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Extended access to nicotine leads to a CRF1 receptor dependent increase in anxiety-like behavior and hyperalgesia in rats

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Abstract

Background—Tobacco dependence is associated with the emergence of negative emotional states during withdrawal, including anxiety and nociceptive hypersensitivity. However, the current animal models of nicotine dependence have focused on the mechanisms that mediate the acute reinforcing effects of nicotine and failed to link increased anxiety and pain during abstinence with excessive nicotine self-administration. Here, we tested the hypothesis that the activation of corticotropin-releasing factor-1 (CRF_1) receptors and emergence of the affective and motivational effects of nicotine abstinence only occur in rats with long access (> 21 h/day, LgA) and not short (1 h/day, ShA) access to nicotine self-administration.

Methods—ShA and LgA rats were tested for anxiety-like behavior, nociceptive thresholds, somatic signs of withdrawal, and nicotine intake after 3 days of abstinence. The role of CRF_1 receptors during abstinence was tested using systemic or intracerebral infusion of MPZP, a CRF1 receptor antagonist, in the central nucleus of the amygdala (CeA).

Results—LgA but not ShA rats exhibited abstinence-induced increases in anxiety-like behavior and nociceptive hypersensitivity, which both predicted subsequent excessive nicotine intake and were prevented by systemic administration of MPZP. Intra-CeA MPZP infusion prevented abstinence-induced increases in nicotine intake and nociceptive hypersensitivity.

Conclusions—These findings demonstrate that the model of short access to nicotine self-administration has limited validity for tobacco dependence, highlight the translational relevance of the model of extended-intermittent access to nicotine self-administration for tobacco dependence, and demonstrate that activation of CRF_1 receptors is required for the emergence of abstinence-induced anxiety-like behavior, hyperalgesia, and excessive nicotine intake.

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Author contributions

Cohen A, George O, and Koob GF contributed to developing the study concept and design, data analysis, and preparation of the manuscript. Cohen A, Treweek J, and Molini Leao R were responsible for conducting nicotine self-administration and related behavioral tests. Edwards S conducted the Von Frey tests, with guidance from Schulteis G. All of the authors critically reviewed the content and approved the final version for publication.

Keywords

addiction; emotion; stress; tobacco; withdrawal

Introduction

Tobacco addiction is the leading avoidable cause of death in the United States (Fellows et al., 2002). Abstinence from nicotine, the main psychoactive ingredient in tobacco, produces robust somatic and affective withdrawal symptoms, including increased anxiety-like behavior and nociceptive hypersensitivity (Hughes et al., 1991; Jackson et al., 2009; Watkins et al., 2000), symptoms that have been associated with increased probability for escalating to chronic heavy smoking (Dierker and Mermelstein, 2010; Doubeni et al., 2010). However, the mechanisms that underlie the negative affective symptoms of withdrawal in the development of excessive nicotine intake are largely unknown due to the lack of appropriate animal models.

Theoretical models have suggested that the recruitment of brain stress systems, such as the corticotropin-releasing factor (CRF)-CRF_1 receptor system in the central nucleus of the amygdala (CeA), are responsible for the transition to tobacco dependence by mediating negative emotional states during abstinence, which in turn produces a powerful driving force to increase and maintain high levels of smoking behavior through negative reinforcement mechanisms (Koob et al., 2008). Models based on chronic passive nicotine administration reliably produce anxiety-like behavior and nociceptive hypersensitivity during withdrawal (Jackson et al., 2009; Wilmouth and Spear, 2006) that can be prevented by administration of a CRF_1 receptor antagonist (Bruijnzeel et al., 2012; Marcinkiewcz et al., 2009; Bruijnzeel et al., 2009), but such noncontingent models preclude any investigation that links the mechanisms of negative emotional states with excessive nicotine self-administration. In contrast, studies that used short access to nicotine self-administration have failed to demonstrate increased anxiety-like behavior and nociceptive hypersensitivity associated with excessive nicotine intake after spontaneous withdrawal (Abreu-Villaca et al., 2006; Irvine et al., 2001; Manhaes et al., 2008), leading to the hypothesis that the negative emotional state of withdrawal does not contribute to nicotine self-administration (Irvine et al., 2001). An alternative hypothesis is that the model of short access to nicotine self-administration has limited validity for tobacco dependence because longer durations of daily access to nicotine are required to produce the neuroadaptations that underlie abstinence-induced anxiety-like behavior and nociceptive hypersensitivity and lead smokers to excessive smoking behavior through negative reinforcement.

To test this hypothesis, we measured anxiety-like behavior, nociceptive hypersensitivity, somatic signs of withdrawal, and nicotine intake using fixed- and progressive-ratio schedules of reinforcement after 3 days of abstinence from either short (1 h/day) or long (21–23 h/day) access to nicotine self-administration. We then investigated whether abstinence-induced anxiety-like behavior, nociceptive hypersensitivity, and excessive nicotine intake are mediated by the activation of CRF_1 receptors selectively in LgA and not ShA rats using systemic and intra-CeA administration of a specific CRF_1 antagonist, *N*,*N*-

bis(2-methoxyethyl)-3-(4-methoxy-2-methylphenyl)-2,5-dimethyl-pyrazolo(1,5α)pyrimidin-7-amine (MPZP).

Materials and Methods

Animals

Seventy-eight male Wistar rats (250–275 g; Charles River, Hollister, CA) were group-housed and maintained on a 12°h/12 h light/dark cycle with *ad libitum* access to food and water. All of the animal procedures were approved by The Scripps Research Institute Institutional Animal Care and Use Committee and were in accordance with National Institutes of Health guidelines.

Drugs

Nicotine hydrogen tartrate salt (Sigma, Natick, MA) was dissolved in saline, and the pH was adjusted to 7.4. Nicotine doses are expressed as free base. Mecamylamine hydrochloride (Sigma) was dissolved in saline and administered intraperitoneally (i.p.; 1.5 mg/kg). The CRF_1 antagonist MPZP was synthesized at The Scripps Research Institute by Dr. Kim Janda and dissolved in 20% hydroxypropyl β-cyclodextrin (HBS; Cavitron; Wayzata, MN) in isotonic saline at pH 4.5 before being injected subcutaneously (s.c.; 20 mg/kg/1 ml) 45 min before testing. This systemic dose of MPZP was previously shown to suppress nicotine intake post-abstinence (George et al., 2007). For bilateral intracranial (i.c.) microinjections, MPZP was dissolved in 20% HBC, diluted in artificial cerebrospinal fluid (aCSF), and administered immediately before testing. The dose of MPZP (0.07 ng/0.3 μl/hemisphere) was chosen because at this concentration (∼585 nM), MPZP blocks more than 90% of CRF_1 receptor binding (Richardson et al., 2008) and because this concentration is similar to other non-peptide CRF_1 receptor antagonists, with similar pharmacological profiles, that are known to be behaviorally active when injected into the CeA in rats (Ji et al., 2007).

Surgeries

The animals underwent either intravenous catheterization alone or together with bilateral cannula placement in the CeA. Detailed descriptions of both procedures have been reported previously (George et al., 2007). For cannula placements, the rats were placed in a Kopf stereotaxic instrument, and guide cannulae (26 gauge; Plastics One, Roanoke, VA) were inserted bilaterally above the left and right CeA (coordinates relative to bregma: anterior/posterior, −2.6 mm; medial/lateral, ±4.2 mm; dorsal/ventral, −5.2 mm from skull surface; Paxinos and Watson, 1998). Internal injection cannulae (33 gauge) extended 2.0 mm beyond the tip of the guide cannula when inserted.

Operant chambers and self-administration

The rats were tested in self-administration operant chambers as described previously (O'Dell and Koob, 2007). Specifically, the chambers (Med Associates, St. Albans, VT) were kept on a light/dark cycle inside chambers with continuous white noise. The exit port of the catheter fitting was connected to a polyethylene tube enclosed in a protective metal spring. The spring was suspended inside the chamber through its attachment to a swivel mounted on a

balance arm, and nicotine was delivered to the tubing via a syringe pump (Razel Scientific Research Instruments, St. Albans, VT).

Operant sessions were conducted using two retractable levers (i.e., active and inactive) that extended 1 inch into the chamber. Each response on the active lever resulted in the delivery of nicotine (0.03 mg/kg/0.1 ml; fixed-ratio 1) over 1 s. A 28 V white cue light was illuminated above the active lever at the onset of the nicotine infusion and ended following a 20 s timeout, during which responses were recorded but did not induce drug delivery. The chambers were fitted with a pellet dispenser with a swing door mounted between the two levers on the front wall of the chamber, allowing the subjects to obtain a 45 mg chow pellet (Precision, Formula A/I from Research Diets, Lancaster, NH) upon each nosepoke responses. Water (0.1 ml) was delivered into a metal cup upon each nosepoke response to a separate hole located on the back of the chamber. The rats were trained to nosepoke for food and water in a 23 h session after recovery from the catheter implantation surgery, without access to the active lever that was later associated with nicotine delivery.

Deprivation effect: basic protocol

In all of the experiments, the rats were first given access to nicotine self-administration for 1 h per day (acquisition; see Fig. 1 for timeline) until they exhibited at least twice as many active lever presses as inactive lever presses in a session and less than 20% variation in the number of infusions over two consecutive sessions (7–12 days total). Subsequently, the subjects self-administered nicotine either in a short access (ShA, 1 h/day) condition or long access condition (LgA, 21–23 h/day). To allow more flexibility and improve utilization of operant chambers while allowing time for cleaning, the rats were tested for 21–23 h/day. Extensive studies in our laboratory have shown that negligible differences exist between 21 h and 23 h access in terms of nicotine intake and anxiety-like behavior because very few, if any, infusions occur after 21 h. None of the rats had access to food or water during the first hour of self-administration. LgA rats were allowed to nosepoke for food and water beginning at the second hour of each self-administration session. In each of the following weeks, LgA and ShA rats self-administered nicotine for 4 days (Monday 10:00 AM to Friday 7:00 AM) followed by 3 days of abstinence from nicotine (Friday-Monday) in their home cage. Nicotine intake (fixed-ratio schedule) was recorded every session, and specific behavioral tests and pharmacological manipulations were conducted either 3 h following termination of the fourth daily session (i.e. Friday 10:00 AM; “pre-abstinence”) or following the 3 days of abstinence days (i.e. Monday 10:00 AM; “post-abstinence”).

Experiment 1. Nicotine self-administration and somatic withdrawal symptoms following abstinence from nicotine self-administration

Twenty-two rats acquired nicotine self-administration (7 acquisition days) and subsequently self-administered nicotine for 1 h/day (n = 14) or 23 h/day (n = 8) for 6 weeks. Each week, the rats alternated between 4 days/sessions of nicotine self-administration and 3 days of abstinence as described above. During the last 4 weeks of the study, the motivation for nicotine (progressive-ratio schedule) and mecamylamine-precipitated somatic signs of withdrawal were measured post-abstinence (Monday 10:00 AM), immediately prior to the

post-abstinence self-administration session. Only one of these behaviors was measured each week in a Latin-square design.

Experiment 2. Anxiety-like behavior and mechanical hypersensitivity after 72 h abstinence from nicotine self-administration: Effects of systemic MPZP

Following 12 days of acquisition, 38 rats were allowed to self-administer nicotine for 21 h/day ($n = 23$) or 1 h/day ($n = 15$) for 14 weeks, as in Experiment 1. Eight additional rats did not undergo intravenous surgeries and remained in their home cages as naive controls for the measurement of anxiety-like behavior and mechanical hypersensitivity. In week 10, all of the rats were evaluated for anxiety-like behavior in the elevated plus maze post-abstinence, with rats receiving either 20 mg/kg MPZP or vehicle prior to testing. Tests for anxiety-like behavior were not repeated with the same rat because the effect of experience on performance in these tests. However, to verify that anxiogenic-like behavior in LgA rats was attributable to abstinence and not merely chronic nicotine, LgA rats were compared pre-abstinence (Friday 10:00 AM) with ShA rats on a different test of anxiety, the open field.

To examine whether abstinence-induced increases in nicotine intake were driven by the emergence of CRF_1-dependent mechanical nociceptive hypersensitivity, we tested paw withdrawal thresholds using the Von Frey test pre-abstinence (Friday 10:00 AM) and post-abstinence (Monday 10:00 AM of weeks 12 and 13), with vehicle or MPZP (20 mg/kg) administered prior to post-abstinence testing in a random order.

Experiment 3. Effects of intra-CeA MPZP on nicotine self-administration and mechanical hypersensitivity after 72 h abstinence from nicotine self-administration

Ten naive rats were implanted with bilateral intracranial cannulae aimed at the CeA (see above). All of the rats acquired nicotine self-administration (7 days) and continued on a LgA (21 h) self-administration schedule identical to the previous experiments (4 days of self-administration, 3 days of abstinence) for 8 weeks. During weeks 3–4, the rats were habituated to the intracranial infusion procedure with “sham injections.” Immediately prior to the initiation of the fifth and sixth post-abstinence self-administration sessions, vehicle or MPZP (0.07 ng/0.3 μl/hemisphere) was infused into the CeA in a within-subjects Latin-square design. A KD Scientific microinfusion pump (connected to a 10 μl Hamilton syringe) was used for MPZP or vehicle infusions at a rate of 0.3 μl/min, and the injection cannula was left in the guide cannula for two additional minutes to allow for adequate diffusion of the solution.

MPZP or vehicle was infused again in a counterbalanced order immediately prior to the seventh and eighth post-abstinence days, with paw withdrawal threshold testing conducted following the microinfusion. The rats were then decapitated, and their cannula placements were verified. The intravenous catheters of two of the animals failed before completing both mechanical hypersensitivity tests, and these animals were excluded from the analysis of this measure. Two animals with incorrect cannula placements were excluded from the analysis.

Detailed procedure for behavioral tests

Somatic signs of nicotine withdrawal—The rats received saline or mecamylamine (1.5 mg/kg, i.p.) and were placed in an opaque plastic cylindrical container (30 × 29 cm). Thirty minutes later, they were observed for 10 min for somatic signs of withdrawal according to the method developed by Malin et al. (1992) and O'Dell et al. (2007). The rats were observed for blinks, body shakes, chews, cheek tremors, escape attempts, foot licks, gasps, writhes, genital licks, hops, head shakes, ptosis, scratches, teeth chattering, and yawns. Multiple successive counts of any sign required a distinct pause between episodes. The observer was blind to the drug treatment of each subject.

Progressive-ratio schedule of reinforcement—In these sessions, the response requirement for reinforcement was increased according to the following sequence: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, etc. (Richardson and Roberts, 1996). The progressive-ratio sessions lasted for either a maximum of 6 h or until 1 h elapsed without the delivery of a reinforcer. The last ratio completed during the session was defined as the breakpoint.

Anxiety-like behavior—The rats were tested in two different tests, one pre-abstinence (open field) and the other 72 h post-abstinence (elevated plus maze) to avoid proactive interference. The open field (100 cm × 100 cm) was placed in a quiet room dimmed to provide 10–20 lux of illumination in the center of the open field. The rats were placed in the center of the apparatus and allowed to explore freely for 10 min. The dependent variables were the time spent in the center, periphery, and corners of the open field.

The elevated plus maze consisted of four arms (50 cm length × 10 cm width) elevated 100 cm above the floor. Two of the arms had 40 cm high dark walls (closed arms), and two had 0.5 cm high ledges (open arms). The arms were angled at 90° to each other. The apparatus was placed in a quiet room dimmed to provide 10–20 lux of illumination on the open arms and < 0.5 lux within the closed arms. The rats were placed in the center of the maze facing a closed arm and removed after 5 min. The apparatus was wiped with water and dried between tests.

Paw withdrawal (mechanical nociceptive) threshold testing—The evaluation of mechanical sensitivity was performed as previously described (Chaplan et al., 1994). Briefly, the rats were acclimated for 15 min in elevated cages with a wire mesh floor. A series of von Frey filaments was applied perpendicularly to the plantar surface of the hindpaw for 3 s. A sharp withdrawal of the hindpaw indicated a positive response. The stimulus was incrementally increased until a positive response was observed and then reduced until a negative result was observed to determine a pattern of responses to apply to the statistical method of Dixon (1980). The 50% paw withdrawal threshold was determined by the formula $Xf + k\delta$, in which Xf indicates the last von Frey filament employed, k indicates the Dixon value that corresponds to the response pattern, and δ indicates the mean difference between stimuli.

Statistical analysis

The data were subjected to either two-way repeated-measures analysis of variance (ANOVA; Time × Access) or two-way ANOVA (Drug × Access), followed by the Newman-Keuls multiple comparison *post hoc* test. In all cases, a normality test and equal variance test were performed before the ANOVA to ensure its validity. When normality was violated, a log transformation ($Y = log\ (X+1)$) was used to ensure homogeneity of error variance. *t*-tests and Pearson correlations were used when appropriate.

Results

Experiment 1

Abstinence increases nicotine self-administration behavior in LgA rats—By the end of the acquisition phase, rats designated for the LgA condition and ShA condition showed similar levels of nicotine self-administration, food self-administration, and water intake (table 1). There was no correlation between food or water intake at this time point and concurrent or future nicotine self-administration.

After being given differential access to nicotine, LgA rats showed higher nicotine intake than ShA, and abstinence-induced increases in nicotine intake were observed in LgA but not ShA rats. Fig. 2A–B represents the pattern of nicotine self-administration during the pre-abstinence (average of Tuesday-Friday) *vs*. post-abstinence (Monday) sessions. A two-way repeated-measures ANOVA revealed a significant Time × Access interaction ($F_{1,20} = 126.26$, $p < 0.001$), with a significant post-abstinence increase in nicotine consumption in LgA rats ($p < 0.01$) but not ShA rats. In LgA rats, post-abstinence nicotine self-administration was highest during the first hour of the session and gradually decreased (Fig. 2C). Post-abstinence food consumption was not significantly different from pre-abstinence consumption in LgA rats ($p > 0.05$; data not shown).

Following 3 days of abstinence, LgA rats exhibited higher self-administration under a progressive-ratio schedule ($t_{20} = 2.12$, $p < 0.05$) compared with ShA rats (Fig. 2D). Nicotine intake on the fixed-ratio schedule was highly correlated with the breakpoints on the progressive-ratio schedule ($r = 0.84$, $p < 0.05$). In contrast to the post-abstinence differences in breakpoints between LgA and ShA rats, the injection of mecamylamine induced similar signs of withdrawal ($F_{1,20} = 25.2$, $p < 0.05$; Fig. 2E and table 2) between the LgA and ShA groups ($F_{1,20} = 0.01$, $p > 0.05$). There was no correlation between the magnitude of precipitated somatic signs and nicotine intake ($r = 0.14$, $p = 0.53$) or the motivation for nicotine on a progressive-ratio schedule ($r = 0.07$, $p = 0.72$).

Experiment 2

Abstinence-induced anxiety-like behavior is mediated by CRF_1 receptors and predicts nicotine self-administration in LgA rats—Pre-abstinence anxiety-like behavior was similar between LgA and ShA rats, reflected by a similar amount of time spent in the center (LgA, 7 ± 4 s; ShA, 8 ± 3 s; $p > 0.05$), periphery (LgA, 357 ± 68 s; ShA, 429 ± 49 s; $p > 0.05$), and corners (LgA, 218 ± 74 s; ShA, 180 ± 47 s; $p > 0.05$) of the open field. However, on the post-abstinence elevated plus maze test (Table 3) LgA rats demonstrated

increased anxiety-like behavior compared with ShA rats and drug-naive rats, reflected by a lower percentage of time spent on the open arms of the elevated plus maze ($F_{2,25} = 7.71$, $p < 0.01$). Newman-Keuls *post hoc* tests confirmed that unlike the LgA rats, ShA rats did not differ from drug-naive control rats ($p > 0.05$). Systemic administration of MPZP attenuated anxiety-like behavior in LgA rats, without affecting ShA rats, reflected by a Drug × Access interaction in the percent time spent on the open arms. This interaction did not reach significance when the ANOVA was conducted on the absolute percentage scores (P=0.06; table 3). However, as the percentage score data deviated from normality, ANOVA was conducted on the normalized (log transformation) percentage scores, resulting in a significant Drug × Access interaction ($F_{1,34} = 4.82$, $p < 0.05$; Fig. 3A). MPZP also increased locomotor behavior in the elevated plus maze, with a main effect of Drug ($F_{1,33} = 4.91$, $p < 0.05$) on the total distance travelled on the apparatus, but this effect was similar in ShA and LgA rats, reflected by no Drug × Access interaction ($p > 0.05$). Finally, the percent time spent on the open arms of the elevated plus maze was negatively correlated with post-abstinence nicotine self-administration ($r = -0.53$, $p < 0.05$; Fig. 3B).

Abstinence-induced mechanical hypersensitivity is mediated by CRF_1 receptors and predicts nicotine self-administration in LgA rats—Mechanical nociceptive thresholds in the naive rats used in this study were similar to those reported in rats of comparable ages (Ririe and Eisenach, 2006). Similar to the lack of anxiety-like behavior between ShA and LgA rats before abstinence, there was no significant difference between paw withdrawal thresholds in LgA and ShA rats (LgA, 82.3 ± 5.23 g; ShA, 88.7 ± 6.74 g; $p > 0.05$) at the pre-abstinence time point (Friday 10:00 AM). However, LgA rats exhibited post-abstinence paw withdrawal thresholds that were lower than ShA rats and drug-naive controls ($F_{2,22} = 15.37$, $p < 0.001$; Fig. 4A). Newman-Keuls *post hoc* tests confirmed that unlike the LgA rats, ShA rats did not differ from drug-naive control rats ($p > 0.05$). Systemic administration of MPZP prevented the decreased paw withdrawal thresholds in LgA rats, without affecting ShA rats, reflected by a significant Drug × Access interaction ($F_{1,15} = 7.96$, $p < 0.02$; Fig. 4A). In vehicle-pretreated LgA rats, post-abstinence paw withdrawal thresholds were negatively correlated with their nicotine intake in the subsequent 21 h self-administration session ($r = -0.65$, $p < 0.05$; Fig. 4B).

Experiment 3

Abstinence-induced increases in nicotine self-administration and mechanical hypersensitivity are mediated by CRF_1 receptors in the CeA—LgA rats infused post-abstinence with MPZP into the CeA demonstrated significantly lower levels of nicotine self-administration ($t_7 = 2.99$, $p < 0.02$) and higher mechanical nociceptive thresholds ($t_5 = 3.17$, $p < 0.02$) than vehicle-treated rats (Fig. 5). As descried above, LgA rats were allowed to nosepoke for 45 mg chow food pallets throughout each self-administration session. Indicating the lack of sedative or locomotor-impairing effect of MPZP, food intake was not significantly different following vehicle (20.73 g ± 3.19) or MPZP pretreatment (24.19 g± 0.86). Two animals were excluded from the analysis because of incorrect cannula placements (open circle in Fig. 5A).

Discussion

This study demonstrated that after 3 days of abstinence, LgA rats exhibited (*i*) increased nicotine intake, (*ii*) higher breakpoints for nicotine on a progressive-ratio schedule, (*iii*) increased anxiety-like behavior, which correlated with excessive nicotine intake upon renewed access, and (*iv*) nociceptive hypersensitivity that also correlated with excessive nicotine intake upon renewed access. A systemically injected small-molecule CRF_1 receptor antagonist prevented the abstinence-induced increases in anxiety-like behavior and nociceptive hypersensitivity in LgA rats without affecting ShA rats. Finally, when injected into the CeA, the CRF_1 antagonist reduced nociceptive hypersensitivity and prevented excessive nicotine self-administration.

Increased nicotine intake and motivation for nicotine following 72 h of abstinence

The present results confirm previous studies that reported increased nicotine intake in LgA but not ShA rats after 2–3 days of abstinence (George et al., 2007; O'Dell and Koob, 2007; Cohen et al., 2012). While the increased responding was most significant during the first 4 h after renewed access, LgA rats exhibited higher cumulative nicotine intake during the entire circadian cycle, with a reduction of peak intake only during periods of sleep (light cycle), as observed in heavy smokers (Benowitz et al., 1982). In addition to increased intake, LgA rats exhibited increased motivation for nicotine under a progressive-ratio schedule. Progressive-ratio responding for nicotine after training with a fixed-ratio 1 schedule of reinforcement is usually very low compared with other drugs, such as psychostimulants (Risner and Goldberg, 1983); however, progressive-ratio responding was still higher in LgA rats than in ShA rats and was highly correlated with abstinence-induced increases in nicotine intake under a fixed-ratio 1 schedule of reinforcement. Some have argued that an increase in responding on a progressive-ratio schedule reflects an element of compulsivity, in which the animals persist in responding despite adverse consequences (i.e., higher work load; Deroche-Gamonet et al., 2004). Others have argued for a more reinforcement-efficacy explanation (Chiodo and Roberts, 2009). Nevertheless, in both cases, the animals with higher progressive-ratio responding showed increased motivation to seek and take nicotine.

LgA but not ShA rats exhibit key features of tobacco dependence

Nicotine intake at levels that approach those demonstrated by LgA rats during pre-abstinence sessions (0.99 mg/kg) have been reported in some studies, in which rats were allowed only 2 h of daily nicotine self-administration (e.g., Feltenstein et al., 2012), leading to hourly rates of infusions that are actually lower in LgA than ShA rats and suggesting that extending access to 21–23 h/day does not produce more dependence than short access. However, studies that used short access with such high intake are usually associated with food restriction or pretraining for food responding on the drug lever to obtain higher intakes, suggesting that this high intake is not driven by nicotine dependence but by other confounding factors. Although ShA rats in the present study had a higher hourly average rate of infusions than LgA rats, ShA rats did not exhibit any of the key aspects of tobacco dependence, such as abstinence-induced anxiety-like behavior, nociceptive hypersensitivity, excessive nicotine intake, and increased progressive-ratio responding. Moreover, recent studies have demonstrated that 1–12 h/day access to nicotine self-administration was not

associated with increased brain reward thresholds during withdrawal (Paterson and Markou, 2004; Paterson et al., 2008), whereas 22 h/day access was associated with pronounced elevations of brain reward thresholds during withdrawal, which was correlated with compulsive seeking during extinction (Harris et al., 2011). These results demonstrate that although the short access model may have good validity for investigating the acute reinforcing effect of nicotine, it has very limited validity for modeling various components of tobacco dependence. Investigations of the neural basis of nicotine dependence and drug development studies for smoking cessation should instead focus on LgA models.

Anxiety-like behavior and mechanical hypersensitivity but not somatic signs of withdrawal are associated with increased nicotine self-administration

Somatic signs of withdrawal were precipitated post-abstinence in both LgA and ShA rats using the nicotinic receptor antagonist mecamylamine. Nicotine has a very short half-life in rats ($T_{1/2}$ = 45 min; Matta et al., 2007), and the precipitated somatic signs of withdrawal likely reflect the blockade of endogenous cholinergic neurotransmission.

These results demonstrate that short access to nicotine is sufficient to produce somatic signs of withdrawal post-abstinence. However, since only one dose of mecamylamine was tested it can not be ruled out that differences between LgA and ShA rats in somatic withdrawal signs could be observed with lower doses. In the present study, we found that post-abstinence nicotine intake did not correlate with the somatic signs of withdrawal, further suggesting that the severity of the somatic symptoms is not a key factor in driving nicotine intake in LgA rats. A similar dissociation has been observed between somatic signs and another motivational measure of nicotine withdrawal, intracranial self-stimulation (Skjei and Marjou, 2003; Watkins et al., 2000), and similar dissociations have been observed for other drugs of abuse, such as opioids (Schulteis et al., 1994).

Post-abstinence anxiety-like behavior and nociceptive hypersensitivity were higher in LgA rats compared with ShA rats and drug-naive rats, and both correlated with nicotine self-administration upon renewed access to the drug. Heightened sensitivity to nociceptive stimuli during abstinence from chronic nicotine has been demonstrated in both human smokers (John et al., 2009) and rodents (Schmidt et al., 2001) after noncontingent nicotine administration. The present results expand these findings by demonstrating that this nociceptive hypersensitivity is not observed in ShA rats and only in LgA rats that exhibit increased motivation for nicotine. Note that we have not tested affective signs after mecamylamine-induced withdrawals. Further testing will be necessary to see if the difference between ShA and LgA rats can also be observed after precipitated withdrawal.

The increases in anxiety-like behavior and nociceptive hypersensitivity contrast with previous studies that reported a lack of a positive relationship between anxiety-like behavior and nicotine intake using other animal model of nicotine exposure (e.g., Jackson et al., 2009; Schmidt et al., 2001). Instead, we found that elevated levels of anxiety and mechanical hypersensitivity during abstinence predict excessive nicotine intake but only in LgA rats. Considering that anxiety-like behavior and mechanical sensitivity were measured before access to nicotine, changes in subsequent nicotine intake could not have affected directly these measures suggesting that anxiety-like behavior and hyperalgesia may be causally

related to the increase in nicotine intake. Furthermore, no difference in anxiety-like behavior between LgA and ShA rats was observed in the open field test after the end of the nicotine session, suggesting that the increased anxiety-like behavior in LgA rats after abstinence was attributable to the deprivation period. An alternative hypothesis would be that the elevated plus maze was not sufficiently sensitive to detect increases in anxiety-like behavior in LgA rats before abstinence since short access rats spent only 8 seconds in the center of the open field it may be difficult to detect an anxiogenic effect. Nonetheless, these results demonstrate that even if there is a constitutive increase in anxiety-like behavior, it is of small magnitude, and abstinence dramatically increases anxiety-like behavior that then represents a powerful negative reinforcer for excessive nicotine intake.

CRF_1 receptors mediate nicotine abstinence-induced anxiety-like behavior and nociceptive hypersensitivity

Chronic nicotine induces the desensitization and upregulation of nicotine receptors (Quick and Lester, 2002), including receptor subtypes known to be involved in withdrawal-induced pain and anxiety (e.g., 4 and α7; De Biasi and Dani, 2011). Such alterations in nicotinic receptors may then drive the negative effects of nicotine abstinence through interactions with various brain mechanisms, including the CRF- CRF_1 system. Systemic administration of the CRF_1 antagonist MPZP has previously been shown to attenuate the post-abstinence increase in nicotine self-administration in LgA rats (George et al., 2007). In the present study, pretreatment with MPZP at the same dose blocked abstinence-induced anxiety-like behavior and nociceptive hypersensitivity in LgA rats.

Infused into the CeA, MPZP at a concentration that blocks more than 90% of CRF_1 receptor binding (Richardson et al., 2008) also reduced the abstinence-induced increases in nicotine intake and nociceptive hypersensitivity, establishing a direct causal relationship between CeA CRF_1 receptor activity and compulsive nicotine seeking in LgA rats. The specific effect of MPZP on nociceptive hypersensitivity in LgA rats is consistent with the ability of CRF receptor antagonists administered systemically or directly into the CeA to attenuate the nociceptive hypersensitivity associated with neuropathic and inflammatory pain (Hummel et al., 2010; Ji and Neugebauer, 2007) and extends the evidence for a central role for CRF_1 receptors in negative emotional states (Phelps and LeDoux, 2005). Furthermore, CRF_1 receptor antagonists generally do not have anxiolytic-like effects like diazepam unless a stressor is imposed (Menzaghi et al., 1994). Indeed, the dose of MPZP used in the current study was previously shown to have anxiolytic effects in drug-naïve rats in the defensive burying test (Richardson et al., 2008). However, CRF-antagonists have been shown to reduce anxiety like behavior in the defensive burying test, but not in the elevated plus maze (Basso et al., 1999). These results suggest that the CRF system in the CeA is activated only under conditions of severe stress (e.g. electric footshock, drug withdrawal) that is capable to promote reinstatement of drug seeking (Shalev and Shaham, 2010).

Such results and the fact that MPZP has no effect in ShA rats suggest that CRF_1 receptor activation in LgA rats during abstinence represents a required neuroadaptation to develop anxiety, pain, and excessive nicotine intake and suggests the potential value of CRF_1 receptor antagonists in the treatment of tobacco addiction.

In summary, the present results demonstrate that abstinence from nicotine self-administration produces anxiety-like behavior and nociceptive hypersensitivity and that both anxiety and hypersensitivity predict excessive nicotine intake when access to the drug is renewed. However, this phenomenon is only observed in LgA rats and not in ShA rats, providing an explanation for the lack of an association between anxiety-like behavior and nicotine intake in short access models and demonstrating the robust translational relevance of the long access model for understanding the neurobiology of tobacco dependence. Moreover, the CRF-CRF_1 system, particularly in the CeA, is required for the emergence of these negative emotional states, suggesting that targeting the CRF-CRF_1 system may counteract the negative emotional states associated with abstinence and relieve this prominent aspect of nicotine craving.

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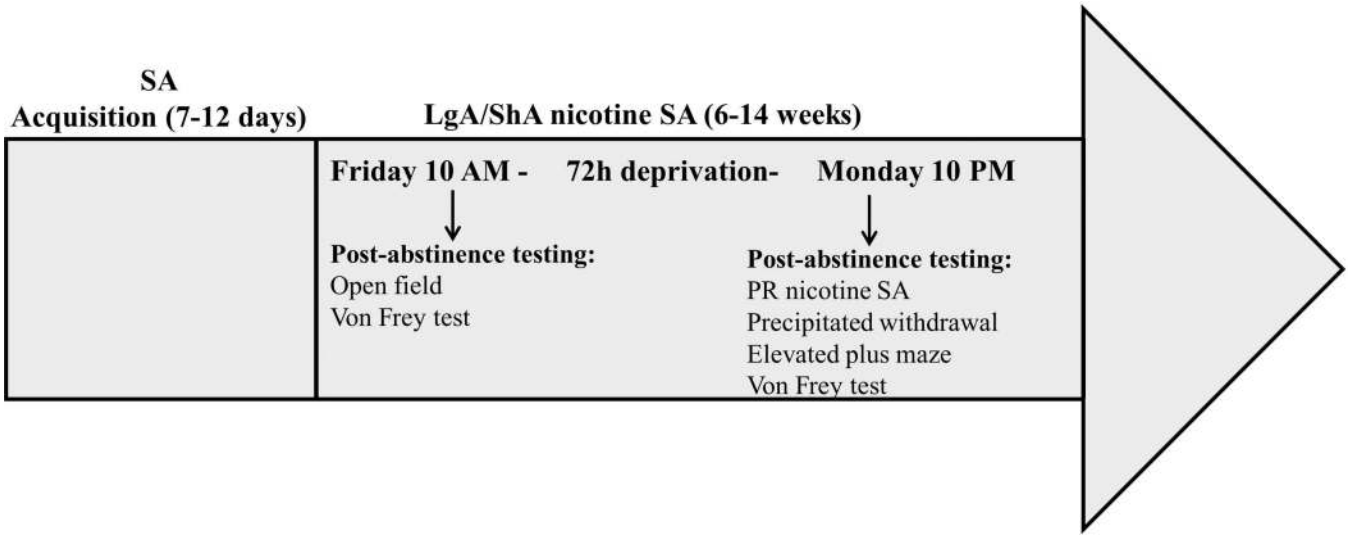

Figure 1.

Experimental design. In all of the experiments, the rats were first given access to nicotine for 1 h per day (acquisition) until nicotine self-administration was stabilized and then separated into two groups given either short access (ShA, 1 h/day) or long access (LgA, 21–23 h/day) to nicotine. In Experiments 2–3 (B, C), LgA rats self-administered nicotine for 21 h instead of 23 h to allow time to run the ShA rats in the same boxes. Each week, in all of the experiments, LgA and ShA rats self-administered nicotine for 4 days/sessions (Monday 10:00 AM to Friday 10:00 AM), followed by 3 days of abstinence from nicotine (Friday-Monday). Behavioral testing was performed before and/or following 72 h of abstinence at 10:00 AM. SA, self-administration; LgA, long access; ShA, short access; PR, progressive-ratio; FR, fixed-ratio.

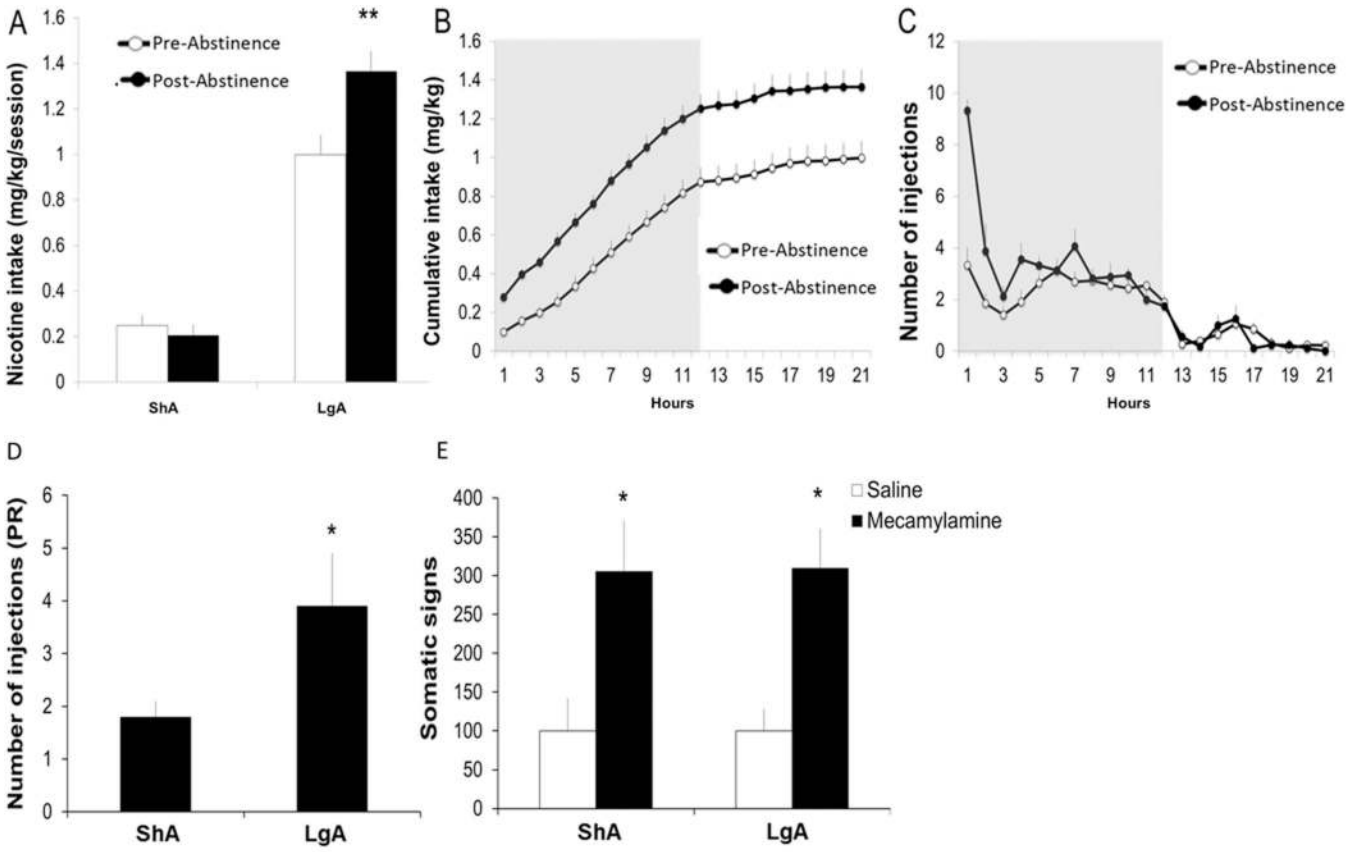

Figure 2.

Following 72 h of abstinence, LgA rats (n = 8) increased their nicotine intake and exhibited higher motivation for nicotine than ShA rats (n = 14). However, both groups had similar levels of mecamylamine-induced somatic signs of withdrawal. Panels A-C describe the patterns of nicotine self-administration during the pre-abstinence daily intake sessions (i.e., Tuesday-Friday) *vs*. the post-abstinence sessions (i.e., Monday), with the data referring to the average performance during the first 2 weeks of the study. (A) Total nicotine intake. (B) Cumulative nicotine intake during the 23 h session in LgA rats. (C) Hour-by-hour nicotine infusions in LgA rats. (D) Breakpoints on a progressive-ratio (PR) schedule of reinforcement. (E) Mecamylamine-induced somatic signs of withdrawal after 72 h of abstinence. *$p < 0.05$, **$p < 0.01$.

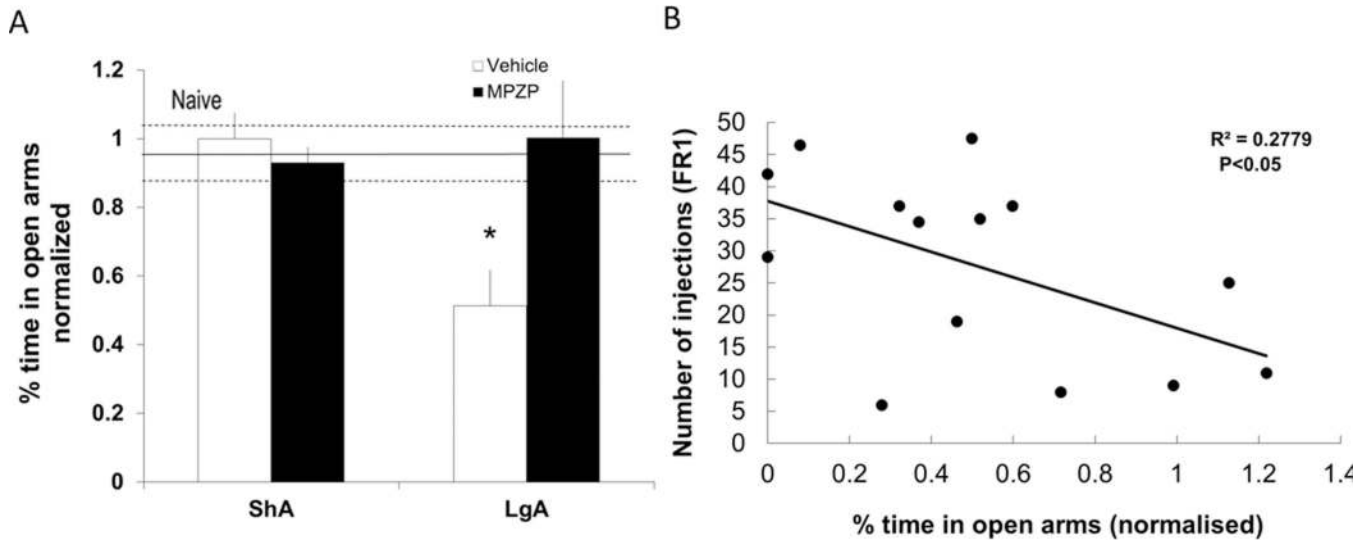

Figure 3.

Anxiety-like behavior in the elevated plus maze is increased following 72 h of abstinence from nicotine self-administration and is CRF_1 receptor-dependent. (A) Percentage of time spent on the open arms of the elevated plus maze (normalized with a log transformation; $Y = log[x+1]$) after 72 h of abstinence in rats pretreated with vehicle (LgA, $n = 14$; ShA, $n = 8$) or MPZP (LgA, $n = 9$; ShA, $n = 7$) and naive rats ($n = 7$). $^*p < 0.05$, compared with LgA-Vehicle. For r**aw values see table 3**. (B) Correlation between the percentage of time spent on the open arms (normalized) and nicotine self-administration after 72 h of abstinence.

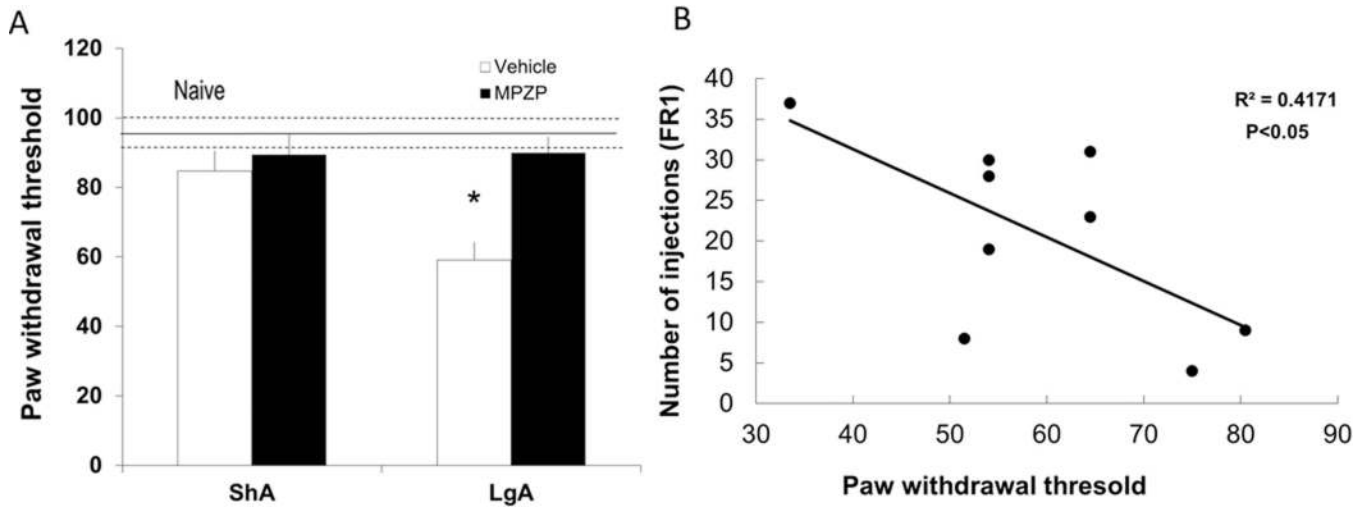

Figure 4.

Mechanical hypersensitivity is increased following 72 h of abstinence from nicotine self-administration and is CRF_1 receptor-dependent. (A) Paw withdrawal thresholds (grams) tested after 72 h of abstinence in LgA rats ($n = 9$) and ShA rats ($n = 8$) following administration of either MPZP or vehicle and naive rats ($n = 8$). *$p < 0.05$, compared with ShA rats. (B) Correlation between paw withdrawal thresholds and nicotine self-administration (fixed-ratio 1) in LgA rats after 72 h of abstinence. *$p < 0.05$. The data are expressed as mean ± SEM.

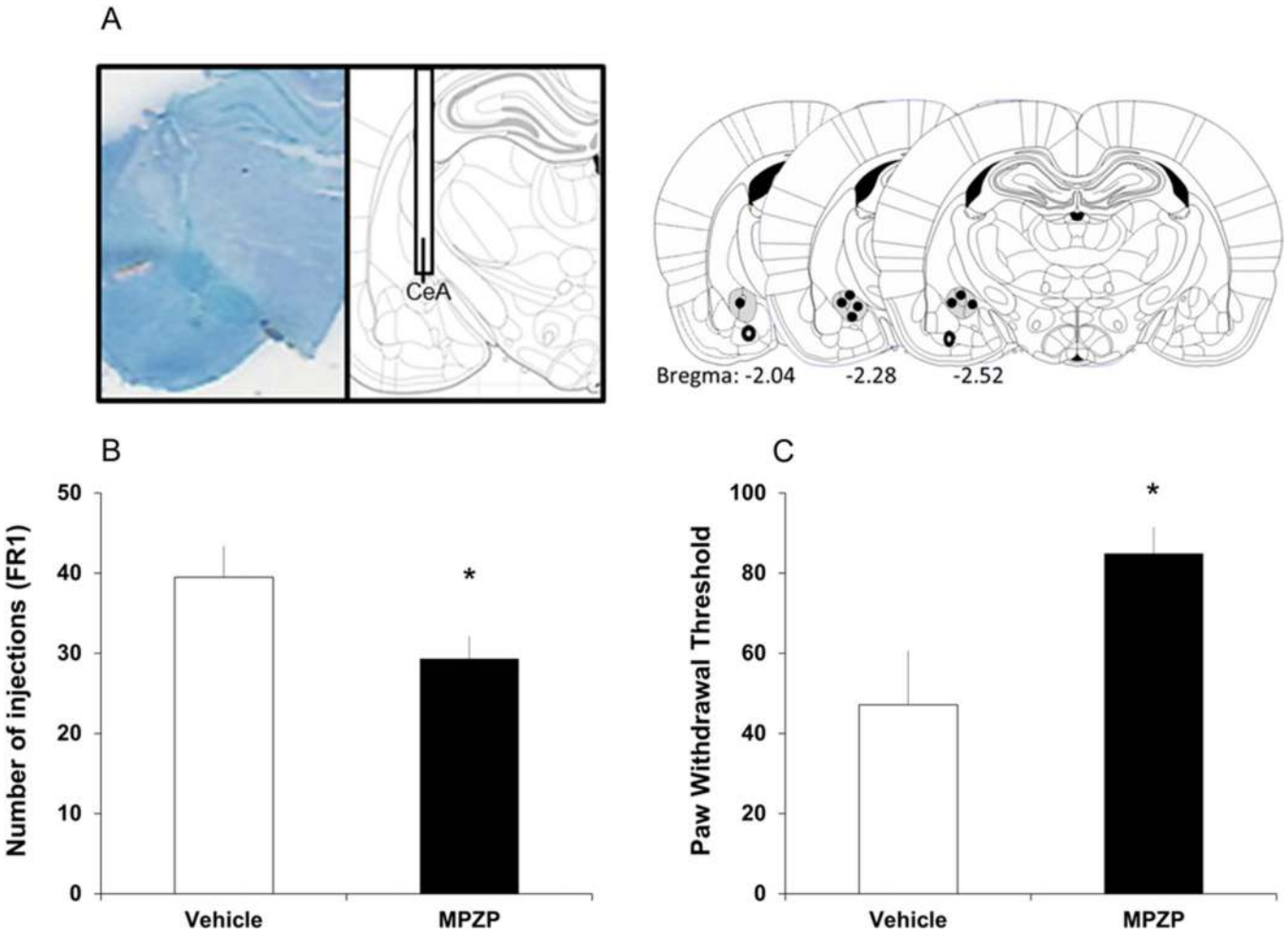

Figure 5.

(A) MPZP microinfusions into the CeA attenuated the abstinence-induced increase in nicotine intake and mechanical hypersensitivity. (B) Nicotine self-administration ($n = 8$) and (C) paw withdrawal thresholds ($n = 6$) in LgA rats after 72 h of abstinence from nicotine self-administration. The data are expressed as mean ± SEM. *$p < 0.02$.

Table 1

Responses for nicotine, food and water prior to division to access conditions

Group	Nicotine lever presses (FR1)	Inactive lever presses	Food intake (g)	Water intake (ml)
LgA	7.71 ±1.73	3.28 ± 0.99	39.65 ± 6.2	64.15 ± 2.57
ShA	6.79 ± 1.01	2.14 ± 0.55	37.63 ± 1.39	60.42 ± 3.28

Table 2

Somatic signs of withdrawal

		ShA		LgA		
	Signs	**mean**	**sem**	**mean**	**sem**	
	blinks	1.8±0.4		1.8±0.5		ns.
	chews	0.0±0.0		0.0±0.0		ns.
	escape attempts	1.5±0.5		2.0±0.0		ns.
	foot licks	3.0±2.0		1.0±n/a		ns.
	hops	5.0±n/a		0.0±0.0		ns.
	head shakes	0.0±0.0		1.0±n/a		ns.
	ptosis	0.0±0.0		0.0±0.0		ns.
	scratches	0.0±0.0		0.0±0.0		ns.
Saline	**total**	**2.0±0.8**		**1.9±0.8**		ns.
	blinks	5.4±1.3		2.9±0.9		ns.
	chews	1.5±0.5		3.0±1.2		ns.
	escape attempts	1.0±n/a		1.0±n/a		ns.
	foot licks	1.0±0.0		3.0±n/a		ns.
	hops	1.0±0.0		2.0±1.0		ns.
	head shakes	2.0±0.6		3.0±0.6		ns.
	ptosis	1.0±n/a		1.0±n/a		ns.
	scratches	1.0±0.0		1.0±n/a		ns.
Mecamylamine	**total**	***7.7±1.6**		***5.9±1.4**		ns.

*p <0.05

vs. saline

note: n/a = not available (only 1 animal showed a sign)

Table 3

Elevated plus maze absolute data

	Duration in zone (sec)			
Group	**Open arms**	**Closed arms**	**Center**	**% in open arms**
LgA-MPZP	45.6 ± 14.6	199 ± 27.2	56.7 ± 11.8	15.2 ± 4.9
LgA-Vehicle	*10.4 ±4.2	254 ±18.2	33.9 ±15.8	*3.5 ± 1.4
Naïve	26.3 ± 4.6	246 ± 8.2	27.8 ± 5.6	8.7 ± 1.5
ShA-MPZP	27.6 ± 6.1	200 ±13.8	71.2 ± 10.6	9.2 ±2.1
ShA-Vehicle	29.9 ± 5.6	230 ± 7.1	42.5 ± 8.9	10.1 ± 1.9
	Distance traveled (cm)			
LgA-MPZP	1041 ± 429	9090 ± 1175	1314 ± 165	11 ± 4.8
LgA-Vehicle	192 ± 87	7839 ± 840	836 ± 230	2.1 ± 1
Naïve	879 ± 263	11195 ± 529	1063 ± 107	6.7 ± 1.9
ShA-MPZP	597 ± 167	9138 ± 1000	1259 ± 118	6 ± 1.7
ShA-Vehicle	454 ± 106	7963 ± 605	1009 ± 211	4.8 ± 1.1
	Entries into zone			
LgA-MPZP	5.1 ± 1.7	13 ± 1.7	16.8 ± 1.4	14.1 ± 4.6
LgA-Vehicle	2.7 ± 1.4	11.1 ± 2.1	12.8 ± 2.6	8.1 ± 3
Naïve	2.8 ± 0.4	13.8 ± 0.7	15.6 ± 1.3	8.6 ± 1
ShA-MPZP	4.3 ± 0.9	13.1 ± 1.7	16.7 ± 2	13 ± 2.2
ShA-Vehicle	3.6 ± 0.9	9.2 ± 1.3	11.9 ± 2.1	13.8 ± 1.7

* $p < 0.05$

Vs. Naïve and ShA-Vehicle