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Reduced ethanol drinking following selective cortical interneuron deletion of the GluN2B NMDA receptors subunit

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Abstract

N-methyl-D-aspartate receptors (NMDAR) are involved in the regulation of alcohol drinking, but the contribution of NMDAR subunits located on specific neuronal populations remains incompletely understood. The current study examined the role of GluN2B-containing NMDARs expressed on cortical principal neurons and cortical interneurons in mouse ethanol (EtOH) drinking. Consumption of escalating concentrations of EtOH was measured in mice with GluN2B gene deletion in either cortical principal neurons (GluN2B^{CxNULL}) or interneurons (GluN2B^{InterNULL}), using a two-bottle choice paradigm. Results showed that GluN2B^{InterNULL}, but not GluN2B^{CxNULL}, mice consumed significantly less EtOH, at relatively high concentrations, than non-mutant controls. In a second paradigm in which mice were offered a 15% EtOH concentration, without escalation, GluN2B^{CxNULL} mice were again no different from controls. These findings provide novel evidence for a contribution of interneuronal GluN2B-containing NMDARs in the regulation of EtOH drinking.

Graphical Abstract

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Mice with genetic deletion of GluN2B were generated.

Self-administration of ethanol was measured using a two-bottle choice paradigm.

Keywords

alcohol; glutamate; addiction; cortex; interneuron

Introduction

The transition from moderate to excessive alcohol use is a defining feature of dependence, but the neural mechanisms regulating elevated alcohol (ethanol (EtOH)) drinking remain incompletely understood. One of the primary mechanisms through which EtOH exerts its actions on the brain is disruption of glutamatergic signaling. In particular, EtOH pharmacologically antagonizes the *N*-methyl-D-aspartate receptor (NMDAR) (Lovinger et al., 1989; Woodward, 2000) and causes alterations in NMDAR expression and function that have been implicated in the behavioral effects of acute and chronic EtOH, respectively (Bachteler et al., 2005; Holmes et al., 2013; Nagy, 2004; Radke et al., 2015a; Seif et al., 2013; Seif et al., 2015; Vengeliene et al., 2005; Vengeliene et al., 2008; Wang et al., 2007; Wang et al., 2010; Wills et al., 2015).

NMDARs are composed of four subunits, only one of which is obligatory (GluN1), and differential expression of the remaining NMDAR subunits alters the receptor's sensitivity to EtOH. NMDARs containing the GluN2B subunit are generally more sensitive to EtOH's antagonist actions (Wills et al., 2012; Woodward, 2000). GluN2B-containing NMDARs are further implicated in the functional effects of chronic EtOH by increased expression of the subunit in cortical, limbic and striatal regions in rodents repeated exposed to EtOH (de Ferron et al., 2015; Follesa and Ticku, 1995; Hardy et al., 1999; Kalluri et al., 1998; Kash et al., 2009; Kroener et al., 2012; McGuier et al., 2015; Narita et al., 2000; Nelson et al., 2005; Nimitvilai et al., 2015; Pian et al., 2010; Rani and Ticku, 2006; Wang et al., 2010) and in the hippocampus of human alcoholics (Enoch et al., 2014). There is also evidence that pharmacologically blocking GluN2B (via systemic ifenprodil administration) can reduce EtOH drinking in rats (Vengeliene et al., 2005).

The existing literature suggests that GluN2B is an important target of EtOH and a modulator of EtOH-related behaviors, but does not parse the critical brain regions and neuronal populations where these actions occur. To address this issue in the current study, we determined EtOH drinking behavior in mutant mice with deletions of GluN2B in specific neuronal populations. Our results reveal a novel role for GluN2B in cortical interneurons but not principal neurons in regulating EtOH drinking.

Materials and methods

Subjects

Subjects were adult male and female mice housed in a temperature- ($72 \pm 5^\circ \text{F}$) and humidity- ($45 \pm 15\%$) controlled vivarium under a 12 hour light/dark cycle (lights on at 0600 h). GluN2B-floxed mice were generated as previously described and backcrossed repeatedly to C57BL/6J to produce a congenic background (Brigman et al., 2010). Three separate Cre-driver transgenic lines were used to delete GluN2B in specific brain regions and neuronal subtypes when crossed with the GluN2B-floxed mice: CaMKII α -Cre (Tsien et al., 1996) to delete GluN2B on cortical and CA1 hippocampal principal neurons (GluN2B^{CxNULL}) (Brigman et al., 2010), Ppp1r2-Cre (Belforte et al., 2010) to delete GluN2B on forebrain interneurons (predominantly parvalbumin-positive) (GluN2B^{InterNULL}) (Brigman et al., 2015; Kiselycznyk et al., 2015), and Rgs9-Cre (Dang et al., 2006) to delete GluN2B on striatal medium spiny neurons (GluN2B^{StNULL}) (Radke et al., 2015b) (Figure 1A). Data from the latter mutants are not reported due to insufficient numbers (see below). Both the CaMKII α -Cre and Ppp1r2-Cre reporter lines were generated on a C57BL/6 background while the Rgs9-Cre line had a mixed C57BL/6, 129/Sv, and BALB/c background (Belforte et al., 2010; Dang et al., 2006; Tsien et al., 1996).

For each of the three mutant lines, homozygous GluN2B-floxed/Cre-negative sires were bred with homozygous GluN2B-floxed/Cre-positive dams to produce GluN2B-deficient mice and Cre-negative (GluN2B^{WT}) littermate controls. Drinking levels did not differ between the GluN2B^{WT} mice from each of the 3 breeding lines; therefore, these groups were combined into a single GluN2B^{WT} control group for analysis. However, due to insufficient numbers of male GluN2B^{StNULL} mice, this mutant line was excluded. All experimental procedures were approved by the National Institute on Alcohol Abuse and Alcoholism Animal Care and Use Committee and followed the US National Institutes of Health guidelines outlined in ‘Using Animals in Intramural Research.’

Two-bottle EtOH drinking

A standard 2-bottle choice test was used to measure EtOH drinking (Boyce-Rustay and Holmes 2006(DePoy et al., 2015). Mice were individually housed in ‘Space Saver’ cages (Model 1145T, Tecniplast, Buguggiate, Italy) with lids fitted for 2 fluid bottles (Model 1145T482SUDB Polysulfone cage-top, Tecniplast). One bottle was filled with EtOH in water (v/v) and the other with tap water according to the following schedule: 0, 3, 6, and 9% EtOH for 4 days each and 11, 13, and 15% EtOH for 6 days each (Figure 1A). These bottles were available 24 hours per day except brief periods when solutions were changed. Solution changes occurred every 2 days, during the time when the EtOH and water bottles (and the

mice) were weighed. The left/right position of the bottles was switched at each solution-change to control for any side bias. Food was available *ad libitum*. EtOH consumption and percent EtOH preference ($=\text{EtOH consumption}/\text{total fluid consumption} \times 100$) were calculated, correcting for evaporation and spillage measured from two empty cages.

On the basis of our results showing altered consumption of the higher EtOH concentrations in GluN2B^{InterNULL}, but not GluN2B^{CxNULL}, mice (see Results), we tested an additional cohort of GluN2B^{CxNULL} mice, along with GluN2B^{WT} littermate controls, for drinking of the highest EtOH concentration (15%) without prior escalation. Mice were tested as above, with the exception that only the single 15% EtOH concentration was offered over a 2-week test period.

Statistical analysis

Drinking data were analyzed using 1-way or 2-way (EtOH-concentration $\times$ genotype, or EtOH/water solution $\times$ genotype) analysis of variance (ANOVA), with repeated measures for concentration. Follow-up comparisons were performed using the Holm-Sidak *post hoc* test. Age and body weight data were analyzed with 1-way ANOVA followed with Dunnett's test for multiple comparisons. Analyses were conducted using GraphPad Prism 6.04 (GraphPad Software Inc, La Jolla, CA, USA). The threshold for statistical significance was set at $P < .05$.

Results

The average age and body weight of each mutant line at the start of testing is given in Table 1. GluN2B^{CxNULL} mice were older than GluN2B^{WT} controls ($P < .05$) but there were no differences between genotypes in weight.

EtOH consumption was significantly affected by EtOH concentrations (main effect of concentration: $F_{5,170} = 47.30$, $P < .0001$) (Figure 1B). *Post hoc* tests revealed GluN2B^{InterNULL} mice drank significantly less EtOH than GluN2B^{WT} controls ($P < .05$) at the 9% and 15% concentrations. EtOH drinking in GluN2B^{CxNULL} mice did not differ from GluN2B^{WT} controls at any concentration. Water consumption was lower in GluN2B^{CxNULL} mice compared to controls ($P < .05$) at the 15% EtOH concentration (Figure 1C). Percent preference for EtOH over water was significantly affected by EtOH concentration (main effect of concentration: $F_{6,204} = 3.67$, $P < .01$, Figure 1D), but *post hoc* tests did not find significant genotype differences at any given concentration.

Averaging data across EtOH concentrations indicated a significant effect of EtOH solution on consumption (main effect of EtOH/water solution: $F_{1,34} = 140.50$, $P < .01$). *Post hoc* tests found significantly lower drinking in GluN2B^{InterNULL} mice ($P < .05$), relative to GluN2B^{WT} controls (Figure 1E), whereas water consumption (Figure 1F) nor EtOH preference (Figure 1G) differed between genotypes.

In a second cohort of GluN2B^{CxNULL} mice (for age and body weight, see Table 2) there was no significant difference between genotypes in 15% EtOH or water drinking, or percent preference for EtOH over water (Table 3). Females drank more 15% EtOH than males (main

effect of sex: $F_{1,30} = 30.20$, $P < .001$), but did not differ in water consumption or EtOH preference.

Discussion

Previous studies strongly implicate the NMDAR GluN2B subunit in various neural and behavioral actions of EtOH, but have not defined the specific neuronal circuits involved. The current findings extend the current literature by measuring EtOH drinking in a two-bottle choice test in mice with gene deletion of GluN2B in two distinct neuronal populations. The major findings were that GluN2B deletion in cortical (mainly parvalbumin-positive) interneurons decreased drinking, whereas deletion in cortical principal neurons did not alter drinking in either of two versions of the two-bottle choice paradigm.

NMDARs, including those which have GluN2B, are key modulators of cortical interneuronal activity, influencing local neuronal synchrony and the output of cortical projections (Cardin et al., 2009; Cho et al., 2015; Courtin et al., 2014; Hanson et al., 2013; Uhlhaas and Singer, 2010). Moreover, disrupting cortical parvalbumin-positive interneuron activity alters cognitive flexibility and conditioned fear, while exciting these cells expedites extinction of a rewarded operant behavior (Bissonette et al., 2015; Cho et al., 2015; Courtin et al., 2014; Sparta et al., 2014). Thus, one mechanism by which GluN2B interneuron deletion might alter EtOH drinking is via the dysregulation of cortical outputs to downstream midbrain, limbic or striatal regions involved in the control of EtOH drinking (Barker et al., 2015). A more thorough examination of functional responses to EtOH in these brain regions would go towards testing this hypothesis. It would also be important to test whether this could be a mechanism that generalizes to other rewards. Although we have previously shown that responding for a food reward is unchanged in GluN2B^{InterNULL} mice (Radke et al., 2015b), behavioral responses to drugs of abuse have not yet been assessed in these mice.

Further work is also needed to define the contribution of GluN2B-expressing principal neurons in EtOH drinking. A previous study found that mutant mice with forebrain-wide GluN2B principal neurons deletion exhibited an exaggerated locomotor stimulant response to EtOH and an attenuated depressant response (Badanich et al., 2011). Although EtOH drinking was not measured in Badanich and colleagues' study, their findings do demonstrate a functional role for GluN2B on principal neuronal in at least some EtOH behaviors.

One caveat to the comparison of EtOH drinking phenotypes in the two mutant lines tested here is that GluN2B deletion is more widespread in the GluN2B^{CxNULL} mutants than in the GluN2B^{InterNULL} line. Prior studies have shown that GluN2B deletion likely extends throughout the hippocampus in the interneuron mutant line (Belforte et al., 2010), whereas it is likely largely restricted to the dorsal hippocampal CA1 subregion in the principal neuron mutant, at the ages tested (Brigman et al., 2013; Brigman et al., 2010). This raises the possibility that the greater extent of GluN2B deletion accounts for the differing phenotypes in the mutant lines, rather than the loss of the subunit on specific neuronal populations. A related issue is that the lack of specificity of the interneuron deletion for parvalbumin-positive cells per se means that the drinking phenotype in these mutants cannot be solely attributed to these cells at the exclusion of other interneuronal classes.

Notwithstanding these issues, an effect of GluN2B deletion on decreasing EtOH drinking in mice is consistent with prior pharmacological work in rats. For example, Wang and colleagues have reported reduced EtOH operant self-administration after microinfusion of GluN2B-selective antagonist, ifenprodil, into the dorsomedial striatum (Wang et al., 2007; Wang et al., 2010), and Vengeliene and colleagues have shown that systemic ifenprodil administration decreased EtOH drinking during withdrawal in EtOH-dependent rats (Vengeliene et al., 2005). Interestingly, in contrast to subunit-unselective NMDAR channel blockers, the actions of GluN2B antagonists on drinking do not appear to extend to effects on acute EtOH place preference or EtOH intoxication (Boyce-Rustay and Cunningham, 2004; Boyce-Rustay and Holmes, 2005; Palachick et al., 2008). These data demonstrate a selective profile of GluN2B antagonists, and taken together with the findings of the current study, provide further support for the importance of the GluN2B as a regulator of EtOH drinking and as a potential novel target for treating alcohol use disorders.

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Highlights

- The NMDA receptor GluN2B subunit was deleted in different neuronal populations
- Drinking of increasing concentrations of EtOH was measured using a 2-bottle test
- Deletion in cortical interneurons, not principal neurons, reduced EtOH drinking
- These data define specific GluN2B-expressing populations mediating EtOH drinking

Figure 1. Effects of GluN2B deletion in specific neuronal populations on EtOH drinking
(A) GluN2B was deleted in cortical principal neurons (GluN2B^{CxNULL}; n=6) or cortical interneurons (GluN2B^{CxNULL}; n=10). **(B)** GluN2B^{InterNULL} mice had lower drinking at the 9% and 15% concentrations, as compared to GluN2B^{WT} (n=21) controls. **(C)** GluN2B^{CxNULL} drank less water than GluN2B^{WT} controls while 15% EtOH was available. **(D)** Percent preference for EtOH over water did not differ between genotypes. **(E)** Average EtOH drinking across EtOH concentrations was lower in GluN2B^{InterNULL} mice, relative to GluN2B^{WT} controls. Genotypes did not differ in average water drinking **(F)** or average

percent preference for EtOH over water (**G**). Data are means $\pm$ SEM. * $P < .05$ versus GluN2B^{WT}

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Table 1

Age and body weight of mice at the beginning of drinking escalating concentrations of EtOH.

	GluN2B^{WT}	GluN2B^{CxNULL}	GluN2B^{InterNULL}
N (males)	21 (12)	6 (3)	10 (6)
Age (weeks)	16.10 ± 1.48	25.50 ± 2.62 ^{**}	16.50 ± 1.60
Weight (g)	26.90 ± 1.15	22.50 ± 1.21	28.54 ± 1.71

^{**}
 $p < 0.01$, significantly different from WT

Table 2

Age and body weight of GluN2B^{CxNULL} mice at the beginning of drinking a 15% concentration of EtOH.

	GluN2B ^{WT}	GluN2B ^{CxNULL}
N (males)	18 (7)	16 (10)
Age (weeks)	9.28 ± 0.33	8.94 ± 0.34
Weight (g)	21.77 ± 0.98	22.34 ± 0.99

Table 3

EtOH drinking in GluN2B^{CxNULL} mice offered a 15% concentration of EtOH.

	GluN2B ^{WT}		GluN2B ^{CxNULL}	
	Male	Female	Male	Female
Ethanol drinking (g/kg/day) **	8.05 ± 0.74	12.31 ± 0.94	8.76 ± 0.67	13.68 ± 1.40
Water drinking (g/day)	1.58 ± 0.29	1.66 ± 0.18	1.35 ± 0.12	1.07 ± 0.28
Preference for ethanol vs. water (%)	53.87 ± 7.62	54.88 ± 3.27	58.18 ± 3.24	68.23 ± 6.51

 $p < 0.001$, significant main effect of sex