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Differential effects of $\alpha 4\beta 2$ nicotinic receptor antagonists and partial-agonists on contextual fear extinction in male C57BL/6 mice

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Abstract

Rationale—Numerous studies have attributed the psychopathology of post-traumatic stress disorder (PTSD) to maladaptive behavioral responses such as an inability to extinguish fear. While exposure therapies are mostly effective in treating these disorders by enhancing extinction learning, relapse of PTSD symptoms is common. Although several studies indicated a role for cholinergic transmission and nicotinic acetylcholine receptors (nAChRs) in anxiety and stress disorder symptomatology, very little is known about the specific contribution of nAChRs to fear extinction

Objectives—In the present study, we examined the effects of inhibition and desensitization of $\alpha 4\beta 2$ nAChRs via a full antagonist (Dihydro-beta-erythroidine, Dh β E) and two $\alpha 4\beta 2$ nAChR partial-agonists (varenicline and sazetidine-A) on contextual fear extinction, locomotor activity, and spontaneous recovery of contextual fear in mice.

Methods—We trained and tested the subjects in a contextual fear extinction as well as an open field paradigm and spontaneous recovery following injections of Dh β E, varenicline and sazetidine-A.

Results—Our results demonstrated that lower doses of Dh β E (1 mg/kg) and sazetidine-A (0.01 mg/kg) enhanced contextual fear extinction whereas higher doses of varenicline (0.1 mg/kg) and sazetidine-A (0.1 mg/kg) resulted in impaired contextual fear extinction. However, the higher dose of sazetidine-A (0.1 mg/kg) decreased locomotor activity, which may contribute to increased freezing response observed during fear extinction. Finally, we found that the low dose of Dh β E, but not sazetidine-A, also decreased spontaneous recovery of contextual fear following fear extinction.

Conclusions—Overall, these results suggest that inhibition and desensitization of $\alpha 4\beta 2$ nAChRs enhance extinction of contextual fear memories. This suggests that modulation of $\alpha 4\beta 2$

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nAChRs may be employed as an alternative pharmacological strategy to aid exposure therapies associated with PTSD by augmenting contextual fear extinction processes.

Keywords

Nicotinic receptors; fear extinction; spontaneous recovery; PTSD

The lifetime prevalence of experiencing a traumatic event is nearly 90% (Breslau et al., 1998). Exposure to traumatic events can lead to negative health outcomes, including post-traumatic stress disorder (PTSD). Individuals with PTSD exhibit exaggerated fear responses to otherwise safe environments. Repeated exposure to a fear-associated stimulus or context should extinguish the fear response; however, individuals with PTSD experience difficulty in extinguishing fear (Rothbaum and Davis 2003; VanElzakker et al. 2014). PTSD is commonly treated through exposure therapy, a behavioral treatment strategy based on fear extinction. However, exposure therapy has high dropout rates due to the unpleasant experience of repeated exposure (Imel et al. 2013) and this treatment method is susceptible to relapse (Craske and Mystkowski 2006).

Individuals with PTSD exhibit high rates of comorbidity with nicotine dependence (Breslau et al. 2004). The relationship between nicotine and PTSD is bidirectional in that smoking increases the likelihood of developing PTSD and the rate of smoking can increase after an event that triggers PTSD (Koenen et al. 2005; Breslau et al. 2003, 2004). When compared to individuals who report never experiencing a traumatic event, the prevalence of nicotine dependence is nearly 23% higher for those with PTSD (Ziedonis et al. 2008). Studies suggest nicotine may increase the amount of intrusive memories related to trauma (Hawkins and Cogle 2013). Similarly, in animal studies, nicotine has been shown to alter fear memories (see Kutlu and Gould 2015 for a review). Hippocampus-dependent types of fear conditioning, such as contextual and trace fear conditioning, are enhanced by acute nicotine (Gould and Wehner 1999; Gould 2003; Gould and Higgins 2003; Gould and Lommock 2003; Gould et al. 2004; Davis et al. 2005, 2006; Davis and Gould 2006). In addition to enhancing contextual fear learning, studies from our lab have shown that nicotine administration also hindered fear extinction in mice (Kutlu and Gould 2014; Kutlu et al. 2016a; 2017a,b). Specifically, acute nicotine administration impairs encoding and consolidation of contextual fear extinction memories (Kutlu and Gould 2014; Kutlu et al. 2017a) and enhances spontaneous recovery of contextual fear memories (Kutlu et al. 2016b). Combined with human studies, these results indicate a strong influence of nicotine on acquisition and extinction of fear memories.

Nicotine is an agonist of nicotinic acetylcholine receptors (nAChRs), a class of ligand gated ion channels located throughout the central and peripheral nervous system (Cordero-Erausquin et al. 2000). The low-affinity $\alpha 7$ and high-affinity $\alpha 4\beta 2$ nAChRs are dominant in the brain regions critical for long-term memory formation such as hippocampus (Alkondon and Albuquerque 1993; Wada et al. 1989; Seguela et al. 1993). Previously, we have shown that $\alpha 4\beta 2$, but not $\alpha 7$, nAChRs are necessary for the impairing effects of acute nicotine on contextual fear extinction (Kutlu et al. 2016a). $\alpha 4\beta 2$ nAChRs show high affinity nicotine binding and desensitize slowly (Fenster et al., 1997; Cordero-Erausquin et al. 2000),

suggesting that activation of $\alpha 4\beta 2$ nAChRs via nicotine may be responsible for the extinction deficits. Importantly, these results also indicate that inhibition of $\alpha 4\beta 2$ nAChRs may lead to enhancement of contextual fear extinction. Therefore, in the present study, $\alpha 4\beta 2$ nAChR partial-agonists and an antagonist (varenicline, sazetidine-A, and Dihydro-beta-erythroidine, Dh β E) were tested in order to examine the effects of $\alpha 4\beta 2$ nAChR inhibition on contextual fear extinction.

Methods

Subjects

Eight-week-old male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were group-housed in a colony room and maintained on a non-reversed 12 hour light/dark cycle and behavioral experiments were carried out in the light phase. Subjects had *ad libitum* access to food and water. Training and testing took place between 9:00 am and 6:00 pm. All behavioral procedures were approved by the Temple University Institutional Animal Care and Use Committee.

Apparatus

Contextual fear conditioning training, testing, and spontaneous recovery took place in 4 identical chambers ($18.8 \times 20 \times 18.3$ cm) within sound-attenuating boxes (MED Associates, St. Albans, VT). A ventilation fan located at the back of each box produced a background noise (65 dB). A white noise conditioned stimulus (CS, 85 dB) was produced by a speaker within the right wall of the chambers. Each chamber was composed of Plexiglas walls and ceiling with metal grid floors (0.20 cm and 1.0 cm apart) connected to a shock generator, which produced a 2 s long, 0.57 mA foot-shock unconditioned stimulus (US). Both the conditioned and unconditioned stimuli were controlled by an IBM-PC compatible computer running MED-PC software. The open field testing took place within a Plexiglas arena ($49.5 \text{ cm} \times 59.7 \text{ cm}$). Between subjects, the fear conditioning chambers and open field arena were cleaned with 70% ethanol.

Drugs and Administration

Mice received 2 doses of either varenicline (0.01 or 0.1 mg/kg, freebase weight; Tocris, Minneapolis, MN, # 3754), Dh β E (1 or 5 mg/kg tartrate weight; Tocris, Minneapolis, MN, #2349), or sazetidine-A (0.01 or 0.1 mg/kg, freebase weight; Tocris, Minneapolis, MN, # 2736) or vehicle. All compounds were prepared in saline. Varenicline doses are roughly correspond to medium to high doses of oral varenicline doses tested in humans (Faessel et al. 2006). Dh β E was administered subcutaneously 25 mins prior to behavioral testing whereas both varenicline and sazetidine-A were administered intraperitoneally 10 mins prior to behavioral testing. All doses, routes of administration, and injection times are based on previous studies from our laboratory (Davis and Gould 2006; Turner et al. 2013). All injection volumes were 10 ml/kg.

Behavioral Procedures

During fear conditioning training, mice were placed in the conditioning chambers for 120 s to assess baseline freezing. Following Kutlu and Gould (2014), subjects received two CS-US

freezing levels at the end of the extinction phase were removed from analysis as freezing levels above this level may indicate incomplete extinction.

Results

Antagonism of $\alpha 4\beta 2$ nAChR with Dh β E dose-dependently enhances contextual fear extinction

First, we administered Dh β E prior to each extinction session to examine the effects of $\alpha 4\beta 2$ antagonism on contextual fear extinction. A repeated measures ANOVA demonstrated that both the Drug $\times$ Trial interaction ($F(10,130) = 1.917$, $p < 0.05$) and Drug main effect ($F(2,26) = 5.130$, $p < 0.05$) were significant. Furthermore, the simple main effect of Drug was significant for the low dose of Dh β E (1 mg/kg; $F(1,19) = 9.726$, $p < 0.01$) but not for the high dose of Dh β E (5 mg/kg; $F(1,17) = 0.123$, $p > 0.05$), which suggests that Dh β E dose-dependently affects contextual fear extinction. That is, while the low dose of Dh β E augments contextual fear extinction, the high dose Dh β E has no effect (Figure 2A–B). These results suggest that partial inhibition of $\alpha 4\beta 2$ nAChRs may augment contextual fear extinction.

$\alpha 4\beta 2$ nAChR partial-agonist varenicline impairs contextual fear extinction

In addition to $\alpha 4\beta 2$ nAChR antagonist Dh β E, we also investigate whether varenicline, a partial agonist of $\alpha 4\beta 2$ nAChRs that keeps the receptor activation at a sub-maximal level, has any effect on contextual fear extinction. A repeated measures ANOVA showed that both the Drug $\times$ Trial interaction ($F(10,185) = 2.285$, $p < 0.05$) and Drug main effect ($F(2,37) = 11.821$, $p < 0.01$) were significant. Furthermore, the simple main effect of Drug was significant for Saline/Var 0.1 mg/kg comparison ($F(1,25) = 16.482$, $p < 0.01$) but not for Saline/Var 0.01 mg/kg comparison ($F(1,24) = 0.087$, $p > 0.05$), suggesting that the effect is mainly driven by the high dose. Therefore, our results show that the high dose of varenicline impairs contextual fear extinction whereas the low dose of varenicline did not have an effect (Figure 2C–D).

$\alpha 4\beta 2$ nAChR partial-agonist sazetidine-A has bi-directional effects on contextual fear extinction

Given that sazetidine-A uniquely desensitizes $\alpha 4\beta 2$ nAChRs without activating these receptors, we also examine the effects of sazetidine-A on contextual fear extinction. A repeated measures ANOVA revealed that for sazetidine-A both the Drug $\times$ Trial interaction ($F(10,95) = 7.111$, $p < 0.01$) and the Drug main effect ($F(2,19) = 28.749$, $p < 0.01$) were significant. Furthermore, the simple main effect of Drug was significant both for Saline/Saz-A 0.1 mg/kg ($F(1,13) = 23.528$, $p < 0.01$) and for Saline/Saz-A 0.01 mg/kg ($F(1,12) = 9.577$, $p < 0.01$). This suggests that sazetidine-A has both impairing and enhancing effects on contextual fear extinction depending on the administered dose (Figure 2E–F). That is, the low sazetidine-A dose enhanced whereas the high dose impaired contextual fear extinction.

A high dose of sazetidine-A decreases locomotor activity

While our results showed that nAChR partial agonists varenicline and sazetidine-A as well as antagonist Dh β E affected freezing behavior during contextual fear extinction, it is

possible that altered locomotor activity may contribute to these effects. Therefore, we ran the same group of mice in an open field paradigm following varenicline (0.1 mg/kg, 0.01 mg/kg, or saline), sazetidine-A (0.1 mg/kg, 0.01 mg/kg, or saline), and Dh β E (1 mg/kg, 5 mg/kg, or saline) injections. Our results showed that the Drug main effect was significant for varenicline ($F(2,37) = 3.886$, $p < 0.05$) and sazetidine-A ($F(2,19) = 47.874$, $p < 0.01$) but not for Dh β E ($F(2,26) = 1.917$, $p > 0.05$). Tukey post-hoc tests revealed that the differences between the high dose (0.1 mg/kg) and low dose (0.01 mg/kg) of sazetidine-A groups as well as the difference between the high dose of sazetidine-A group and vehicle controls were significant ($ps < 0.05$). The high dose varenicline group also showed significantly reduced levels of locomotor activity compared to the group that received the low dose of varenicline ($p < 0.05$). However, the high and low dose varenicline groups' locomotor activity failed to differ from saline controls ($ps > 0.05$). These results show that the full antagonist of $\alpha 4\beta 2$ nAChRs Dh β E does not affect locomotor activity (Figure 3A) suggesting the enhancement of contextual fear extinction observed following Dh β E administration is specific to extinction learning. In contrast, the high dose of the $\alpha 4\beta 2$ nAChR partial agonist sazetidine-A and the high dose of varenicline altered locomotor function (Figure 3B&C), which may contribute to the increased freezing response observed during contextual fear extinction.

Dh β E reduces spontaneous recovery of extinguished contextual fear

Finally, we examined whether the doses of Dh β E (1 mg/kg) and sazetidine-A (0.01 mg/kg) that were effective in enhancing contextual fear extinction enhanced retrieval of extinction memories and consequently reduced spontaneous recovery. We only focused on Dh β E and sazetidine-A, but not varenicline, due to lack of effect of varenicline on fear extinction. Separate one-way ANOVAs conducted using %freezing during re-testing showed that the main effect of Drug was significant for Dh β E (5.442, $p < 0.05$), but not for sazetidine-A ($F(1,14) = 0.242$, $p > 0.05$), which demonstrates that Dh β E reduces spontaneous recovery (Figure 4A) whereas sazetidine-A has no effect (Figure 4C). However, the Drug main effect was not significant for %Rebound scores for either Dh β E ($F(1,13) = 2.461$, $p > 0.05$; Figure 4B) or sazetidine-A ($F(1,14) = 0.240$, $p > 0.05$; Figure 4D) suggesting a transient effect of Dh β E on spontaneous recovery. Overall, in addition to their modulatory role in encoding of contextual fear extinction, our results indicate a potential role of $\alpha 4\beta 2$ nAChRs in the retrieval of extinction memories.

Discussion

The results of the present study demonstrate that the nAChR antagonist Dh β E and partial-agonists varenicline and sazetidine-A alter contextual fear extinction and spontaneous recovery of contextual fear. Whereas Dh β E and sazetidine-A dose dependently enhanced contextual fear extinction, varenicline administration resulted in impaired contextual fear extinction. In addition, only Dh β E, at the dose that was effective in enhancing contextual fear extinction, decreased spontaneous recovery of contextual fear whereas sazetidine-A did not affect spontaneous recovery. These results indicate that inactivation of $\alpha 4\beta 2$ nAChRs results in enhancement of contextual fear extinction. This hypothesis is also supported by previous work from our lab. For example, we found that acute administration of nicotine, an agonist of high-affinity $\alpha 4\beta 2$ nAChRs, impaired contextual fear extinction but did not affect

general freezing behavior (Kutlu & Gould 2014; Kutlu et al. 2016a; 2017a,b). In addition, acute nicotine also augmented spontaneous recovery of extinguished contextual fear while not altering recall of unextinguished fear memories (Kutlu et al. 2016b, 2017b). We also showed that knockout mice that lacked functional $\alpha 4$ or $\beta 2$ nAChR subunits, but not $\alpha 7$ subunits, did not exhibit acute nicotine-induced impairment of contextual fear extinction (Kutlu et al. 2016a). In the Kutlu et al. (2016a) study, we observed a modest enhancement of contextual fear extinction in $\beta 2$ nAChR knockout mice (Kutlu et al. 2016a; Figure 2). However, this effect was not as robust as we achieved with $\alpha 4\beta 2$ nAChR antagonism, which suggests that deletion of both $\alpha 4$ and $\beta 2$ nAChR subunits may be required to achieve this effect in nAChR knockout mice. Together with our previous research, these results strongly suggest that $\alpha 4\beta 2$ nAChRs modulate contextual fear extinction processes.

It is important to note that partial agonists of $\alpha 4\beta 2$ nAChRs may have potential side-effects. For example, varenicline has been shown to cause several side effects in humans including fatigue, abnormal dreams, and dry mouth (Patterson et al. 2009). In parallel, we showed that the higher dose of sazetidine-A reduced locomotor activity in an open field paradigm. We also showed a trending effect of the higher varenicline dose on locomotor activity, though a previous study from our laboratory showed that the same doses of varenicline did not induce freezing behavior in the absence of prior footshock presentations (Raybuck et al. 2008). The doses of varenicline and sazetidine-A that altered or trended to alter locomotion also impaired fear extinction suggesting that effects on locomotor activity could contribute to increased freezing response observed in these groups. Previous studies using 129SvJ-C57BL/6J hybrid mice did not report locomotor effects of sazetidine-A at 0.1 mg/kg dose (Turner et al. 2010; 2013). This suggests a potential susceptibility of C57BL/6J mice for the locomotor effects of $\alpha 4\beta 2$ nAChR partial-agonists. Nevertheless, our data showed that Dh β E enhanced contextual fear extinction and reduced spontaneous recovery without affecting locomotor activity, which indicates that full antagonism of $\alpha 4\beta 2$ nAChRs may be required for successful augmentation of fear extinction memories.

In this study we selected 3 compounds due to their effects on $\alpha 4\beta 2$ nAChRs. Varenicline, a partial agonist of $\alpha 4\beta 2$ nAChRs, limits nicotinic receptor activation at a submaximal level (Coe et al. 2005; Rollema et al. 2007). Sazetidine A is also a partial agonist of $\alpha 4\beta 2$ nAChRs; however, it desensitizes $\alpha 4\beta 2$ nAChRs without activating them (Xiao et al. 2006; Zwart et al. 2008). Finally, Dh β E is a direct antagonist that deactivates receptors by blocking the binding of acetylcholine to the receptors (Luetje et al. 1990). Our data indicate that Dh β E and sazetidine-A enhanced fear extinction whereas varenicline resulted in impaired fear extinction. This suggests that even limited activation of $\alpha 4\beta 2$ nAChRs, as could occur with varenicline may contribute to deficits in contextual fear extinction. Although the present study did not aim to investigate the involvement of downstream targets, we recently showed that during contextual fear extinction, acute nicotine disrupted phosphorylation of ventral hippocampal cell signaling kinases associated with memory consolidation such as ERK1/2 and JNK1 (Kutlu et al. 2017a). In addition, we showed that acute nicotine augments spontaneous recovery of contextual fear by enhancing neuronal activity in the ventral hippocampus and basolateral amygdala and reducing neuronal activity in the infralimbic cortex (Kutlu et al. 2016b). Given that nicotine, an agonist of $\alpha 4\beta 2$ nAChRs, impairs contextual fear extinction and the opposite effect is achieved with inhibition of $\alpha 4\beta 2$

nAChRs, it is possible that alterations in ventral hippocampal cell signaling cascades may also underlie the enhancing effects of Dh β E and sazetidine-A on contextual fear extinction and recovery. However, future studies are necessary to validate these hypotheses.

It is also important to note that all three compounds tested in the present study also act on other nAChR receptor subtypes. For example, varenicline is also a full agonist of α 7 nAChRs and shows high efficacy at α 3 β 4 and α 3 β 2 receptors, though at lower potency than for α 4 β 2 nAChRs (Mihalak et al. 2006). Similarly, sazetidine-A exhibits differential effects on α 4 β 2 nAChR subtypes; acting as an agonist of (α 4)2(β 2)3 pentamers, but as an antagonist of (α 4)3(β 2)2 pentamers (Zwart et al. 2008). These unique characteristics may shed light on the mechanisms underlying the differential effects of varenicline, sazetidine-A, and Dh β E on contextual fear extinction. For instance, it is possible that varenicline's action on α 7 nAChRs counteracts the potential enhancing effects of its α 4 β 2 nAChR partial-agonism on contextual fear extinction. Therefore, future studies that will test these compounds in combination with agonists and antagonists of other nAChR subtypes are needed to determine the main site of action critical for mediating fear extinction memories.

The clinical use of nicotinic agents such as varenicline (Chantix®, Pfizer, Mission, KS) is generally limited to smoking cessation (Gibbons and Mann 2013). However, there are reports showing that these compounds have cognitive and emotional effects in humans (Patterson et al. 2009; Loughhead et al. 2010). For example, a recent report showed that varenicline had destabilizing effects on mental health of veterans with PTSD (Campbell and Anderson 2010), suggesting potential adverse effects of nicotinic agents on mental health. Nevertheless, our results suggest that inhibition and desensitization of α 4 β 2 nAChRs may be helpful for PTSD treatment as they potentially enhance fear extinction and reduce recovery of fear memories. Therefore, α 4 β 2 nAChR compounds that decrease nAChR activation may provide a new line of pharmacotherapies to aid exposure therapy for PTSD. Investigating α 4 β 2 nAChR antagonists and partial-agonists may be especially important given the inadequate development of new medications of for PTSD (PTSD Psychopharmacology Working Group; Krystal et al. 2017).

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A **B**

Figure 1. The schematic of experimental designs

While each box represents a phase of the experiment, the syringe represents Dh β E, varenicline, sazetidine-A, or saline injections and the lightning bolt symbol indicates the presentations of the footshocks.

Figure 2. Effects of DhβE, varenicline, sazetidine-A on contextual fear extinction

Normalized and raw %freezing scores across initial testing and 5 extinctions sessions. Panel A–B: Effects of acute injections of DhβE (DhβE 1 and DhβE 5 mg/kg) and saline on contextual fear extinction. Panel C–D: Effects of acute varenicline (Var 0.01 mg/kg and Var 0.1 mg/kg) and saline injections on contextual fear extinction. Panel E–F: Effects of acute sazetidine-A (Saz-A 0.01 mg/kg and Saz-A 0.1 mg/kg) and saline injections on contextual fear extinction. Error bars show standard error of the mean. (*) denotes significant differences between lower dose drug groups and saline controls at p value <0.05. (#) denotes significant differences between higher dose drug groups and saline controls at p value <0.05.

Figure 3. Locomotor activity following Dh β E, varenicline, sazetidine-A

Distance traveled (inches) in an open field paradigm. Panel A: Effects of acute injections of Dh β E (Dh β E 1 and Dh β E 5 mg/kg) and saline on locomotor activity. Panel B: Effects of acute varenicline (Var 0.01 mg/kg and Var 0.1 mg/kg) and saline injections on locomotor activity. Panel C: Effects of acute sazetidine-A (Saz-A 0.01 mg/kg and Saz-A 0.1 mg/kg) and saline injections on locomotor activity. Error bars show standard error of the mean. (*) denotes Tukey post-hoc results showing significant differences between drug groups and saline controls with p value <0.05.

Figure 4. DhβE decreases spontaneous recovery of contextual fear following extinction
 %freezing and %Rebound scores during re-testing 7 days following the last fear extinction session Panel A: Effects of low dose DhβE (DhβE 1 mg/kg) on spontaneous recovery of contextual fear when administered prior to re-testing. Panel B: %Rebound values for the DhβE 1 mg/kg and saline controls. Panel C: Effects of low dose sazetidine-A (Saz-A 0.01 mg/kg) on spontaneous recovery of contextual fear. Panel D: %Rebound values for the Saz-A 0.01 mg/kg and saline controls. Error bars show standard error of the mean. (*) denotes significant main effect of Drug with p value <0.05.