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Nicotine Withdrawal-Induced Deficits in Trace Fear Conditioning in C57BL/6 Mice: A Role for High-Affinity $\beta 2$ Subunit-Containing Nicotinic Acetylcholine Receptors

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Abstract

Nicotine alters cognitive processes that include working memory and long-term memory. Trace fear conditioning may involve working memory during acquisition while also allowing the assessment of long-term memory. The present study used trace fear conditioning in C57BL/6 mice to investigate the effects of acute nicotine, chronic nicotine, and withdrawal of chronic nicotine on processes active during acquisition and recall 24 hours later and examine the nicotinic acetylcholine receptor subtypes (nAChRs) involved in withdrawal-deficits in trace fear conditioning. During training, acute nicotine (0.09 mg/kg) enhanced, but chronic nicotine (6.3 mg/kg/day, 13 days) and withdrawal of chronic nicotine (6.3 mg/kg/day, 12 days) had no significant effect on acquisition of trace conditioning. At recall, acute treatment enhanced conditioning while chronic nicotine had no effect and withdrawal of chronic nicotine resulted in deficits. Antagonist precipitated withdrawal was used to characterize the nAChRs involved in the withdrawal deficits. The low-affinity nAChR antagonist MLA (1.5, 3, 9 mg/kg) had no effect on trace fear conditioning, but the high-affinity nAChR antagonist DH β E (3 mg/kg) precipitated deficits in trace fear conditioning if administered at training or training and testing, but not if administered at testing alone. The $\beta 2$ nAChR subunit is involved in the withdrawal effects as withdrawal of chronic nicotine produced deficits in trace fear conditioning in wildtype but not in $\beta 2$ knockout mice. Thus, nicotine alters processes involved in both acquisition and long-term memory of trace-fear conditioning, and high-affinity $\beta 2$ subunit containing nAChRs are critically involved in the effects of nicotine withdrawal on trace fear conditioning.

Keywords

Working Memory; Learning; Addiction; Cognition; Hippocampus

Introduction

Nicotine addiction, primarily in the form of cigarette smoking, is responsible for massive health and financial costs to both the individual and society (CDC, 2005; Rice, 1999). Great progress has been made in developing smoking cessation therapies; however, even with current cessation therapies most smokers fail to quit (Schnoll & Lerman, 2006). Part of the reason smokers fail to quit may be that nicotine withdrawal produces deficits in learning and memory. Nicotine withdrawal-induced deficits in learning-related processes may be particularly detrimental because, in addition to the fact that removal of these symptoms by reinstatement of drug use could serve as a negative reinforcer for smoking, these symptoms could result in deficits in the learning of adaptive behaviors that facilitate smoking cessation (Brega *et al.*, 2008; Gutkin *et al.*, 2006).

Nicotine withdrawal produces deficits in a number of tasks involving working memory and long-term memory. For instance, abstinence from smoking produces deficits in working memory, as demonstrated in the N-Back task (Jacobsen *et al.*, 2005; Mendrek *et al.*, 2006; Xu *et al.*, 2005), serial letter recall (Blake & Smith, 1997), and visuospatial working memory (Sacco *et al.*, 2005). Additionally, nicotine withdrawal selectively produces deficits in contextual fear conditioning, without affecting delay fear conditioning (Davis & Gould, 2007; Davis *et al.*, 2005; Gould, 2006; Portugal & Gould, 2007; Portugal *et al.*, 2008). Both working memory and contextual fear conditioning critically involve the hippocampus (Kim *et al.*, 1993; Logue *et al.*, 1997; McEchron *et al.*, 1998; Phillips & LeDoux, 1992; Yoon *et al.*, 2008). Thus, it may be that nicotine withdrawal selectively produces deficits in hippocampus-dependent processes.

Trace fear conditioning involves the association of a conditioned stimulus (CS) and an unconditioned stimulus (US), but the two stimuli are temporally separated by a trace interval. Temporal separation of the stimuli engages the hippocampus (Misane *et al.*, 2005). Lesions to the dorsal hippocampus produce deficits in trace fear conditioning (Bangasser *et al.*, 2006; Burman *et al.*, 2006; Fendt *et al.*, 2005; McEchron *et al.*, 1998; Quinn *et al.*, 2002; Quinn *et al.*, 2008; Rogers *et al.*, 2006; Trivedi & Coover, 2006). Additionally, inhibition of neuronal signaling (Misane *et al.*, 2005; Quinn *et al.*, 2005; Seo *et al.*, 2008; Wanisch *et al.*, 2005), signal transduction pathways (Runyan & Dash, 2004; Villarreal & Barea-Rodriguez, 2006), and protein synthesis in the hippocampus produce deficits in trace fear conditioning (Weitemier & Ryabinin, 2004). Further, in humans trace fear conditioning activates the hippocampus (Büchel *et al.*, 1999; Knight *et al.*, 2004). In addition to requiring hippocampal integrity, trace fear conditioning is thought to necessitate working memory to maintain a memory trace of the CS during conditioning. Trace fear conditioning can be disrupted by tasks that interfere with working memory (Carter *et al.*, 2003) or attention (Han *et al.*, 2003) and strength of trace fear conditioning is associated with subjects' awareness of the CS-US association (Knight *et al.*, 2006; Weike *et al.*, 2007). Thus, trace fear conditioning depends upon the hippocampus and may involve working memory. These properties of trace fear conditioning combined with the fact that trace fear conditioning is easily performed in many species, including humans (Büchel *et al.*, 1999; Hamm & Weike, 2005; Han *et al.*, 2003; Knight *et al.*, 2004; Knight *et al.*, 2006; Weike *et al.*, 2007; Weike *et al.*, 2008), suggests that trace fear conditioning may be a good model in which to investigate the effects of acute nicotine, chronic nicotine and withdrawal of chronic nicotine on hippocampus-dependent learning.

To investigate the effects of nicotine on trace fear conditioning, we compared the effects of acute nicotine, chronic nicotine, and withdrawal from chronic nicotine on trace fear conditioning in mice, examining changes that occur during training and changes that occur at testing 24 hours later. The acute (0.09 mg/kg) and chronic (6.3 mg/kg/day, 14 days) doses of nicotine were chosen because they produce similar plasma nicotine levels and those levels are within the range reported in smokers (Benowitz, 1988; Davis *et al.*, 2005; Henningfield & Keenan, 1993). In addition, the effects of nicotine on behavior may depend on the nAChRs subtypes involved. Nicotinic acetylcholine receptors are grouped into two classes, low-affinity nAChRs, which are homomeric and consist of the $\alpha 7$ subunit and high-affinity nAChRs which are heteromeric and consist of a combination of α and β subunits (Changeux & Taly, 2008; Hogg & Bertrand, 2003; McGehee, 1999). Thus, to characterize the nicotinic acetylcholine receptor (nAChR) subtypes involved in the effects of withdrawal of chronic nicotine on trace fear conditioning, we attempted to precipitate withdrawal deficits by administering low-affinity or high-affinity nAChR antagonists to mice receiving chronic nicotine treatment. The precipitated withdrawal studies were followed by withdrawal studies in $\beta 2$ nAChR subunit KO mice.

Materials and Methods

Subjects

Subjects of this study were 257, 8–12 week old male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) and 30 male $\beta 2$ KO and littermate WT mice. $\beta 2$ KO and WT mice, bred from heterozygous mice provided by Dr. Beaudet (Baylor College of Medicine), were originally backcrossed onto a C57BL/6J background, for details see (Xu *et al.*, 1999). Mice were housed in groups of four in standard colony cages, maintained on a 12h light/dark cycle with lights on at 7:00 am, and allowed *ad libitum* access to food and water. All procedures were approved by the Temple University Animal Care and Use Committee.

Materials

Apparatus—Training of trace fear conditioning was conducted in conditioning chambers (model 307AW, Med Associates, St. Albans, VT) housed in sound attenuating boxes. The chamber floors were composed of 18 stainless steel bars connected to a shock generator and scrambler (Med-Associates) through which a 2 second, 0.57 mA footshock US was administered. Speakers attached to the right wall of each chamber provided a 30 second, 85 dB white noise CS. Ventilation fans, providing background noise (69 dB), were mounted on the right wall of each sound-attenuating box. Stimulus administration was controlled by a computer running Med-PC software (Med-Associates).

Testing of trace fear conditioning was conducted in conditioning chambers situated in sound attenuating boxes located in a different room than that used for training. The testing chambers were distinct from the training chambers and had white plastic floors, stainless steel sides, and Plexiglas panels for the front, rear and lid. Additionally, a novel olfactory cue (artificial vanilla extract) was applied to paper toweling placed below each of the chambers. Ventilation fans mounted on the right wall of the sound attenuating chambers provided background noise. A Grason-Stradler noise generator (model 901B, West Concord, MA) generated the white noise CS through 3-inch speakers mounted on the left side of each of the conditioning chambers.

Drugs—Nicotine hydrogen tartrate salt (Sigma-Aldrich, St. Louis, MS) was administered both chronically and acutely. Chronic nicotine was administered through osmotic minipumps (model 1002; Alzet, Cupertino, CA) at a dose of 6.3 mg/kg/day (freebase), at a rate of 0.25 μ l/h. Dosing of chronic nicotine administration was based on previous studies showing deficits in contextual fear conditioning following spontaneous (Davis & Gould, 2007; Davis *et al.*, 2007) and precipitated nicotine withdrawal (Portugal *et al.*, 2008). Acute nicotine was administered at 0.09 mg/kg ip (freebase) 5 minutes prior to training and testing of trace fear conditioning. Dosing of acute nicotine was based on studies showing enhancement of both trace (Davis & Gould, 2007; Gould *et al.*, 2004) and contextual fear conditioning by acute nicotine (Davis *et al.*, 2005; Gould & Lewis, 2005; Gould & Lommock, 2003; Gould & Stephen Higgins, 2003; Gould & Wehner, 1999; Gould, 2003; for review see Gould, 2006) and studies showing that 10 minutes after ip administration this dose produces plasma nicotine levels similar to those produced by the chronic nicotine dose, which are also comparable to those reported in smokers (Davis *et al.*, 2005).

Dihydro- β -erythroidine (DH β E, Sigma-Aldrich), a high-affinity nAChR antagonist, was administered subcutaneously at 3 mg/kg, 25 minutes prior to training and/or testing of trace fear conditioning. Methyllycaconitine (MLA, Sigma-Aldrich), a low-affinity nAChR antagonist, was administered subcutaneously at 1.5, 3, and 9 mg/kg, 25 minutes prior to training and testing of trace fear conditioning. Dosing for DH β E and MLA was based on

previous studies (Davis & Gould, 2006; Portugal *et al.*, 2008). All drugs were dissolved in physiological saline and acute administrations were given at a volume of 10 ml/kg.

Procedure

Surgical—On day 1, osmotic minipumps were implanted subcutaneously via an inter-scapular incision, as described in (Davis *et al.*, 2005). Incisions were closed with wound clips. In chronic nicotine and saline treated mice, pumps were left in through training and testing, which occurred on days 13 & 14, respectively. In chronic nicotine and saline withdrawn mice, pumps were removed on day 12, 24 hours before training. Mice were anesthetized with isoflurane (Abbott Laboratories, North Chicago, IL) and surgical procedures were conducted under sterile conditions.

Behavioral—Behavioral procedures were based on previous studies from our lab (Davis & Gould, 2007; Gould *et al.*, 2004). Acute drugs were administered 5 (nicotine) or 25 (MLA & DH β E) minutes prior to the start of training and testing. All training or testing only drug injections were matched with saline injections on the opposite day. Training of trace fear conditioning was conducted during a single 16 minute training session wherein the mice were presented 5 CS-US pairings separated by a variable inter-trial-interval (90–120 seconds). CS-US pairings consisted of a 30 second, 85 dB white-noise CS, followed by a 30 second trace interval, after which a 2 second 0.57 mA footshock US was presented. The training session began with the activation of the house light and terminated 30 seconds following the last US presentation. During training, baseline freezing (the first 120 seconds in the chamber) and freezing during the five trace-intervals was recorded. Acquisition of the trace-CS-US association during training was quantified by observing freezing during the 30 second trace-interval, resulting in 3 observations per CS-US presentation, and a total of 15 observations per session; this measure is based on Rogers and colleagues (2006). To ensure sufficient statistical power, for analysis each subject's trace-interval freezing was grouped into a single bin. Twenty-four hours following training, mice were tested for cued freezing. First, mice were placed in the testing chambers and observed three minutes for freezing to an altered context, then the CS was presented and mice were observed three minutes for cued freezing.

During both training and testing, mice were observed for freezing with a time sampling procedure wherein one second observations were taken at ten second intervals (Gould & Wehner, 1999). Freezing was defined as a lack of movement excepting respiration (Blanchard & Blanchard, 1969).

Experimental Design

To compare the effects of acute nicotine, chronic nicotine, and withdrawal from chronic nicotine on trace fear conditioning, we administered acute saline or nicotine, chronic saline or nicotine, or withdrew chronic saline or nicotine from C57BL/6 mice ($n = 8–11$). To determine if low-affinity nAChRs are involved in nicotine withdrawal effects on trace conditioning we attempted to precipitate withdrawal effects in chronic nicotine-treated mice with the low-affinity nAChR antagonist MLA (Alkondon *et al.*, 1992). First, to rule out general drug effects, we administered MLA at a range of doses to mice treated with chronic saline ($n = 8–9$). Then to determine if MLA could precipitate nicotine withdrawal-induced deficits, we administered MLA across the same dose range to mice treated with chronic nicotine ($n = 12–14$), and compared conditioning to a chronic saline treated control group. To determine if high-affinity nAChRs were involved in withdrawal deficits in trace conditioning, we attempted to precipitate deficits with the high-affinity antagonist DH β E (Williams & Robinson, 1984). First, to rule out general effects of DH β E on trace conditioning, we administered DH β E (3mg/kg) at training, testing, or both training and

testing to chronic saline treated mice ($n = 9-12$). Then, we administered DH β E at training, testing, or both training and testing to chronic nicotine treated mice ($n = 9-14$) and compared these groups to a chronic saline treated group. By administering DH β E at either training or testing we were able to determine if withdrawal affected processes underlying acquisition or retrieval of trace conditioning, administration at both training and testing was included as a control for state-dependent effects. DH β E precipitated deficits in chronic nicotine treated mice. However, DH β E antagonizes a number of high-affinity nAChR subtypes, such as $\alpha 4\beta 2$, $\alpha 3\beta 4$, $\alpha 3\beta 2$, and $\alpha 4\beta 4$ receptors (Harvey & Luetje, 1996; Williams & Robinson, 1984). Thus, to determine if the $\beta 2$ nAChR subunit was involved in nicotine withdrawal effects on trace fear conditioning, we withdrew chronic nicotine or chronic saline from $\beta 2$ nAChR subunit KO mice and WT littermates ($n = 7-8$).

Analysis

Data were analyzed with either a 1-way or 2-way ANOVA. Homogeneity of variance was tested with the Levene statistic (Cohen, 2001). Tukey post hoc tests were conducted on data sets meeting the homogeneity of variance assumption. Games-Howell post hoc tests were conducted on data sets not meeting the homogeneity of variance assumption (Maxwell & Delaney, 2003). Differences between groups meeting $p < 0.05$ are reported as significant. Analyses were conducted with SPSS 13 (SPSS Inc. Chicago, IL).

Results

Effects of Acute Nicotine, Chronic Nicotine, and Withdrawal from Chronic Nicotine on Trace Conditioning

The effects of acute nicotine (Figure 1A), chronic nicotine (Figure 1B), and withdrawal from chronic nicotine (Figure 1C) on trace fear conditioning were examined with a 2-way ANOVA (Drug $\times$ Administration). The drug conditions were either saline or nicotine, and the administration conditions were either acute, chronic or withdrawal of chronic. There was no effect of drug or administration and no interaction on baseline freezing or freezing to an altered context. However, there was a significant effect of administration [$F(2,50) = 9.682$, $p < 0.05$], and a significant drug administration interaction [$F(2,50) = 3.657$, $p < 0.05$] on freezing during the trace-interval at training. Planned comparisons, between drug conditions in the different administration conditions showed that acute nicotine enhanced freezing compared to acute saline during the trace-interval, while chronic nicotine and chronic saline did not differ, and withdrawal of chronic saline and withdrawal of chronic nicotine were not significantly different. Additionally, there was a significant effect of administration [$F(5,51) = 11.048$, $p < 0.05$] and a significant interaction between drug and administration [$F(5,51) = 16.927$, $p < 0.05$] on cued freezing at testing. Tukey's post hoc analysis revealed that acute nicotine treated mice froze significantly more to the CS presentation than all other groups, and that chronic-nicotine withdrawn mice froze significantly less to the CS than all other groups (Figure 1A, C). Thus, during training, acute nicotine enhanced freezing during the trace interval, while chronic nicotine and withdrawal from chronic nicotine had no significant effect; and at recall 24 hours later, mice treated with acute nicotine had enhanced trace fear conditioning, chronic nicotine had no effect, and withdrawal of the same dose of chronic nicotine produced deficits in trace conditioning.

Effects of MLA on Trace Fear Conditioning in Chronic Nicotine and Chronic Saline treated Mice

To determine if $\alpha 7$ nAChRs are involved in the effects of withdrawal of chronic nicotine on trace conditioning, we attempted to precipitate withdrawal deficits in chronic nicotine treated animals with the low-affinity nAChR antagonist MLA. First, to rule out effects of MLA on trace fear conditioning, MLA was administered to chronic saline treated mice. A 1-

way ANOVA showed no significant effects of 0, 1.5, 3, or 9 mg/kg MLA on baseline, trace-interval, altered, or cued freezing (Figure 2A). Second, to determine if MLA precipitated withdrawal deficits in trace fear conditioning, the same doses were administered to chronic nicotine treated mice, with a chronic saline treated group included for comparison. A 1-way ANOVA showed no significant effects of MLA on baseline, trace-interval, or cued freezing but a significant effect on altered freezing [$F(4, 57) = 2.619$, $p < 0.05$], however, post hoc analysis revealed no significant differences (Figure 2B). Together these findings suggest that low-affinity nAChRs are not critically involved in the effects of chronic nicotine withdrawal on trace fear conditioning. Somatic nicotine withdrawal symptoms have been precipitated by MLA at 1, 3, and 7.5 mg/kg in mice treated chronically with 24 mg/kg/day of nicotine for 14 days (Damaj *et al.*, 2003), thus our results are not due to use of an ineffective dose of MLA.

Effects of DH β E on trace fear conditioning in chronic nicotine and chronic saline treated mice

To determine if high-affinity nAChRs are involved in the effects of withdrawal of chronic nicotine on trace conditioning, we attempted to precipitate withdrawal deficits in chronic nicotine treated animals with the high-affinity nAChR antagonist DH β E. First, we administered DH β E to chronic saline treated mice during trace fear conditioning. A 1-way ANOVA showed no effect of DH β E (3 mg/kg) if administered at training, testing, or both training and testing on baseline, trace-interval, altered, or cued freezing (Figure 3A). Second, we administered DH β E (3 mg/kg) to chronic nicotine treated mice prior to training, testing, or both training and testing of trace fear conditioning, with a chronic saline treated group included for comparison. A 1-way ANOVA showed a significant effect of DH β E on cued freezing [$F(3,38) = 10.162$, $p < 0.05$], but not on baseline, trace-interval, or altered freezing (Figure 3B). Post-hoc analysis showed that chronic nicotine treated mice administered DH β E at training or at both training and testing showed deficits in trace conditioning compared to chronic saline treated controls and chronic nicotine treated mice that did not receive DH β E. Mice treated with DH β E only at testing did not differ from saline treated controls. Thus, DH β E precipitated deficits in trace conditioning in chronic nicotine treated mice, but had no effect on trace conditioning in chronic saline treated mice. Additionally, DH β E precipitated deficits if administered prior to training or prior to both training and testing of trace conditioning. These results suggest that high-affinity nAChRs may be involved in processes underlying the withdrawal deficits, and that antagonism of these receptors during chronic nicotine administration produces deficits in processes related to acquisition and/or consolidation of trace conditioning.

Effects of spontaneous nicotine withdrawal in β 2 nAChR subunit KO mice

To determine if β 2 subunit containing nAChRs were involved in chronic nicotine withdrawal-induced deficits in trace fear conditioning, we withdrew either chronic saline or chronic nicotine from β 2 subunit KO mice and from WT mice. Trace fear conditioning data were analyzed with a two-way ANOVA, comparing effects of genotype and drug condition. There were no main effects or interactions on measures of baseline, trace-interval, or altered freezing, and there was no main effect of drug or genotype on trace conditioning. However, there was a significant drug $\times$ genotype interaction on trace conditioning [$F(3,26) = 4.24$, $p < 0.05$] (Figure 4). Post-hoc analysis showed that nicotine withdrawal produced deficits in cued freezing in WT mice but did not produce deficits in KO mice. Thus, the β 2 nAChR subunit is critical for the development of nicotine withdrawal deficits in trace fear conditioning.

Discussion

The primary findings of this study are threefold. First, acute nicotine, chronic nicotine, and withdrawal of chronic nicotine, at doses that produce matched plasma nicotine levels (Davis *et al.*, 2005), have different effects on trace fear conditioning assessed 24 hours after acquisition. Specifically, acute administration enhances trace conditioning; chronic administration has no effect, which suggests the development of tolerance; and withdrawal of chronic nicotine produces deficits in trace conditioning. Importantly, the doses of nicotine used produce plasma nicotine concentrations within the range reported in smokers (Benowitz, 1988; Davis *et al.*, 2005; Henningfield & Keenan, 1993); thus, these findings may model what occurs in smokers. Second, high-affinity, $\beta 2$ subunit containing nAChRs are critical for the development of nicotine withdrawal-induced deficits in trace fear conditioning. The high-affinity nAChR (*e.g.*, $\alpha 4\beta 2$ nAChR) antagonist DH β E precipitated deficits in trace fear conditioning in chronic nicotine-treated mice, while the low-affinity nAChR (*e.g.*, $\alpha 7$ nAChR) antagonist MLA did not. Further supporting a role for high-affinity nAChRs in withdrawal deficits in trace fear conditioning, $\beta 2$ nAChR subunit KO mice did not show deficits in trace fear conditioning following withdrawal of chronic nicotine unlike WT mice. Third, nicotine withdrawal disrupts acquisition and/or consolidation of trace fear conditioning. DH β E precipitated deficits if administered at training or at both training and testing of trace fear conditioning but not if administered only at testing, which suggests that deficits occur in processes related to acquisition of trace fear conditioning, but not in processes related to retrieval of trace fear conditioning.

Effects of nicotine on trace fear conditioning could occur through enhancement of working memory, which is thought to support acquisition of the trace-CS-US relationship during trace conditioning (Carter *et al.*, 2003; Han *et al.*, 2003). Alternately, effects of nicotine on trace fear conditioning could reflect changes in processes associated with long-term memory formation. Enhancement of working memory may be reflected in increased freezing during the trace-interval at training, whereas enhancement of long-term memory effects would be evident as increases in cued freezing 24 hours post training. Our finding that freezing during the trace interval during training is enhanced by acute nicotine suggests that acute nicotine may enhance working memory, while chronic nicotine had no effects on trace-interval freezing suggesting that tolerance developed to the effects of nicotine during acquisition. Withdrawal of chronic nicotine did not produce significant deficits in trace-interval freezing at training. Thus, it may be that while acute nicotine enhances working memory in this task, chronic nicotine results in tolerance reflected by lack of enhancement.

Previous studies have found that in non-spatial working memory tasks acute nicotine enhances working memory but with chronic treatment tolerance develops to this effect and withdrawal produces deficits in working memory (Ernst *et al.*, 2001; Jacobsen *et al.*, 2004; Jacobsen *et al.*, 2005; Jacobsen *et al.*, 2006; Jacobsen *et al.*, 2007; Kumari *et al.*, 2003; Mendrek *et al.*, 2006; Rusted & Trawley, 2006; Rycroft *et al.*, 2006; Spinelli *et al.*, 2006; Xu *et al.*, 2006). The results of the current study are consistent with these findings. However, in tasks examining spatial working memory, as assessed with the radial arm maze, a different pattern emerges; both acute nicotine and chronic nicotine enhance performance and some studies report that the enhancement remains after removal of chronic nicotine (Levin *et al.*, 1990; Levin *et al.*, 1992; Levin *et al.*, 1993; Levin *et al.*, 1996; Levin *et al.*, 1999). These findings suggest that the cognitive processes underlying spatial versus nonspatial tasks may involve neural substrates that are differentially affected by nicotine.

While both trace fear conditioning and radial arm maze depend on the hippocampus, effects of nicotine on these tasks may depend on different parts of the hippocampus. Effects of nicotine on radial arm maze depend upon nicotinic activity in the ventral hippocampus

(Felix & Levin, 1997; Kim & Levin, 1996; Levin *et al.*, 1999). Whereas trace fear conditioning depends largely on the medial prefrontal cortex (Blum *et al.*, 2006; McLaughlin *et al.*, 2002; Quinn *et al.*, 2008; Runyan & Dash, 2004; Runyan *et al.*, 2004) and dorsal hippocampus (Bangasser *et al.*, 2006; Burman *et al.*, 2006; Fendt *et al.*, 2005; McEchron *et al.*, 1998; Quinn *et al.*, 2002; Quinn *et al.*, 2008; Rogers *et al.*, 2006; Trivedi & Coover, 2006; Yoon & Otto, 2007); although some studies have implicated the ventral hippocampus in trace fear conditioning as well (Rogers *et al.*, 2006; Yoon & Otto, 2007). Thus, it is possible that nAChRs within the dorsal hippocampus mediate the effects of nicotine on trace fear conditioning. If this is the case then one might expect chronic nicotine treatment to differentially alter nAChRs in the dorsal hippocampus versus the ventral hippocampus. Indeed, the effects of chronic nicotine on nAChRs in these two brain areas are different. Abdulla and colleagues (1996) administered nicotine once a day for 10 days and found upregulation of nAChRs in the dorsal, but not in the ventral hippocampus. In addition, it is possible that the effects of nicotine on these tasks involve different nAChR subtypes.

The present study examined the nAChRs involved in the effects of withdrawal from chronic nicotine on trace fear conditioning and found that the high-affinity nicotinic antagonist DH β E precipitates nicotine withdrawal deficits in trace fear conditioning. This is consistent with a number of studies examining different symptoms of nicotine withdrawal. For instance, in chronic nicotine-treated rodents, DH β E precipitates increases in brain reward threshold, produces deficits in 5-choice serial reaction time task performance, and produces deficits in contextual fear conditioning (Epping-Jordan *et al.*, 1998; Kenny & Markou, 2005; Portugal & Gould, 2008; Shoaib & Bizarro, 2005; Skjei & Markou, 2003; Stoker *et al.*, 2008; Watkins *et al.*, 2000). Additionally, the present finding that DH β E precipitates deficits in trace fear conditioning if administered at training but not at testing is consistent with a previous report that nicotine withdrawal disrupts acquisition, but not recall of contextual fear conditioning (Portugal *et al.*, 2008). While some studies report that DH β E precipitates somatic nicotine-withdrawal symptoms (Damaj, 2003; Malin, 1998), other studies have failed to find this effect (Epping-Jordan, 1998; Stoker, 2008; Watkins, 2000a). However, DH β E is an antagonist for multiple high-affinity nAChR sub-types (Williams & Robinson, 1984; Harvey & Leutje, 1996; Harvey *et al.*, 1996), and it seems that precipitation of somatic withdrawal symptoms occurs through DH β E's action on β 4 subunit-containing nAChRs and not on β 2 subunit-containing nAChRs (Salas *et al.*, 2007). Thus, it may be that the β 4 subunit is involved in somatic withdrawal symptoms, while the β 2 subunit is more involved in cognitive and affective withdrawal symptoms. The involvement of β 2 containing nAChRs in nicotine withdrawal-associated cognitive deficits is supported by the present finding that spontaneous nicotine withdrawal-induced deficits in trace conditioning do not occur in β 2 KO mice and by a recent report that β 2 KO mice fail to show nicotine withdrawal-induced deficits in contextual fear conditioning (Portugal *et al.*, 2008).

The role of high-affinity nAChRs in nicotine withdrawal deficits in trace fear conditioning contrasts with the effects of the low-affinity nicotinic antagonist MLA in chronic nicotine treated animals. While MLA can precipitate somatic withdrawal signs in chronic nicotine treated rodents (Damaj, 2003; Salas, 2007), MLA does not precipitate increases in brain reward threshold or deficits in 5-choice serial reaction time task performance (Markou, 2001; Shoaib, 2005). These findings are consistent with the present finding that MLA does not precipitate nicotine withdrawal deficits in trace fear conditioning. Collectively, these findings and those reviewed in the previous paragraph suggest that high-affinity β 2 subunit-containing nAChRs are critical to the development of cognitive and affective nicotine withdrawal symptoms, while low-affinity nAChRs may be critical to the development of somatic withdrawal symptoms. Establishing the nAChR subtypes involved in withdrawal symptoms is only a first step in understanding nicotine withdrawal. The opponent-process

theory may explain the behavioral effects of nicotine as administration transitions from acute to chronic and from chronic to withdrawal.

The opponent-process theory predicts that a biological system reacts to perturbation (A-process) by counteracting the perturbation (B-process) and maintaining homeostasis (Solomon & Corbit, 1973). In the case of drugs of abuse, the A-process typically has a positive/rewarding effect. This A-process pushes the system away from its inherent homeostatic set point. To maintain homeostasis, the system reacts with a B-process (opponent-process), which counteracts the initial effects of the drug (A-process), returning the system to equilibrium or baseline (see Figure 5). Thus, if a drug has a positive hedonic effect, the system upon which it acts should react in a negative way, minimizing the effects of the drug and maintaining homeostasis. These effects could occur through multiple mechanisms; for instance, drug receptors could be inactivated, reducing drug effects, or a competing system could increase activity resulting in a net decrease of drug effect. The opponent-process theory also describes the time course of these processes (Solomon & Corbit, 1974). The initial drug effect (A-process) occurs quickly, while the B-process (opponent-process) has a slower onset and typically a slower offset. In the absence of the A-process, the B-process (opponent-process) remains, resulting in negative symptoms. In other words, during chronic drug administration, tolerance develops through the onset of the B-process and discontinuation of drug administration produces withdrawal symptoms.

The effects of nicotine on cognition and affect fit well with the opponent-process theory. This statement holds true for trace fear conditioning, various working memory dependent tasks in humans (Ernst *et al.*, 2001; Jacobsen *et al.*, 2004; Jacobsen *et al.*, 2005; Jacobsen *et al.*, 2006; Jacobsen *et al.*, 2007; Kumari *et al.*, 2003; Mendrek *et al.*, 2006; Xu *et al.*, 2005; Xu *et al.*, 2006), contextual fear conditioning in mice (Davis *et al.*, 2005) and for nicotine's effects on brain reward threshold (Epping-Jordan *et al.*, 1998; Huston-Lyons & Kornetsky, 1992; Johnson *et al.*, 2008; Stoker *et al.*, 2008; Watkins *et al.*, 2000). As demonstrated in the present study, for example, acute nicotine administration enhances trace fear conditioning; chronic administration leads to the development of tolerance, suggesting an opponent process minimizes the cognitive enhancing effects of nicotine; and withdrawal of chronic nicotine removes the cognitive enhancing effects of nicotine but not the opponent-process, revealing a deficit. Further, the fact that DH β E precipitates deficits in trace conditioning and the fact that β 2 KO mice do not display withdrawal deficits suggest that high-affinity β 2-containing nAChRs are critically involved in the cognitive deficits associated with nicotine withdrawal. Since the acute, enhancing effects of nicotine are also mediated by these receptors (Davis & Gould, 2007), it seems likely that β 2-containing nAChRs mediate the A-process and in chronic nicotine treated mice DH β E blocks the A-process, revealing the B-process. In β 2 KO mice, there is no A-process and therefore no B-process develops with chronic nicotine treatment. The opponent-process theory has been applied to habitual behavior associated with nicotine use (Solomon & Corbit, 1973; Ternes, 1977), psychostimulant drugs (Koob *et al.*, 1997), opiates (Koob *et al.*, 1989), and the rewarding effects of nicotine (Gutkin *et al.*, 2006), but to our knowledge this is the first application of opponent-process theory to the cognitive effects of nicotine. A better understanding of the opponent-process (B-process) responsible for the withdrawal effects of nicotine on cognition will aid in the development of new smoking cessation therapies.

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Abbreviations

nAChR	Nicotinic Acetylcholine Receptor
MLA	Methyllycaconitine
DHβE	Dihydro- <i>beta</i> -erythroidine
KO	Knockout
WT	Wildtype
US	Unconditioned Stimulus
CS	Conditioned Stimulus
ANOVA	Analysis of Variance

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Figure 1.

Effects of acute nicotine, chronic nicotine, and withdrawal of chronic nicotine on trace fear conditioning. Acute nicotine (0.09 mg/kg) enhances trace-interval and cued freezing, but has no effect on baseline or altered freezing (A). Chronic nicotine (6.3 mg/kg/day) has no effect on baseline, trace-interval, altered, or cued freezing (B). Withdrawal of chronic nicotine (6.3 mg/kg/day, 24 hours prior to training) produces deficits in cued freezing, but has no effect on baseline, trace-interval, or altered freezing (C). Significant difference ($p < 0.05$) from saline treated groups denoted with (*), data are reported as mean $\pm$ standard error of the mean.

Figure 2.

Effects of MLA on trace fear conditioning in chronic saline and chronic nicotine treated mice. MLA (1.5, 3, 9 mg/kg) did not alter baseline, trace-interval, altered, or cued freezing in chronic saline (A) or chronic nicotine (B) treated mice. Thus, the low-affinity nAChR antagonist MLA failed to precipitate nicotine withdrawal-induced deficits in trace fear conditioning. Data are reported as mean $\pm$ standard error of the mean.

Figure 3.

Effects of DHβE on trace fear conditioning in chronic saline and chronic nicotine treated mice. The high-affinity nAChR antagonist DHβE (3 mg/kg) had no effect on baseline, trace-interval, altered, or trace cued freezing in chronic saline treated mice if administered at training, testing, or both training and testing (A). However, in chronic nicotine treated mice DHβE (3 mg/kg) precipitated deficits in cued freezing if administered prior to training or prior to both training and testing, but had no effect on baseline, trace-interval, or altered freezing (B). Significant difference ($p < 0.05$) from saline treated groups denoted with (*), data are reported as mean $\pm$ standard error of the mean.

Figure 4.

Nicotine withdrawal in $\beta 2$ KO mice. Withdrawal of chronic nicotine (6.3 mg/kg/day, 24 hours prior to training) produced deficits in cued freezing in WT mice, but did not affect baseline, trace-interval, or altered freezing. However, in $\beta 2$ nAChR subunit KO mice, withdrawal of chronic nicotine had no effect on cued freezing, baseline freezing, or altered freezing. Significant difference ($p < 0.05$) from saline treated groups denoted with (*) data are reported as mean $\pm$ standard error of the mean.

Opponent-Process and the Effects of Nicotine on Cognition

Figure 5.

Nicotine, cognition, and the opponent-process theory. The opponent process theory explains the effects of nicotine on cognitive processes. Deflection from baseline reflect either an improvement (+) in cognition or a deficit (–). Acute nicotine enhances learning (A-process), but during chronic nicotine treatment, adaptation occurs resulting in an opponent-process (B-process) that negates the enhancing effects of nicotine on cognition. Withdrawal of chronic nicotine treatment removes the A-process, leaving the opponent-process and revealing cognitive deficits.