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## Excitatory conditioning to the interoceptive nicotine stimulus blocks subsequent conditioning to an exteroceptive light stimulus.

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### Abstract

Previous research has shown that a nicotine conditional stimulus (CS) can compete with (i.e., overshadow) a brief light CS. Another form of competition, blocking, has not yet been examined with the nicotine CS. Groups of rats were assigned to an element training condition. For the N+ group, during each daily 2-hr element training session, there were ten intravenous nicotine infusions (0.03 mg/kg) followed 30-sec later with 4-s access to sucrose. In the N- group, nicotine and sucrose presentations were explicitly unpaired. The chamber alone group (C alone) had no stimulus presentations. Element training was followed by compound training in all groups. A 30-sec houselight was included during the time between the nicotine infusion and paired sucrose delivery. Non-reinforced element presentations assessed relative control of the goal tracking conditioned response (CR). The N+ group showed a higher proportion of CR control by the nicotine than the light. The opposite pattern was found in the N- and C alone groups indicating that nicotine CS controlled less of the CR than the light. Thus, excitatory conditioning with the nicotine CS blocked later conditioning to the light. This finding adds to literature examining the interaction between interoceptive drug CSs and other environmental stimuli.

### Keywords

appetitive conditioning; blocking; interoceptive cue competition; drug discrimination; nicotine; overshadowing

The interoceptive effects of nicotine have been shown to serve as a contextual conditional stimulus (CS) in an appetitive Pavlovian conditioning task. In these studies, rats were injected subcutaneously with nicotine or saline before placement in a conditioning chamber. On nicotine sessions, liquid sucrose (the unconditioned stimulus; US) was intermittently available. On intermixed saline sessions, sucrose was not available. This discrimination is readily acquired as indicated by an increase in anticipatory head entries into the sucrose receptacle [i.e., goal-tracking; (1; 2)] on nicotine compared to saline sessions [e.g., 3]. This discriminated goal-tracking task has been helpful in studying behavioral and neuropharmacological processes involving the nicotine stimulus [cf. 4; 5]. However, the

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contextual nature of subcutaneous nicotine administration has limited each session to a single trial – the time before the first sucrose delivery on nicotine sessions (equivalent time on saline sessions) so that the index of anticipatory responding is not confounded by sucrose delivery. For example, in experiments in which there are 36 deliveries of sucrose in a single 20-min session – a standard number in our laboratory – the measure of conditioning can only be evaluated after every 36 nicotine-sucrose pairings. As assessment of conditioning is not possible after each nicotine-sucrose pairing, this arrangement poses some experimental limitations. One example comes from a set of studies examining possible dose-dependent differences in nicotine-evoked conditioned responding. In that research, we compared rate of acquisition of a 0.1, 0.2, and 0.4 mg/kg nicotine CS [6; 7]. Although there were dose-dependent differences in several measures (e.g., extinction), the rate of acquisition was similar across groups (i.e., within 10-12 intermixed nicotine and saline sessions). This translates into at least 180 nicotine-sucrose pairings (5 sessions × 36 deliveries per session). Perhaps if conditioning could be assessed following each nicotine-sucrose pairing, dose-dependent differences in acquisition would have emerged. Further, the ability to measure conditioning after each trial would allow us to determine the effects of manipulating specific within-session trials on responding several times within a single session – an experimental design more analogous to ‘traditional’ conditioning research that uses discrete stimuli such as illumination of a light or onset of a tone.

With this and other limitations in mind that have been described previously [cf. 8], we developed a task that used short, intravenous (IV) nicotine infusions as a CS [4; 8; 9]. Briefly, rats were infused with IV nicotine or saline (36 µl over 1 s). On nicotine trials, 30 sec after the infusion, liquid sucrose was available for 4 sec; sucrose was not available on intermixed saline sessions. Goal-tracking increased in the 30 sec that intervened between the nicotine infusion and sucrose; no such increase was seen after saline infusions. The goal-tracking conditioned response (CR) evoked by the IV nicotine CS was blocked by the central and peripheral nicotinic acetylcholine receptor antagonist mecamylamine, but not the predominately peripheral nicotinic acetylcholine receptor antagonist hexamethonium [10; 11], indicating the CS effects of IV nicotine are mediated by receptors in the central nervous system [8].

How the nicotine stimulus interacts with other co-presented stimuli is only beginning to be investigated. In humans, nicotine is rarely, if ever, experienced without the presence of other stimuli. These stimuli could include tactile cues of a cigarette or a lighter, the visual cues of an ashtray or cigarette pack, or the smell and taste of inhaled smoke. From this perspective, the stimulus effects of nicotine are part of a more complex multimodal compound stimulus with interoceptive and exteroceptive elements. Because of this potential complexity of a compound stimulus formed between nicotine and other stimulus elements, an important associative property to examine is competition for control of the CR between the interoceptive nicotine stimulus and an exteroceptive stimulus. Two important forms of cue competition include overshadowing and blocking. In overshadowing, two stimulus elements are trained as a compound stimulus. Each element of the compound can acquire some control over the CR. The amount of behavioral control exerted by each stimulus element is presumably based on the salience (perceptibility) of that element relative to the other element [12-14]. We have previously shown that the IV nicotine stimulus competes with an exteroceptive light stimulus when trained as a compound CS [15]. In those experiments, cue-lights near the dipper receptacle were presented during the 30-sec interval between nicotine infusion and access to sucrose. Tests of the nicotine and light elements individually revealed that the goal-tracking CR evoked by the light, was dose-dependently reduced (i.e., overshadowed) by co-presentation with the nicotine stimulus.

In blocking, one of the stimulus elements is first paired with the US. Following acquisition of the CR, a second stimulus is added to the initial stimulus to create a compound CS. This compound CS is then repeatedly paired with the US. Subsequent testing on the different elements of the compound would result in the added stimulus controlling less responding than if the stimulus had been trained alone [16]. Additionally, the originally-trained stimulus should control more conditioned responding than the stimulus that had been subsequently added. Investigation of blocking between nicotine and exteroceptive stimuli has not yet been conducted. The present experiment sought to fill this gap by assessing whether previous excitatory conditioning with the nicotine CS would block subsequent conditioning to an exteroceptive light CS. We expect the nicotine CS to block conditioning to the light CS given our recent demonstration of overshadowing using this light plus nicotine compound stimulus [15].

Male Sprague-Dawley rats ( $348 \pm 2$  g at surgery) from Harlan Industries (Indianapolis, IN) were housed individually in clear  $48.3 \times 26.7 \times 20.3$  cm ( $l \times w \times h$ ) polycarbonate tubs lined with aspen shavings. The colony was humidity- and temperature-controlled and maintained on a 12-h light:dark cycle; sessions were conducted during the light portion of the cycle. Water was continuously available in the home cage. Food was restricted to 20 g after each daily session except when noted.

Sessions were conducted in twenty conditioning chambers (ENV-008CT; Med Associates, Inc., Georgia, VT) measuring  $30.5 \times 24.1 \times 21.0$  cm ( $l \times w \times h$ ). Each chamber was enclosed in a light- and sound-attenuating cubicle fitted with a fan to provide airflow and mask noise. A houselight with two bulbs (28 V, 100 mA each) was mounted on the back wall of the cubicle. The houselight was centered side-to-side, 23.5 cm above the top of the conditioning chamber, and 5 cm below the ceiling of the cubicle. Chamber sidewalls were aluminum; the ceiling and front and back walls were clear polycarbonate. Chambers were equipped with a recessed receptacle ( $5.2 \times 5.2 \times 3.8$  cm;  $l \times w \times d$ ) on the right sidewall. A dipper arm raised a 0.1-ml cup of 26% sucrose solution (w/v) in the receptacle. An infrared emitter/detector unit, located 1.2 cm into the receptacle and 3 cm from the floor, monitored head entries into the dipper. A second infrared emitter/detector unit was mounted 14.5 cm from the sidewall containing the receptacle and was positioned 4 cm above the rod floor. This unit provided a measure of chamber activity. Each chamber had a computer-controlled variable-speed syringe pump (Med-Associates, PMH-100VS) that allowed nicotine to be delivered IV. Pumps were located outside the sound-attenuating cubicle. Each chamber contained a spring leash attached to a balanced metal arm with a swivel. Tygon® tubing (AAQ04103; VWR, West Chester, Pennsylvania) extended from a 5-ml syringe mounted on the syringe pump through the leash to attach to the catheter. A personal computer with Med Associates interface and software (Med-PC for Windows, version IV) controlled stimulus presentations and recorded dipper entries and chamber activity.

Before surgery, rats were handled for at least 3 min per day for 3 days. Food was removed after handling on the last day. Dipper training in the conditioning chambers began the following day. Three 50-min sessions were conducted on three consecutive days with each session not starting until a rat's first dipper entry. The probability of receiving 4-s access to sucrose decreased from 0.167 to 0.05 per 60 s over the three sessions (ca. from 2.5 to 0.75 sucrose deliveries per min).

Catheter surgery occurred within two days of the last preliminary training session. Each rat was anesthetized with an intraperitoneal (IP) injection (1 ml/kg) of ketamine hydrochloride (100 mg/ml) followed by an IP injection (0.6 ml/kg) of xylazine hydrochloride (20 mg/ml) purchased from Midwest Veterinary Supply (Des Moines, IA). One end of a silicon catheter was implanted into the external left jugular. The other end was positioned under the skin

such that it exited just below the scapula via a back-mount. The catheter was accessible by a metal cannula. To manage post-surgical pain, buprenorphine hydrochloride (0.1 mg/kg; Sigma, St. Louis, MO) was injected SC immediately following surgery and once more approximately 12 h after surgery. The catheter was flushed twice a day for the duration of the experiment with 0.1 ml of sterile saline mixed with heparin (30 U/ml; Midwest Veterinary Supply). The first five post-surgical flushes also contained streptokinase (ca. 7000 U/ml) to break down any clots that may start to form in the catheter. Rats were allowed five to six days of recovery in their home cage with free access to food before beginning the experiment. Catheter patency was assessed with a 0.05 ml IV infusion of xylazine (20 mg/ml) at pre-established points in each study [cf. 15; 17]. Only rats with patent catheters were included in analyses.

Following the surgical recovery period, rats were assigned to a training group ( $n = 14$  for C alone;  $n = 10$  for N+;  $n = 11$  for N-) irrespective of preliminary training performance. Nicotine is denoted by N. Chamber is denoted by C. The + indicates the stimulus was paired with sucrose, and the - indicates the stimulus was explicitly unpaired with sucrose during element training. More specifically, during the element phase of training, the N+ group received a 1-s nicotine infusion [(-)-nicotine hydrogen tartrate (Sigma), mixed in 0.9% sterile saline, adjusted to pH  $7.0 \pm 0.2$  with NaOH, infusion of  $36 \mu\text{l}$  over 1 s at 0.03 mg (base)/kg/infusion] followed 30 s later by 4-s access to sucrose; 10 such nicotine CS-sucrose US pairings occurred in each 2-h session. Stimulus presentations were separated by an average of 11 min (range 8-14 min) [cf. 8]. The N- group received the same number of nicotine and sucrose presentations as group N+ except that a nicotine infusion did not occur within 4 min of any sucrose presentations (i.e., unpaired control). This control was used to control for exposure to the CS and the US in a manner that does not produce excitatory conditioning. The C alone group was transported and exposed to the chamber like the other 2 groups, but these rats did not receive nicotine or sucrose in this phase. This element training phase continued for 8 sessions.

The blocking phase began 24 h after the last element training session. All three groups in this phase received identical compound stimulus training in which the nicotine-light compound stimulus was repeatedly paired with sucrose. That is, each 1-s infusion of nicotine was followed by 30-s illumination of the houselight; offset of the light coincided with 4-s access to sucrose. As in the previous phase, the 10 CS presentations per 2-h session were separated by an average of 11 min (range 8-14 min). Training continued until there was no difference between the groups.

Subsequent testing of the nicotine and light elements of the compound CS was conducted across two sessions. In each of these sessions there were three test stimulus presentations intermixed among seven standard compound stimulus training trials. The element test presentations were not followed by sucrose. The order of element testing was counterbalanced such that approximately half of the rats in a group had the light tested in the first test session and nicotine in the second session. The remaining rats had nicotine tested first, then the light.

For the element and compound training phases, the average elevation score for each session was used to evaluate conditioning. The elevation score for each trial was calculated by taking the number of dipper entries during the 30-s CS presentation minus the number of dipper entries in the 30-s interval before the CS presentation. The elevation score allows for measurement of change above a normalized baseline and indicates whether conditioned responding is specific to a particular stimulus [e.g., 18-20]. For the element tests, the elevation scores were converted into a proportion of total measure (i.e., response ratio). That is, each element was divided by the amount of responding on both elements [e.g., proportion

nicotine = elevation nicotine / (elevation nicotine + elevation light)]. Training and test phases were analyzed using Group  $\times$  Session repeated measures analyses of variance (ANOVAs) followed by Fisher's protected tests. Statistical significance was declared at  $p < .05$  for all tests.

As shown in Figure 1a, group N+ acquired a robust nicotine-evoked CR during element training, whereas N- group displayed relatively little responding; group C alone did not have stimulus presentations during this phase [Group:  $F(1,19) = 25.35, p < .001$ ; Session:  $F(7,133) = 11.12, p < .001$ ; Group  $\times$  Session:  $F(7,133) = 6.73, p < .001$ ]. Follow-up comparisons revealed more conditioned responding in the N+ than the N- group for sessions 2 through 8. By the end of the compound conditioning phase, in which all groups had the nicotine+light compound repeatedly paired with sucrose, groups N- and C alone had a CR comparable to group N+. Notably, the acquisition of this CR in group C alone was faster than in group N- [Group:  $F(2,32) = 8.16, p = .001$ ; Session:  $F(5,160) = 35.79, p < .001$ ; Group  $\times$  Session:  $F(10,160) = 3.93, p < .001$ ]. In session 1, the N+ group had the highest responding; the N- and C alone group did not differ. In sessions 2, 3, and 5, there was no difference between the N+ and C alone groups and both these groups had greater responding than the N- group. In session 4, N+ group was higher than the C alone group, and the C alone group was higher than the N- group. Finally, in the last compound CS training session before testing, there were no differences among the groups.

We also measured total dipper entries to determine whether there were similarities between groups that receive sucrose. Total dipper entries in the 2 hr sessions decreased across element training for all groups (Figure 1b), with group C alone having substantially lower responding than groups N+ and N- [Group:  $F(2,32) = 31.71, p < .001$ ; Session:  $F(7, 224) = 20.37, p < .001$ ; Group  $\times$  Session:  $F(14,224) = 2.42, p = .004$ ]. There was no overall group difference between the N+ and N- groups, suggesting similar sucrose seeking in both groups during this phase. The interaction likely reflects that the C alone group was lower than the N+ and the N- groups, and the N+ and N- groups were similar except for session 3 when N+ was significantly higher than N-. During compound stimulus training, total dipper entries in group C alone were higher than group N+ or N- [Group:  $F(2,32) = 10.97, p < .001$ ; Session and Group  $\times$  Session:  $F_s < 1$ ]. As an index of locomotor activity, the number of infrared beam breaks in the chamber was also measured. Chamber activity during element training was lower in group C alone than in group N+ or N- [Figure 1c; Group,  $F(2, 32) = 9.01, p = .001$ ; Session and Group  $\times$  Session:  $F_s < 1$ ]. However, in the compound stimulus training phase, there were no significant differences in chamber activity ( $F_s \leq 1.65, p_s \geq .15$ ).

Figure 2 shows the proportion of total responding for each element during testing. Groups N- and C alone had a higher proportion of responding to the light than the nicotine. In contrast, the N+ group had a greater proportion of responding to the nicotine element. Further, the proportion of responding to nicotine was higher in group N+ than in the C alone control group; the proportion of responding to the light was lower in the N+ group than in the C alone control group [Group:  $F < 1$ ; Element:  $F(1,32) = 2.86, p = .10$ ; Group  $\times$  Element:  $F(2,32) = 12.56, p < .001$ ].

This data pattern indicates blocking of conditioning to the light CS in group N+. That is, only the group that had an excitatory conditioning history with nicotine as the CS before the compound conditioning phase (i.e., group N+) had less conditioned responding to the light element than the nicotine element during testing. This result extends previous blocking research. Studies examining blocking between a drug and an exteroceptive stimulus are particularly lacking. In an operant conditioning experiment investigating blocking using shock avoidance in the T-maze, Järbe and Johansson [21] initially trained rats on an interoceptive pentobarbital discriminative stimulus. This pentobarbital training blocked

subsequent control of responding by a light or dark exteroceptive visual discriminative stimulus. The results of the current experiment add to this sparse literature by demonstrating that nicotine can also block a visual stimulus in a Pavlovian drug discriminated goal-tracking task. The current findings also add blocking to the list, along with overshadowing [15], of cue competition phenomenon involving nicotine as an interoceptive stimulus element.

Acquisition of the CR in the element phase for group N+ proceeded as expected based on the results of previous IV nicotine CS experiments [8]. Also replicating previous research, when nicotine and sucrose presentations were unpaired in the same session (group N-), there was no evidence of excitatory conditioning to the nicotine stimulus. Interestingly, in the compound stimulus training phase, acquisition of conditioned responding in group N- was slower than group N+ or C alone. There are a number of possible explanations for the slower acquisition in group N-. For instance, rats may have not learned to access the sucrose in the N- group because the deliveries were not signaled (i.e., preceded by an experimenter-presented CS). In that case, subsequent acquisition may be slowed because they would have received less sucrose, especially in the earlier sessions of the compound training phase. This account appears unlikely given that accessing the receptacle while the dipper arm was raised was comparable between groups N+ and N-. For example, on the first session of the element phase, the N+ group accessed 64% of deliveries; the N- group accessed 75%. On session 8 of the element training phase (i.e., last day), the N+ group accessed 95% of the deliveries; the N- group accessed 96%. Further, the N+ and N- groups had a comparable number of total dipper entries across sessions, suggesting similar rates of goal-tracking evoked by the sucrose stimulus.

Alternatively, the retarded acquisition of conditioning in the N- group may reflect a US-preexposure effect, a CS-preexposure (latent inhibition) effect, and/or acquired conditioned inhibitory effects of the nicotine. One, or a combination of these effects, may have slowed expression of the conditioned response in the nicotine+light compound CS conditioning phase. Briefly, the US-preexposure effect refers to slowed acquisition of conditioned responding resulting from previous experience with the US—sucrose in the present study [e.g., 22; 23]. The CS-preexposure (or latent inhibition) effect refers to slowed acquisition of conditioned responding that results from a previous history with the CS alone—nicotine in the present research [e.g., 24; 25]. Finally, the conditioned inhibitory effect refers to explicitly presenting the CS so that it is temporally separated from the US thereby imbuing the CS with inhibitory characteristics as a result of the CS signaling the absence of the US that can retard subsequent excitatory conditioning [e.g., 26; 27]. The design and groups used in the current experiment controlled for exposure to the CS, US, chamber, handling, etc. and allow us to make conclusions regarding blocking of conditioning to a light CS by the nicotine CS. Future work will need to be directed at assessing the mechanism behind slower acquisition in group N-.

The results of the current experiment extend our understanding of drug stimuli and their relation to other environmental and predictive stimuli [9]. That is, drug stimuli appear to follow established associative learning principles [28; 29]. These demonstrations of cue competition between an interoceptive IV nicotine stimulus and exteroceptive light stimuli exemplify the similarities, rather than the differences, between these types of stimuli. Exteroceptive stimulation is generally considered to be generated from 'outside' the organism and perceived through vision, hearing, taste, smell, or touch. These stimuli can be lights [e.g., 30-32], sounds [e.g., 30; 33], tactile cues [e.g., 32], and environmental contexts [e.g., 34]. Presumably these stimuli can evoke shared experiences with any organism within range of the stimulus presentation [35]. Interoceptive stimulation, on the other hand, is considered to originate from 'inside' the organism. These stimuli can include schedule-

generated aftereffect cues [e.g., 30], temporal intervals [e.g., 29; 31; 33], reward-related aftereffects [e.g., 31], physiological states such as hunger or satiety [e.g., 36], and notably, drug states [e.g., 34; 37; 38]. They also tend to be more personal or private in nature [e.g., 39], experienced individually regardless of the proximity of other organisms.

In spite of these distinctions, many researchers have argued that exteroceptive and interoceptive stimuli can be studied in the same manner [see 29; 39-45]. Indeed, for perception of an exteroceptive stimulus such as a light, there needs to be some sort of internalized representation of the stimulus that evokes an interoceptive change in state [29; 38]. Catania [46] claimed that the functional properties of drug cues are like any other stimulus such as a light or noise with the major difference between them being how the stimulus is presented – when stimuli are presented by an experimenter, drugs must be delivered inside the organism in some manner, whereas a light is simply turned on, yet it is perceived by the eyes and represented within the brain as a notable stimulus. As such, there has been some research conducted showing that interoceptive and exteroceptive stimulation can function similarly in behavioral paradigms.

An interesting distinction between a drug interoceptive stimulus like nicotine and a light exteroceptive stimulus is that the nicotine, once delivered, cannot readily be ‘turned off’. As such, in the present experiment we conceptualize the nicotine stimulus as the perceptible increase in stimulus effects that accompany the IV infusion rather than the mere presence versus absence of nicotine [cf. 8]. During the tests of the light alone in this experiment, there would be some nicotine that could influence responding evoked by the light. Note that this influence, if anything, would work against observing blocking because it would inflate responding to the light element. Further, we consider this ‘increase above baseline’ to be comparable to the human condition in that following a smoker's loading dose with their first cigarette of the day, every subsequent puff/cigarette throughout the day increases and maintains desired blood/brain levels of nicotine [47; 48]. Therefore, experiencing exteroceptive stimuli that are also associated with nicotine-associated USs completely outside the influence of nicotine is unlikely. Ultimately, examination of how drug stimuli form relations with other environmental stimuli could help inform modern theories of drug use and addiction, as well as inform treatment approaches [e.g., 49-51].

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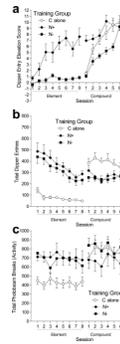
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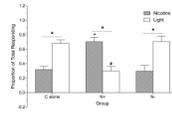
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**Fig. 1.**

Panel (a) shows the mean elevation scores ( $\pm 1$  SEM) of acquisition for element and compound CS training for the 3 groups. Panel (b) shows mean total dipper entries ( $\pm 1$  SEM) across each training session for the 3 groups. Panel (c) shows mean chamber activity counts ( $\pm 1$  SEM) for each training session in all groups. Significant effects are described in the text.



**Fig. 2.**

The panel shows the proportion of total responding for nicotine and light (+1 SEM) on the element tests. \* indicate significant difference between nicotine- and light-evoked responding within a group. + indicates significant difference from nicotine-evoked responding in the C alone group. # indicates significant difference from light-evoked responding in the C alone group.