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Emergence of Dormant Conditioned Incentive Approach by Conditioned Withdrawal in Nicotine Addiction

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Abstract

Background—Nicotine is one of the determinants for the development of persistent smoking and this maladaptive behavior is characterized by many symptoms, including withdrawal and nicotine seeking. The process by which withdrawal affects nicotine seeking is poorly understood.

Method—The impact of a withdrawal-associated cue on nicotine (0.2 mg/kg) conditioned place preference (CPP) was assessed in male C57BL/6J mice (n = 8 - 17/group). To establish a cue selectively associated with withdrawal distinct from those associated with nicotine, a tone was paired with withdrawal in their home cages; mice were chronically exposed to nicotine (200 µg/mL for 15 d) in their home cages from drinking water, and received the nicotinic acetylcholine receptor antagonist mecamylamine (2.5 mg/kg) to precipitate withdrawal in the presence of a tone. The effect of the withdrawal-associated tone on nicotine CPP was then evaluated in the place conditioning apparatus after a delay, when nicotine CPP spontaneously disappeared.

Results—A cue associated with precipitated withdrawal reactivated the dormant effect of nicotine-associated cues on CPP. This effect occurred during continuous exposure to nicotine, but not during abstinence.

Conclusions—A conditioned withdrawal cue could directly amplify the incentive properties of cues associated with nicotine. This observation extends the contemporary incentive account of the role of withdrawal in addiction to cue-cue interaction.

Keywords

nicotine addiction; extinction; Pavlovian conditioning; withdrawal; conditioned place preference; cue reactivity

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Introduction

Approximately one-fifth of the world population smokes tobacco products; half of these smokers are expected to die prematurely from tobacco-related diseases (1). Once smoking is initiated, it continues for a lifetime in a subpopulation of smokers (2) and is one of the most difficult forms of addiction to break (3). Nicotine is responsible for sustained tobacco smoking, as shown by reports that smokers gradually reduce smoking of de-nicotinized cigarettes (4) and humans will self-administer nicotine (5).

While many factors are likely to underlie persistent smoking, it is thought that smokers maintain smoking to avoid and escape withdrawal symptoms associated with reduced nicotine effects, a process termed negative reinforcement (6). Although it has not been conclusively demonstrated that smoking is causally controlled by negative reinforcement, smokers exhibit a number of features that are consistent with this account. Active smokers increasingly experience craving and negative affect until they consume the next cigarette (7-10). Moreover, regardless of whether smokers are abstinent, acute nicotine administration reduces basal and cue-induced craving and increases quit rates (11). Cue reactivity, by which smoking-related cues control smoking behavior, might also include negative reinforcement, as cues associated with smoking elicit negative affect when smokers do not expect to be able to smoke (12).

However, negative reinforcement does not fully account for other aspects of smoking behavior. Active smokers report intense craving without nicotine deprivation (7,13). Similarly, exposure to smoking-associated cues evokes an increase in craving, positive mood, and more importantly, cigarette seeking in active smokers, provided that they expect to be able to smoke (12). These observations are generally consistent with the incentive account of addiction: the addictive substance itself, and cues associated with the addictive substance, provide incentive properties and reinstate seeking (14).

Although smokers consistently correlate craving with smoking (15), craving, as reported subjectively, does not provide a clear case to test the incentive vs. negative reinforcement accounts of addiction. What is subjectively self-reported as craving -- during smoking, abstinence, and cue exposure -- includes both a desire for nicotine's pleasant effects and a desire to avoid withdrawal and abstinence (16,17). This duality presumably stems from the ways smokers reason, and the ways reasoning is measured and recorded. Self-reported subjective feelings might not accurately reveal genuine motives, as humans could be unaware of the underlying motivation processes (18). Questionnaires are often not designed to unequivocally differentiate these two processes. Moreover, it is difficult to identify and use smoking-related cues (e.g., an unlit or lit cigarette, imagination of smoking, and video images of smoking) in experimental settings that are selectively associated with either withdrawal or nicotine-like effects.

In experimental animals, nicotine-like effects and withdrawal can be manipulated independently. However, evidence is lacking to support the negative reinforcement account of nicotine seeking in experimental animals. Precipitated withdrawal *decreases* nicotine self-administration in rats (19), and cues selectively associated with precipitated withdrawal subsequently elicit *avoidance* in rats chronically exposed to nicotine (20). It is not known how withdrawal and withdrawal-associated cues *enhance* nicotine seeking. By contrast, the incentive properties of nicotine have been clearly demonstrated. Experimental animals readily self-administer nicotine without withdrawal (21,22). Cues that are associated with nicotine, but not withdrawal, sustain nicotine-seeking and reinstate extinguished nicotine self-administration in experimental animals (21,23,24).

As there is a paucity of evidence supporting the role of negative reinforcement in nicotine seeking in experimental animals, we asked whether withdrawal and conditioned withdrawal have an effect on nicotine cue approach via processes other than negative reinforcement.

Methods and Materials

Nicotine CPP

Groups 1-3—We used a place conditioning paradigm to establish an association between the approach-inducing effects of nicotine and visual and tactile cues in mice (see Supplementary Material, Animals). The apparatus was identical to that described in our previous studies (25-27). Each compartment of a three-compartment place conditioning apparatus contained distinct visual and tactile cues. Light intensities were adjusted so that individual mice preferred one of the two large compartments during a 15-min preconditioning session (Day 1; see Supplementary Material, Biased CPP procedure). On Day 2, mice were confined to the two large compartments during two 30-min conditioning sessions immediately after they received two injections of saline 5 h apart (Group 1) or a single injection of nicotine (0.2 free base mg/kg, s.c.) and a single injection of saline 5 h apart (Groups 2 and 3) (see Fig. 1A; see Drugs section in the Supplement). We chose this nicotine dose because it has been shown to produce the most robust CPP in our place conditioning apparatus (25,26). A nicotine injection was always paired with the initially non-preferred compartment during conditioning. Conditioned preference was tested without an injection during a 15-min postconditioning session on the next day (Day 3, Groups 1 and 2) or 16 days later (Day 18, Group 3). An observer blinded to experimental conditions recorded the amounts of time animals, in these groups and all other groups, spent in the two large compartments. The time each animal spent in the initially non-preferred compartment minus that in the initially preferred compartment was calculated for the pre-conditioning and post-conditioning days and used for analysis (see Statistical Analysis section in the Supplement).

Groups 4-11—Mice in Groups 4, 6-11 were pre-tested and conditioned with nicotine and saline, as described above for Group 3; Group 5 received saline injections in both compartments during conditioning. Mice were then singly housed and given continuous nicotine (200 free base µg/mL, Groups 4, 5, 7-11; see Drugs section in the Supplement) or water (Group 6) from single water bottles in their home cages from Day 3 (see Fig. 2A). The amount of nicotine solution consumed and body weight were measured every 3 days. On the 15th day of chronic nicotine exposure (i.e., Day 17), mice were brought in their home cages to a dark separate room, injected with mecamylamine hydrochloride (0 or 2.5 free base mg/kg, s.c., Groups 4-10; see Drugs section in the Supplement) or LiCl salt (240 mg/kg, i.p., Group 11; see Drugs section in the Supplement), and left for 30 min in the presence or absence of a tone (10 kHz, 80 dB) generated by an NCH tone generator (NCH Swift Sound, Canberra, Australia). Because mice might mitigate the precipitated withdrawal by consuming more nicotine from drinking water, the water bottles were removed during the 30-min period.

On Day 18, the mice were presented with the tone in the place conditioning apparatus and tested for nicotine CPP during continuous access to oral water (Group 6) or nicotine (Groups 4, 5, 7-9, and 11) in their home cages, or 24 h after termination of oral nicotine administration in home cages (Group 10). No acute nicotine injection was given on the test day for any group. Two speakers were placed at the far ends of the two compartments of the place conditioning apparatus, so that the sound levels (10 kHz, 80 dB) were equal in the two compartments.

Groups 12-14—To evaluate the impact of spontaneous withdrawal on nicotine CPP, one additional group of mice was tested 24 h after termination of oral nicotine administration (200 free base $\mu\text{g/mL}$, Group 12) (see Fig. 4A). One control group was tested while mice still had access to oral nicotine (Group 13). Another group of mice consumed water instead of nicotine throughout the study (Group 14). These three groups did not receive a tone or mecamylamine at any time. Otherwise, the procedure was identical to that used for Group 4.

Conditioned Place Aversion (CPA)

Although somatic withdrawal signs could potentially confirm mecamylamine-induced withdrawal, we chose to examine a motivational withdrawal sign in withdrawal-induced CPA. In humans, somatic withdrawal signs are not considered a critical element for craving (28). In rodents, somatic withdrawal signs are not correlated with motivational aspects of nicotine addiction (29,30). Moreover, adolescent rodents do not exhibit robust somatic withdrawal signs (31). We chose LiCl as a control, as mice quickly learn to avoid a place associated with LiCl due to its action in the brain to induce visceral illness (32) independently of addiction-related pathways (33).

Light intensities of the place conditioning apparatus were adjusted so that, on a group basis, mice spent indistinguishable amounts of time in the two large compartments during a 15-min preconditioning session (Day 1 or 14; see Fig. 3A). The compartment paired with mecamylamine or LiCl was counterbalanced during two 30-min conditioning sessions (Day 2 or 15).

To establish withdrawal-induced CPA, two groups of mice were first exposed to chronic water or nicotine (200 free base $\mu\text{g/mL}$) from single water bottles in their home cages. These mice were tested for their baseline preference/avoidance of the two compartments of the place conditioning apparatus on the 14th day. On day 15, mice were injected with mecamylamine hydrochloride (2.5 free base mg/kg , s.c.; see Drugs section in the Supplement) and vehicle 5 h apart for conditioning in the two compartments. Mice were tested for CPA 24 h after conditioning while they still had access to nicotine or water in their home cages. The time each animal spent in the drug-paired compartment minus that in the vehicle-paired compartment was calculated for the pre-conditioning and post-conditioning days and used for analysis (see Statistical Analysis section in the Supplement).

A third group of mice was injected with LiCl salt (240 mg/kg , i.p.) and vehicle 5 h apart during conditioning and tested on the next day. Otherwise, the procedure was identical to that used for the Group 2 of CPP.

Results

Time-dependent disappearance of nicotine CPP

Animals learn to approach stimuli that are associated with addictive substances in the place conditioning paradigm (34,35). To investigate nicotine cue reactivity in mice, we first established an association between the approach-inducing effects of nicotine and the visual and tactile cues of the place conditioning apparatus, using Pavlovian conditioning (Fig. 1A), which produces cue reactivity in human smokers (36).

Mice shifted their aversion to preference following conditioning [$F(1,27) = 15.11$, $P = 0.0006$], but this effect depended on the experimental condition [Group, $F(2,27) = 6.85$, $P = 0.0039$; Group $\times$ Conditioning, $F(2,27) = 9.14$, $P = 0.0009$] (Fig. 1B). When mice were injected with saline alone during conditioning in each of two large compartments of a place conditioning apparatus, their initial avoidance of one compartment did not shift (Fig. 1B, Group 1). Following a single pairing of nicotine with the initially non-preferred

compartment during conditioning, mice shifted their preference to the nicotine-paired compartment, resulting in CPP (Fig. 1B, Group 2). When a separate set of mice was tested 16 days after nicotine conditioning (i.e., Day 18; see Fig. 1A, Group 3), they did not exhibit CPP (Fig. 1B, Group 3). Group 3 did not experience extinction, as repeated testing in the absence of nicotine was not done. Taken together, these observations show that a single pairing is sufficient to establish nicotine CPP. This CPP is apparently absent 16 days after place conditioning, without an active extinction process.

Nicotine CPP revealed by a conditioned withdrawal cue

To establish a cue selectively associated with withdrawal distinct from those associated with nicotine, mice were chronically exposed to nicotine in their home cages, and the nicotinic acetylcholine receptor antagonist mecamylamine was used to precipitate withdrawal in the presence of a tone in their home cages (Fig. 2A). Moreover, precipitated withdrawal was not present when an acute nicotine injection was used to establish CPP during conditioning. This experimental design prevented an accidental association between the effects of nicotine and the tone, and between precipitated withdrawal and the visual/tactile cues of the place conditioning apparatus. The effect of the withdrawal-associated tone on the apparently absent CPP on the delayed test day was examined in the place conditioning apparatus. On the test day, the tone was presented so that it was equally detectable in both compartments. No acute nicotine injection was given on the test day.

Although the overall group effect [$F(7, 85) = 4.19, P = 0.0005$] and conditioning effect [$F(1, 85) = 9.43, P = 0.0029$] were significant, animals shifted initial aversion to preference based on the condition [Group $\times$ Conditioning, $F(7, 85) = 3.27, P = 0.004$] (see Fig. 2B). Mice exhibited CPP in the presence of the withdrawal-associated tone (Fig. 2B, Group 4) at the time when approach behavior was otherwise absent (compare with Fig. 1B, Group 3). This effect required the formation of an association between cues and nicotine-induced effects during conditioning, because when saline was injected in both compartments during conditioning, the withdrawal-associated tone did not shift the initial bias in the two compartments (Fig. 2B, Group 5). This control group further rules out the possibility that chronic exposure to nicotine or the withdrawal-tone pairing experience alone shifted an initial bias in the place-conditioning apparatus.

A tone did not evoke CPP when a tone-mecamylamine pairing was given in the absence of chronic nicotine exposure (Group 6). This indicates that the re-appearance of nicotine CPP required the tone be paired with precipitated withdrawal, but not the effects of mecamylamine *per se*. This enabling effect required selective association between the tone and withdrawal. When mecamylamine (Group 7), a tone (Group 8), or both (Group 9) were omitted during withdrawal conditioning, the tone subsequently had no effect on dormant nicotine CPP.

Group 4 and Groups 5, 7-9 consumed indistinguishable amounts of nicotine [$F(4, 53) = 2.49, P = 0.0542$] (Fig. 2C). This observation excludes the possibility that Group 4 differed from the other groups in nicotine intake.

The reactivation by the withdrawal-associated tone of nicotine CPP occurred while mice still had access to oral nicotine in home cages (see Group 4). To determine whether the enabling effect of a withdrawal-associated cue could occur during spontaneous withdrawal, we tested the impact of the withdrawal-associated cue on dormant nicotine CPP following termination of chronic oral nicotine consumption in home cages (Fig. 2A, Group 10). The tone failed to elicit re-appearance of nicotine CPP during abstinence (Fig. 2B, Group 10), indicating that the enabling effect of the withdrawal-associated cue required continued nicotine exposure on the test day.

Aversiveness of withdrawal-associated cues

To evaluate the nature of aversiveness required for the re-emergence of nicotine CPP, we investigated the impact of a tone paired with LiCl. The presence of a tone, paired with LiCl in home cages, did not subsequently cause dormant nicotine CPP to reappear in the place conditioning apparatus (Fig. 2B, Group 11). This indicates that the impact of withdrawal cannot be generalized to this aversive event.

Nicotine withdrawal includes somatic withdrawal symptoms in experimental animals, but somatic withdrawal signs are unlikely to contribute to the motivational process underlying persistent smoking (28). To compare the intensity of negative motivational effects associated with withdrawal and LiCl, we established conditioned behaviors induced by these two aversive stimuli in the place conditioning paradigm in a separate set of animals (Fig. 3A). When one of the two large compartments of the place conditioning apparatus was paired with mecamylamine-induced withdrawal in mice chronically exposed to nicotine or with LiCl in nicotine-naïve mice, they showed indistinguishable levels of conditioned place aversion (CPA); mecamylamine *per se* did not induce CPA in nicotine-naïve mice [Group, $F(2,23) = 3.68$, $P = 0.0411$; Conditioning, $F(1,23) = 9.64$, $P = 0.005$; Group x Conditioning, $F(2,23) = 1.52$, $P = 0.2394$] (Fig. 3B). Taken together, these data show that although LiCl and precipitated withdrawal induce quantitatively indistinguishable levels of conditioned aversion, withdrawal conferred unique aversive properties to cues that enabled nicotine CPP to reappear.

Spontaneous withdrawal and dormant nicotine CPP

We next examined whether the effect of the cue-evoked withdrawal can be generalized to spontaneous withdrawal during abstinence (see Fig. 4A). Mice were given chronic nicotine (Groups 12 and 13) or water (Group 14) via oral intake in home cages and tested after termination of chronic nicotine (Group 12) or during continuous access to oral nicotine (Group 13) or water (Group 14). No tone was present during conditioning or testing. None of these experimental groups shifted their initial aversion to preference following nicotine CPP conditioning [Group, $F(2,24) = 0.26$, $P = 0.7762$; Conditioning, $F(1,24) = 4.15$, $P = 0.0529$; Group x Conditioning, $F(2,24) = 0.45$, $P = 0.6445$] (see Fig. 4B, Groups 12 and 13). These data indicate that the enabling effect of the withdrawal-associated cue cannot be generalized to a state of spontaneous withdrawal.

Discussion

Our results show that nicotine CPP, established by a single pairing, spontaneously waned without exposure to the place conditioning apparatus. A withdrawal-associated tone reactivated dormant nicotine CPP. The tone acquired this enabling effect through Pavlovian conditioning with precipitated withdrawal. While the precise nature of the tone that reactivated dormant nicotine CPP remains unclear, the tone acquired such properties because it was paired with precipitated withdrawal; a tone that was not paired with precipitated withdrawal did not evoke dormant nicotine CPP. The emergence of dormant nicotine CPP occurred during continuous exposure to nicotine, but not during a state of spontaneous withdrawal. Moreover, dormant nicotine CPP was not reactivated by a state of spontaneous withdrawal alone. Although LiCl and withdrawal induced indistinguishable levels of conditioned aversion, a tone paired with LiCl failed to evoke dormant nicotine CPP. Taken together, our data identify the precise condition under which nicotine cue reactivity re-emerges: the unique aversive properties of a conditioned withdrawal cue reactivate dormant nicotine CPP during continuous exposure to nicotine.

It has been demonstrated that when a once-acquired conditioned stimulus is repeatedly presented without an unconditioned stimulus, such conditioning is no longer expressed as a behavior (i.e., extinguished), but the memory of this behavior remains intact (37). Our observations extend this account into a behavior that spontaneously disappears without an active extinction procedure.

Dormant nicotine CPP was not reinstated by a cue associated with the conditioned aversive effects of LiCl (Group 11) or by spontaneous withdrawal (Group 12). This observation appears to contradict the general roles of stressful events (e.g., foot shock) to reinstate extinguished morphine and cocaine CPP (38). This apparent discrepancy might reflect the differences between nicotine and the other addictive substances, as well as quantitative and qualitative differences in stress induced by foot shock and a LiCl-associated cue or spontaneous withdrawal. Alternatively, stressful events might reinstate extinguished, but not dormant, behaviors, as nicotine CPP was not actively extinguished by repeated presentations of conditioned stimuli without drug in our study.

Cues learned under the influence of a certain state can be recalled when the animal is in a similar state. However, the retrieval of dormant nicotine CPP cannot be adequately explained by priming-induced or cue-triggered reinstatement. No acute nicotine injection was given on the test day. Moreover, continuous oral nicotine intake (Group 13) or nicotine-associated cues (Group 3) alone failed to reactivate dormant nicotine CPP. Conversely, although the conditioned withdrawal cue evoked dormant nicotine CPP, it cannot serve as a reminder of nicotine CPP; the latter was established before withdrawal was induced and a conditioned withdrawal cue was established (Group 4).

It could be argued that negative reinforcement played a role in the re-emergence of dormant nicotine CPP. Negative reinforcement, by definition, is the process by which a behavior occurs more frequently when that behavior alleviates or terminates an aversive stimulus. Our experimental design rules out the possibility that animals acquired conditioning via negative reinforcement. First, an animal's behavior had no effect on a tone-withdrawal pairing. Second, a tone and precipitated withdrawal were not present during the acquisition of nicotine CPP.

Negative reinforcement does not adequately account for the expression of dormant nicotine CPP during the delayed testing. The withdrawal-associated tone was presented in both the previously saline- and nicotine-paired compartment on the delayed test day, providing no directionality as to approaching which compartment reduces conditioned withdrawal. Moreover, dormant CPP emerged when the withdrawal-associated tone was presented and the animal's CPP did not alleviate or terminate the tone.

It could still be argued that animals experienced conditioned nicotine-like effects when approaching the previously nicotine-paired compartment during the delayed testing, and these effects countered conditioned withdrawal-like effects induced by the tone. However, mice did not exhibit any preference for the previously nicotine-paired compartment on the delayed test day without a conditioned withdrawal cue (Fig. 1B, Group 3). This indicates that cues previously associated with nicotine no longer evoked nicotine-like effects, at least to the extent that this effect reveals itself as a behavior.

One plausible alternative explanation is that conditioned withdrawal augmented the incentive values of nicotine conditioned cues. The contemporary incentive account of addiction posits that withdrawal augments instrumental drug seeking behavior by enhancing the unconditioned incentive value of the addictive substance (39,40). Our data extend this account into Pavlovian conditioned behavior that is controlled by cue-cue interaction alone

by demonstrating that a *conditioned* withdrawal cue augments the incentive value of *conditioned* nicotine cues.

Previous studies with opiates have demonstrated that withdrawal-associated cues influence incentive behavior during extinction (41-43). These studies presented a cue and withdrawal (41), or cues previously associated with withdrawal (42), while animals were engaged in opiate taking. These experiments examined how such cues subsequently affect opiate seeking behavior during extinction. In another study (43), after morphine CPP was first extinguished by repeated exposure to the CPP apparatus, mice were exposed to all compartments of the apparatus in the presence of withdrawal. Although withdrawal did not alter the extinguished value of conditioned incentive cues, this procedure enabled CPP to subsequently re-appear.

Our study differs from these studies in four critical aspects. First, previous studies presented withdrawal or withdrawal-associated cues when animals received the addictive substance (41,42). In our study, withdrawal or withdrawal-associated cues were not present while mice received nicotine in the place conditioning apparatus. Our study was designed to evaluate the effects of withdrawal-associated cues on the incentive properties of nicotine-conditioned cues, not nicotine itself. Second, while the study by Lu and colleagues (43) demonstrated how *precipitated* withdrawal altered the conditioned incentive values of CPP cues, our study was designed to examine how a *conditioned* withdrawal cue alters the conditioned incentive values of CPP cues. Third, to prevent the same set of cues from being associated with both the incentive value of the addictive substance and withdrawal, our study separately paired different sets of cues with either nicotine or precipitated withdrawal. This procedure also prevented involvement of negative reinforcement in nicotine CPP conditioning. Fourth, while behaviors were actively extinguished by repeatedly presenting conditioned cues in the absence of the drug in the previous studies, such a procedure was not applied in the present study. The memory processes underlying the dormant and extinguished CPP might differ and the spontaneous waning drug memory might represent a distinct form of memory.

As the evocation by a withdrawal-associated cue of dormant CPP occurred when mice were still continuously exposed to nicotine (Group 4), but not after oral nicotine administration was terminated (Group 10), such an action of withdrawal-associated cues is likely to occur during continuous exposure to nicotine. While actively smoking, smokers experience negative affect between cigarettes (10). Cues that a smoker encounters between cigarettes might awaken the dormant effects of nicotine-associated cues on sustained nicotine-seeking. As retrieval of cue reactivity renders its memory labile and amenable to genuine extinction (44), our results indicate a time window that can be exploited as a therapeutic target to eliminate nicotine cue reactivity in active smokers.

The majority (~70-86%) of smokers experience the most intense craving immediately after quitting, but experience progressively less craving as abstinence continues (45-48). Our observation that mice showed robust nicotine CPP one day after conditioning, but not 16 days later is consistent with these clinical findings. The dormant form of nicotine cue reactivity might underlie nicotine seeking as a long-lasting, potent process. This process is likely to be easily acquired as it was established by a single injection of nicotine.

Cue-induced craving, one aspect of drug cue reactivity, is not unique to persistent smoking and is seen in many other forms of addiction (49). Our finding provides a novel means to understand incentive motivation in addiction and examine the genetic and neuronal substrates underlying cue reactivity in both experimental animals and humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. (A) Experimental design. White boxes, pre- and post-conditioning tests; black boxes, nicotine-conditioning sessions (0 and 0 mg/kg or 0 and 0.2 free base mg/kg, s.c.; black arrows). Note that an acute nicotine injection was not given on the test day. (B) Nicotine CPP is present 1 day after conditioning (Group 2; No Delay) but not 16 days after conditioning (Group 3; Delay). Nicotine was paired with the initially non-preferred compartment. ** $P < 0.01$, pre- versus post-conditioning tests, as determined by Newman-Keuls post-hoc comparisons. All data were normally distributed, as determined by the Jarque-Bera test of normality (P values ranging from 0.507 to 0.823). Time difference, time spent in the initially non-preferred compartment minus that in the initially preferred compartment; black bars, pre-conditioning test; gray bars, post-conditioning test. $N = 9 - 12$ per group.

Fig. 2.

(A) Experimental design. White boxes, pre- and post-conditioning tests; black boxes, nicotine-conditioning sessions (0 and 0 mg/kg or 0 and 0.2 free base mg/kg, s.c.; black arrows); black horizontal lines, chronic nicotine administration (0 or 200 free base $\mu\text{g/mL}$) in home cage; black arrow heads, injection with vehicle, mecamylamine (2.5 free base mg/kg, s.c.) or LiCl salt (240 mg/kg, i.p.); speaker symbol, tone. All groups were presented with the tone on the test day. (B) Withdrawal-associated cue elicits reappearing of CPP (Group 4). Nicotine was paired with the initially non-preferred compartment. Time difference, time spent in the initially non-preferred compartment minus that in the initially preferred compartment; Nicotine, an acute nicotine injection during conditioning; Chronic nicotine, access to nicotine from drinking water in home cages; Mecamylamine, a mecamylamine injection paired with a tone or with no tone in home cages; LiCl, a LiCl injection paired with a tone in home cages. No mecamylamine injection was given to this group; Tone, a tone presented following a mecamylamine, LiCl or vehicle injection in home cages; Oral nicotine at test, access to nicotine from drinking water in home cages on the test day. $**P < 0.01$, pre- versus post-conditioning tests, as determined by Newman-Keuls post-hoc comparisons. All data were normally distributed, as determined by the Jarque-Bera test of normality (P values ranging from 0.228 to 0.794). $N = 8 - 17$ per group. (C) Nicotine intake. Nicotine intake for Groups 4, 5, 7, 8, and 9 was calculated from the total volume of nicotine-containing water consumed. All data were normally distributed, as determined by the Jarque-Bera test of normality (P values ranging from 0.628 to 0.835).

Fig. 3.

(A) Experimental design. Aversive effects of LiCl and mecamylamine-induced withdrawal were evaluated in the place conditioning paradigm. Nicotine-naïve mice were used for LiCl (240 mg/kg, i.p., N = 6) conditioned place aversion (LiCl CPA). For withdrawal-induced CPA, mice were continuously exposed to nicotine (200 free base μ /mL, Mec, Nic, N = 12) or water (Mec, Water, N = 8), given mecamylamine (2.5 free base mg/kg, s.c.) and confined to the two place conditioning compartments on the 15th day of chronic nicotine or water administration, and tested on the 16th day. Time difference, time spent in the compartment paired with the drug minus that in the compartment paired with vehicle; white boxes, pre- and post-conditioning tests; black boxes, conditioning sessions; black horizontal line, chronic nicotine administration (0 or 200 free base μ /mL) in home cages; black arrows, acute injections with LiCl and vehicle; black arrow heads, acute injections with mecamylamine and saline. (B) Mecamylamine-induced withdrawal and LiCl established indistinguishable levels of CPA. N.S., not significantly different CPA induced by LiCl versus mecamylamine-precipitated withdrawal. * $P < 0.05$ and ** $P < 0.01$, pre-conditioning versus post-conditioning tests, as determined by Newman-Keuls post-hoc comparisons. All data were normally distributed, as determined by the Jarque-Bera test of normality (P values ranging from 0.444 to 0.987).

Fig. 4.

(A) Experimental design. White boxes, pre- and post-conditioning tests; black boxes, nicotine-conditioning sessions (0 and 0.2 free base mg/kg, s.c.; black arrows); black horizontal lines, chronic nicotine administration (0 or 200 free base μ mL) in home cages. Mice were tested 24 hr after oral nicotine was substituted by plain water (Group 12), while they still had access to nicotine in drinking water in home cages (Group 13), or while they continuously had access to plain water in home cages (Group 14). No group was presented with a tone during conditioning or on the test day. (B) Without the withdrawal-associated cue, CPP remains dormant following abstinence (Group 12), continuous nicotine exposure (Group 13), or continuous water exposure (Group 14). Nicotine was paired with the initially non-preferred compartment. Time difference, time spent in the initially non-preferred compartment minus that in the initially preferred compartment; Chronic nicotine, access to nicotine from drinking water in home cages; Oral nicotine at test, access to nicotine from drinking water in home cages on the test day. All data were normally distributed, as determined by the Jarque-Bera test of normality (P values ranging from 0.383 to 0.999). $N = 9$ per group.