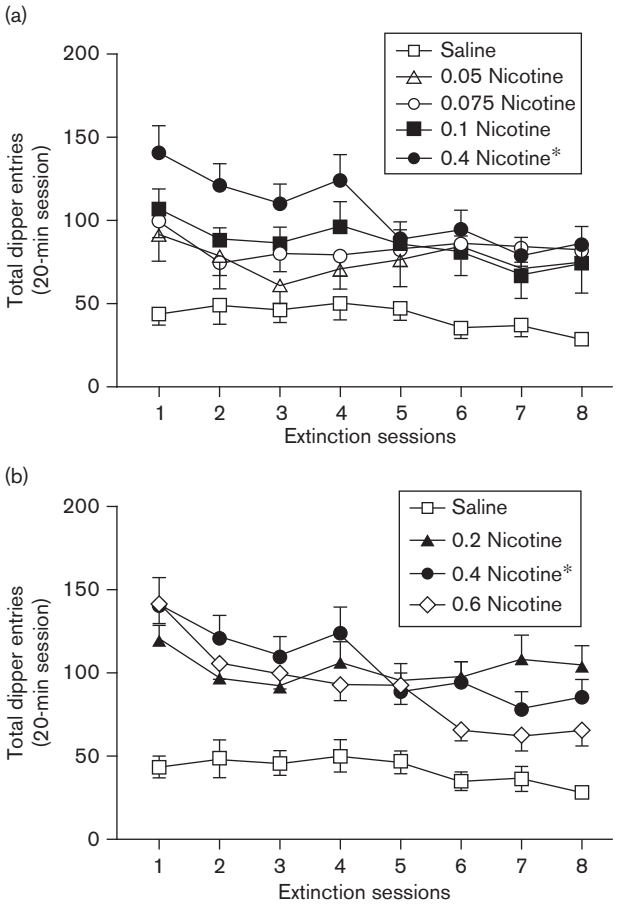
Results from the transfer test, in which all rats were tested with 0.4 mg/kg nicotine, are shown in Fig. 2. There were significant main effect of Bin ($F_{4,420} = 75.30$, $P < 0.001$; $LSD_{mmd} = 3.10$) and Group ($F_{6,105} = 2.42$, $P = 0.032$), and a significant Group × Bin interaction ($F_{24,420} = 2.16$, $P = 0.001$), indicating that the pattern of responding differed between groups across the session (Fig. 2a and b). A one-way ANOVA on the first 4-min bin (Fig. 2c) revealed that responding in groups 0.075, 0.1, 0.2, and 0.6 mg/kg nicotine did not differ from that of the 0.4 mg/kg group ($LSD_{mmd} = 11.76$), indicating full transfer of extinction early in the test. Saline and 0.05 mg/kg were significantly higher than all the other groups. The same pattern of responding was seen in the second 4-min bin ($LSD_{mmd} = 10.55$; Fig. 2d). For the remaining bins, there were no longer differences in responding across groups.

Experiment 2: transfer of extinction after training with 0.2 mg/kg nicotine

Discrimination training

For the end of discrimination training with 0.2 mg/kg nicotine, the three-way ANOVA revealed only a significant main effect of Condition (nicotine vs. saline; $F_{1,105} = 593.66$, $P < 0.001$). Rates of dipper entries on each of the four final nicotine sessions [0.22 (±0.008), 0.22 (±0.009), 0.23 (±0.008), and 0.21 (±0.009)] were higher than those on saline sessions [0.06 (±0.004), 0.05 (±0.003), 0.05 (±0.003), 0.05 (±0.003)].

Fig. 1



Total dipper entries for the eight 20-min extinction sessions (no sucrose reinforcement) in which rats, trained with 0.4 mg/kg nicotine, received saline, 0.05, 0.075, 0.1, or 0.4 mg/kg nicotine (a); saline, 0.2, 0.4, or 0.6 mg/kg nicotine (b). *Training dose of nicotine.

Extinction training

Figure 3a shows extinction with 0.05, 0.075, and 0.1 mg/kg nicotine, and Fig. 3b shows extinction with 0.4 and 0.6 mg/kg nicotine; saline and the 0.2 mg/kg training dose of nicotine are displayed on both figures. There was a significant main effect of Session ($F_{7,735} = 15.22$, $P < 0.001$; $LSD_{mmd} = 6.38$), indicating that dipper entries decreased across extinction training. There was also a significant main effect of Group ($F_{6,105} = 11.05$, $P < 0.001$), and Group × Session interaction ($F_{42,735} = 2.09$, $P < 0.001$), suggesting that the pattern of extinction differed between nicotine doses. Relative to 0.2 mg/kg

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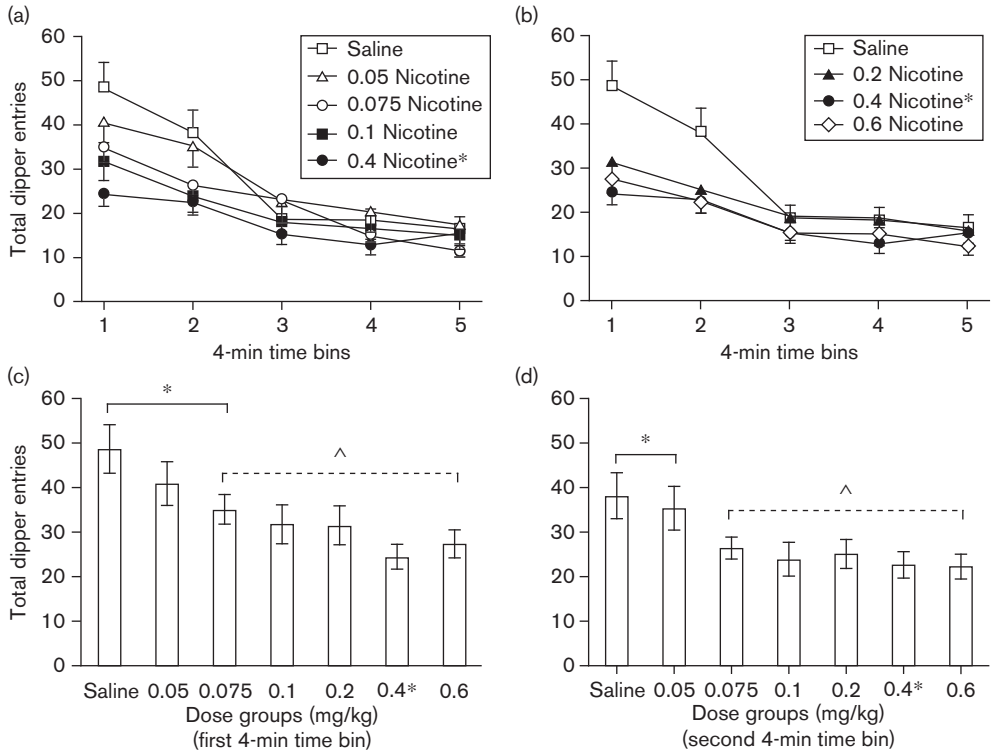
Fig. 2



Total dipper entries per 4-min bins on the transfer test for rats trained and transfer tested on 0.4 mg/kg nicotine for rats that experienced extinction training with saline, 0.05, 0.075, 0.1, and 0.4 mg/kg nicotine (a); and with saline, 0.2, 0.4, and 0.6 mg/kg nicotine (b). Total dipper entries for the first 4-min time bin (c). Total dipper entries for the second 4-min time bin (d). *Significant difference for nicotine; ^Significant difference from saline ($P < 0.05$).

nicotine, responding evoked by saline was lower on all extinction sessions ($LSD_{mmd} = 16.867$); 0.4 mg/kg never differed significantly from the training dose. In addition, groups 0.05 and 0.6 mg/kg had lower responding than the training dose on the first three sessions; 0.075 mg/kg differed on sessions 1 and 3. The remaining differences were on session 7, when 0.05 and 0.075 mg/kg nicotine evoked lower responding than the 0.2 mg/kg training dose; 0.075 mg/kg was also lower than the training dose on session 8.

Transfer testing

Results from the transfer test, in which all rats were tested with 0.2 mg/kg nicotine, are shown in Fig. 4. There were significant main effects of Bin ($F_{4,420} = 95.50$, $P < 0.001$; $LSD_{mmd} = 2.59$) and Group ($F_{6,105} = 2.30$, $P = 0.040$), and a significant Group × Bin interaction ($F_{24,420} = 5.41$, $P < 0.001$), indicating that the pattern of responding differed between doses across bins (Fig. 4a and b). A one-way ANOVA on the first 4-min bin (Fig. 4c) revealed that group 0.4 mg/kg did not differ from group 0.2 mg/kg and that responding in group 0.4 mg/kg was significantly lower than the saline group ($LSD_{mmd} = 9.69$), indicating full transfer of extinction. Groups that previously had extinction with 0.075, 0.1, and 0.6 mg/kg nicotine differed from the saline and the 0.2 mg/kg group, indicating partial transfer of extinction. However, the group that had extinction with 0.05 mg/kg nicotine showed no difference compared with the saline group, indicating no transfer of extinction with this low dose of nicotine. By the second 4-min bin (Fig. 4d), groups 0.1, 0.4, and 0.6 mg/kg had lower responding relative to saline, but did not differ from group 0.2 mg/kg ($LSD_{mmd} = 8.67$). From the third bin on, there were no longer any differences in responding across groups.

Between-subjects generalization curve

The design of the current experiments provides us with a unique opportunity to construct a between-subjects generalization curve for 0.4 and 0.2 mg/kg nicotine. These generalization curves can be compared with the extinction and transfer of extinction findings reported here, as well as to published reports using the more common within-subject approach with brief nonreinforced tests. To do so, dipper entries from the first 4 min of the first day of extinction training were extracted for each nicotine dose and the ED_{50} was calculated using the ascending portion of the dose–effect curve. Figure 5 shows the mean number of dipper entries (±SEM) for each dose (group) that received

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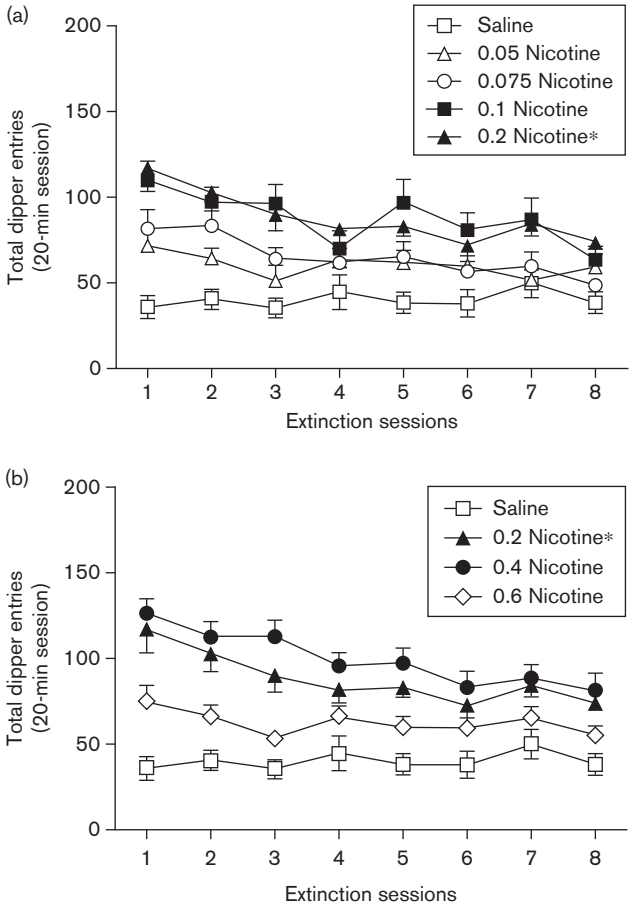
Fig. 3



Total dipper entries for the eight 20-min extinction sessions (no sucrose reinforcement) in which rats, trained with 0.2 mg/kg nicotine, received saline, 0.05, 0.075, 0.1, or 0.4 mg/kg nicotine (a); saline, 0.2, 0.4, or 0.6 mg/kg nicotine (b). *Training dose of nicotine.

discrimination training with 0.4 (Fig. 5a) or 0.2 mg/kg nicotine (Fig. 5b). For rats that received the 0.4 mg/kg nicotine training dose, a one-way ANOVA revealed a significant main effect of Dose ($F_{6,105} = 12.17$, $P < 0.001$). Relative to the training dose, responding was lower for saline and the 0.05 and 0.6 mg/kg doses ($LSD_{mmd} = 10.87$); the ED_{50} was 0.1 mg/kg. For rats that received the 0.2 mg/kg nicotine training dose, there was a significant main effect of Dose ($F_{6,105} = 12.47$, $P < 0.001$). Relative to the 0.2 mg/kg training dose, responding was lower for saline and the 0.05, 0.4, and 0.6 mg/kg doses ($LSD_{mmd} = 12.70$); the ED_{50} was 0.05 mg/kg.

Discussion

We recently introduced repeated extinction and transfer of extinction as methodological tools for studying interoceptive conditioning and the similarity of two ligands (Reichel *et al.*, 2010). The former approach, repeated extinction, asks about the persistence of responding controlled by the ligand of interest despite the absence of the reinforcer. The latter approach, transfer of extinction, assesses how well this learning regarding this nonreinforcement generalizes back to the training stimulus. The findings and hence conclusions using these approaches can differ dramatically from the standard stimulus substitution protocols used in drug discrimination research. Those protocols try to prevent or minimize learning regarding the test ligands (see the Introduction section and Bevins *et al.*, 2012 for more on this point). The present research extended earlier work by applying these approaches to two widely studied doses of nicotine (0.2 and 0.4 mg/kg) and examining the effects of higher and lower doses. As detailed further in the following narrative, there are some notable similarities; 0.2 mg/kg is similar to a 0.4 mg/kg training dose across approaches. There are also some notable differences; 0.05 mg/kg does not show partial substitution in the transfer of extinction test.

One of the major advantages of the standard stimulus generalization protocol is that it allows for repeated testing that generates within-subjects dose–effect curves. In research with the DGT task used here, this testing approach typically finds that 0.1 and 0.2 mg/kg nicotine fully substitute for the 0.4 mg/kg training dose; the 0.05 mg/kg dose evokes a partial CR and the 0.6 mg/kg dose produces motor impairment (i.e. fewer infrared beam breaks in the chamber), thus precluding any conclusion regarding its similarity to 0.4 mg/kg nicotine (e.g. Murray and Bevins, 2007a; Murray *et al.*, 2010; Struthers *et al.*, 2009). When a 0.2 mg/kg nicotine training dose is used, the 0.1 mg/kg dose substitutes fully for the nicotine stimulus and 0.05 mg/kg substitutes partially. However, 0.4 mg/kg produces motor impairment and, accordingly, 0.6 mg/kg is not assessed in those studies (e.g. Murray and Bevins, 2007a, b; Reichel *et al.*, 2007). Until the current study, the 0.075 mg/kg dose has not been assessed in this DGT task.

As noted in the Results section, the design of the present study provided us with the opportunity to generate complete between-subjects dose–effect functions for the 0.2 and the 0.4 mg/kg nicotine stimulus (Fig. 5). These between-subjects dose–effect curves replicate those from the previously published within-subjects dose–effect curves described in the previous paragraph. This comparability suggests two points regarding conclusions derived from the within-subjects approach to testing. First, the additional training and more extensive nicotine exposure required by a within-subjects approach does not appear to affect the overall nature of the nicotine dose–effect curve. Second, the brief and repeated 4-min substitution tests without reinforcement in the within-subjects studies also do not have a substantive effect on the nicotine dose–effect curve. These conclusions, of course, are restricted to the brief 4-min substitution test

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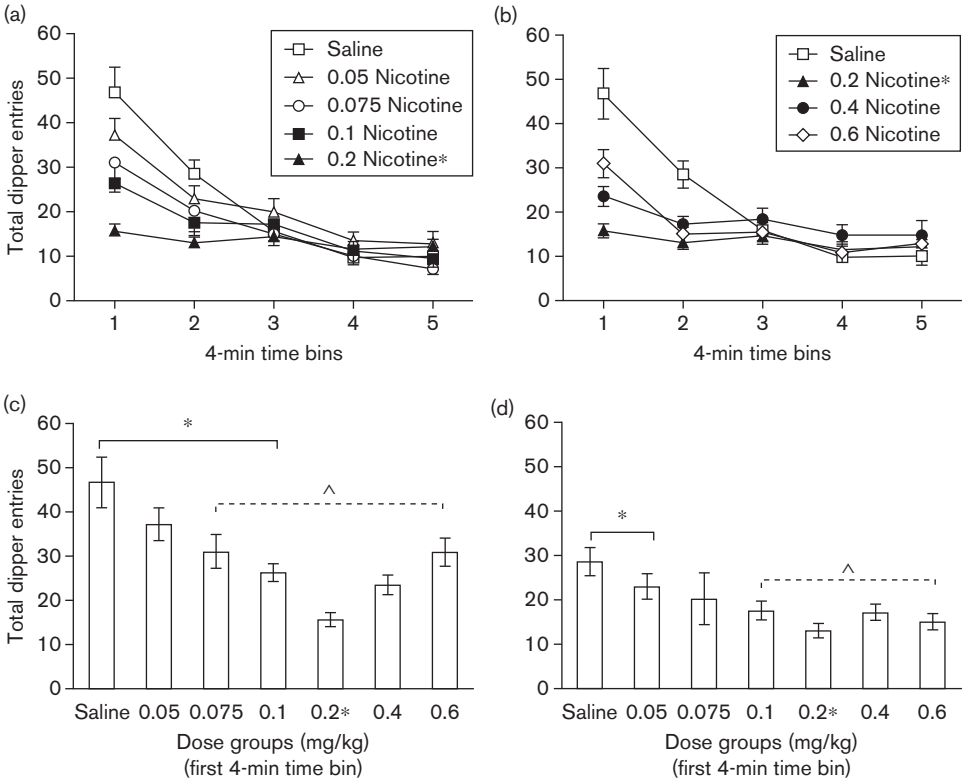
Fig. 4



Total dipper entries per 4-min bins on the transfer test for rats trained and tested on 0.2 mg/kg nicotine for rats that experienced extinction training with saline, 0.05, 0.075, 0.1, and 0.2 mg/kg nicotine (a); and with saline, 0.2, 0.4, and 0.6 mg/kg nicotine (b). Total dipper entries for the first 4-min time bin (c). Total dipper entries for the second 4-min time bin (d). *Significant difference for nicotine; ^Significant difference from saline ($P<0.05$).

in which reinforcement is withheld. In addition, the result from the between-subjects dose–effect curves obtained using the DGT task also parallel findings using operant drug discrimination procedures (cf. Stolerman *et al.*, 1984). Specifically, there was increased sensitivity to nicotine for those rats trained on the lower dose (0.2 mg/kg) as measured by the lower ED_{50} value compared with those trained on the higher dose (0.4 mg/kg).

Of primary interest in the present report is whether conclusions regarding stimulus similarity shifts with testing approach. Repeated nonreinforced presentations of nicotine in 20-min sessions resulted in a rapid decrease in conditioned responding (i.e. goal tracking) for lower nicotine doses (0.05 and 0.075 mg/kg) and a systematic decrease in conditioned responding for higher doses (0.1, 0.2, 0.4, and 0.6 mg/kg) that were examined. When the training dose was 0.4 mg/kg nicotine, this approach revealed that 0.05 and 0.075 mg/kg only partially substituted for the training dose. That is, conditioned responding was lower than 0.4 mg/kg on the first four extinction sessions. All other doses were statistically comparable with 0.4 mg/kg training dose. The conclusion regarding partial substitution with 0.05 mg/kg, and full substitution with 0.1 and 0.2 mg/kg, is consistent with the 4-min generalization testing approach (within-subjects or between-subjects). However, the partial substitution at 0.075 mg/kg differs from the full substitution found using the between-subjects 4-min testing protocol. Perhaps the most striking finding with repeated extinction is that the 0.6 mg/kg dose of nicotine was similar to the 0.4 mg/kg dose. Previous attempts to assess this higher dose using the more standard testing protocol had failed given that a decrease in chamber activity, defined as breaking an infrared beam that bisects the chamber, accompanied a decrease in goal-tracking (Murray and Bevins, 2009). A similar decrease in chamber activity early in the first session was seen here, but this effect quickly dissipated. Thus, this repeated extinction test protocol may be especially useful when the ligand of interest has motoric effects in the more standard brief testing approach.

In the repeated extinction tests, only the 0.1 and 0.4 mg/kg doses of nicotine fully substituted for the 0.2 mg/kg training dose. The remaining doses (0.05, 0.075, and 0.6 mg/kg) evoked less conditioned responding on at least three of the extinction sessions. The conclusion regarding partial substitution with 0.05 mg/kg, and full substitution with 0.1 mg/kg nicotine, is consistent with the 4-min generalization testing approach (within-subjects or

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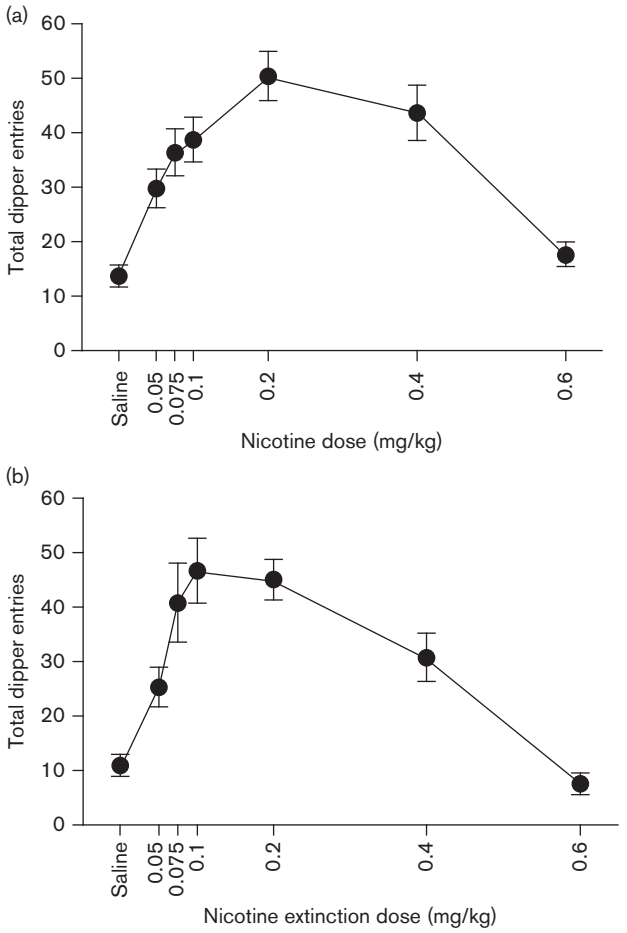
Fig. 5



Dose–effect curves calculated for the first 4 min of the first extinction trial for rats trained with 0.4 mg/kg nicotine (a) and 0.2 mg/kg nicotine (b). During extinction training rats received saline, 0.05, 0.075, 0.1, 0.2, 0.4, or 0.6 mg/kg nicotine.

between-subjects). However, the partial substitution at 0.075 mg/kg differs from the full substitution found using the between-subjects 4-min testing protocol. Also, we are able to suggest using this approach that the stimulus effects of 0.4 mg/kg nicotine are similar to those of 0.2 mg/kg, but 0.6 mg/kg may have some distinct stimulus elements as this dose only partially substituted for the 0.2 mg/kg training dose (see later). Neither dose had motor-impairing effects beyond the first extinction session.

In the transfer of extinction test, where all rats were challenged with their training dose of nicotine, the generalization of the extinction learning history occurred across a wide range of doses. For the 0.4 mg/kg training dose, only rats that had extinction with 0.05 mg/kg did not show complete transfer of extinction in the first part of the test session. Similar to the repeated extinction tests, extinction learning with the dose of nicotine higher than the training dose (0.6 mg/kg) produced full generalization in the transfer test. This reinforces an earlier point regarding the use of these alternative test procedures to evaluate stimulus similarity of a ligand or doses of a drug that may have some motor-impairing effects in the more standard testing protocol. Given that the transfer test uses the training dose of nicotine, any concerns regarding motor impairment influencing the outcome and, hence, interpretation, are negated. When 0.2 mg/kg nicotine was the training dose, transfer of extinction in the first 4 min of the test was complete only for the group that had extinction with 0.4 mg/kg; partial transfer was seen at 0.075, 0.1, and 0.6 mg/kg. Full transfer of extinction was seen at these doses by the second 4-min bin.

Since Thorndike (1913), many have theorized that stimuli are comprised of elemental components in which each component acquires its own associations and that the behavior reflects the summated associative strength of these elements (Spence, 1936; Spence, 1937; Konorski, 1948; Estes, 1950; Blough, 1975; Wagner, 2008). Nicotine as an interoceptive stimulus is presumably a complex polymodal event with its elements reflecting the neurobiological processes on which the drug acts directly or indirectly (Balster, 1988; Stolerman *et al.*, 1999; Bevins and Murray, 2011). Because receptor specificity can vary with drug dose, the interoceptive stimulus effects of a low dose of a drug can differ from a higher dose in its general intensity, its neurobiological elements, and in the salience of those particular stimulus elements (Bevins and Murray, 2011). We would not be surprised to find that some of the elements, control over appetitive behavior may be more susceptible to extinction than others. In fact, an across-experiment comparison suggests that extinction was greater in the group that received 0.2 mg/kg in training and in extinction than in the group that received 0.4 mg/kg in training and in testing (e.g. compare Fig. 2c with Fig. 4c). This finding is consistent with earlier research directly comparing rate of extinction across different nicotine doses (Murray and Bevins, 2007b). In that study, the persistence of conditioned responding despite nonreinforcement was higher for the group trained with 0.4 mg/kg than those trained with 0.2 or 0.1 mg/kg nicotine. These findings suggest that the intensity, and perhaps the neurobiological elements, differ between the 0.4 and 0.2 mg/kg doses. Indeed, this somewhat greater extinction with 0.2 mg/kg nicotine may explain why more differences were found in the transfer test. Simply, the baseline level of conditioned responding was lower, thus providing a greater window statistically in which to see a difference in responding early in the transfer test.

In summation, different approaches to assessing interoceptive conditioning and the similarity of ligands or doses of a ligand can sometimes lead to different

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conclusions (see also Kaempf and Kallman, 1987; Reichel *et al.*, 2010; Bevins *et al.*, 2012). One advantage of the repeated extinction and the transfer of extinction protocols revealed in the present research was that doses traditionally producing motor impairment could be evaluated. Of course, an important disadvantage of these two approaches to assessing stimulus generalization is that they use between-subjects designs that require many more animals. Regardless, the disparities in findings, and hence conclusions, prompts us to ask whether the different approaches provide different insights into processes relevant to interoceptive conditioning and nicotine addiction? Would one particular approach, or some combination of approaches, be especially useful for medication development? Reichel *et al.* (2010) found that ABT-418 and varenicline (i.e. the smoking cessation aid Chantix) prompt full substitution in 4-min generalization tests (cf. Damaj *et al.*, 1995; Paterson *et al.*, 2010; Jutkiewicz *et al.*, 2011). However, only varenicline continued to prompt partial substitution in the repeated extinction and the transfer of extinction tests. In the present report, similar dissociations were found when doses of nicotine higher than the training dose were used, as well as with the 0.05 mg/kg dose. Future research will need to continue to examine the similarities and differences across approaches, as well as identify behavioral and neural processes underlying the different forms of substitution. We do not expect these processes to be completely identical.

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Conflicts of interest

There are no conflicts of interest.

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