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NMDA receptor GluN2A subunit deletion protects against dependence-like ethanol drinking

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Abstract

The *N*-methyl-D-aspartate receptor (NMDAR) is mechanistically involved in the behavioral and neurophysiological effects of alcohol, but the specific role of the GluN2A subunit remains unclear. Here, we exposed mice with constitutive GluN2A gene knockout (KO) to chronic intermittent ethanol vapor (CIE) and tested for EtOH consumption/preference using a two-bottle choice paradigm, as well as NMDAR-mediated transmission at basolateral amygdala synapses via *ex vivo* slice electrophysiology. Results showed that GluN2A KO mice attained comparable blood EtOH levels in response to CIE exposure, but did not exhibit the significant increase in EtOH drinking that was observed in CIE-exposed wildtypes. GluN2A KO mice also showed no alterations in BLA NMDAR-mediated synaptic transmission after CIE, relative to air-exposed, whereas C57BL/6 J mice showed an attenuated synaptic response to GluN2B antagonism. Taken together, these data add to mounting evidence supporting GluN2A-containing NMDARs as a mechanism underlying relative risk for developing EtOH dependence after repeated EtOH exposure.

Keywords

Alcohol; CIE; NMDA; Glutamate; Addiction; Amygdala

There is compelling evidence that glutamate signaling through the *N*-methyl-D-aspartate receptor (NMDAR) is a crucial mechanism contributing to the behavioral and neurophysiological effects of acute and chronic alcohol [1–3]. One outstanding issue that remains unresolved, however, is the relative roles of the specific subunits (GluN2A-D, GluN3-B) that constitute, in various combinations with the obligatory GluN1 subunit, the heteromeric NMDAR. However, a convergence of recent data from human and rodent

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Appendix A. Supplementary data

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genetic studies [4–8] and rodent pharmacological [9–13] work point to a key role for GluN2A in the effects of alcohol. Here, we sought to extend current understanding of the role of GluN2A in mediating behavioral and neurophysiological sequelae of chronic EtOH. To this end, we exposed mice with constitutive GluN2A gene knockout (KO) to CIE and tested for related increases in EtOH consumption [9,14–16] and NMDAR-mediated synaptic transmission in the BLA, a limbic brain region where glutamatergic neuroplasticity may play a role in the development and maintenance of an addictive phenotype [17].

Subjects used were adult (> 4 months old) female and male mice, housed 2 per cage, in a temperature- (72 ± 5 °F) and humidity- ($45 \pm 15\%$) controlled vivarium under a 12 h light/dark cycle (lights on at 0600 h). GluN2A KO mice were generated as previously described [18–21] and repeatedly backcrossed to the C57BL/6 J strain. Mice heterozygous for the GluN2A mutation were bred, in house, to produce KO mice and wildtype (WT) littermate controls. Male C57BL/6 J mice used for slice recordings were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The numbers of mice used in each experiment are given in the figure legends. All experimental procedures were approved by the National Institute on Alcohol Abuse and Alcoholism Animal Care and Use Committee and the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill, and followed the US National Institutes of Health guidelines outlined in ‘Using Animals in Intramural Research.’

Chronic EtOH exposure was achieved using a vapor inhalation procedure, as previously described [16,22]. Mice were removed from the home cages and individually or pair housed in standard mouse cages in Plexiglas vapor chambers ($60 \times 36 \times 60$ cm, PlasLabs, Lansing, MI) (up to 6 cages/chamber) and exposed to continuous vaporized ethanol (EtOH). EtOH was volatilized by passing air through a vaporization stone submerged in EtOH (95%) and mixed with fresh air to deliver 19–22 mg EtOH/L of air at a rate of ~ 10 L/min. CIE-induced blood EtOH concentrations (BECs) were confirmed weekly via blood samples taken from dedicated ‘sentinel’ mice exposed to EtOH simultaneously with the test mice. Sentinel mice were pricked with a lancet on the submandibular vein to collect the blood into heparinized capillary tubes. Blood samples were then centrifuged for 30 min at 15,000 rpm at 4 degrees Celsius prior to determination of sentinel BECs using an Analox AM1 alcohol analyzer (Analox Instruments USA, Lunenburg, MA, USA). Sentinels were age- and genotype-specific to the animals being exposed in each of the respective vapor chambers.

In order to induce intoxication and produce stabilized BECs, the EtOH group received intraperitoneal (i.p.) injections of 71.6 mg/kg of the alcohol dehydrogenase inhibitor pyrazole (Sigma, St. Louis, MO) combined with 1.5 g/kg 20% (v/v) EtOH, in a volume of 10 mL/kg body weight, prior to being placed into the vapor chambers. Vapor exposure lasted 16 h per day (in at 1700 h, 1 h before start of the 12-hr circadian dark phase, out at 0900 h), followed by an 8-h withdrawal period. For 1 cycle of exposure, there were 4 consecutive days of intermittent exposure (Monday-Friday) and then a longer, 80-h, withdrawal (Friday-Monday). This cycle was repeated 4 times. Air controls also received an injection of pyrazole, 68.1 mg/kg, to control for this treatment, before being placed into dedicated air vapor chambers (located adjacent to the EtOH chambers) which received vaporized air at ~ 10 L/min for the same periods described for the CIE-exposed group.

EtOH consumption was measured using a two-bottle choice paradigm, as previously described [16,23]. Mice were individually housed in ‘Space Saver’ cages (Model 1145 T, Tecniplast, Buguggiate, Italy) with lids fitted for 2 fluid bottles [Model 1145 T482SUDB Polysulfone cage-top to accommodate 2 water bottles (top flow design; Tecniplast)]: contained 15% (v/v) EtOH in water and the other contained tap water. Every 2 days, bottle position was switched, body weight was recorded and EtOH and water consumption determined by measuring the change in weight, correcting for evaporation and spillage (i.e., the average loss of fluid measured from bottles in two empty ‘dummy’ cages was subtracted from the amount drank). Food was available *ad libitum* throughout.

Physiological and behavioral data were analyzed via Student’s *t* tests or two-way analysis of variance (ANOVA) followed by Sidak’s multiple comparison *post hoc* test, using GraphPad Prism 7.01 (GraphPad Software Inc, La Jolla, CA, USA). The threshold for statistical significance was set at $P < .05$.

Pre-CIE consumption and preference (=amount drank from the EtOH-containing bottle / amount drank from both bottles * 100) was measured for 14 days to provide a reference for post-CIE drinking. Mice were assigned to CIE exposure groups by matching the groups for average pre-CIE drinking. EtOH was not available for drinking (water bottle only) during CIE exposure. Beginning on the third day (72 h) after CIE exposure (as in prior studies [9,15,16] to avoid confounding effects of acute withdrawal on behavior, such as transient aversion to EtOH), the mice were again presented with the water and EtOH-containing bottles for an additional 7 days to measure post-CIE consumption and preference. For a schematic of the experimental design and timeline, see Fig. 1A. We confirmed that the CIE exposure parameters used produced similar BECs in GluN2A KO and WT sentinel mice (Fig. 1B) and that body weight was also similar between genotypes, both during the pre-CIE and post-CIE periods (Fig. 1C).

We then examined EtOH drinking and preference both prior to and following CIE. EtOH consumption did not differ between genotypes during the pre-CIE period, but differed in a genotype and treatment dependent manner, post-CIE (genotype x treatment interaction: $F_{1,34} = 5.01$, $P < .05$). *Post hoc* analysis showed that the amount of EtOH consumed was significantly higher in WT mice exposed to CIE than in WT mice exposed to air ($P < .05$), whereas GluN2A KO mice showed no such difference (Fig. 1D). EtOH consumption levels were also significantly higher in CIE-exposed WT mice than CIE-exposed KO mice (post hoc test: $P < .05$) and higher in WT mice post-CIE, as compared to pre-CIE (post hoc test: $P < .05$). Pre-CIE, EtOH preference was similar between genotypes, but differed as a function of genotype post-CIE (genotype x treatment interaction: $F_{1,34} = 5.84$, $P < .05$). EtOH preference was significantly higher in CIE-exposed WT mice than similarly-exposed GluN2A KO mice (post hoc test: $P < .05$) and preference was higher in WT mice post-CIE as compared to pre-CIE $F_{2,44} = 18.52$, $P < .01$) (Fig. 1E). Baseline and post-CIE water consumption did not differ irrespective of genotype or treatment group (Table 1). As previously reported [16,35,36], air controls exhibited an increase in EtOH consumption compared to pre-exposure baseline (Fig. 1D). One plausible explanation for this increase is that pre-vapor drinking experience, followed by a period of forced abstinence (i.e., during the air exposure), produces a modest deprivation-like increase in EtOH consumption [14].

To explore potential neural correlates of the GluN2A KO EtOH drinking phenotype, we next examined synaptic transmission in BLA neurons of these mice after CIE, using ex vivo slice physiology, as previously described [24,25]. Naïve cohorts of GluN2A KO and C57BL/6 J mice were exposed to CIE, as described above, and 72–96 h later anaesthetized with isoflurane and decapitated (for a schematic of the experimental design and timeline, see Fig. 2A and B). The brain was quickly removed and transferred to ice-cold, oxygenated sucrose artificial cerebrospinal fluid (ACSF) [(in mM): 194 sucrose, 20 NaCl, 4.4 KCl, 2 CaCl₂, 1 MgCl₂, 1.2 NaH₂PO₄, 10 glucose, and 26 NaHCO₃ saturated with 95% O₂/5% CO₂]. Three-hundred µm thick coronal slices containing the BLA were prepared using a tissue slicer Leica vibratome 1200S (Buffalo Grove, IL, USA). Slices were incubated in a heated holding chamber maintained at approximately 32 °C containing normal ACSF [(in mM): 124 NaCl, 4.4 KCl, 2 CaCl₂, 1.2 MgSO₄, 1 NaH₂PO₄, 10.0 glucose, and 26.0 NaHCO₃ saturated with 95% O₂/5% CO₂]. Slices were then transferred to a submerged recording chamber (Warner Instruments, Hamden, CT, USA) and perfused with heated (28–30 °C), oxygenated ACSF maintained at ~2 mL/min and incubated for 30 min.

Principal neurons in the BLA were identified using infrared differential interference contrast enhanced microscopy (Olympus, Waltham, MA, USA). Borosilicate electrodes with a pipette resistance between 4–6 MΩ were pulled with a Flaming-Brown micropipette puller (Sutter Instruments, Novato, CA, USA). Whole-cell patch clamp recordings were performed in voltage clamp mode with a cesium gluconate based internal solution [(in mM): 117 Cs-gluconate, 20 HEPES, 0.4 EGTA, 5 TEA, 2 MgCl₂·6H₂O, 4 disodium ATP, and 0.4 disodium GTP, pH =7.3, 290 mOsm]. QX-314.HCl was added at 1 mg/ml to block voltage gated sodium channels.

To isolate NMDAR current, neurons were held at +40 mV in the presence of picrotoxin (25 µM, Abcam, Cambridge, MA, USA) and NBQX disodium salt (10 µM, Abcam) to block GABA-A receptors and AMPA/kainate receptors, respectively. To record evoked NMDAR-mediated excitatory postsynaptic current (NMDAR-EPSC), a bipolar nichrome electrode was placed in the BLA dorsal to the recorded neurons. NMDAR-EPSCs were evoked at 0.167 Hz for 100–150 micro-seconds using current pulses. After establishing a stable baseline over 5 min, 2 µM Ro 25–6981 maleate (Tocris, Minneapolis, MN, USA) was bath applied for 10 min. The peak amplitude of the evoked response was normalized to the baseline period.

Temporal summation of NMDAR was examined by measuring the responses to bursts of stimulation (10 pulses) at 10 Hz. The amplitude of the subsequent pulses was normalized to the 1st pulse. Decay kinetics for NMDAR-EPSC was calculated by fitting the average normalized baseline and post-drug responses using 2 exponential simplex function in Clampfit 10.6 and the weighted Tau (Tw) was calculated using the equation: $Tw = (T1 * A1) + (T2 * A2)$; where T1 and T2 are the 2 time constants and A1 and A2 are their relative amplitudes. Input resistance, holding current and series resistance were monitored throughout the experiments and cells in which changes in series resistance were greater than 20% were excluded from the analysis. All signals were acquired using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA), digitized at 10 kHz and low-pass filtered at 3 kHz and analyzed in pClamp 10.6.

Results showed there was no difference between CIE- and air-exposed GluN2A KO mice in the pooled normalized amplitude of NMDAR-mediated currents (*i.e.*, temporal summation) at BLA synapses (Fig. 2C and D). The decay kinetics of NMDAR-mediated EPSCs was also similar between the groups (Fig. 2E). Our next step was to assess whether the function of the GluN2B-containing NMDARs was affected by CIE in the GluN2A KO mice. The time course and overall inhibition of NMDAR-mediated EPSCs following application of the GluN2B antagonist, Ro 256981, was not affected by CIE-exposure, as compared to air-exposure, in these mice (Fig. 2F–H). Finally, the decay kinetics of Ro 25–6981 -induced inhibition were also no different between CIE- and air-exposed GluN2A KO mice (Fig. 2I and J).

For comparison, BLA recordings were also conducted in CIE- and air-exposed C57BL/6 J mice. There were no differences observed in the neurophysiological measurements made, with the exception that the decrease in decay kinetics of NMDAR-mediated EPSCs produced by Ro 25–6981 was attenuated in CIE-exposed counterparts (Supplementary Fig. S1). Given this effect of the GluN2B antagonist was absent in the GluN2A KO mice, these data offer initial evidence that GluN2A deletion may perturb CIE-induced adaptations in other NMDAR subtypes within the BLA. The contribution of GluN2B to NMDAR-mediated transmission observed in wildtype mice was greater than that from GluN2A KO counterparts. In contrast to our findings, others have reported a greater contribution of GluN2B to NMDAR-mediated transmission in GluN2A knockout mice [35–37]. Of note, however, these earlier studies examined brain regions other than the amygdala (*i.e.*, the dorsal striatum and bed nucleus of the stria terminalis). As such, in addition to methodological and species differences, there could be as yet unidentified regional differences accounting for these apparent discrepancies. The conclusions regarding the contribution of NMDAR subunits remains tentative in lieu of replication and additional investigation.

To reiterate, the major findings of these various behavioral and physiological results were that GluN2A KO mice did not exhibit increases in EtOH drinking or alterations in Ro 25–6981 mediated alteration of decay kinetics of NMDAR-mediated synaptic transmission following CIE exposure. These data add to growing support for GluN2A-containing NMDARs as an important mechanism underlying the neural and molecular adaptations that drive EtOH dependence. For instance, the current data align with rodent work showing that selection of high two-bottle choice EtOH preference in (heterogeneous stock-collaborative cross) mice and (high alcohol drinking) rats associates with the *Grin2a* gene locus [7,8] and differential nucleus accumbens expression of *Grin2a* [26]. Speaking to the translational relevance of these observations in rodents, variation in the human *GRIN2A* gene has been associated with alcohol dependence [4–6], but not alcohol cue-induced prefrontal cortical (PFC) BOLD activation [30].

We have previously reported that GluN2A KO mice show normal two-bottle choice EtOH drinking and behavioral (stimulant, ataxic, hypothermic, sedative/hypnotic) responses to acute EtOH challenge, but loss of EtOH conditioned place preference and attenuated sedative/hypnotic responses to co-administration of the NMDAR antagonist, MK801, and EtOH [27–29]. In terms of repeated EtOH exposure, there are reports of upregulation of

GluN2A-mediated synaptic transmission and protein levels in the medial PFC, orbitofrontal cortex, BLA, and bed nucleus of the stria terminalis [9–12], while other studies find no change or downregulation in cortex, nucleus accumbens or hippocampus [11,31–34]. While significant variation, not only in brain region examined, but also the type and duration of exposure and the timing of the analysis relative to withdrawal complicates direct comparison of these findings, taken together they indicate dynamic alterations in GluN2A in response to chronic EtOH.

In this context, in a clear parallel with the current data, we recently found that GluN2A KO mice did not develop tolerance to the EtOH's sedative/hypnotic challenge after CIE exposure [13]. Adding to these data, we show here an absence of the marked alterations in synaptic transmission that have been repeatedly documented in the BLA and proposed as a critical substrate for EtOH-induced changes in, for example, anxiety-like behavior [17]. Tolerance is one of the hallmarks of EtOH-dependence and could explain why we failed to observe a post-CIE induced increase in EtOH consumption and preference, and alterations in NMDAR-mediated synaptic transmission in NR2A KO mice. Whether this physiological phenotype also contributes to the loss of CIE-induced EtOH-dependence we found in the current study remains to be fully investigated in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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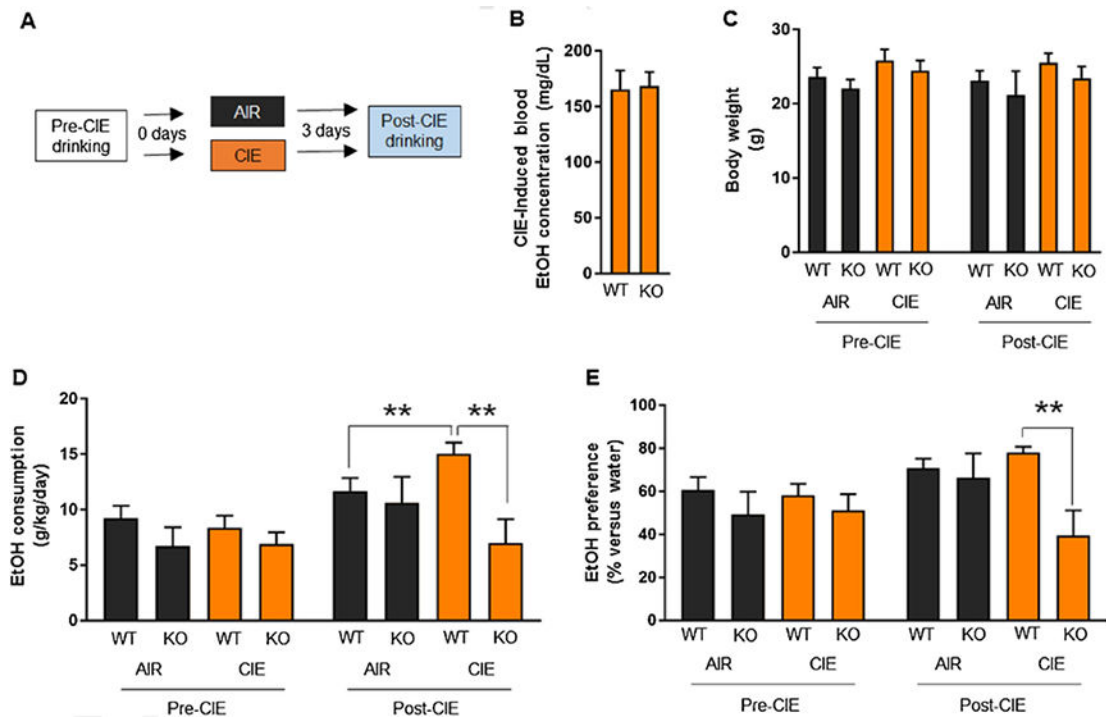


Fig. 1. Absence of CIE-potential of EtOH drinking in GluN2A knockout mice.

(A) Schematic of the timeline for pre-CIE EtOH drinking, CIE (or air) exposure and post-CIE drinking in GluN2A knockout (KO) mice and their wild-type (WT) litter mate controls.

(B) CIE-induced blood EtOH concentration (BECs) did not differ between genotypes.

(C) Body weight did not differ between genotypes, irrespective of CIE-exposure.

(D) Pre-CIE EtOH consumption did not differ between genotypes. Post-CIE EtOH consumption was

significantly higher in CIE-exposed WT mice, as compared to either air-exposed WT mice or CIE-exposed KO mice.

(E) Pre-CIE EtOH preference did not differ between genotypes. Post-CIE EtOH preference was significantly higher in CIE-exposed WT mice relative to

CIE-exposed KO mice. Data are mean \pm SEM. $n = 6-12$ per genotype per treatment group.

* $P < .05$ WT AIR versus WT CIE or WT CIE versus KO CIE.

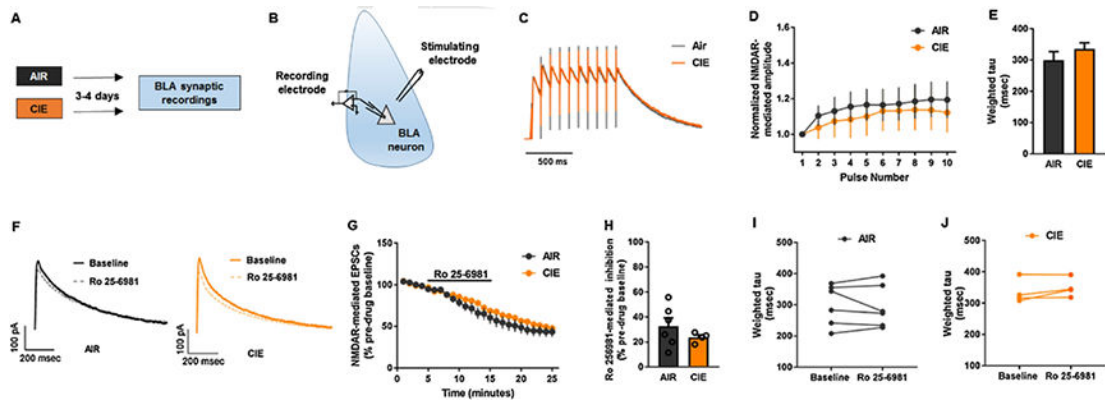


Fig. 2. Absence of CIE effects on NMDAR-mediated synaptic transmission in the BLA of GluN2A KO mice.

(A) Schematic of the timeline for CIE (or air) exposure and post-CIE recordings of synaptic transmission in the BLA of GluN2A knockout (KO) mice. (B) Cartoon of procedure for synaptic recordings in BLA principal neurons. Example normalized traces (C) and pooled normalized amplitude data (D) showing similar temporal summation (from 10 stimulation pulses at 10 Hz) of NMDAR-mediated currents in CIE- and air-exposed GluN2A KO mice ($n = 11-15$ cells per treatment group from 3 to 4 mice). (E) Pooled data showing similar decay kinetics of NMDAR-mediated excitatory post-synaptic currents (EPSCs) in CIE- and air-exposed GluN2A KO mice ($n = 4-6$ cells per treatment group). (F) Example traces showing similar NMDAR-mediated EPSCs after application of the GluN2B antagonist, Ro 25-6981 (2 μ M), in CIE and air-exposed GluN2A KO mice ($n = 4-6$ cells per treatment group from 3 to 4 mice per genotype). (G) Similar time course of Ro 25-6981-induced reduction in NMDAR-mediated EPSC amplitude in CIE and air-exposed GluN2A KO mice. (H) Averaged data showing the reduction in NMDAR-mediated EPSC amplitude during minutes 10–15 after Ro 25-6981 application. Paired data showing the absence of effects of Ro 25-6981 on decay kinetics of NMDAR-mediated excitatory post-synaptic currents (EPSCs) in air-exposed (I) and CIE-exposed (J) GluN2A KO mice ($n = 4-6$ cells per treatment group from 3 to 4 mice per genotype). Data are mean \pm SEM.

Table 1.

Water consumption during two-bottle choice experiment. Neither genotype or CIE exposure significantly altered water consumption (n = 6–12 per genotype per treatment group). Data are mean \pm SEM.

Baseline water consumption (g/day)				Post-CIE water consumption (g/day)			
WT		NR2A KO		WT		NR2A KO	
Air	CIE	Air	CIE	Air	CIE	Air	CIE
1.1 \pm 0.2	1.2 \pm 0.2	1.2 \pm 0.2	1.6 \pm 0.3	0.9 \pm 0.1	0.9 \pm 0.1	0.8 \pm 0.2	1.6 \pm 0.3

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