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Impairment of contextual fear extinction by chronic nicotine and withdrawal from chronic nicotine is associated with hippocampal nAChR upregulation

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Abstract

Chronic nicotine and withdrawal from chronic nicotine have been shown to be major modulators of fear learning behavior. Moreover, recent studies from our laboratory have shown that acute nicotine impaired fear extinction and safety learning in mice. However, the effects of chronic nicotine and withdrawal on fear extinction are unknown. Therefore, the current experiments were conducted to investigate the effects of chronic nicotine as well as withdrawal from chronic nicotine on contextual fear extinction in mice. C57BL6/J mice were given contextual fear conditioning training and retention testing during chronic nicotine administration. Mice then received contextual fear extinction either during chronic nicotine or during withdrawal from chronic nicotine. Our results showed that contextual fear extinction was impaired both during chronic nicotine administration and subsequent withdrawal. However, it was also observed that the effects of prior chronic nicotine disappeared after 72 h in withdrawal, a timeline that closely matches with the timing of the chronic nicotine-induced upregulation of hippocampal nicotinic acetylcholine receptor (nAChR) density. Additional experiments found that 4 days, but not 1 day, of continuous nicotine administration upregulated hippocampal nAChRs and impaired contextual fear extinction. These effects disappeared following 72 h withdrawal. Overall, these experiments provide a potential link between nicotine-induced upregulation of hippocampal nAChRs and fear extinction deficits observed in patients with anxiety disorders, which may lead to advancements in the pharmacological treatment methods for this disorder.

Keywords

Nicotine; Nicotinic receptors; Extinction; PTSD

1. Introduction

The bi-directional relationship between nicotine dependence and anxiety and stress-related disorders such as Post-traumatic stress disorder (PTSD) has been well documented (Breslau

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et al., 2003, 2004; Feldner et al., 2007; Koenen et al., 2005). These studies showed that PTSD patients show significantly higher rates of nicotine dependence compared to the healthy population (Lasser et al., 2000; Ziedonis et al., 2008) and development of PTSD triggers higher rates of smoking initiation and increased number of cigarettes smoked daily (Breslau et al., 2003, 2004). While the increase in nicotine consumption can be explained by its anxiolytic properties (Ericson et al., 2000; O'Neill and Brioni, 1994), there is also evidence suggesting that nicotine may aggravate PTSD symptomatology as studies found that nicotine intake increased trauma-related intrusive memories (Hawkins and Cougle, 2013) and fear response to trauma-related scripts (Calhoun et al., 2011). In line with human studies, studies using animal models of fear learning have also shown that nicotine alters fear memory (see Kutlu and Gould, 2015 for a review). These studies repeatedly demonstrated that acute nicotine selectively enhanced hippocampus-dependent forms of fear conditioning, such as contextual and trace fear conditioning, but does not affect hippocampus-independent cued fear conditioning (Gould and Wehner, 1999; Gould, 2003; Gould and Stephen Higgins, 2003; Gould and Lommock, 2003; Gould et al., 2004; Davis et al., 2005, 2006; Davis and Gould, 2006). On the other hand, multiple studies showed that while chronic nicotine administration did not affect fear learning, withdrawal from chronic nicotine resulted in deficits in hippocampus-dependent fear learning (Portugal and Gould, 2009; Portugal et al., 2012a; André et al., 2008; Davis et al., 2005; Raybuck and Gould, 2009). Together with human studies, these results clearly suggest that nicotine alters fear learning and memory.

While one of the major manifestations of PTSD is heightened emotional response to the environmental stimuli that trigger trauma-related memories, there is evidence showing that PTSD patients also show deficits in fear extinction (Blecher et al., 2007; Michael et al., 2007; Milad et al., 2009; Waters et al., 2009). Therefore, we recently examined the effects of nicotine on contextual fear extinction. Our results showed that acute nicotine administration impaired encoding and retrieval of contextual fear extinction memories without affecting general freezing behavior measured in a novel context (Kutlu and Gould, 2014; Kutlu et al., in press). In addition, we also found that the impairing effects of acute nicotine on contextual fear extinction require high-affinity $\alpha 4\beta 2$, but not low-affinity $\alpha 7$, nicotinic acetylcholine receptors (nAChRs, Kutlu et al., 2016). These results suggest that cholinergic mechanisms in the brain may critically modulate contextual fear extinction memories.

Although understanding the effects of acute nicotine on contextual fear extinction informs us on the mechanisms that are responsible for the involvement of nAChRs in extinction process, the majority of PTSD patients are chronic smokers (Lasser et al., 2000; Ziedonis et al., 2008) and therefore exposed to nicotine chronically. Therefore, it is imperative to examine the effects of chronic nicotine and withdrawal from chronic nicotine on contextual fear extinction in order to fully capture the potential effects of nicotine dependence on fear extinction. In the present study, we tested contextual fear extinction in mice that received one of multiple doses of chronic nicotine (3.1, 6.3, and 12.6 mg/kg/ d) and or were withdrawn from chronic nicotine treatment. In addition, in order to test if nAChR upregulation may be involved in the effects of chronic nicotine and withdrawal effects, we examined the effects of different lengths of chronic nicotine administration (1 day vs. 4 days) on contextual fear extinction as well as on hippocampal nAChR binding. We

hypothesize that chronic nicotine administration will impair contextual fear extinction and this effect will be carried over during withdrawal. In addition, we expect that the length of chronic nicotine administration required for this effect will correlate with the length of chronic nicotine treatment necessary for hippocampal nAChR upregulation.

2. Method

2.1. Subjects

Subjects were adult (8–10 weeks old) male C57BL6/J mice (Jackson Laboratory, Bar Harbor, ME) group housed in a colony room maintained on a 12 h light/dark cycle with ad-libitum access to water and food. All training and testing occurred between 9:00 a.m. and 6:00 p.m. Behavioral procedures used in this study were approved by the Temple University Institutional Animal Care and Use Committee.

2.2. Apparatus

Training for acquisition and extinction of contextual fear conditioning took place in 4 identical chambers (18.8 × 20 × 18.3 cm) with Plexiglass doors on the front wall of the chambers placed in sound attenuating boxes (MED Associates, St. Albans, VT). Ventilation fans, which produce a background noise (65 dB), were located at the back wall of the chambers. Another set of speakers mounted on the right wall of the chambers provided a white noise (85 dB) auditory conditioned stimulus (CS). A 2 s long, 0.57 mA foot-shock unconditioned stimulus (US) was delivered via metal grids (0.20 cm and 1.0 cm apart) on the chamber floors, which were connected to a shock generator. All stimuli were controlled by an IBM-PC compatible computer running MED-PC software.

2.3. Behavioral procedures

Behavioral procedures were based on our previous reports examining the effects of acute nicotine on contextual fear extinction (Kutlu and Gould, 2014; Kutlu et al., 2016). The dependent variable for behavioral experiments was freezing response to the context, which was defined as the absence of voluntary movement except respiration (Davis et al., 2007; Kutlu and Gould, 2014). A time sampling method was employed to detect freezing response where mice were observed and scored every 10 s for a duration of 1 s as freezing or active (Blanchard and Blanchard, 1972). Mice were first given contextual fear conditioning training, in which they received two white noise-footshock (0.57 mA) pairings. Twenty-four hours after training, mice were returned to the training context for the retention test in order to measure initial freezing to the context. Then all mice received a single daily session of contextual fear extinction for 5 days where mice were reintroduced to the training context in the absence of foot-shocks in order to reduce conditioned freezing response. All behavioral testing was performed in the light phase of the dark/light cycle.

2.4. Osmotic mini-pump surgeries

In order to mimic long-term nicotine exposure in smokers, nicotine hydrogen tartrate salt (Sigma, St. Louis, MO) dissolved in saline or saline alone was subcutaneously delivered via osmotic mini-pumps (Alzet, Model 1002, Durect, Cupertino, CA). Mini-pumps for both chronic nicotine and saline infusions were surgically inserted through an incision made on

the lower back of the mouse while mice were under 2–5% isoflurane anesthetic. Surgeries were performed under sterile conditions. Mice in the chronic nicotine experiment received continuous nicotine (3.1, 6.3, or 12.6 mg/kg/day) or saline for 11 days prior to behavioral testing and throughout contextual fear training, retention test, and extinction sessions for a total of 18 days. For the withdrawal experiments, both saline and nicotine mini-pumps were removed before extinction sessions to induce spontaneous withdrawal. Therefore, withdrawal experiment mice received 13 days of chronic nicotine (3.1, 6.3, or 12.6 mg/kg/day) or saline during contextual fear training and retention test but not during extinction sessions. Finally, the second set of withdrawal experiments established the minimum duration of nicotine exposure for the withdrawal-associated effects; mice either received 1 day or 4 days of nicotine (12.6 mg/kg/day; 1 d NIC and 4 d NIC groups) or saline (1 d SAL and 4 d SAL groups) between the retention test and extinction session. These mice were implanted with mini-pumps right after the retention test and the mini-pumps were removed 24 h prior to the first extinction session. For the receptor binding experiments, mice were implanted with mini-pumps and given 4 d of saline, 1 d or 4 d of 12.6 mg/kg/day nicotine to determine the hippocampal receptor binding levels following the minimum length of chronic nicotine administration resulting contextual fear extinction impairment. We chose the 12.6 mg/kg/day dose for these experiments as it was the only effective dose for the withdrawal effects. The nicotine doses are reported as freebase weights and chosen based on the previous research from our laboratories showing deficits in contextual fear learning during withdrawal with these doses (Portugal et al., 2012b). In addition, previous reports also showed that the range of doses we chose for these studies resulted in plasma nicotine and cotinine levels comparable to the levels observed in human smokers (Murrin et al., 1987; Benowitz, 1988, 1996; Benowitz et al., 1989; Davis et al., 2005; Portugal et al., 2012b; Cole et al., 2015) without leading to potentially confounding somatic withdrawal.

2.5. Nicotinic acetylcholine receptor binding

Brains were harvested following either 4 days of chronic saline, 1 day of chronic nicotine, 4 days of chronic nicotine and 24 h withdrawal or 4 days of chronic nicotine and 72 h withdrawal, and dorsal and ventral hippocampi were dissected for radioligand binding. Tissues were homogenized in Lysis Buffer (5 mM Tris + 5 mM EDTA + 5 mM EGTA) and centrifuged at 100,000 g for 30 min at 4 °C. Pellets were resuspended in Lysis Buffer and centrifuged again. Pellets were re-suspended in Tris/10% sucrose buffer (50 mM Tris-HCl/pH7.4 and 10% sucrose) and the resulting membrane preparation was used for binding studies. Membranes were incubated with [³H]Epibatidine ([³H]EB) (~2 nM) (specific activity 54.1 Ci/mmol, PerkinElmer, Boston, MA) for 1 h at room temperature. We chose to use [³H]EB for our receptor binding assay because [³H]EB has much higher affinity for all heteromeric nAChR subtypes (e.g., $\alpha 4\beta 2$ nAChRs) in the brain than for homomeric nAChRs (e.g., $\alpha 7$ nAChRs; Avalos et al., 2002; Houghtling et al., 1995; Marks et al., 1998; Xiao and Kellar, 2004) and our previous experiments suggested that the effects of nicotine on contextual fear extinction require $\alpha 4\beta 2$ but not $\alpha 7$ nAChRs (Kutlu et al., 2016). Non-specific binding was determined in the presence 300 μ M of nicotine. Bound and free [³H]EB was separated by filtration over GF/B filters under vacuum using a 24-well cell harvester (Brandel Co, Gaithersburg, MD). GF/B filters were pre-soaked for 1 h with 0.2% polyethyleneimine and 0.01% bovine serum albumin in 50 mM Tris-HCl buffer (pH 7.4).

Radioactivity in filters was determined by use of a liquid scintillation counter (Tri-Carb 2810 TR, PerkinElmer). Specific binding was calculated as the difference between total binding and nonspecific binding and expressed as fmol/mg tissue (Portugal et al., 2012b; Wilkinson et al., 2013).

2.6. Statistical analysis

Following previous reports on the effects of nicotine on contextual fear extinction (Tian et al., 2008; Kutlu et al., 2016), we converted freezing response to “normalized %freezing”, where each freezing response measured during extinction sessions was normalized to the initial freezing response measured during the retention test (freezing $\times$ 100/initial freezing). This method ensures that potential between-group baseline differences in contextual freezing do not affect subsequent fear extinction curves. For each behavioral experiment, we used a 2 (Drug) $\times$ 6 (Trial) Repeated measures ANOVAs at $\alpha = 0.05$. Initial freezing levels during the retention test were analyzed using a one-way ANOVA. Tukey post-hoc tests were used for all between-group comparisons where the independent variable had more than 2 levels (nicotine groups vs. the saline controls). Following procedures from our previous studies examining the effects of nicotine on contextual fear extinction (Kutlu and Gould, 2014; Kutlu et al., 2016), 1 mouse from the chronic nicotine experiment (Saline group) and 4 mice from the withdrawal experiment (1 from Nic 3.1 mg/kg/d, 1 from Nic 6.3 mg/kg/d and 2 from 12.6 mg/kg/d groups) were removed from analysis as their freezing levels were 2 standard deviations above the group means during at least 1 extinction session. Also following Kutlu et al. (2016), 3 mice from the additional withdrawal experiments (1 from 1 d Saline, 1 from 4 d Nicotine, and 1 from 4 d Saline group) were removed from the study because their initial freezing responses were lower than 15% during the retention test. This acquisition criterion, as used in our and others' previously published work (Kutlu et al., 2016, in press; Corcoran et al., 2005) is essential to any studies that examines extinction behavior. This is because, in the absence of this criterion, it would be impossible to determine if extinction occurs when the initial freezing levels are at baseline levels of freezing. We chose 15% freezing as our cut-off point because this level of freezing was found to be generalized to a novel context that was not paired with a foot-shock before (Kutlu and Gould, 2014). The receptor binding data was analyzed using a one-way ANOVA followed by Dunnett post-hoc tests to compare all treatment groups to saline-treated controls in each brain regions. Group sizes were indicated in figure captions. All statistical analyses were run using SPSS 21.

3. Results

3.1. Chronic nicotine impairs contextual fear extinction

We first examined the effects of continuous chronic nicotine administration during extinction on contextual fear extinction performance. Mice received a total of 18 days of chronic nicotine during which they were trained and tested in contextual fear conditioning after the first 11 days of administration (Fig. 1A). Although a one-way ANOVA on the retention test freezing showed that the main effect of drug was significant ($F_{(3,29)} = 2.973$, $p < 0.05$), follow-up Tukey post-hoc tests found that no groups significantly differed from the saline controls ($ps > 0.05$), which is in line with previous research showing that chronic

nicotine does not alter contextual fear conditioning (Davis et al., 2005; Portugal et al., 2012b). Therefore, any differences found between nicotine and saline controls during extinction cannot be attributed to differences in initial contextual fear conditioning performance. Following contextual fear conditioning training and retention test, mice received single daily contextual extinction sessions for 5 days to examine the effects of chronic nicotine on contextual fear extinction while continuous nicotine administration continued (Fig. 1A). A repeated measures ANOVA showed that Drug $\times$ Trial interaction was significant during extinction ($F(5,145) = 3.779, p < 0.001$). In addition, Tukey post-hoc tests found that the Nic 12.6 mg/kg/d group freezing levels significantly differed from the saline controls during the last 3 extinction sessions while Nic 6.3 mg/kg/d groups differed only during the 2nd and 4th extinction sessions ($p < 0.05$). The Nic 3.1 mg/kg/d group did not differ from the saline controls during any of the extinction session. These results show that chronic nicotine administered prior to and during extinction impaired contextual fear extinction (Fig. 1B). While the most persistent effects were achieved with the higher dose of chronic nicotine (12.6 mg/kg/d), a lower dose of chronic nicotine (6.3 mg/kg/d) was also effective (Fig. 1B).

3.2. Contextual fear extinction is impaired during withdrawal

In order to examine contextual fear extinction during withdrawal from chronic nicotine, mice first received 13 days of chronic nicotine where they were given contextual fear conditioning training and retention test on days 12 and 13 (Fig. 2A). Similar to the chronic nicotine experiment, a one-way ANOVA on the retention test freezing showed that the main effect of drug was significant ($F(3,28) = 3.340, p < 0.05$) but follow-up Tukey post-hoc tests found that no groups significantly differ from the saline controls ($ps > 0.05$). Following the retention test, mini-pumps were removed in order to establish spontaneous withdrawal and mice were tested for contextual fear extinction for 5 days as described above (Fig. 2A). A repeated measures ANOVA showed that Drug $\times$ Trial interaction was significant ($F(5,140) = 4.190, p < 0.001$). Tukey post-hoc tests found that Nic 12.6 mg/kg/d group freezing levels significantly differed from the saline controls during the first 2 extinction sessions while Nic 6.3 mg/kg/d groups differed only during the 5th extinction session ($p < 0.05$). The Nic 3.1 mg/kg/d group did not differ from the saline controls during any of the extinction sessions ($p > 0.05$). These results suggest that nicotine administered prior to extinction had an impairing effect on contextual fear extinction only with the highest dose of nicotine (12.6 mg/kg/d; Fig. 2B). However, withdrawal from a lower dose of nicotine (6.3 mg/kg/d) did not impair contextual fear extinction and even resulted in lower levels of normalized freezing during the last trial of extinction (Fig. 2B), which may be interpreted as enhanced extinction. It is also important note that the effects of prior chronic nicotine disappeared following 72 h in withdrawal. Previous studies from our laboratory showed that 12 day chronic nicotine (6.3 mg/kg/d) administration upregulates hippocampal nAChRs (Gould et al., 2014) and the nAChR density returns to baseline after 72 h (Gould et al., 2012). Thus, chronic nicotine's impairing effects on contextual fear extinction may be a result of upregulated hippocampal nAChRs.

3.3. Contextual fear extinction is impaired following 4 days but not 1 day of nicotine administration

Given that the effects of prior chronic nicotine on contextual fear extinction disappear following 72 h in withdrawal and at least 4 days of chronic nicotine administration is required to achieve significant nAChR upregulation in the hippocampus (Gould et al., 2012, 2014), we hypothesized that 4 days of chronic nicotine administration but not 1 day administration will result in impaired contextual fear extinction. Therefore, in order to test this hypothesis, mice were trained and tested in contextual fear conditioning in the absence of nicotine and then, implanted with osmotic mini-pumps and given either 1 or 4 days of continuous nicotine before receiving contextual fear extinction for 5 days in withdrawal. Separate one-way ANOVAs showed that there were no significant main effects of Drug on the initial freezing levels measured during the retention tests ($F(1,613) = 1.576, p > 0.05$ for the 1 d experiment, $F(1,27) = 0.210, p > 0.05$ for the 4 d experiment). Two separate repeated measures ANOVAs showed that Drug $\times$ Trial interaction was significant following the 4 d nicotine exposure ($F(5,60) = 2.436, p < 0.05$) but not following the 1 d nicotine exposure ($F(5,65) = 0.268, p > 0.05$). Individual one-way ANOVAs for each extinction session found no significant main-effect of drug during any of the extinction sessions ($p > 0.05$). However, 4 d Nic group freezing levels were significantly different from the saline controls during the 2nd extinction session ($F(1,14) = 5.505, p < 0.05$). Overall, this suggests that 4 d of chronic nicotine but not 1 d of nicotine administration resulted in impaired contextual fear extinction (Fig. 3). These results support our hypothesis that hippocampal nAChR upregulation may be required for the impairing effects of chronic nicotine on contextual fear extinction.

3.4. Four days of chronic nicotine exposure upregulates $\alpha 4\beta 2$ nAChRs in dorsal and ventral hippocampus

To further test our hypothesis linking hippocampal nAChR and contextual fear extinction impairments, we performed [^3H]Epibatidine binding to $\alpha 4\beta 2$ nAChR receptor in membranes prepared from these brain regions of contextual fear conditioning naïve mice following either 4 days of chronic saline, 1 day of chronic nicotine, 4 days of chronic nicotine and 24 h withdrawal or 4 days of chronic nicotine and 72 h withdrawal. Two separate one-way ANOVAs showed that there was a significant main effect of nicotine treatment for both dorsal (dHPC, $F(3,28) = 3.057, p < 0.05$) and ventral (vHPC, $F(3,28) = 5.027, p < 0.05$) hippocampus. In addition, Dunnett post-hoc tests showed that for both dHPC and vHPC, the groups that received 4 days of chronic nicotine and tested 24 h later significantly differed from the control group that received saline ($ps < 0.05$), but not the groups that received 1 day of nicotine or the groups that received 4 days of nicotine but tested 72 h later ($ps > 0.05$). Therefore, our results show that 4 d, but not 1 d, of nicotine administration at the 12.6 mg/kg/d dose followed by 24 h withdrawal resulted in upregulated nAChR binding in dHPC and vHPC, whereas nAChR upregulation returned to baseline following 72 h of withdrawal (Fig. 4). The timeline of the upregulation and return to baseline of hippocampal nAChRs closely match with the timeline of the chronic nicotine-induced impairment of contextual fear extinction.

4. Discussion

The results of the present study showed that higher (12.6 mg/kg/d) and lower doses (6.3 mg/kg/d) of chronic nicotine impaired contextual fear extinction. Similarly, contextual fear extinction was also impaired during withdrawal from the higher dose of nicotine (12.6 mg/kg/d) but lower doses (6.3 and 3.1 mg/kg/d) did not impair contextual extinction. Interestingly, the effect of the higher dose of chronic nicotine disappeared following 72 h of withdrawal. In addition, 4 days of chronic nicotine administration, but not 1 day, resulted in impaired contextual fear extinction and upregulated hippocampal nAChRs. In parallel, we also found that both contextual fear extinction impairment and hippocampal nAChR upregulation following 4 days of chronic nicotine disappeared after 72 h withdrawal. Overall, these results suggest that chronic nicotine may lead to fear memories that are more resistant to extinction and this effect may be a result of upregulated hippocampal nAChRs.

Chronic nicotine has been shown to have no effect on contextual fear conditioning (Portugal and Gould, 2009; Portugal et al., 2012a; André et al., 2008; Davis et al., 2005; Raybuck and Gould, 2009). Thus, the results of the present study demonstrating that chronic nicotine impairs contextual fear extinction seem to be in contrast with these results. However, it is important to note that contextual fear acquisition and extinction are inherently different behavioral paradigms and they recruit different brain circuits (Morgan and LeDoux, 1995; Quirk et al., 2006; Quirk and Mueller, 2008). For example, during fear extinction, medial prefrontal cortex receives input from the hippocampus and actively suppresses the amygdala, which is required to establish inhibitory control and consequently reduce conditioned emotional response (Quirk et al., 2006). Also, even though both behavioral paradigms are considered as new memory formation, the most striking difference between these paradigms is that fear acquisition requires the organisms to learn a neutral stimulus as fearful whereas in fear extinction the organism needs to counteract the previously learned fear association and learn that the previously fearful stimulus is now safe. Therefore, unlike contextual fear conditioning, contextual fear extinction requires a certain degree of behavioral flexibility. Interestingly, previous results by Ortega et al. (2013) showed that chronic nicotine impairs cognitive flexibility in mice. Thus, it is possible that chronic nicotine leads to difficulty in updating the established memories.

In addition to the current report, multiple studies from our laboratory demonstrated that chronic nicotine results in upregulation of nAChRs in the hippocampus (Gould et al., 2012, 2014; Portugal et al., 2012b; Wilkinson et al., 2013). For example, Gould et al. (2014) showed that 6.3 mg/kg/d dose of nicotine required at least 4 days of chronic administration for significant high-affinity nAChR upregulation in the dorsal hippocampus. In addition, Gould et al. (2012) showed that the high-affinity nAChR upregulation in the whole hippocampus as a result of 12 days of chronic nicotine exposure at the 6.3 mg/kg/d dose went back to baseline following 72 h withdrawal. These results are in agreement with the results of the present study showing that 12.6 mg/kg/d chronic nicotine for 4 days, but not for 1 day, resulted in significant high-affinity nAChR upregulation in both dorsal and ventral hippocampus and the upregulation was absent following 72 h withdrawal. Interestingly, Wilkinson et al. (2013) measured high-affinity nAChR binding in the hippocampus in another strain of C57BL6 mice (C57BL6/NTac) following 12 days of 18 mg/kg/d chronic

nicotine administration and found significant upregulation in the dorsal but not in ventral hippocampus after 48 h of withdrawal. There might be multiple possible reasons for these contradicting results. First, genetic and behavioral differences between BL6 substrains are well-documented (Bryant et al., 2008; Mekada et al., 2009; Zurita et al., 2011) and therefore the lack of nAChR upregulation in the ventral hippocampus may be a manifestation of differences between C57BL6/J and C57BL6/NTac genetic makeup. It is also possible that the duration of nAChR regulation is different between dorsal and ventral hippocampus. For example, if upregulation in the ventral hippocampus is maintained for a shorter period of time in comparison to the dorsal hippocampus, nAChR density in this subregion may have returned to baseline following 48 h of withdrawal in the Wilkinson et al. (2013) study but still have been upregulated after 24 h of withdrawals as shown in the present study. Nevertheless, future studies are needed in order to clarify the underlying mechanisms for these contrasting results.

It is important to note that although contextual fear extinction is largely impaired during abstinence from the 12.6 mg/kg/d dose, abstinence from a lower dose of chronic nicotine (6.3 mg/kg/d) enhanced extinction (Fig. 2B). Previous studies have also documented some beneficial effects of nicotine abstinence on visuo-spatial working memory (George et al., 2002) and anxiety levels (West and Hajek, 1997) in humans. Therefore, it is possible that nicotine abstinence may improve cognition after treatment with lower doses. In addition, based on our receptor binding results, this effect lays outside a potential nAChR upregulation window, which suggests that a secondary mechanism may play a role in this effect.

As mentioned above, nicotine dependence is significantly more prevalent among people diagnosed with PTSD (Lasser et al., 2000; Ziedonis et al., 2008). Our results suggest that this high rate of nicotine exposure may have adverse effects on PTSD symptomatology. Specifically, it is possible that chronic nicotine exposure may prolong the course of the disorder by interfering with extinction of trauma-related fear memories and exposure therapy. Our results also suggest that these negative effects may be associated with high-affinity nAChR upregulation in memory-related brain regions such as the hippocampus. Interestingly, in parallel with these hypotheses, there is evidence showing that alterations of nAChRs may be involved in PTSD symptomatology (Czermak et al., 2008; Xie et al., 2012; Schulz et al., 2013). For example, Schulz et al. (2013) found that prenatal stress exposure in rats resulted in upregulated high-affinity $\alpha 4\beta 2$ nAChRs in the hippocampus during adulthood. In addition, using the radiotracer [^{123}I]5-IA-85380 and single-photon emission computed tomography (SPECT), Czermak et al. (2008) showed that non-smoker PTSD patients showed a significantly higher density of $\beta 2$ nAChRs in the amygdala and hippocampus compared to non-smoker healthy individuals. This study also importantly found that upregulation of $\beta 2$ nAChRs in these brain regions were positively correlated with PTSD symptoms such as re-experiencing. Therefore, together with the studies suggesting a relationship between stress and hippocampal nAChR density, our results suggest that chronic nicotine may interfere with contextual fear extinction via similar nAChR alterations caused by stress and trauma. Nevertheless, it is one of the limitations of the present study that our receptor binding assay captures both cell surface nAChRs and nAChRs that are expressed intracellularly. Consequently, it is hard to know that if the total receptor binding reported

here comes from nAChRs that are already incorporated into the cell surface or if it reflects total nAChRs expressed as a whole. Thus, future studies are required to establish the link between nicotine dependence and fear extinction deficits in PTSD patients as well as the specific characteristics of chronic nicotine-induced nAChR upregulation within the hippocampus.

Overall, our results show that chronic nicotine exposure impairs contextual fear extinction and this effect persists during withdrawal while disappearing as nAChR upregulation decreases. This suggests that hippocampal high-affinity nAChR upregulation may be critically involved in fear-related symptoms of PTSD. Therefore, these findings may lead to a better understanding of PTSD, nicotine addiction, and comorbidity between them while also suggesting potential novel pharmacological treatment strategies to alleviate nicotine's negative effects on PTSD symptomatology.

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Fig. 1.

Chronic nicotine impairs contextual fear extinction. Panel A: The schematic of the experimental design and drug treatment. While each box represents a phase of the experiment, the lightning bolt symbol indicates the presentations of the footshocks. Panel B: Effects of chronic nicotine administration (3.1, 6.3, and 12.6 mg/kg/d) on contextual fear extinction ($n = 8-9$ per group). Panel C: Raw freezing scores. Error bars indicate Standard Error of the Mean (SEM). (*) denotes significant differences between Nic 12.6 mg/kg/d group and saline controls and (#) denotes significant differences between Nic 6.3 mg/kg/d and saline controls at the $p < 0.05$ level.

Fig. 2.

Contextual fear extinction is impaired during withdrawal. Panel A: The schematic of the experimental design and drug treatment. While each box represents a phase of the experiment, the lightning bolt symbol indicates the presentations of the footshocks. Panel B: Effects of withdrawal from chronic nicotine administration (3.1, 6.3, and 12.6 mg/kg/d) on contextual fear extinction ($n = 7-10$ per group). Panel C: Raw freezing scores. Error bars indicate Standard Error of the Mean (SEM). (*) denotes significant differences between Nic 12.6 mg/kg/d group and saline controls and (#) denotes significant differences between Nic 6.3 mg/kg/d and saline controls at the $p < 0.05$ level.

Fig. 3.

Contextual fear extinction is impaired following 4 days but not 1 day of nicotine administration. Panel A: Effects of withdrawal from 1 day of chronic nicotine administration (12.6 mg/kg/d) on contextual fear extinction ($n = 7-8$ per group). Panel B: Raw freezing scores Panel C: Effects of withdrawal from 4 days of chronic nicotine administration (12.6 mg/kg/d) on contextual fear extinction ($n = 7$ per group). Panel D: Raw freezing scores. Error bars indicate Standard Error of the Mean (SEM). (*) denotes significant differences between the Nic groups and saline controls at the $p < 0.05$ level.

Fig. 4.

4 days of chronic nicotine upregulates nAChRs in dorsal and ventral hippocampus. nAChR binding was performed on membranes of the dHPC and vHPC following 1 day chronic saline administration, 1 day chronic nicotine (12.6 mg/kg/d) and 24 h withdrawal, 4 days of chronic nicotine (12.6 mg/kg/d) and 24 h withdrawal, and 4 days of chronic nicotine (12.6 mg/kg/d) and 72 h withdrawal ($n = 7-9$ per group). Error bars indicate Standard Error of the Mean (SEM). (*) denotes significant differences between the Nic groups and saline controls at the $p < 0.05$ level.