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Potentiated startle as a measure of the negative affective consequences of repeated exposure to nicotine in rats

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Abstract

Rationale—Elevated acoustic startle amplitude has been used to measure anxiety-like effects of drug withdrawal in humans and animals. Withdrawal from a single opiate administration has been shown to produce robust elevations in startle amplitude (“withdrawal-potentiated startle”) that escalate in severity with repeated exposure. Although anxiety is a clinical symptom of nicotine dependence, it is currently unknown whether anxiety-like behavior is elicited during the early stages of nicotine dependence in rodents.

Objective—The objective of this study is to examine whether, as is the case with opiates, single or repeated exposure to nicotine can produce withdrawal-potentiated startle.

Methods—Rats received daily nicotine injections for 14 days, and startle amplitude was tested during spontaneous withdrawal on injection days 1, 7, and 14.

Results—Elevated startle responding was observed during nicotine withdrawal on days 7 and 14 but not on day 1, was greater at higher nicotine doses, and was reduced by a nicotine replacement injection given during an additional test session on day 15. Additional experiments demonstrated that nicotine withdrawal-potentiated startle was reduced by the α_2 -adrenergic agonist clonidine and that precipitated withdrawal-potentiated startle could not be induced by injection of the nicotinic acetylcholine receptor antagonist mecamylamine.

Conclusions—These results suggest that nicotine withdrawal escalates in severity across days, similar to the previously reported escalation of opiate withdrawal-potentiated startle. Potentiated startle may be a reliable measure of withdrawal from different classes of abused drugs and may be useful in the study of the early stages of drug dependence.

Keywords

Anxiety; Withdrawal; Nicotine; Startle; Sensitization; Addiction

Numerous signs and symptoms of psychological distress have been reported during tobacco abstinence in dependent smokers (Hughes et al. 1991; Hughes and Hatsukami 1986). The negative affective consequences of tobacco withdrawal, such as anxiety, are likely due to withdrawal from nicotine, the primary addictive ingredient (e.g., Hughes et al. 1984; West et al. 1984). Given the role that avoidance of these consequences plays in relapse to smoking (Piasecki et al. 1997, 1998, 2000), it is important to identify neural mechanisms involved in nicotine withdrawal.

Studies of withdrawal from continuously infused nicotine have provided valuable insight into mechanisms involved in a single, severe nicotine withdrawal episode, but they are not well-suited for examining the multiple withdrawal episodes that occur as a result of intermittent, discrete nicotine exposures. Due to the intermittent nature of drug-taking behavior in the earliest stages of dependence, it is important to examine the effects of repeated withdrawal episodes across multiple acute drug exposures. Such investigations may lead to a better characterization of neural adaptations involved in the emergence of nicotine dependence (Harris and Gewirtz 2005).

Recently, a procedure has been developed using the acoustic startle reflex to measure the anxiety-like aspects of repeated opiate withdrawals. Elevated acoustic startle responding provides a reliable, cross-species measure of fear and anxiety (e.g., Walker et al. 2003). Moreover, potentiated startle has also been observed during withdrawal from drugs of abuse such as ethanol (Krystal et al. 1997; Rassnick et al. 1992) and opiates (Harris and Gewirtz 2004; Kalinichev and Holtzman 2003; Stine et al. 2001) in both human and animal subjects. Robust elevations in acoustic startle responding have also been reported in rats upon cessation of chronic, continuous nicotine infusion (Helton et al. 1993, 1997; Rasmussen et al. 1996, 1997, 2000). Harris, et al. (2004) observed an escalation in the level of startle potentiation across daily withdrawals from morphine, suggestive of increasingly severe withdrawal episodes across repeated opiate exposures. The goal of the current study was to determine whether similar escalations in the severity of withdrawal could be observed across repeated nicotine injections.

Few studies have examined the effect of repeated withdrawals from discrete nicotine exposures, but there is evidence for increased anxiety-like behavior after withdrawal from daily acute nicotine injection in paradigms such as the elevated-plus maze, the social interaction test, and a drug-discrimination procedure (Bhattacharya et al. 1995; Cheeta et al. 2001; Harris et al. 1986; Irvine et al. 2001). These findings, along with striking similarities between other aspects of nicotine and opiate withdrawal syndromes in both rats (Ise et al. 2000, 2002; Malin et al. 1992, 1996a, b, 1993) and humans (Hughes et al. 1994), supports the prediction that an escalation in withdrawal-potentiated startle severity similar to that for opiates will be observed across multiple nicotine exposures.

In four experiments, spontaneous and precipitated withdrawal from repeated injections of nicotine were examined. Experiment I established the time course of spontaneous withdrawal, measured as potentiation of the startle reflex, and assessed the effects of nicotine replacement. Experiment II assessed the dose-dependence of nicotine withdrawal-potentiated startle. Experiment III examined whether clonidine, an α_2 -adrenergic receptor agonist that reduces morphine withdrawal-potentiated startle in rats (Harris and Gewirtz 2004) and relieves nicotine withdrawal symptoms in humans (Glassman et al. 1984), would reduce nicotine withdrawal-potentiated startle. Experiment IV investigated whether potentiated startle would emerge during withdrawal precipitated by mecamylamine, a nonselective nicotinic acetylcholine receptor (nAChR) antagonist.

Due to the similarities between the injection protocol used in the current study and those used to investigate drug-induced sensitization of locomotor activity (for a review, see Stewart and Badiani 1993), we included measures of the rats' activity levels at the beginning of each startle test session. Although not a standard measure of locomotor activity (due to the small size of the test chambers), activity readings were used to assess whether increases in activity levels immediately after nicotine injection would be observed across days, similar to the sensitization effects observed using conventional measures of locomotor activity (Janhunen et al. 2005).

Materials and methods

Animals

Male albino Sprague-Dawley rats, obtained from Harlan (Indianapolis, IN, USA; experiments I and II) and Charles River (Raleigh, NC, USA; experiments III and IV), were used. Rats were housed in hanging metal cages in groups of four per cage and were maintained on a 12-h light–dark cycle (lights on at 8:00 a.m.) with food and water continuously available. Upon arrival in the colony, rats were allowed a 2-week acclimation period followed by 3 days of handling and habituation to subcutaneous injections (one daily saline injection, 1 ml/kg body weight). All rats weighed between 270 and 370 g at the start of testing, and all tests were run during the light phase of the light–dark cycle. All experimental procedures conformed to the Principles of Laboratory Animal Care (National Institutes of Health publication no. 8023, revised 1996) and Guidelines for the Humane Care and Use of Laboratory Animals of The Institutional Animal Care and Use Committee at the University of Minnesota.

Drugs

(–)-Nicotine hydrogen tartrate salt, clonidine, and mecamylamine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). All drugs were dissolved in saline (0.9% w/v) and injected subcutaneously in a volume of 1 ml/kg body weight. All nicotine doses are expressed as the base and all other doses as the salt. The nicotine solution was titrated to a pH of approximately 7.1 using sodium hydroxide.

Testing apparatus and procedure

Startle reflex amplitude and activity were tested using a stabilimeter device that has been described previously (Rothwell et al. 2009). Briefly, cage displacement proportional to the rat's movement was measured by a piezoelectric accelerometer in the absence of discrete stimuli (*activity trials*) and in response to startle-eliciting noise bursts (*startle trials*). Activity levels for each activity trial were defined as the mean peak-to-peak accelerometer voltage within a 200-ms accelerometer sample, and response amplitude for each startle trial was defined as the mean peak-to-peak voltage during the first 200 ms after onset of the startle stimulus. The startle stimulus consisted of a 50-ms (rise-decay <5 ms) filtered white noise (low pass: 22 kHz) delivered through a high frequency speaker (RadioShack Supertweeter, range 5–40 kHz, Model 40–1310b) located 7 cm from the side of each cage at intensities of 95 or 105 dB.

Each test session consisted of a 5-min period, during which, activity levels were monitored every 10 s in the absence of startle stimuli, followed by a 20-min period of startle testing. Startle stimuli (20 at 95 dB and 20 at 105 dB) were presented at a fixed 30-s interval. The two intensity levels were presented in a pseudorandom order.

Baseline startle measurement

At the start of each experiment, all rats received 2 days of baseline startle measurement. On both days, all rats were injected with saline 1 h after the beginning of the startle test session. The first baseline test day was intended to habituate the rats to the startle testing procedure,

and data from this session were not analyzed. The second baseline test day (subsequently referred to as *baseline*) was used to place animals into treatment groups with approximately equal mean baseline startle amplitude.

Experiment I: time course of spontaneous withdrawal

In this experiment, rats received multiple injections of a single nicotine dose to determine if an escalation in withdrawal-potentiated startle severity would be observed across multiple nicotine exposures. Beginning the day after baseline, rats in one group ($n=12$) received daily saline injections and rats in a second group ($n=12$) received daily nicotine injections (0.25 mg/kg) for a total of 14 days. On days 1, 7, and 14, the rats were given a startle test session 1 h before nicotine or saline injection (referred to as the *pretest*), and four startle test sessions beginning 5 min, 1 h, 2 h, 3 h, and 4 h after the injection (referred to as *posttests*; Fig. 1a). Rats were returned to their home cages between startle test sessions. On all other days of the spontaneous withdrawal procedure, no startle test sessions were conducted; the only treatment was the nicotine or saline injection.

On day 15, a test was included to evaluate whether the potentiated startle effect observed on the previous day was due to nicotine deprivation. A subset of rats ($n=8$ from each group) underwent a startle testing procedure that was identical to that on days 1, 7, and 14 with the following exceptions: animals in the nicotine group received a second nicotine injection (0.25 mg/kg), and animals in the saline group received a second saline injection 10 min prior to the 2-h test session, and there were two additional posttest sessions, taking place 5 and 6 h after the first injection (Fig. 1b).

Experiment II: dose-response evaluation

The purpose of experiment II was to replicate the spontaneous withdrawal effect observed in experiment I and to determine how different doses of nicotine modulate the withdrawal-potentiated startle effect. The procedures over 14 days were the same as those used in experiment I, with the exception that there were four groups of rats ($n=8$ per group), each assigned to a different nicotine dose (0, 0.125, 0.25, or 0.5 mg/kg/day).

Experiment III: effect of clonidine on withdrawal-potentiated startle

The purpose of this experiment was to determine whether nicotine withdrawal-potentiated startle could be blocked by clonidine, an α_2 -adrenergic receptor agonist that has been shown to block affective signs of withdrawal in general (Smith and Aston-Jones 2008) and opiate withdrawal-potentiated startle in particular (Harris and Gewirtz 2004). Rats were assigned to groups that received either saline or nicotine (0.5 mg/kg) daily for 8 days (Fig. 1c). On day 6, all rats were given a startle test session 1 h prior to the nicotine or saline injection, to allow them to re-acclimate to the startle testing procedure. On days 7 and 8, startle test sessions took place 1 h prior to and 2 h after the nicotine or saline injection. All animals received an additional injection 5 min prior to the start of the 2-h test session, which is where the peak withdrawal-potentiated startle effect was observed in experiments I and II. On day 7, this injection was saline for all animals. This test was conducted to make certain that animals were showing normal levels of withdrawal-potentiated startle 2 h after nicotine injection. On day 8, animals within the saline and nicotine groups were assigned to three subgroups that were injected with clonidine (0, 10, or 15 μ g/kg; $n=9$, 6, and 5 per subgroup).

Experiment IV: precipitated withdrawal using mecamlamine

Because the first three experiments suggested that repeated nicotine exposure results in potentiated startle during spontaneous withdrawal, a final experiment examined whether a similar effect could be observed during precipitated withdrawal. Rats received daily injections

of nicotine (0 or 0.5 mg/kg) for 8 days. On day 6, all rats were given a startle test session 1 h prior to the nicotine or saline injection, to allow them to re-acclimate to the startle testing procedure. On days 7 and 8, startle test sessions took place 1 h prior to and 40 min after nicotine injection (Fig. 1d), so that posttest startle amplitude during precipitated withdrawal could be measured before the emergence of spontaneous withdrawal-potentiated startle that was observed at later time points during experiments I and II (cf. Harris and Gewirtz 2004). Five minutes prior to the start of the posttest session on day 7, all animals were injected with saline. On day 8, half of the animals within each nicotine dose were injected with saline ($n=8$ per nicotine dose), and the other half were injected with mecamylamine (1 mg/kg; $n=8$ per nicotine dose).

Statistical analysis

As in previous studies (Harris et al. 2006, 2004; Harris and Gewirtz 2004), the 105-dB startle stimuli resulted in greater startle amplitude than did the 95-dB startle stimuli, but there was no significant effect of startle stimulus intensity on the magnitude of startle potentiation (data not shown; see Walker and Davis 2002). Thus, startle amplitude was averaged across all 40 trials during the test session, creating a single startle amplitude score for each session. Similarly, a single activity score was created for each test session by averaging across all 30 activity readings measured during the first 5 min of the session. To verify that there were no significant between-groups differences in baseline or pretest startle amplitude, these data were subjected to a nicotine dose $\times$ test day analysis of variance (ANOVA), with group as a between-subjects factor and test day as a within-subjects factor.

In all experiments, withdrawal-potentiated startle was quantified as the percent change in startle amplitude between each day's pretest and posttest sessions (Walker and Davis 2002).

Analogous percent change scores were calculated for activity levels. Animals with percent change scores in startle or activity greater than three standard deviations from the population mean were considered outliers and excluded from analyses (Johnson and Wichern 2002).

For experiments I and II, percent change in startle and percent change in activity were analyzed separately using the general linear models procedure in SYSTAT 12 (SYSTAT Software, Inc., Chicago, IL, USA). For all tests, the criterion for significance was set at $p<.05$. Multivariate test statistics (Wilks λ and its approximate F statistic) were used to test the significance of all effects involving repeated measures because multivariate tests do not require the assumption of a spherical covariance matrix across all levels of a repeated measure (Johnson and Wichern 2002; Maxwell and Delaney 1990). In experiments III and IV, planned contrasts were used to test the effect of clonidine or mecamylamine on withdrawal-potentiated startle. These contrasts are based on the results of previous experiments assessing the effect of clonidine on opiate withdrawal-potentiated startle (Harris and Gewirtz 2004).

Results

Experiment I

This experiment examined the time course of spontaneous withdrawal-potentiated startle from a daily dose of nicotine (0.25 mg/kg). Dose $\times$ test day ANOVA was used to evaluate whether there were any differences in startle amplitude across the baseline day and pretests on days 1, 7, and 14 of nicotine injection. The main effects of dose [$F(1, 22) < 1, p > .1$] and day [$F(3, 20) = 2.23, p > .1$], and the dose $\times$ day interaction [$F(3, 20) < 1, p > .1$] were not significant (see Table 1).

Examination of percent change in startle amplitude from pretest to posttest confirmed that startle responding escalates over repeated exposures to nicotine (Fig. 2, left column; significant

nicotine dose $\times$ day $\times$ test session interaction [$\lambda = .39$, $F(8, 15) = 2.88$, $p < .05$]. Follow-up dose $\times$ test session ANOVAs within each day revealed significant dose $\times$ test session interactions on days 7 [$\lambda = .46$, $F(4, 19) = 5.56$, $p < .01$] and 14 [$\lambda = .23$, $F(4, 19) = 16.32$, $p < .001$], but not on day 1 [$\lambda = .68$, $F(4, 19) = 2.28$, $p = .10$]. These interactions were reflective of differences in the shape of startle amplitude vs time curves between the two groups, with significant startle potentiation in the nicotine group, compared to the saline group, during the 2-h test session on days 7 [$F(1, 22) = 4.49$, $p < .05$] and 14 [$F(1, 22) = 8.34$, $p < .01$]. There was also a significant between-group difference during the 5-min test on day 14 [$F(1, 22) = 4.56$, $p < .05$], where startle magnitude was lower in the nicotine group than in the saline group. Presumably, elevated startle magnitude at this time point in the saline group was due to stress caused by the subcutaneous injections (similar to shock-induced sensitization of startle, see Davis 1989), and nicotine reduced this effect, which is consistent with the hypotheses that nicotine is capable of reducing acute stress effects in rats (Acri 1994).

Activity levels increased immediately after nicotine injection on days 7 and 14, suggestive of an escalation in nicotine-induced activity across days, similar to the escalation in withdrawal-potentiated startle (Fig. 2, right column). ANOVA for activity readings revealed a significant dose $\times$ day $\times$ test session interaction [$\lambda = .30$, $F(8, 15) = 4.41$, $p < .01$], and follow-up dose $\times$ time point ANOVAs at each day found significant two-way interactions on days 7 [$\lambda = .59$, $F(4, 19) = 3.32$, $p < .05$] and 14 [$\lambda = .51$, $F(4, 19) = 4.60$, $p < .01$], but not on day 1 [$\lambda = .84$, $F(4, 19) < 1$, $p > .1$]. These interactions were the result of significant effects of nicotine dose during the 5-min test session on days 7 [$F(1, 22) = 13.19$, $p < .01$] and 14 [$F(1, 22) = 25.82$, $p < .001$]. The effect of nicotine dose was not significant at any other time point on days 7 and 14.

On day 15, a second nicotine injection temporarily reversed withdrawal-potentiated startle, delaying its peak from the 2- to the 3-h test session (Fig. 3, upper panel). This result was supported by a significant dose $\times$ time point interaction [$\lambda = .08$, $F(6, 9) = 16.98$, $p < .001$], with a significant effect of dose during the 1-h test session [$F(1, 14) = 6.22$, $p < .05$], no significant effect of dose during the 2-h test session [$F(1, 14) = 2.95$, $p > .1$], and re-emergence of the significant dose effect during the 3-h [$F(1, 14) = 14.16$, $p < .01$] and 4-h [$F(1, 14) = 8.08$, $p < .05$] test sessions. For activity, there was evidence of an increase after both the first and second nicotine injections (Fig. 3, lower panel). The main effect of dose was significant [$F(1, 14) = 5.80$, $p < .05$], but the main effect of test session [$\lambda = .39$, $F(6, 9) = 2.39$, $p > .1$] and the dose $\times$ test session interaction [$\lambda = .44$, $F(6, 9) = 1.89$, $p > .1$] were not significant. Importantly, follow-up tests revealed a significant effect of nicotine dose on activity during the 5-min [$F(1, 14) = 9.05$, $p < .01$] and 2-h [$F(1, 14) = 11.44$, $p < .01$], but not the 1-h [$F(1, 14) = 2.80$, $p > .1$], test sessions.

Experiment II

Data from one animal in the 0.125 mg/kg nicotine group were excluded from analyses as an outlier. As in experiment I, analysis of baseline and pretest startle amplitude found no evidence of a significant main effect of nicotine dose [$F(3, 27) < 1$, $p > .1$] or dose $\times$ test day interaction [$F(9, 60) = 1.40$, $p > .1$], indicating that there were no between-groups differences in startle amplitude prior to nicotine injection on any test day. However, the main effect of test day was significant [$F(3, 25) = 9.81$, $p < .001$; see Table 1]. Use of percent change scores from each test day's pre-injection startle amplitude allowed for assessment of withdrawal-potentiated startle independent of day-to-day variations in baseline startle amplitude (Walker and Davis 2002).

As expected, analysis of percent change in startle from pretest to posttest yielded no significant evidence of withdrawal-potentiated startle on day 1, and there were dose-dependent increases in startle amplitude during spontaneous withdrawal on days 7 and 14 (Fig. 4, left column), as

evidenced by a significant dose $\times$ day $\times$ test session interaction [$\lambda = .43$, $F(8, 22) = 3.65$, $p < .01$], and significant dose $\times$ test session interactions on days 7 [$\lambda = .58$, $F(4, 26) = 4.69$, $p < .01$] and 14 [$\lambda = .52$, $F(4, 26) = 5.88$, $p < .01$], but not on day 1 [$\lambda = .84$, $F(4, 26) = 1.20$, $p > .1$]. Although these results were consistent with experiment 1 in demonstrating an emergence of withdrawal-potentiated startle in the days 7 and 14 tests, the effect appeared to be less robust on day 7 in this experiment. Thus, significant effects of nicotine dose were observed during the 1-h [$F(1, 29) = 4.99$, $p < .05$], 3-h [$F(1, 29) = 8.11$, $p < .01$], and 4-h [$F(1, 29) = 10.96$, $p < .01$] test sessions on day 14. The dose effect during the 2-h test session, where peak withdrawal-potentiated startle was observed in experiment I, approached significance on day 14 [$F(1, 29) = 3.51$, $p = .07$] but not on day 7 [$F(1, 29) = 2.64$, $p > .1$], suggesting some variability in the rate of development of this phenomenon as a function of the number of nicotine exposures.

Experiment II also provided evidence that the increase in activity observed immediately after nicotine injection on days 7 and 14 is dose-dependent (Fig. 4, right column). This result was supported by a dose $\times$ day $\times$ time point interaction that approached significance [$\lambda = .55$, $F(8, 22) = 2.33$, $p = .07$], and significant dose $\times$ time point interactions on days 7 [$\lambda = .41$, $F(4, 26) = 9.39$, $p < .001$] and 14 [$\lambda = .47$, $F(4, 26) = 7.44$, $p < .001$], but not on day 1 [$\lambda = .76$, $F(4, 26) = 2.11$, $p > .1$]. These significant interactions were the result of significant effects of dose during the 5-min test on days 7 [$F(1, 29) = 19.17$, $p < .001$] and 14 [$F(1, 29) = 23.28$, $p < .001$], but at no other time point.

Experiment III

Experiment III examined whether clonidine would dose-dependently reduce withdrawal-potentiated startle from 0.5 mg/kg nicotine. Nicotine dose $\times$ day ANOVA found no significant main effect of dose [$F(1, 38) < 1$, $p > .1$] or dose $\times$ day interaction [$F(3, 36) < 1$, $p > .1$] on baseline and pretest startle amplitude, but there was a significant main effect of day [$F(3, 36) = 11.95$, $p < .001$; see Table 1].

On days 7 and 8 of nicotine injection (0 or 0.5 mg/kg), withdrawal-potentiated startle was assessed 2 h after injection. On day 7, rats injected with 0.5 mg/kg had significantly greater percent change in startle amplitude than rats injected with 0 mg/kg nicotine [0mg/kg mean = -2.12% , SEM = 5.85% ; 0.5mg/kg mean = 25.59% , SEM = 5.50% ; dose effect $F(1, 38) = 11.91$, $p < .001$]. On day 8, planned contrasts in the 0 $\mu\text{g/kg}$ clonidine groups confirmed the presence of withdrawal-potentiated startle following the 0.5 mg/kg nicotine injection [Fig. 5; $F(1, 34) = 17.68$, $p < .001$]. As expected, clonidine dose-dependently reduced startle amplitude in the 0.5 mg/kg nicotine group [linear trend of clonidine dose: $F(1, 34) = 9.271$, $p < .01$], but not in the 0 mg/kg nicotine group [linear trend of clonidine dose: $F(1, 34) < 1$, $p > .1$].

Experiment IV

Experiment IV examined whether mecamylamine-precipitated withdrawal could be observed after 7 days of nicotine injection. Data from one animal in the 0.5 mg/kg nicotine-0 mg/kg mecamylamine group were excluded from analyses as an outlier. Also, technical difficulties resulted in the loss of day 6 pre-injection startle data for 16 animals. Thus, day 6 (which was only used to re-habituate rats to the startle testing procedure) was not included in the nicotine dose $\times$ day ANOVA on baseline and pretest startle amplitude. This ANOVA found no significant main effect of dose [$F(1, 29) < 1$, $p > .1$] or dose $\times$ day interaction [$\lambda = .94$, $F(2, 28) < 1$, $p > .1$], but there was a significant main effect of day [$\lambda = .74$, $F(2, 28) = 4.94$, $p < .05$].

Analysis of percent change in startle from pretest to posttest found that, as expected, the early test session (40 min following 0 or 0.5 mg/kg nicotine) did not reveal significant withdrawal-

potentiated startle on day 7 [0 mg/kg mean = 5.70%, SD = 9.82%; 0.5 mg/kg mean = 24.67%, SD = 6.51%; dose effect $F(1, 29) = 2.52, p > .1$]. On day 8, there was also no evidence of nicotine withdrawal-potentiated startle in any of the four groups of rats [Fig. 6; all $F_s(1, 27) < 1$, all $p_s > .1$].

Discussion

Emergence of negative affective withdrawal symptoms is thought to be an important factor in the transition from initial drug use to dependence and addiction (Koob and Le Moal 2001; Solomon and Corbit 1973). Thus, animal models of these processes may lead to improved understanding of neural mechanisms involved in drug dependence. The withdrawal-potentiated startle procedure developed in the current study, which provides a methodology for measuring anxiety-like withdrawal episodes from repeated exposures to nicotine, may provide a valuable tool for the development of such models.

Several findings from the current study support the validity of withdrawal-potentiated startle as a measure of negative affective aspects of nicotine withdrawal. First, our findings agree with observations that potentiated startle emerges during withdrawal from chronic, continuously infused nicotine (Helton et al. 1993; Rasmussen et al. 1996, 1997, 2000). This suggests that the withdrawal episodes produced in our studies—those that emerge after repeated, intermittent nicotine exposure—are similar to those observed after continuous infusion, which raises the possibility that intermittent nicotine exposure paradigms can be used to study the initial development of the same components of withdrawal that persist through long-term, continuous nicotine exposure (Harris and Gewirtz 2004). Second, withdrawal-potentiated startle is temporarily blocked by re-administration of nicotine, which is also consistent with observations from studies of withdrawal from chronic, continuous nicotine (Helton et al. 1993). This observation is also clinically relevant in that relief from the anxiety-related consequences of abstinence constitutes a critical motivational factor in compulsive smoking behavior (Piasecki et al. 1997). Third, withdrawal-potentiated startle appears to be dose-dependent, with both larger increases and later peaks at higher doses. Fourth, nicotine withdrawal-potentiated startle is blocked by clonidine, an α_2 -adrenergic receptor agonist that has been shown to block withdrawal-potentiated startle from morphine (Harris and Gewirtz 2004) and to reduce affective symptoms and craving in humans withdrawing from tobacco (Glassman et al. 1984). Blockade of withdrawal-potentiated startle by clonidine is also consistent with the hypothesis that increases in central noradrenergic function are involved in the expression of withdrawal from drugs of abuse (Smith and Aston-Jones 2008).

It should be noted that not all studies of withdrawal from chronic, continuous nicotine exposure have observed increases in baseline startle responding (Acri et al. 1995, 1991; Jonkman et al. 2008). Jonkman et al. (2008) reported that withdrawal from nicotine after 28 days of chronic infusion via osmotic minipump did not potentiate the baseline startle response of rats but did increase potentiation of startle by exposure to a bright light (“light-enhanced startle”), another measure of anxiety-like behavior (de Jongh et al. 2003; Walker and Davis 1997). Thus, withdrawal from chronic, continuous nicotine may potentiate responses to stress (e.g., exposure to a bright light), whereas withdrawal from discrete injections of nicotine may produce a more general increase in basal anxiety levels. It is also noteworthy that some studies of continuous nicotine infusion have waited 24 h after the cessation of infusion to examine changes in startle responding (Acri et al. 1995, 1991; Jonkman et al. 2008). In the present study, startle responding was elevated 2 h after the final dose of nicotine but had returned to baseline levels within 4 h of drug exposure. Assessments of withdrawal taken 24 h after nicotine may therefore miss the critical window in which anxiety-like behavior can be detected in the absence of an explicit stressor.

The anxiogenic effect seen in our study is consistent with other measures of negative affect in animals going through nicotine withdrawal. In the elevated-plus maze, rats show a decrease in open arm time following repeated (Irvine et al. 2001) and continuous (Brioni et al. 1994) drug exposure. Additionally, intracranial self-stimulation (ICSS) thresholds are elevated during withdrawal from both continuously infused nicotine (Epping-Jordan et al. 1998) and from repeated nicotine injections (Bozarth et al. 1998). Our results are also consistent with the 45-min half-life of nicotine in the rat (Matta et al. 2007). Withdrawal-potentiated startle was already apparent at 1 h and peaked at 2 h after nicotine administration, a time point at which the majority of the drug is metabolized and therefore displaced from receptors. Combined with the ability of nicotine replacement to reverse the withdrawal-potentiated startle effect, these data suggest that loss of nAChR occupancy is a key event in the production of nicotine withdrawal-potentiated startle.

Surprisingly, we did not find evidence of mecamylamine-precipitated withdrawal-potentiated startle (experiment IV). This observation is inconsistent with the robust precipitated-withdrawal effects observed using other measures of nicotine withdrawal such as somatic signs (e.g., Malin et al. 1994), conditioned place aversion (e.g., Suzuki et al. 1996), or increases in brain-reward thresholds (e.g., Epping-Jordan et al. 1998), and with measures of naloxone-precipitated opiate withdrawal observed in our laboratory (Harris and Gewirtz 2004; Harris et al. 2004). It seems unlikely that the absence of an effect of mecamylamine was related to the dose used (1 mg/kg) because the same dose effectively precipitates withdrawal as assessed through ICSS thresholds (Watkins et al. 2000). This null effect is also unlikely to be related to differences in the properties of mecamylamine's constituent stereoisomers (Papke et al. 2001) since, in a preliminary investigation, we also have found no evidence of withdrawal-potentiated startle after injection of dihydro- β -erythroidine (DH β E), a selective $\alpha_4\beta_2$ nAChR antagonist. Moreover, our results are supported by the recent study of the effects of withdrawal from chronic nicotine on light-enhanced startle, in which higher doses of both mecamylamine and DH β E were also found to be ineffective in precipitating withdrawal (Jonkman et al. 2008).

The fact that startle potentiation in both this and the Jonkman et al. (2008) study was seen during spontaneous but not precipitated withdrawal may suggest that the expression of nicotine withdrawal-potentiated startle is not dependent on decreased activity of nAChRs per se, but rather is the result of downstream mechanisms (perhaps involving norepinephrine release) that do not become activated during the time window examined in experiment IV. In the spontaneous withdrawal procedure, loss of nAChR occupancy began well before the 2-h startle test; in contrast, competitive occupancy by mecamylamine began 5 min before the final startle test session. Allowing more time between mecamylamine injection and startle testing may therefore have been necessary to yield a positive result. Interestingly, the notion that different mechanisms may mediate spontaneous and precipitated nicotine withdrawal also gains support from a recent finding that a corticotropin-releasing factor receptor antagonist blocked changes in ICSS thresholds induced by precipitated withdrawal only (Bruijnzeel et al. 2007).

In addition to its implications regarding nicotine dependence, this study adds to the growing body of evidence that potentiated startle is a reliable measure of the negative affective consequences of withdrawal following repeated episodes of drug exposure. Intermittent exposure to opiates results in escalating levels of withdrawal-potentiated startle across repeated injections, similar to the results of the current study for intermittent nicotine exposure. Opiate withdrawal-potentiated startle is reduced by clonidine, and results from experiment III of the current study indicate that this is also the case for nicotine withdrawal. The affective component of opiate withdrawal appears to be more severe, however, with potentiated startle present during the first withdrawal episode (Harris and Gewirtz 2004) and increasing in severity with subsequent exposures (Harris et al. 2004). In contrast, in the current study, nicotine withdrawal-

potentiated startle was only evident after multiple injections, and the number of injections required varied across experiments. The magnitude of opiate withdrawal is also frequently greater than that of nicotine withdrawal, measured as changes in ICSS thresholds (Kenny and Markou 2005). These findings are consistent with reports that opiate withdrawal is more debilitating than tobacco withdrawal in humans (Hughes et al. 1994).

One of the advantages of using potentiated startle as a measure of withdrawal in animals is that the startle reflex is also a reliable, non-invasive measure of affective changes in humans. Withdrawal-potentiated startle can be used to assess withdrawal states in human addicts and is therefore a promising model for translational research projects. Indeed, elevated startle magnitude has been observed in humans withdrawing from opiates (Stine et al. 2001) and ethanol (Krystal et al. 1997; Saladin et al. 2002), and we are investigating whether similar effects can be observed during tobacco withdrawal (Engelmann and Cuthbert 2008).

Perhaps the most important aspect of withdrawal-potentiated startle is that it allows changes in anxiety to be detected at the earliest stages of addiction. Symptoms of anxiety have been reported in human adolescents after only a few cigarettes (DiFranza et al. 2000), and the severity of such symptoms predicts the likelihood of future nicotine dependence (O'Loughlin et al. 2003). Studies that use the withdrawal-potentiated startle paradigm to identify brain mechanisms involved in the expression of negative affective symptoms of nicotine withdrawal may therefore be important in developing more effective treatments for tobacco dependence in humans, particularly in the vulnerable adolescent population.

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Fig. 1.

Experimental design. **a** In experiments I and II, rats were given daily nicotine injections (experiment I: 0 or 0.25 mg/kg; experiment II: 0, 0.125, 0.25, or 0.5 mg/kg) for 14 days. On days 1, 7, and 14, test sessions took place 1 h prior to the injection and at multiple time points thereafter. **b** On day 15 in experiment I, a second injection of nicotine (same dose as the first injection) took place 1:50 h after the first injection (i.e., at the time point where peak withdrawal-potentiated startle was observed on previous days). **c** In experiment III, rats were given daily nicotine injections for 8 days (0 or 0.5 mg/kg). On day 8, clonidine (0 or 10, or 15 μ g/kg) was given 1:55 h after nicotine injection, and test sessions took place 1 h before and 2 h after the nicotine injection. **d** In experiment IV, rats were given daily nicotine injections for 8 days (0 or 0.5 mg/kg). On day 8, mecamylamine (0 or 1 mg/kg) was injected 35 min after nicotine injection, and startle test sessions took place 1 h prior to and 40 min after nicotine injection. In all experiments, 0 h was defined as the time point of the first nicotine or saline injection. Gray arrows indicate injections. White arrows indicate the commencement of test sessions

Fig. 2.

Daily nicotine injections (0 or 0.25 mg/kg) result in an escalation of withdrawal-potentiated startle severity across days and increased activity immediately after the nicotine injection. Data shown depict percent change in startle amplitude (*left column*) and activity levels (*right column*) from the same-day pretest at 5 min, 1 h, 2 h, 3 h, or 4 h after nicotine injection. Asterisks indicate a significant effect of nicotine dose at that time point (* $p < .05$, ** $p < .01$). $n=12$ per nicotine dose

Fig. 3.

A second nicotine injection, administered immediately prior to the time point where peak withdrawal-potentiated startle was observed on previous days in experiment I, delays the peak of withdrawal-potentiated startle to a later time point (*top panel*) and partially reinstates increased activity levels observed immediately after the first nicotine injection (*bottom panel*). Arrows indicate the timing of the second nicotine injection (1:50 after the first nicotine injection). Asterisks indicate a significant effect of nicotine dose at that time point (* $p < .05$, ** $p < .01$). $n=8$ per nicotine dose

Fig. 4.

The magnitude of nicotine withdrawal-potentiated startle (*left column*) and nicotine-induced increases in activity (*right column*) is dose-dependent. In experiment II, rats were injected with nicotine (0, 0.125, 0.25, or 0.5 mg/kg per day) for 14 days, and test sessions took place on days 1, 7, and 14. Data depict the mean percent change in startle amplitude (*left column*) and activity levels (*right column*) from the same-day pretest during each post-injection test session. Asterisks indicate a significant effect of nicotine dose at that time point (* $p < .05$, ** $p < .01$). $n=8, 7, 8$, and 8 for the 0-, 0.125-, 0.25-, and 0.5-mg/kg groups, respectively

Fig. 5.

Clonidine dose-dependently decreases nicotine withdrawal-potentiated startle. Data shown are the percent change in startle on day 8 from the pre-nicotine test session to the 2-h post-nicotine test session. Immediately prior to the post-nicotine test session, rats assigned to each level of nicotine dose (0 or 0.5 mg/kg) were injected with clonidine (0, 10, or 15 µg/kg). ** Indicates a significant difference between the group receiving 0 mg/kg nicotine followed by 0 mg/kg clonidine and the group receiving 0.5 mg/kg nicotine followed by 0 mg/kg clonidine (planned contrast, $p < .01$). †† Indicates a significant linear trend of clonidine dose within the 0.5 mg/kg nicotine groups (planned contrast; $p < .01$). $n = 9, 6,$ and 5 for each nicotine dose for the 0, 10, and 15 µg/kg clonidine doses, respectively

Fig. 6.

Mecamylamine-precipitated withdrawal-potentiated startle is not evident. Data shown are the percent change in startle on day 8 from the pre-nicotine test session to the 40-min post-nicotine test session. Immediately prior to the post-nicotine test session, rats assigned to each level of nicotine dose (0 or 0.5 mg/kg) were injected with mecamylamine (0 or 1 mg/kg). $n=8$ and 7 for the 0 and 0.5 mg/kg nicotine doses in the groups injected with 0 mg/kg mecamylamine, and $n=8$ and 8 for the 0 and 0.5 mg/kg nicotine doses in the groups injected with 1 mg/kg mecamylamine, respectively

Table 1Mean $\pm$ SEM baseline and pre-injection startle amplitude (arbitrary units)

Nicotine dose ^a	Clonidine dose ^b	Mecamylamine dose ^a	Baseline	Day 1	Day 6	Day 7	Day 8	Day 14
Experiment I								
0			5.64 $\pm$ 0.54	5.09 $\pm$ 0.56		6.09 $\pm$ 0.77		6.61 $\pm$ 0.76
0.25			5.72 $\pm$ 0.25	5.72 $\pm$ 0.38		5.64 $\pm$ 0.41		6.04 $\pm$ 0.35
Experiment II								
0			7.52 $\pm$ 1.42	5.80 $\pm$ 1.04		6.97 $\pm$ 1.33		8.09 $\pm$ 1.17
0.125			6.63 $\pm$ 1.38	6.25 $\pm$ 1.51		6.76 $\pm$ 1.34		7.92 $\pm$ 1.69
0.25			6.74 $\pm$ 0.67	6.42 $\pm$ 0.45		8.42 $\pm$ 0.64		8.80 $\pm$ 1.04
0.5			7.44 $\pm$ 1.75	6.96 $\pm$ 1.16		8.39 $\pm$ 1.88		8.00 $\pm$ 1.40
Experiment III								
0	0		5.78 $\pm$ 0.87		7.14 $\pm$ 0.96	5.80 $\pm$ 0.74	5.04 $\pm$ 0.64	
0	10		4.13 $\pm$ 0.55		4.76 $\pm$ 0.73	4.91 $\pm$ 0.90	4.26 $\pm$ 1.01	
0	15		5.18 $\pm$ 1.45		5.58 $\pm$ 0.99	4.36 $\pm$ 0.47	4.53 $\pm$ 0.76	
0.5	0		6.21 $\pm$ 0.82		7.56 $\pm$ 0.96	6.28 $\pm$ 0.58	5.39 $\pm$ 0.58	
0.5	10		3.78 $\pm$ 0.80		4.31 $\pm$ 0.72	3.29 $\pm$ 0.23	2.96 $\pm$ 0.28	
0.5	15		4.22 $\pm$ 0.71		5.13 $\pm$ 0.77	4.40 $\pm$ 0.72	4.45 $\pm$ 0.82	
Experiment IV								
0		0	5.21 $\pm$ 0.90			6.75 $\pm$ 1.14	6.76 $\pm$ 1.21	
0		1	4.08 $\pm$ 0.65			4.96 $\pm$ 0.89	4.61 $\pm$ 0.78	
0.5		0	4.25 $\pm$ 0.56			5.63 $\pm$ 0.56	5.12 $\pm$ 0.46	
0.5		1	4.95 $\pm$ 0.46			5.82 $\pm$ 0.48	4.94 $\pm$ 0.33	

Pre-injection startle data on day6 in experiment IV were lost due to technical difficulties and are not included in the analysis

^aDose expressed in milligrams/kilogram^bDose expressed in micrograms/kilogram