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The duration of nicotine withdrawal-associated deficits in contextual fear conditioning parallels changes in hippocampal high affinity nicotinic acetylcholine receptor upregulation

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ABSTRACT

A predominant symptom of nicotine withdrawal is cognitive deficits, yet understanding of the neural basis for these deficits is limited. Withdrawal from chronic nicotine disrupts contextual learning in mice and this deficit is mediated by direct effects of nicotine in the hippocampus. Chronic nicotine treatment upregulates nicotinic acetylcholine receptors (nAChR); however, it is unknown whether upregulation is related to the observed withdrawal-induced cognitive deficits. If a relationship between altered learning and nAChR levels exists, changes in nAChR levels after cessation of nicotine treatment should match the duration of learning deficits. To test this hypothesis, mice were chronically administered 6.3 mg/kg/day (freebase) nicotine for 12 days and trained in contextual fear conditioning on day 11 or between 1 to 16 days after withdrawal of treatment. Changes in [^{125}I]-epibatidine binding at cytisine-sensitive and cytisine-resistant nAChRs and chronic nicotine-related changes in α4, α7, and β2 nAChR subunit mRNA expression were assessed. Chronic nicotine had no behavioral effect but withdrawal produced deficits in contextual fear conditioning that lasted 4 days. Nicotine withdrawal did not disrupt cued fear conditioning. Chronic nicotine upregulated hippocampal cytisine-sensitive nAChR binding; upregulation continued after cessation of nicotine administration and the duration of upregulation during withdrawal paralleled the duration of behavioral changes. Changes in binding in cortex and cerebellum did not match behavioral changes. No changes in α4, α7, and β2 subunit mRNA expression were seen with chronic nicotine. Thus, nicotine withdrawal-related deficits in contextual learning are time-limited changes that are associated with temporal changes in upregulation of high-affinity nAChR binding.

1. Introduction

Statistics attest to the problematic nature of nicotine addiction; over 440,000 deaths per year in the US alone are attributed to smoking (CDC, 2002; Mokdad et al., 2004). Perhaps in recognition of this fact, 70% of smokers indicate they want to quit, but unfortunately only 3–5% of the 42% that attempt to quit are successful (Nides, 2008). What remains unknown is why nicotine addiction is so difficult to treat. Interactions between many environmental and genetic factors contribute to the development and maintenance of nicotine addiction. The multiplicity and variability of these interactions most likely give rise to the difficulty in treating nicotine addiction. Thus understanding these factors should advance the development of treatments for nicotine addiction.

Avoidance of withdrawal symptoms is one factor that contributes to the maintenance of smoking and relapse during quit attempts as the severity and the duration of withdrawal symptoms predict relapse (Piasecki et al., 1998, 2000). However, nicotine withdrawal syndrome is complex. While multiple withdrawal symptoms exist, over 65% of smokers cite changes in cognition as a serious withdrawal symptom (Ward et al., 2001). Cognitive changes observed in abstinent smokers include deficits in working memory, verbal memory, digit recall, and associative learning (Jacobsen et al., 2007, 2005; Kleinman et al., 1973; Mendrek et al., 2006; Snyder and Henningfield, 1989).

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We have focused on understanding the neurobiology of nicotine withdrawal-associated changes in learning in the mouse. Mice withdrawn from chronic treatment with a dose of nicotine that produces plasma nicotine levels in the range seen in smokers showed deficits specifically in hippocampus-dependent learning such as contextual fear conditioning, trace conditioning, and spatial object recognition (Davis et al., 2005; Kenney et al., 2011; Raybuck and Gould, 2009). This work is complemented by independent investigations demonstrating that changes in cognition during periods of abstinence predicted relapse for smokers (Patterson et al., 2010; Rukstalis et al., 2005). In order to understand and treat nicotine withdrawal-related changes in cognition, it is important to identify the underlying neural substrates. These withdrawal deficits in learning are mediated by nicotinic acetylcholinergic receptors (nAChRs) containing the β2 subunits (β2* nAChR) but not by those containing the α7 subunit (α7 nAChR) (Davis and Gould, 2009; Portugal et al., 2008). Direct drug infusion studies have demonstrated that nicotine specifically acts in the hippocampus to produce withdrawal-induced changes in learning (Davis and Gould, 2009). While these studies have helped define the cognitive effects of nicotine withdrawal and identified an underlying nAChR subtype and brain region, the duration of these deficits and the critical neural changes responsible for the deficits remain unknown.

With chronic nicotine treatment, nAChRs upregulate (Marks et al., 1983; Schwartz and Kellar, 1983) and this upregulation lasts beyond cessation of nicotine treatment. Abstinent smokers compared to nonsmokers had higher levels of β2* nAChRs, which returned to baseline between 3–12 weeks, and elevated nAChR levels correlated with urge to smoke (Cosgrove et al., 2009; Mamede et al., 2007; Staley et al., 2006). Similarly, a study in mice indicated that α4β2* nAChRs binding sites increased during chronic nicotine treatment and returned to control levels following cessation of treatment; these changes were related to the development and loss of tolerance (Marks et al., 1985). It is not clear if similar changes contribute to cognitive withdrawal deficits.

If nAChR upregulation contributes to withdrawal symptoms, one would predict that the duration of receptor upregulation would parallel the duration of withdrawal symptoms. While this prediction is straightforward, testing it has been confounded by the observation that different brain regions and nAChR subtypes have different upregulation parameters (Collins et al., 1989; Mao et al., 2008; Perry et al., 2007) and a lack of understanding of which brain regions are critically involved in specific withdrawal symptoms. However, withdrawal from chronic nicotine infusion into the hippocampus, but not cortex or thalamus, produced deficits in contextual fear conditioning (Davis and Gould, 2009). Furthermore, infusion of DHβE (an α4β2 nAChR antagonist) into the hippocampus of mice treated systemically with chronic nicotine precipitated withdrawal deficits in contextual fear conditioning (Davis and Gould, 2009). These experiments demonstrate that the hippocampus is necessary and sufficient for nicotine withdrawal-related disruption of contextual fear conditioning. Thus, the prediction emerges that if nAChR upregulation contributes to learning-related withdrawal deficits, then the duration of nAChR upregulation in the hippocampus should parallel the duration of withdrawal deficits in contextual fear conditioning. The present experiments examined the duration of nicotine withdrawal deficits in contextual fear conditioning, the duration of changes in cytisine-sensitive (i.e., α4β2* (Marks et al., 2006); * indicates potential additional nAChRs subunits) and resistant (i.e., α3β2*, α3β4*, and α6β2* (Marks et al., 2006)) nAChR binding sites measured with radiolabeled epibatidine in cortex, hippocampus, and cerebellum after nicotine withdrawal, and chronic nicotine-related changes in hippocampal nAChR subunit mRNA expression.

2. Materials and methods

2.1. Subjects

Male C57BL/6J mice (8 and 12 weeks of age; $n = 5$–13 per group, depending on experiment) (Jackson Laboratory, Bar Harbor, ME) weighing between 20–28 g were group housed four to a cage with *ad libitum* food and water. This strain was chosen because it performs well in fear conditioning (Owen et al., 1997) and is sensitive to the effects of nicotine on learning (Kenney and Gould, 2008). The light–dark cycle was 12:12 h with lights on at 07.00; all testing occurred between 08.00 and 17.00. The Temple University Institutional Animal Care and Use Committee approved all behavioral procedures.

2.2. Apparatus

Training and context testing took place in four identical conditioning chambers ($18 \times 19 \times 38$ cm; Plexiglas walls in the front and back, and stainless steel on the sides) housed in sound-attenuating boxes with a tan interior (MED Associates, St. Albans, VT, USA). Ventilation provided air exchange and background noise (69 dB). A speaker, mounted on the right wall of each chamber, produced the 85-dB white noise conditioned stimulus (CS). The shock unconditioned stimulus (US) was a 0.57 mA foot shock for 2 s delivered via grid floors. A computer running MED-PC software controlled stimuli administration. The CS test, which occurred one hour after contextual testing on test day, took place in a separate room with altered conditioning chambers that differed in size ($20 \times 23 \times 19$ cm), construction, and environmental cues; specifically, the chamber walls were constructed from Plexiglas on all sides, the floors were white plastic, the speakers were mounted on the left wall of each chamber, a vanilla extract olfactory cue was added, and the sound attenuating boxes that housed the chambers had black interior walls. All chambers were cleaned with 70% ethanol before each subject was tested.

2.3. Drug and administration

Mini-osmotic pumps (model 1002; Alzet, Cupertino, CA) containing 100 μl of saline or nicotine bitartrate (Sigma Co., St Louis, MO, USA) dissolved in saline were implanted subcutaneously under aseptic conditions with 5% isoflurane as the anesthetic. Solutions were administered at a rate of 0.25 μl/h to deliver 6.3 mg/kg/day of nicotine freebase. Pumps were removed 12 days after surgery under aseptic conditions with 5% isoflurane as an anesthetic. The dose of nicotine was chosen based on our prior research demonstrating that it produced withdrawal deficits in contextual fear conditioning (André et al., 2008; Davis and Gould, 2007b) with plasma nicotine levels of approximately 13.00 ng/ml (Davis et al., 2005), which is within the range seen in smokers (Henningfield and Keenan, 1993).

2.4. Fear conditioning

Training lasted 5 min and 30 s. Baseline freezing behavior was recorded during the first 120 s of the training session followed by two co-terminating CS (30 s, 85 dB white noise)-US (2 s, 0.57 mA foot shock) presentations at 120 and 270 s. Immediate freezing behavior was recorded during the 120 s intertrial interval. Mice remained in the chamber 30 s after the second CS-US presentation. During training and testing, each mouse was judged as either freezing or active once every 10 s (Gould and Higgins, 2003). Freezing was defined as an absence of visible movement except for respiration (Blanchard and Blanchard, 1969).

Twenty-four hours after training, mice were placed in the training chamber and contextual freezing behavior was recorded for 5 min. An hour after testing for contextual freezing, freezing to the CS was evaluated. Mice were placed in the altered context chamber for 6 min. During the first 180 s (preCS period), freezing in the absence of the CS was assessed to measure generalized freezing. During the final 180 s, freezing to the CS was assessed (cued freezing).

Two sets of mice were tested. The first set established the duration of withdrawal-induced deficits in contextual fear conditioning examining days 1–8, and day 16 after nicotine withdrawal. Previous work demonstrated that nicotine withdrawal disrupts the acquisition but not the recall of contextual fear conditioning (Portugal and Gould, 2009) and therefore withdrawal time points are discussed in relationship to training. The second set of mice was used for the binding studies. Mice were conditioned on the 11th day of chronic treatment or days 1–6 days after nicotine withdrawal. Half of the mice were used for the binding data and the other half were tested to replicate the first set; for the mice behaviorally tested only baseline freezing, immediate freezing, and contextual conditioning were measured because no effects were seen for cued fear conditioning in the first set. The first set of mice and the second set were tested by independent investigators that were blind to experimental conditions. Prior to the study, inter-rater reliability was established at >95%. Behavior was scored live.

2.5. Nicotinic acetylcholine receptor binding

Mice were euthanized and the cortex, cerebellum, and hippocampus were dissected and homogenized in ice-cold hypotonic buffer (NaCl, 14.4 mM;

KCl, 0.2 mM; CaCl2, 0.2 mM; MgSO4, 0.1 mM, HEPES, 2.0 mM; pH = 7.5) using a glass-Teflon tissue grinder. Particulate fraction was obtained by centrifugation at 20,000 g for 10 min in a Sorvall RC-2B centrifuge. Pellets were resuspended in fresh homogenization buffer, incubated at 37 °C for 10 min, and collected after centrifugation. Samples washed three more times by resuspension and centrifugation were stored in homogenization buffer at −70 °C until use.

[^{125}I]-epibatidine binding was measured as described previously (Whiteaker et al., 2000). Frozen, washed pellets were resuspended in the overlying buffer and centrifuged at 20,000 g for 10 min. The supernatant was discarded and the pellet was resuspended in ice-cold solution. Resuspension volume varied among brain regions and was adjusted such that less than 10% of the [^{125}I]-epibatidine was bound to the protein at the highest ligand concentration. α-bungarotoxin binding, which would assess α7 nAChR binding, was not examined because α-bungarotoxin binding returns to baseline almost immediately after chronic treatment (Marks et al., 1985) and because prior work has shown that α7nAChRs are not involved in the effects of nicotine on contextual fear conditioning or nicotine withdrawal-associated deficits in hippocampus-dependent learning (André et al., 2011; Davis and Gould, 2006, 2007a, 2009; Portugal et al., 2008; Raybuck and Gould, 2009).

Samples were incubated in 96-well polystyrene plates for 2 h at room temperature in a final incubation volume of 30 μl with either buffer (total) or buffer containing cytisine (50 nM) or cytisine (100 μM), along with 200 pM [^{125}I]-epibatidine. Buffer composition was (NaCl 144 mM; KCl, 1.5 mM; CaCl2, 2.0 mM; MgSO4, 1.0 mM, HEPES, 25 mM; pH = 7.5). The 100 μM cytisine defines non-specific binding whilst the total and 50 nM cytisine enables determination of cytisine-sensitive and cytisine-resistant binding sites (of note cytisine-resistant sites are a relatively small fraction of total epibatidine binding in cortex and hippocampus) (Marks et al., 1998). After incubation, samples were diluted with 200 μl of ice-cold wash buffer and filtered under vacuum (0.2 atm) onto glass fiber filters treated with 0.5% polyethelenimine (top filter, MFS Type B; bottom filter, TypeA/E, Pall Bioscience). An Inotech Cell Harvester (Inotech Biosystems International, Rockville, MD) collected the samples, which were subsequently washed six times with ice-cold buffer. Filters were transferred to a 96-well scintillation plate and counted using a Wallac TriLux 1450 MicroBeta scintillation counter (PerkinElmer Life and Analytical Sciences, Waltham, MA) at 30% efficiency after the addition of 150 μl of Optiphase Supramix scintillation mixture (PerkinElmer Life and Analytical Sciences, Waltham, MA). Protein was measured using the Lowry method (Lowry et al., 1951) with bovine serum albumin standard.

2.6. RNA extraction and RT-qPCR

The effects of chronic nicotine administration (6.3 mg/kg/day for 12 days, sc) and withdrawal from chronic nicotine administration on hippocampal α4, α7, and β2 nAChR subunit mRNA were measured. On the 12th day of chronic nicotine or saline administration, or 24 h following cessation of chronic treatment, mice were euthanized and whole hippocampi were rapidly dissected, placed in RNA later (Sigma–Aldrich, St. Louis, MO), and stored at −80 °C. Total RNA was isolated from tissue using the RNAqueous Kit (Ambion, Austin, TX) and Turbo DNA Free (Ambion, Austin, TX) was used to remove possible genomic DNA contamination. RNA quantity and purity were assessed using the NanoDrop 2000 (Thermo Scientific,Wilmington, DE). RNA was reverse transcribed and RT-qPCRs were performed using the Power SYBR Green RNA-to-CT 1-step kit (Applied Biosystems, Austin, TX).

RT-qPCRs were performed in triplicate in a 20 μL volume in 96-well plates using 20 μg RNA, 200 nM concentration of primers, 10 uL Power SYBR Green RT-PCR master mix, and 0.16 uL of RT Enzyme mix per well. Reactions were carried out in the 7500 Fast Real-time PCR system (Applied Biosystems, Austin, TX) using the following conditions: reverse transcription for 30 min at 48 °C, and polymerase activation for 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. The mRNA expression levels for each gene of interest were normalized by the geometric mean of two housekeeping genes, GAPDH and HPRT (Vandesompele et al., 2002), and relative expression was calculated with qbase^{PLUS} software via the ΔΔ*Ct* method (Hellemans et al., 2007). All primers (see Table 1) were tested for efficiency (100 ± 10%) prior to use and specificity was examined using melt curves and running RT-qPCR products on an ethidium bromide stained agarose gel visualized via UV fluorescence.

2.7. Statistical analysis

Data was analyzed with two-way ANOVAs (SPSS Version 16.0) and when an interaction or main effect of drug treatment was detected, planned independent samples *t*-tests with Bonferroni corrections were conducted comparing treatment conditions at different time points. Expression data are represented as fold change compared to control group. Results were considered significant at $p \leq 0.05$. All data are expressed as means ± SEM.

Table 1
Primers used for RT-qPCR.

Gene	Forward (5′–3′)	Reverse (5′–3′)
CHRNA4	GAAGCAGGAGTGGCATGACT	GAACAGGTGGGCTTTGGTTA
CHRNA7	CCGTGTACTTCTCCCTGAGC	TCAAAGCGTTCATCTGCACT
CHRNB2	GAGTGTGAGGGAGGATTGGA	GGGAGCTGAGTGGTCAGAGT
Hprt	TTGCTGACCTGCTGGATTACA	CCCCGTTGACTGATCATTACA
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGA

3. Results

3.1. Behavioral results

For the experiment in which mice were conditioned 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, or 16 days after nicotine withdrawal, a 2 × 9 (treatment x time) ANOVA revealed a significant main effect for treatment [$F(1, 150) = 13.56$, $p < 0.05$], time [$F(8, 150) = 2.79$, $p < 0.05$], and a significant interaction between treatment and time [$F(17, 150) = 3.30$, $p < 0.05$]. Planned Bonferroni comparisons revealed that nicotine withdrawal disrupted contextual fear conditioning 1, 2, 3, and 4 days post withdrawal ($p < 0.05$) but no significant effect of nicotine withdrawal was seen at days 5, 6, 7, 8 or 16 (Fig. 1A). There was no significant effect of nicotine withdrawal on baseline freezing, generalized freezing, or freezing to the cue (data for cued shown in Fig. 1B).

For the mice that were behaviorally tested as part of the binding study, a 2 × 7 (treatment x time) ANOVA revealed a significant main

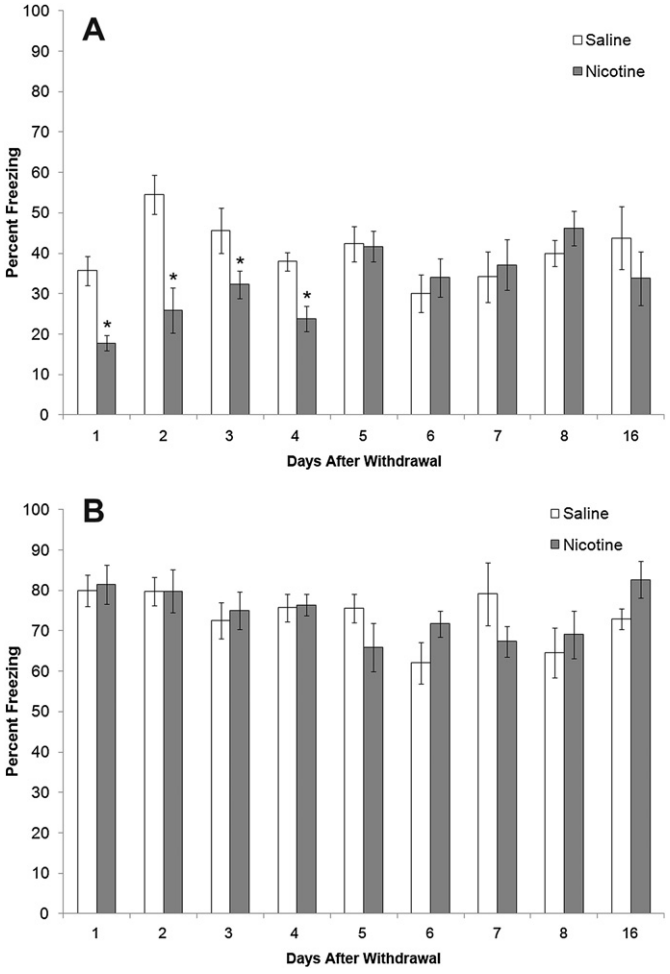

Fig. 1. The time course of contextual and cued fear conditioning after nicotine withdrawal. A) Contextual fear conditioning was examined 1–8, and 16 days after cessation of nicotine treatment; deficits in learning were seen on days 1–4. B) Cued fear conditioning was examined 1–8, and 16 days after cessation of nicotine treatment; no deficits were seen. All time points are separate groups of mice. * = significant difference from saline ($n = 7$–13). Data are presented as mean and standard error of the mean.

effect for treatment [$F(1, 98) = 28.53$, $p < 0.05$], time [$F(6, 98) = 3.24$, $p < 0.05$], and a significant interaction between treatment and time [$F(13, 98) = 3.30$, $p < 0.05$]. Bonferroni corrected post hoc comparisons revealed that withdrawal deficits were again seen at 1, 2, 3, and 4 days after withdrawal ($p < 0.05$), whereas no effects were observed in mice treated with chronic nicotine and mice withdrawn from chronic nicotine for 5 or 6 days (Fig. 2). Furthermore, no effects of chronic nicotine or nicotine withdrawal were observed in baseline freezing or immediate freezing for all groups.

3.2. Nicotinic acetylcholine receptor binding

Cytisine-sensitive and resistant nAChR binding was measured in the cortex, hippocampus, and cerebellum during chronic nicotine treatment and at 1–6 days after mini-osmotic pump removal. For cytisine-sensitive nAChR binding in the hippocampus, a 2×7 (treatment $\times$ time) ANOVA revealed a significant main effect for treatment [$F(1, 98) = 43.67$, $p < 0.05$] and time [$F(6, 98) = 3.05$, $p < 0.05$], whereas the interaction between treatment and time was not significant. Post hoc tests revealed that cytisine-sensitive nAChR binding in the hippocampus was significantly greater in mice treated with chronic nicotine and in mice withdrawn from chronic nicotine for 1, 2, 3, or 5 days ($p < 0.05$; Fig. 3). This upregulation of nAChRs in the hippocampus was consistent with the time course of nicotine withdrawal-related deficits in contextual learning (Figs. 1 and 2). No significant differences in cytisine-resistant binding in the hippocampus were observed at any of the time points tested.

Cytisine-sensitive nAChR binding in the cortex was examined with a 2×7 (treatment x time) ANOVA (see Table 2 for group means and standard error). A significant main effect for treatment [$F(1, 98) = 10.18$, $p < 0.05$] was observed, whereas the main effect of time and the interaction between treatment and time were not significant. For cytisine-resistant nAChR binding in the cortex, a 2×7 (treatment $\times$ time) ANOVA revealed a significant main effect for treatment [$F(1, 98) = 21.68$, $p < 0.05$], but the main effect of time and the interaction between treatment and time were not significant. Planned Bonferroni comparisons revealed that cytisine-sensitive nAChR binding in the cortex was significantly increased

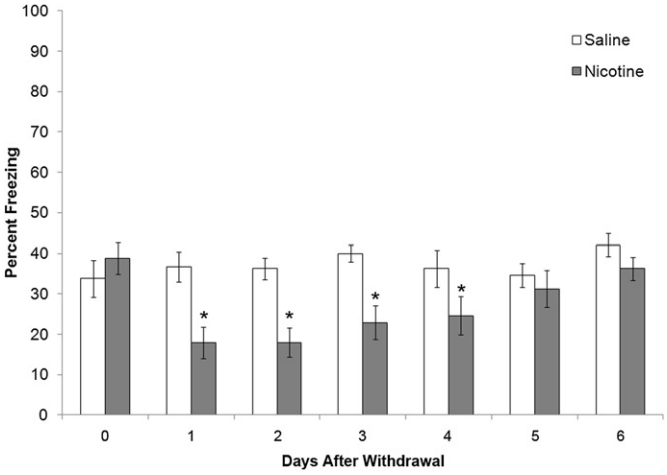

Fig. 2. The effects of chronic nicotine and withdrawal from chronic nicotine on contextual fear conditioning. A second set of animals were tested by an independent investigator to examine behavior on the last day of chronic treatment and to replicate withdrawal findings. Chronic nicotine had no effect on contextual fear conditioning but withdrawal deficits in learning were seen on the first 4 days of withdrawal. All time points are separate groups of mice. * = significant difference from saline ($n = 8$). Data are presented as mean and standard error of the mean.

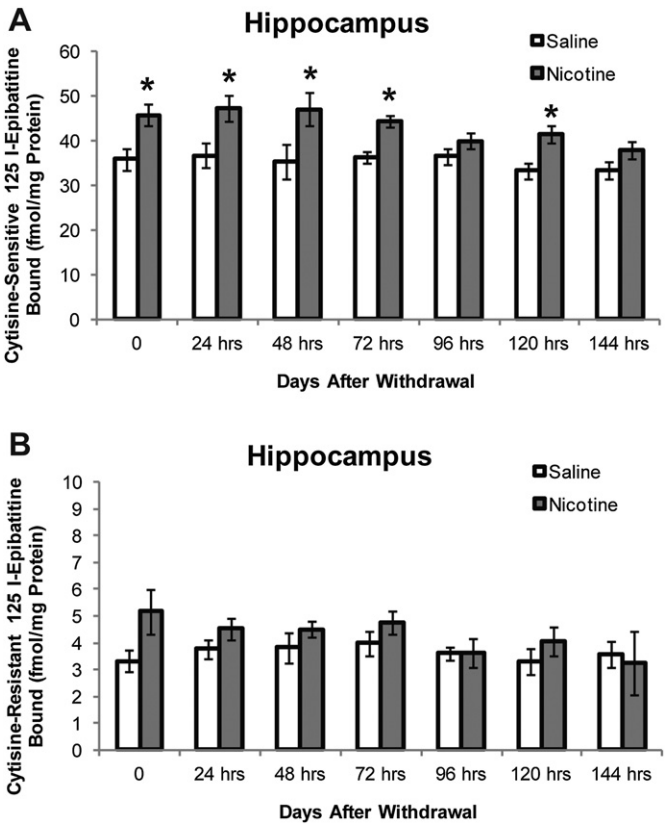

Fig. 3. The time course of cytisine-sensitive and cytisine-resistant epibatidine binding in the hippocampus. Chronic nicotine was associated with significantly increased binding in (cytisine-sensitive), and withdrawal was associated with significantly increased binding in the hippocampus (days 1–3 and 5 cytisine-sensitive). All time points are separate groups of mice. * = significant difference from saline ($n = 8$). Data are presented as mean and standard error of the mean.

Table 2
Cerebellar and cortical cytisine-sensitive and cytisine-resistant nAChR binding data.

	Hours after withdrawal						
	0	24 h	48 h	72 h	96 h	120 h	144 h
Cerebellum							
Cytisine sensitive							
Saline	13.80	15.59	15.17	12.95	10.49	10.84	13.48
Standard error	1.24	1.29	1.27	0.73	1.73	1.09	0.92
Nicotine	***21.71***[a]	18.29	18.16	13.47	***16.65***[a]	14.19	15.30
Standard error	***0.94***[a]	1.10	1.24	0.60	***1.49***[a]	1.56	0.89
Cytisine resistant							
Saline	4.09	4.89	4.72	4.14	5.24	5.50	5.50
Standard error	0.18	0.21	0.20	0.23	0.26	0.29	0.28
Nicotine	4.88	4.62	5.47	4.11	5.86	5.93	5.45
Standard error	0.33	0.47	0.31	0.13	0.53	0.31	0.26
Cortex							
Cytisine sensitive							
Saline	36.26	34.81	37.03	35.35	26.87	39.61	36.90
Standard error	3.90	4.13	6.16	4.86	3.59	4.19	2.74
Nicotine	49.17	40.78	46.34	***51.85***[a]	38.82	40.93	32.45
Standard error	4.84	1.34	4.49	***3.79***[a]	4.46	6.06	5.95
Cytisine resistant							
Saline	4.15	4.43	3.87	4.16	3.86	4.15	3.77
Standard error	0.27	0.32	0.21	0.19	0.23	0.38	0.16
Nicotine	***5.76***[a]	5.13	4.99	***5.65***[a]	4.99	4.25	4.17
Standard error	***0.43***[a]	0.47	0.50	***0.73***[a]	0.24	0.45	0.29

[a] Indicates significant difference between nicotine and saline. Data presented as mean and standard error of the mean.

only 3 days after withdrawal from chronic nicotine, and cytisine-resistant nAChR binding was elevated in mice treated with chronic nicotine and in mice withdrawn from chronic nicotine for 3 days ($p < 0.05$).

In the cerebellum, cytisine-sensitive nAChR binding was examined with a 2 × 7 (treatment × time) ANOVA (see Table 2 for group means and standard error). A significant main effect for treatment [$F(1, 98) = 32.59$, $p < 0.05$], time [$F(6, 98) = 6.12$, $p < 0.05$], and a significant interaction between treatment and time [$F(13, 98) = 2.31$, $p < 0.05$] were observed. Subsequent Bonferroni post-hoc tests revealed that cytisine-sensitive nAChR binding was significantly increased in mice treated with chronic nicotine and in mice withdrawn from chronic nicotine for 4 days relative to saline treated mice ($p < 0.05$). Cytisine-resistant binding in the cerebellum was not significantly different between groups. Changes in nAChR binding in the cortex and cerebellum were not consistent with the effects of nicotine withdrawal on contextual fear conditioning.

3.3. Quantification of nAChR mRNA expression on the 12th day of chronic nicotine administration and following one day of withdrawal from chronic nicotine administration

The effect of chronic nicotine exposure on hippocampal nAChR mRNA expression was examined with RT-qPCR. Changes in CHRNA4, CHRNA7, and CHRNB2 mRNA levels were assessed on the 12th day of chronic nicotine (6.3 mg/kg/day, sc) or saline administration. No significant change in mRNA expression was seen for any of the receptor subtypes examined.

The effect of 24 h of withdrawal from chronic nicotine administration on hippocampal CHRNA4, CHRNA7, and CHRNB2 mRNA expression were also assessed. No change in mRNA level expression was seen for any of the nAChR subtypes examined (p's > 0.05).

4. Discussion

The results from this study aid in understanding the impact of nicotine withdrawal on learning and the underlying neural changes. It was demonstrated that withdrawal from chronic nicotine produces a deficit in the learning of contextual information that remained for four days after cessation of nicotine administration. Chronic nicotine treatment upregulated nAChRs in the cerebellum, cortex, and hippocampus but only the duration of nAChR upregulation in the hippocampus paralleled the duration of the withdrawal-associated deficits in contextual learning. Finally, chronic nicotine did not produce any significant changes in α4, α7, or β2 nAChR subunit mRNA expression in the hippocampus.

In smokers, cessation of smoking is associated with cognitive deficits that include altered associative learning and working memory (Hughes, 2007; Jacobsen et al., 2007, 2005; Mendrek et al., 2006). While these deficits are a major feature of the nicotine withdrawal syndrome, they are not global deficits affecting all forms of cognition but instead affect selected cognitive tasks. This is in agreement with the present study that found that withdrawal from chronic treatment with a dose of nicotine that produces plasma nicotine levels in mice similar to those seen in smokers (Davis et al., 2005) resulted in a time-limited disruption of contextual fear conditioning with no effect on cued fear conditioning. The withdrawal deficit in contextual learning was no longer present by the 5th day after cessation of nicotine treatment. The fact that the withdrawal deficits were time-limited demonstrates that they are not due to long-lasting neurotoxic effects but instead resemble withdrawal symptoms in humans that dissipate with time (Hughes et al., 1994). In addition, the finding that withdrawal disrupted contextual but not cued fear conditioning adds to a growing literature that, at least in the mouse model, nicotine withdrawal disrupts hippocampus-dependent learning but not hippocampus-independent learning as contextual fear conditioning requires the hippocampus whereas cued fear conditioning does not (Fanselow et al., 1994; Logue et al., 1997; Phillips and Ledoux, 1992). This hippocampal specificity for learning-related withdrawal deficits has been shown for other learning tasks such as trace conditioning and spatial object recognition (Kenney et al., 2011; Raybuck and Gould, 2009), and direct drug infusion studies have confirmed that chronic nicotine is acting directly in the hippocampus to produce the withdrawal symptoms when treatment ceases (Davis and Gould, 2009). Because nicotine effects in the hippocampus are necessary and sufficient to produce the withdrawal-related learning deficits, neurobiological changes that underlie these deficits must occur in the hippocampus.

As discussed earlier, chronic nicotine treatment is associated with upregulation of nAChRs (Marks et al., 1983; Schwartz and Kellar, 1983), and one interesting possibility is that this upregulation contributes to withdrawal-related deficits in learning. Central nervous system nAChRs are broadly divided into two groups, α7 nAChRs and non-α7 nAChRs. Non-α7 nAChRs show a longer lasting upregulation than α7 nAChRs, which return to baseline almost immediately after cessation of nicotine treatment (Marks et al., 1985). The non-α7 nAChRs, which are measured by high affinity epibatidine binding, can be further subdivided by sensitivity to cytisine binding. Cytisine-sensitive binding is associated with α4β2* nAChRs whereas cytisine-resistant binding is associated with α3β2*, α3β4*, and α6β2* nAChRs (Marks et al., 2006). In the present study, chronic treatment with nicotine increased cytisine-sensitive binding in the cerebellum and hippocampus and cytisine-resistant binding in the cortex, suggesting that different nAChR subtypes are upregulated in the cortex compared to cerebellum and hippocampus. The reversal of upregulation after cessation of chronic nicotine treatment varied among brain regions. In cerebellum, cytisine-sensitive binding returned to baseline immediately with only significantly more binding seen on the 4th day after withdrawal. In cortex, cytisine-resistant binding returned to baseline immediately, with only significantly more binding seen on the 4th day after withdrawal; a significant difference in cytisine-sensitive binding in cortex was seen only on the 3rd day after withdrawal.

Of the three areas examined, the binding in the hippocampus may be the most interesting. Cytisine-sensitive binding in the hippocampus remained elevated after withdrawal with significantly increased levels of binding seen in the nicotine withdrawn mice on the 1st, 2nd, 3rd, and 5th days after cessation of nicotine treatment. No change in cytisine-resistant binding, which was at a lower level than cytisine-sensitive binding, was seen in the hippocampus. The duration of increased cytisine-sensitive binding in the hippocampus was highly similar to the duration of nicotine withdrawal-associated deficits in contextual learning. This finding fits well with our prior data demonstrating that direct nicotine effects in the hippocampus mediate withdrawal-related deficits in contextual learning (Davis and Gould, 2009), that direct infusion of acute nicotine into the hippocampus enhances learning (Davis et al., 2007; Gulick and Gould, 2009; Raybuck and Gould, 2010), and acute nicotine ameliorates withdrawal-related deficits in hippocampus-dependent learning (Davis et al., 2005). Together, current and prior research suggests that changes in hippocampal nAChR function, potentially α4β2 nAChRs, may be an important contributing factor to these deficits. However, it should be noted that learning deficits dissipated by day 5 but hippocampus binding was elevated at day 5, suggesting that either a specific level of upregulation must occur before deficits are seen or other factors contribute to the deficits.

No changes in α4, α7, and β2 nAChR subunit mRNA expression in the hippocampus on the 12th day of chronic nicotine treatment

were found. The α4, α7, and β2 nAChR subunits were targeted because they show high expression in the hippocampus (Baddick and Marks, 2011) and hippocampal β2* nAChRs are involved in nicotine withdrawal learning deficits (Davis and Gould, 2009; Portugal et al., 2008; Raybuck and Gould, 2009). Our finding replicates prior *in situ* hybridization work that found no chronic nicotine-associated change in hippocampal (and other areas) α4 or β2 mRNA levels (Marks et al., 1992; Pauly et al., 1996); though a study using northern blot analysis of PC12 cells found an increase in β2 mRNA and a decrease in α3 mRNA with chronic nicotine treatment (Madhok et al., 1995). Thus, upregulation of α4β2 nAChRs may involve an increase in receptor protein through post-translational events that may include changes in nAChR maturation and trafficking (Darsow et al., 2005; Kane et al., 2004; Lomazzo et al., 2011; Rezvani et al., 2007).

Based on current results and prior studies, a model of a potential underlying mechanism of nicotine withdrawal-related deficits in hippocampus-dependent learning can be put forth. This model is extrapolated from an earlier model proposed by Dani and Heinemann (1996) in which during periods of low nicotine levels, upregulated nAChRs would be excessively responsive to ACh. Chronic nicotine treatment results in an upregulation of β2* nAChRs (Marks et al., 1983; Schwartz and Kellar, 1983) but also a desensitization of nAChRs (Gentry and Lukas, 2002). This combined upregulation and desensitization that occurs while nicotine is present may help to maintain a functional homeostatic state for the nACh system and thus support proper cognitive functioning. When nicotine is no longer present for an extended period of time (i.e., nicotine withdrawal), the desensitized nAChRs may return to a functional state while upregulation persists. This could result in a hyper-sensitive nACh system, which could produce cognitive deficits. In support, nicotine withdrawal was associated with a persistent increase in hippocampal CA1 pyramidal cell activity (Penton et al., 2011). Furthermore, *in vitro* studies have shown that nAChRs return from a desensitized state in minutes to hours (see Gentry and Lukas, 2002 for review). *In vivo*, Pietilä et al. (1998) found that a 7 week chronic nicotine treatment produced an increase in cortical nAChR binding that lasted over 3 days after cessation of chronic treatment but tolerance to the acute locomotor stimulatory effects of nicotine only lasted 1 day. As it has been proposed that tolerance is related to nAChR desensitization (Robinson et al., 2007), these findings thus suggest that upregulation lasts longer than desensitization.

If nicotine withdrawal deficits in learning and cognition are due to a hyper-sensitive nAChR system that results from functional upregulation, then drugs that ameliorate withdrawal symptoms should desensitize or dampen nAChR signaling. It has been shown that nicotine, varenicline, and bupropion ameliorate nicotine withdrawal-associated deficits in hippocampus-dependent learning (Davis et al., 2005; Portugal and Gould, 2007; Raybuck et al., 2008). Nicotine could ameliorate deficits through desensitizing nAChRs, bringing the system back toward baseline. Similarly, varenicline is a partial agonist and thus could ameliorate the withdrawal deficits by occupying nAChRs. Papke and colleagues (2011) provide support for this position by demonstrating that while varenicline provided weak tonic activation of the nACh system, it also decreased the effects of full agonists. Finally, bupropion is a noradrenergic reuptake inhibitor but also a noncompetitive nAChR antagonist (Slemmer et al., 2000). The nAChR antagonist property of bupropion could dampen nAChR response during withdrawal alleviating withdrawal symptoms, if the symptoms are due to a hyper-sensitive nACh system. These results would suggest that a nAChR antagonist could work as a smoking cessation aid. In support, Rose and colleagues (1994) compared the effectiveness of the nicotine patch versus the nicotine patch paired with administration of the nAChR antagonist mecamylamine on maintenance of abstinence and found that patients receiving the paired treatment had a 9 times higher rate of abstinence at 12 months after quitting. The current data and these studies support a model of nicotine withdrawal where withdrawal deficits in cognition are related to increased sensitivity of the nACh system; however, this model is not without limitations.

In summary, the present results suggest that changes in nAChR upregulation may be an important factor in nicotine withdrawal-related deficits in hippocampus-dependent learning. Upregulation of hippocampal nAChRs and withdrawal deficits in hippocampus-dependent learning shared a common time course. The present results and prior work (Davis and Gould, 2009; Portugal et al., 2008; Raybuck and Gould, 2009) suggest that these effects are mediated by β2* nAChRs. This is in agreement with results from studies of abstinent smokers showing a relationship between β2* nAChR concentration and cravings to smoke to reduce withdrawal symptoms (Staley et al., 2006). However, it should be noted that not all withdrawal symptoms are mediated by β2* nAChRs; for example, β4*, α5*, and α7 nAChRs, but not β2* nAChRs, contribute to somatic withdrawal symptoms (Jackson et al., 2008; Salas et al., 2007, 2004). Because different withdrawal symptoms involve different nAChRs and brain regions and because upregulation varies across brain regions and nAChR types, a future question is whether nAChR upregulation is universally associated with all withdrawal systems. Finally, a caveat is that the current results show a correlational relationship but have not demonstrated causation. Further *in vitro* and *in vivo* studies on the role of receptor-related changes in withdrawal are needed.

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