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Research article

The effect of varenicline on binge-like ethanol consumption in mice is β4 nicotinic acetylcholine receptor-independent

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HIGHLIGHTS

- Genetic ablation of β4 nicotinic acetylcholine receptor subunit does not affect ethanol drinking in mice in the drinking in the dark paradigm.
- The acetylcholine receptor partial agonist varenicline reduces binge-like ethanol drinking following long-term exposure in mice.
- The effect of varenicline in reducing binge-like ethanol drinking is independent of β4 nicotinic acetylcholine receptor subunit.

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ABSTRACT

Background: Our laboratory has previously shown that the smoking-cessation agent varenicline, an agonist/partial agonist of α4β2*, α3β4*, α3β2*, α6β2* (* indicates the possibility of additional subunits) and α7 subunits of nicotinic acetylcholine receptors (nAChRs), reduces ethanol consumption in rats only after long-term exposure (12 weeks). As compounds having partial agonistic activity on α3β4* nAChRs were shown to decrease ethanol consumption in rodents, we assessed here the involvement of the β4 subunit in the effect of varenicline in the reduction of short- and long-term binge-like ethanol drinking in mice.
Methods: We used the well-validated drinking-in-the-dark (DID) paradigm to model chronic binge-like ethanol drinking in $\beta4^{-/-}$ and $\beta4^{+/+}$ littermate mice and compare the effect of intraperitoneal injection of varenicline (2 mg/kg) on ethanol intake following short- (4 weeks) or long-term (12 weeks) exposure.
Results: Drinking pattern and amounts of ethanol intake were similar in $\beta4^{-/-}$ and $\beta4^{+/+}$ mice. Interestingly, our results showed that varenicline reduces ethanol consumption following short- and long-term ethanol exposure in the DID. Although the effect of varenicline on the reduction of ethanol consumption was slightly more pronounced in $\beta4^{-/-}$ mice than their $\beta4^{+/+}$ littermates no significant differences were observed between genotypes.
Conclusion: In mice, varenicline reduces binge-like ethanol consumption both after short- and long-term exposure in the DID and this effect is independent of β4 nAChR subunit.

1. Introduction

The neuronal nicotinic acetylcholine receptors (nAChRs) are a superfamily of ligand gated ion channels, activated primarily by the endogenous neurotransmitter acetylcholine (ACh) [1]. These receptors are expressed throughout many brain regions, most notably the mesolimbic dopaminergic pathway, with twelve neuronal nAChR subunits (α2–α10 and β2–β4) identified. While the α4, β2, and α7 subunits, which form either heteromeric (α4* and β2*) or homomeric (α7) receptor arrangements (* indicates the possibility of additional subunits), have been shown to be expressed extensively throughout the brain, the α2, α3, α6, β2, β3, or β4 subunits, which either form simple (α3β4) or complex receptor subtypes (α3β2β4* or α3β3β4*), are found in localized regions and have more specific functions [11].

A growing body of evidence suggests that nAChRs are important mediators of addiction and represent significant pharmacotherapeutic targets for the treatment of alcohol use disorders (AUDs)

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[4]. Varenicline has been initially described as a smoking-cessation agent displaying partial agonist activity at nAChRs, with high affinity at α4β2* and low affinity at α3β4*, α3β2*, α6β2* subunits, and a low affinity full agonist activity at α7 nAChRs [6]. Our laboratory, as well as others, have shown that varenicline reduces ethanol seeking and consumption in rats [2,8,27], mice [12,26], monkeys [15] and humans [22]. Furthermore, two independent double-blind Randomized Controlled Trials (RCTs) have shown that varenicline significantly reduces alcohol consumption in human alcoholics, as compared to placebo [7,16], suggesting that varenicline is a potentially viable option for the treatment of alcohol use disorders.

In long-term (12 weeks) heavy drinking rats, we have previously shown that α4β2* nAChRs have a minor role in ethanol-mediated behaviours, whereas partial activation of α3β4* nAChRs significantly reduces ethanol drinking and seeking [5]. In line with this, a recent study revealed that over-expression of α5, α3 and β4 nAChRs subunits reduces ethanol drinking in mice [10]. Interestingly, we have previously shown that the α5 subunit does not alone modulate ethanol consumption [26] in mice, suggesting that partial activation of α3β4* nAChRs may play a role in the effect of varenicline in reducing ethanol drinking behaviours following long-term exposure.

In this study, we utilised β4 knockout (CHRNB4$^{-/-}$ or β4$^{-/-}$) mice to determine the involvement of the β4 nAChR subunit in the effects of varenicline in reducing heavy ethanol consumption following both short-term (4 weeks) and long-term (12 weeks) exposure. By using the well described drinking-in-dark (DID) paradigm that promotes high ethanol intake and pharmacologically relevant blood ethanol concentrations (BECs) in mice [24], we have shown that the effect of varenicline in the reduction of alcohol intake after short- and long-term exposure is independent of the β4 nAChR subunit.

2. Materials and methods

2.1. Animals and housing

Male β4$^{+/+}$ and matching β4$^{-/-}$ littermate mice were generated from heterozygous breeding pairs. The β4 mice were provided by Dr. Jerry Stitzel (Institute for Behavioral Genetics, University of Colorado), and had been backcrossed at least 10 generations on a C57BL/6J background. All transgenic mice used were healthy and similar in appearance to their wild type (WT) littermates. Genotyping was performed using polymerase chain reaction (PCR) as previously described [25]. For the drinking experiments, male β4$^{+/+}$ and matching β4$^{-/-}$ littermate mice (5 weeks old), were individually housed in standard cages (Techniplast) on a reverse 12 h light/dark cycle room (lights off at 9:00 a.m.) with *ad libitum* access to food and water. Mice were habituated to the housing conditions for one week before the start of the drinking experiments. This study was carried out in accordance with the recommendations of National Health and Medical Research Council (NHMRC) guidelines to promote the wellbeing of animals used for scientific purposes and the Australian code for the care and use of animals for scientific purposes. The protocol was approved by the Queensland University of Technology Animal Ethics Committee and the University of Queensland Animal Ethics Committee.

2.2. Drinking-in-the-dark (DID) paradigm

The mice were trained to consume 20% (v/v) ethanol using the well validated DID paradigm [24]. Briefly, mice were presented with one bottle of 20% (v/v) ethanol and one bottle of filtered water for a 2 h period (12 p.m.–2 p.m.), 3 h into the dark cycle, five days a week. The sides for ethanol and water bottles were switched every presentation to control for side preference. Two bottles of filtered water were available at all other times. All fluids were presented in 50 ml, graduated, plastic centrifuge tubes (Corning Centristar, NY, USA) fitted with rubber stoppers and a 2.5 in. stainless-steel sipper tube with double ball bearings (Ancare). Bottles were weighed prior to and at 30 min and 2 h after presentation, and measurements were taken to the nearest 0.1 g (g). Mouse weights were measured daily to calculate the adjusted g/kg intake. Only mice consuming over 1.1 g/kg of ethanol in 30 mins were included in the study.

2.3. Drug testing

Following short-term (4 weeks) or long-term (12 weeks) consumption of 20% (v/v) ethanol, we evaluated the acute effects of varenicline administration (vehicle and 2 mg/kg, s.c.) on ethanol intake. All drugs were prepared on the day of the experiment and administered to mice in a volume of 10 μl/g, 30 min before presentation of the bottles. Each injection was given seven days apart using a Latin square design, thus each animal served as its own control.

2.4. Blood ethanol concentration

Blood Ethanol Concentration (BEC) was tested at 30 mins following ethanol presentation. Tail blood samples were collected in tubes containing 10 μl of EDTA. Whole blood was centrifuged at 4 °C for 20 min at 4000 rpm and the serum was separated into aliquots. Samples were stored at −80 °C until running the BEC assay. Analysis was done using the nicotinamide adenine dinucleotide (NAD)-ethanol dehydrogenase (ADH) spectrophotometric assay [29]. All reagents used in this assay were purchased from Sigma-Aldrich (St. Louis, MO). BECs were computed against a standard calibration curve. All samples and standards were run in triplicate.

2.5. Drugs and chemicals

Varenicline tartarate (7,8,9,10-Tetrahydro-6,10-methano-6*H*-pyrazino[2,3-*h*] [3] benzazepine tartrate, Tocris/Invitro Technologies, Victoria, Australia) was dissolved in saline. The 20% ethanol (v/v) solution was prepared using 100% food grade ethyl alcohol (Recochem, SA, Australia) and filtered water.

2.6. Statistics

Statistical analyses were carried out using GraphPad Prism 6 (Graph Pad Software Co., San Diego, CA, USA). Statistical comparisons were performed using an unpaired two-tailed student's *t*-test or two-way analysis of variance (ANOVA) followed by a Bonferroni multiple comparisons post-test. A p value of <0.05 was considered significant. All values are expressed as mean ± SEM.

3. Results

3.1. 20% ethanol intake, preference and blood ethanol concentration (BEC) in β4$^{-/-}$ and matching β4$^{+/+}$ littermate mice

To assess the baseline intake of 20% ethanol in β4$^{+/+}$ and β4$^{-/-}$ mice, we trained the two genotypes to consume 20% ethanol for 12 weeks (60 exposures) using the DID paradigm. We found that both the genotypes consumed similar amounts of 20% ethanol and had a baseline consumption of 3.5 g/kg of ethanol (β4$^{+/+}$: 3.46 ± 0.07 g/kg and β4$^{-/-}$: 3.57 ± 0.10 g/kg, n = 12–14, unpaired *t*-test, p = 0.39, Fig.1A). In addition, both the genotypes had similar ethanol preference (74%) which showed a pattern of escalation with increased exposures (Fig. 1B). We also measured BEC in the two genotypes at 30 mins after ethanol presentation. We found no difference in

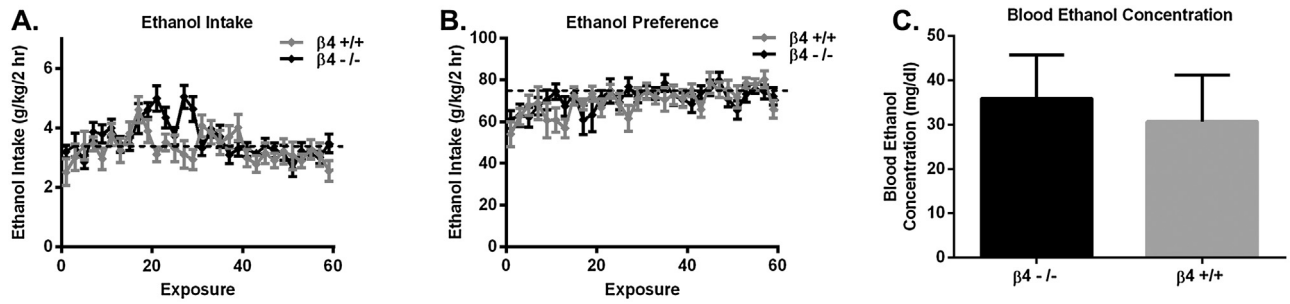

Fig. 1. Intake, preference and blood ethanol concentration of 20% ethanol in $\beta4^{-/-}$ and matching $\beta4^{+/+}$ littermate mice.
Stable drinking levels of 20% ethanol in $\beta4^{-/-}$ and matching $\beta4^{+/+}$ mice at 2 h over a period of 60 exposures (A). The values are expressed as mean ethanol intake (g/kg) ±SEM at each drinking session, n = 12–14. Stable 20% ethanol preference at 2 h over a period of 60 exposures in $\beta4^{-/-}$ and matching $\beta4^{+/+}$ mice (B). The values are expressed as mean percentage of ethanol intake (%) ±SEM at each drinking session, n = 12–14. The blood ethanol concentrations were not different between $\beta4^{-/-}$ and matching $\beta4^{+/+}$ mice at 30 mins following ethanol presentation (C). The values are expressed as mean blood ethanol concentration (mg/dl) ±SEM (unpaired two-tailed student's *t*-test), n = 12–14.

BEC between $\beta4^{+/+}$ and $\beta4^{-/-}$ mice indicating that both the genotypes consumed similar levels of 20% ethanol ($p = 0.7213$, unpaired two-tailed student's *t*-test, Fig. 1C).

3.2. Effect of systemic injections of varenicline on short-term and long-term ethanol consumption in $\beta4^{-/-}$ and matching $\beta4^{+/+}$ littermate mice

After achieving stable levels of 20% ethanol intake following short- and long-term ethanol exposure, we evaluated the effects of systemic administration of varenicline (vehicle and 2 mg/kg, s.c.) on ethanol intake in $\beta4^{+/+}$ and $\beta4^{-/-}$ mice. Following short-term drinking, two-way ANOVA showed a significant effect of treatment on consumption of 20% ethanol at 30 min [$F(1, 16) = 78.34$, $p < 0.0001$, Fig. 2A] and 2 h [$F(1, 16) = 44.68$, $p < 0.0001$, Fig. 2B] in the two genotypes, but no significant effect of genotype at 30 min ($F(1, 16) = 3.412$, $p = 0.0833$) or 2 h ($F(1, 16) = 0.002236$, $p = 0.9629$). Bonferroni's post hoc analysis revealed that at 30 mins, varenicline (2 mg/kg) significantly reduced ethanol consumption in $\beta4^{+/+}$ (***$p = 0.0002$, Fig. 2A) and $\beta4^{-/-}$ (****$p < 0.0001$) mice and, at 2 h varenicline (2 mg/kg) significantly reduced ethanol consumption in $\beta4^{+/+}$ (**$p = 0.0032$) and $\beta4^{-/-}$ (****$p < 0.0001$) mice.

Following long-term drinking, two-way ANOVA showed a significant effect of treatment on consumption of 20% ethanol at 30 min [$F(1, 14) = 46.87$, $p < 0.000$, Fig. 2C] and 2 h [$F(1, 14) = 39.64$, $p < 0.0001$, Fig. 2D]. No significant effect of genotype was observed at 30 min ($F(1, 14) = 0.06597$, $p = 0.8010$) or 2 h ($F(1, 14) = 0.1310$, $p = 0.7228$). Bonferroni's post hoc analysis revealed that at 30 mins, varenicline (2 mg/kg) significantly reduced ethanol consumption in $\beta4^{+/+}$ (**$p = 0.0040$) and $\beta4^{-/-}$ (****$p < 0.0001$) mice and, at 2 h varenicline (2 mg/kg) significantly reduced ethanol consumption in $\beta4^{+/+}$ (**$p = 0.0071$) and $\beta4^{-/-}$ (***$p = 0.0002$) mice. Varenicline had no effect on water consumption in the two genotypes following short-term drinking at 30 mins ($F(1, 17) = 1.382$, $p = 0.2559$) and 2 h ($F(1, 17) = 0.5647$, $p = 0.4626$, Table 1), and long-term drinking at 30 mins ($F(1, 14) = 2.915$, $p = 0.1098$) and 2 h ($F(1, 14) = 0.4729$,

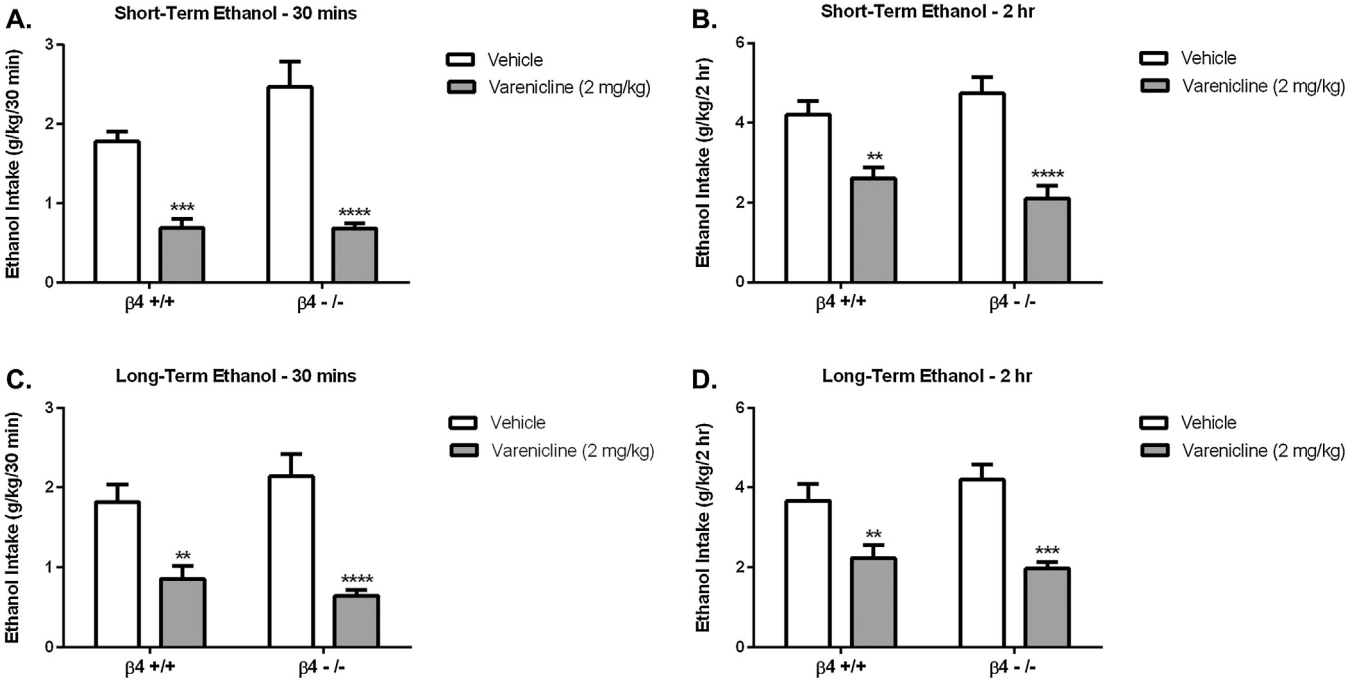

Fig. 2. Effect of varenicline on 20% ethanol following short-term and long-term exposure in $\beta4^{-/-}$ and matching $\beta4^{+/+}$ mice.
Varenicline significantly decreased ethanol consumption following short-term exposure at 30 mins (A) and 2 h (B) in $\beta4^{-/-}$ and matching $\beta4^{+/+}$ mice. Varenicline also significantly decreased ethanol consumption following long-term exposure at 30 mins (C) and 2 h (D) in $\beta4^{-/-}$ and matching $\beta4^{+/+}$ mice. Varenicline (2 mg/kg, s.c.) was administered 30 min before the start of the drinking session. Values are expressed as mean ethanol consumed (g/kg) ±SEM (two-way ANOVA followed by Bonferroni's post hoc test). **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ as compared with vehicle in the two genotypes, ($n = 12–14$ per treatment).

Table 1
Effect of varenicline on consumption of ethanol, water and ethanol preference at 2 h.

Ethanol Exposure	Genotype	Treatment	Parameters Tested				
			Ethanol		Water		Preference
4 weeks	WT	VEH	4.20 ± 0.33	** $p = 0.0032$	0.40 ± 0.09	NS $p > 0.9999$	86.09 ± 3.66
		VAR	**2.60 ± 0.27**		0.43 ± 0.10		79.98 ± 3.52
	KO	VEH	4.73 ± 0.40	**** $p < 0.0001$	0.46 ± 0.12	NS $p = 0.4812$	85.83 ± 3.44
		VAR	**2.10 ± 0.32**		0.29 ± 0.07		81.67 ± 5.34
12 weeks	WT	VEH	3.63 ± 0.41	** $p = 0.0071$	0.49 ± 0.16	NS $p > 0.9999$	84.05 ± 4.30
		VAR	**2.23 ± 0.32**		0.47 ± 0.12		75.92 ± 5.44
	KO	VEH	4.20 ± 0.37	*** $p = 0.0002$	0.47 ± 0.16	NS $p = 0.8301$	84.75 ± 4.96
		VAR	**2.23 ± 1.97**		0.33 ± 0.09		79.96 ± 4.97

Values are expressed in mean ethanol consumed (g/kg) ± SEM or mean water consumed (ml/100 g) ± SEM or mean percentage of preference of ethanol over water (%) ± SEM. **: p < 0.01; ***: p < 0.001 and ****: p < 0.0001 analysed by a One-way ANOVA analysis of variance followed by Bonferroni post-hoc comparisons test.
Values in bold represent a significant effect of varenicline treatment as compared to vehicle.

$p = 0.5029$, Table 1). Furthermore, varenicline also caused a reduction in the ethanol preference in these animals although, the difference was non-significant in the two genotypes as seen in the short-term testing at 30 mins ($F(1, 17) = 0.12191$, $p = 0.6457$) and 2 h ($F(1, 17) = 0.1512$, $p = 0.2355$, Table 1), and long-term testing at 30 min ($F(1, 14) = 0.4782$, $p = 0.5005$) and 2 h ($F(1, 14) = 2.934$, $p = 0.1088$, Table 1).

4. Discussion

Here, we have been able to show for the first time that β4 nAChR subunits are not necessary or required for ethanol binge drinking behaviour in mice. While the involvement of various α and β nAChR subunits in ethanol consumption has been well documented, the β4 nAChR subunit has received less attention. Studies using knockout mice have shown that acute ethanol consumption in the DID paradigm is significantly reduced in mice that do not express the α4 nAChR subunit, likely by abolishing ACh-mediated potentiation of dopamine neurons in the ventral tegmental area [17]. Conversely, the rewarding effect of ethanol in the conditioned place preference paradigm is enhanced [19] in mice expressing a hypersensitive α4 nAChR subunit (Leu9′Ala knock-in mouse line) [9,28]. Similarly, previous studies have also suggested that α6 subunits are involved ethanol-induced sedation [14] and expression of hypersensitive α6 subunit-containing nAChRs was shown to increase alcohol reward-related behaviours [23], probably by increasing ethanol-induced activation of the dopamine reward pathway [18]. Furthermore, a sex-dependent effect on ethanol consumption was observed in mice lacking the α7 subunit in females, but not males [13]. While previous studies have reported the involvement of α5 subunits in ethanol-induced sedation [14,26], it is likely that α5 (and β3) nAChR subunits are not involved in alcohol-drinking behaviours [14,26]. In line with this, we have reported here that genetic ablation of β4 nAChR subunits does not alter the acquisition or long-term maintenance of ethanol binge consumption in the drinking-in-the-dark (DID) paradigm.

While varenicline was designed to be selective for α4β2* nAChRs at low doses, at high concentrations it has been shown to be a partial agonist at α6β2* nAChRs, a full agonist at α3β4 and α7 nAChRs, as well as an agonist at 5-HT3 receptors [3,20,21]. In short-term exposed mice in the DID paradigm, low doses of varenicline (0.1 and 0.3 mg/kg) were shown to significantly reduce ethanol consumption [12]. This effect was abolished in mice lacking the α4 nAChR subunit, suggesting that α4 nAChR subunit is necessary and sufficient for the reducing effect of varenicline on short-term alcohol drinking [12]. However, in a previous work, we have shown that following long-term exposure to ethanol (3–5 months), only high (1 and 2 mg/kg) but not low doses (0.3 mg/kg) of varenicline reduced ethanol seeking and consumption in operant self-administration and intermittent access paradigms, respectively [27]. Furthermore, this effect was shown to be independent of β2, α5 and α7 nAChR subunits [13,26]. As we further observed that partial agonists at α3β4* nAChRs significantly reduce ethanol but not sucrose intake [5], we investigated here the contribution of β4 subunit in the effect of a high dose of varenicline (2 mg/kg) in reducing binge ethanol drinking following short- (4 weeks) and long-term (12 weeks) exposure. We showed that following short- or long-term ethanol exposure, no significant differences were observed between mice lacking the β4 subunit and their wild type littermate controls, suggesting that β4 subunit is not involved in varenicline's effect on the reduction of binge-like ethanol consumption. Although the effect of varenicline on the reduction of ethanol consumption was slightly more pronounced in $\beta4^{-/-}$ mice than their $\beta4^{+/+}$ littermates, no significant differences were observed between the genotypes.

By further uncovering the mechanisms by which varenicline mediates the reduction of ethanol consumption, we will be more equipped to elucidate potential pharmacotherapies for the treatment of AUDs.

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