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Knock-in mice expressing an ethanol-resistant GluN2A NMDA receptor subunit show altered responses to ethanol

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Abstract

Background: N-methyl-D-aspartate receptors (NMDARs) are glutamate-activated, heterotetrameric ligand-gated ion channels critically important in virtually all aspects of glutamatergic signaling. Ethanol inhibition of NMDARs is thought to mediate specific actions of ethanol during acute and chronic exposure. Studies from our laboratory, and others, identified ethanol-sensitive sites within specific transmembrane (TM) domains involved in channel gating as well as those in sub-domains of extracellular and intracellular regions of GluN1 and GluN2 subunits that affect channel function. In this study, we characterize for the first time the physiological and behavioral effects of ethanol on knock-in mice expressing a GluN2A subunit that shows reduced sensitivity to ethanol.

Methods: A battery of tests evaluating locomotion, anxiety, sedation, motor coordination and voluntary alcohol intake were performed in wild-type mice and those expressing the GluN2A A825W knock-in mutation. Whole-cell patch clamp electrophysiological recordings were used to confirm reduced ethanol sensitivity of NMDAR-mediated currents in two separate brain regions (mPFC and the cerebellum) where the GluN2A subunit is known to contribute to NMDAR-mediated responses.

Results: Male and female mice homozygous for the GluN2A(A825W) knock-in mutation showed reduced ethanol inhibition of NMDAR-mediated synaptic currents in mPFC and cerebellar neurons as compared to their wild-type counterparts. GluN2A(A825W) male but not female mice were less sensitive to the sedative and motor-incoordinating effects of ethanol and showed a rightward shift in locomotor stimulating effects of ethanol. There was no effect of the mutation on ethanol-induced anxiolysis or voluntary ethanol consumption in either male or female mice.

Conclusions: These findings show that expression of ethanol-resistant GluN2A NMDARs results in selective and sex-specific changes in the behavioral sensitivity to ethanol.

Keywords

NMDA receptor; GluN2A subunit; alcohol sensitivity; cerebellum; medial prefrontal cortex; alcohol consumption

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Introduction

NMDARs play a fundamental role in normal neuronal function, survival and development as well as synapse formation, synaptic transmission and plasticity (Traynelis, Wollmuth et al. 2010). Disruption of their function by disease, drugs and ethanol has profound effects on brain function and behavior. Thus, it is critical to understand how ethanol inhibits channel function and what processes regulate the ethanol sensitivity of these receptors. Acutely, behaviorally relevant concentrations of ethanol inhibit currents through NMDARs and significant changes in the expression and localization of these channels are observed in animals and humans that are ethanol dependent (Szumlinski and Woodward 2014). During withdrawal from chronic ethanol, hyperactivation of up-regulated NMDARs contributes to behavioral excitability and may drive aspects of craving and relapse to ethanol drinking (Tzschentke and Schmidt 2003, Hwa, Besheer et al. 2017).

Site-directed mutagenesis studies have identified ethanol-sensitive sites within specific transmembrane (TM) domains involved in channel structure and gating and in extracellular and intracellular regions of GluN1 and GluN2 subunits that modulate channel function (Ronald, Mirshahi et al. 2001, Honse, Ren et al. 2004, Ren, Salous et al. 2007, Ren, Salous et al. 2008, Xu, Smothers et al. 2015, Smothers and Woodward 2016). In particular, these studies identified a phenylalanine in the TM3 domain of the GluN1 subunit (F639) and residues in the TM4 domain of GluN2 subunits including alanine 825 (A825) in GluN2A that influence the ethanol inhibition of NMDARs (Honse, Ren et al. 2004, Xu, Smothers et al. 2015, Smothers and Woodward 2016). For example, the ethanol inhibition of recombinant NMDARs is dramatically reduced when alanine is substituted for phenylalanine 639 (F639A) in the GluN1 subunit (Ronald, Mirshahi et al. 2001) or if tryptophan replaces alanine at position 825 in the GluN2A (A825W) subunit (Honse, Ren et al. 2004, Smothers and Woodward 2016). Based on these findings, we generated the first line of knock-in mice, GluN1(F639A), that express ethanol-resistant NMDARs (den Hartog et al. 2013) and results from these studies suggest that NMDARs mediate discrete actions of ethanol and regulate patterns of ethanol drinking in a protocol-dependent manner. Based on these findings, we generated and have begun testing additional genetically modified mice that express ethanol resistant GluN2 subunits. In this study, we provide the first behavioral characterization of a line of knock-in mice that express the A825W mutation in the TM4 domain of the GluN2A subunit. After validating that neuronal NMDA responses in GluN2A(A825W) mice were less sensitive to ethanol, we examined whether this mutation would alter behaviors in male and female mice commonly associated with ethanol including sedation, motor activity and coordination, anxiety and drinking.

Materials and Methods

Subjects

To generate GluN2A(A825W) mice, a portion of a bacterial artificial chromosome (clone bmQ274M03; Source Bioscience, Nottingham, UK) encompassing exons 11 and 12 and intervening introns was subcloned into the pBluescript vector followed by insertion of a floxed neomycin cassette 643 bp past exon 11 (Figure 1). Site-directed mutagenesis was used to replace the alanine codon (GCT) at position 825 with a tryptophan codon (TGG).

The pBS-GRIN2A-A825W-neo construct was linearized with NotI, ethanol precipitated, resuspended in TE and electroporated into R1 ES cells (Nagy, Rossant et al. 1993) as described previously (Homanics, Ferguson et al. 1997). G418 and ganciclovir resistant clones were screened for gene targeting by Southern blot analysis of BamHI digested DNA using a 5' probe that was external to the gene targeting vector. Correctly targeted ES cell clones were injected into C57BL/6NcrJ blastocysts (Charles River Laboratories, Wilmington, MA) and chimeric offspring were mated to Ella-Cre females (catalog number 003724; mixed C57BL/6J ; C57BL/6N background; Jackson Labs, Bar Harbor, ME) to remove the Neo cassette. Cre+, neo- F1 KI mice were crossed with C57BL/6J mice (catalog number 000664; Jackson Labs, Bar Harbor, ME) to generate Cre- KI heterozygous mice that were then used for breeding. Mice from Het X Het crosses were genotyped by PstI digestion of polymerase chain reaction from tail-derived DNA. Polymerase chain reactions spanning exon 11 included forward (GCCAAAGGCCAGCAAAGCTCAAGA) and reverse (AACTGCCCTGTGTTGTTCTGCACCT) primers. Age-matched littermate male and female WT and A825W homozygous mutant mice were used in all studies. After weaning, mice were housed with *ad libitum* access to rodent chow (Purina 5V75, Lab Diet, St. Louis, MO) and water with 12-h light/dark cycles (lights on at 9:00 AM) and standard corn cob bedding. Mice were tested on multiple behavioral assays with at least one-week recovery between ethanol administrations. Voluntary alcohol consumption as well as electrophysiological recordings were performed in cohorts of naïve mice separate from those used in behavioral assays. Mice that participated in behavioral assays were grouped housed and separated only during acclimation periods and testing. Acclimation periods consisted of 30 min exposure to the environmental conditions (i.e. room lighting, background noise) of every specific test. All experiments using mice were approved by the MUSC or the University of Pittsburgh Institutional Animal Care and Use Committees and conformed to NIH guidelines for the use of animals in biomedical research.

Electrophysiology

Coronal (300 µm) and sagittal slices (200–250 µm) containing the prefrontal cortex or cerebellum (intravermal), respectively were prepared from WT and A825W male and female mice (8–16 weeks old). Following anesthesia with isoflurane, animals were euthanized and brains were removed and placed in ice-cold oxygenated (95% O₂, 5% CO₂) sucrose-containing buffer containing (in millimolar) 200 sucrose, 1.9 KCl, 1.2 NaH₂PO₄, 6 MgCl₂, 0.5 CaCl₂, 0.4 ascorbate, 10 glucose, 25 NaHCO₃, adjusted to 305 to 315 mOsm. Slices were cut using a VT1000 vibrating microtome (Leica Biosystems, Buffalo Grove, IL) with a double-walled chamber through which cooled (1–4°C) solution was circulated. Slices were collected and transferred to a chamber filled with aCSF solution containing (in mM): NaCl (125), KCl (2.5), NaH₂PO₄ (1.4), CaCl₂ (2), MgCl₂ (1.3), glucose (10), ascorbic acid (0.4) and NaHCO₃ (25); osmolarity 310–320 mOsm. After at least 1 hour incubation at room temperature, whole-cell patch-clamp recordings were acquired using an Axon 700B amplifier (Molecular Devices, San Jose, CA) controlled by AxographX software (Axograph, Sydney, AUS). Recording pipettes (resistance of 2–3 MΩ) were filled with internal solution containing: (in mM): 120 CsCl, 2 MgCl₂, 10 HEPES, 2 EGTA, 2 Na₂ATP, 0.3 Na₃GTP, (pH 7.25–7.35). Recordings of NMDAR-EPSCs were performed in the presence of 50 µM picrotoxin and 10 µM 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo(f)quinoxaline-7-

sulfonamide (NBQX, Abcam Biochemicals, Cambridge, MA) to block GABA_A and AMPA receptors, respectively. For prefrontal cortex recordings, deep-layer pyramidal neurons in the prelimbic sub-region were held at +40 mV and NMDAR EPSCs were evoked by electrical stimulation (300–500 μ A) using a glass micropipette placed about 150 μ m from the soma of the patch-clamped pyramidal PFC neuron. For cerebellar Purkinje neurons, climbing fiber synapses were stimulated in the granule cell layer about 150 μ m from the soma of the whole-cell voltage-clamped (–70 mV) Purkinje neuron. The recording aCSF was magnesium free and was supplemented with 25 μ M glycine to facilitate NMDAR activation (Zamudio-Bulcock, Homanics et al. 2018). 50 μ M DL-2-Amino-5-phosphonopentanoic acid (DL-APV; Abcam Biochemicals, Cambridge, MA) was used to verify NMDAR-mediated currents.

Behavioral Testing

Latency/Duration of Loss of Righting Reflex—Mice were injected with ethanol (3.5 g/kg; i.p.) and the latency to loss of righting reflex (LORR) was measured as the time from injection until mice were unable to right themselves within 30 s of being placed in a supine position. The duration of the LORR was measured from the onset of the LORR until the time that mice regained their ability to right themselves twice within a 30s period.

EtOH Metabolism—Mice were injected with ethanol (3.5 g/kg; ip) and blood samples were taken from the retro-orbital sinus at 30, 120 and 240 min post-injection. The study was repeated a week later and samples were taken at 60, 120 and 180 min post-injection. Blood ethanol concentration (BEC) values were determined with a colorimetric alcohol oxidase assay (Pava, Blake et al. 2012) and were expressed as milligram of ethanol per deciliter of blood.

Motor coordination—One day prior to testing, mice were trained to remain on a fixed speed (5 rpm) rotating rod (Ugo Basile, Gemonio, Italy) using a 1 minute trial period. On the testing day, mice were given i.p. ethanol (2.5 g/kg) and were for rotarod performance 20 minutes later and every ten minutes thereafter. In between trials, mice were placed back in their homecage.

Locomotor excitation/inhibition—Prior to ethanol administration, mice were placed in an opaque open field box (40 $\times$ 40 $\times$ 40 cm square) and spontaneous locomotor activity (measured as total distance traveled) was recorded using Anymaze video tracking software (Stoelting Co.). All locomotor activity testing sessions were done one week apart and mice were injected 5 minutes before being placed in the activity chamber for 10 min. Baseline locomotor activity was first tested in all mice following treatment with saline. Mice were then tested weekly following treatment with saline or different concentrations of ethanol (0.75, 1.5, or 3.0 g/kg; i.p.) in a Latin-Square design so that each mouse received all doses.

Anxiety—The elevated zero maze (Med-Associates, St. Albans, VT) was used to assess the anxiolytic effects of ethanol. Mice were administered either saline or an anxiolytic dose of ethanol (1.25 g/kg; i.p.) and 5 min later placed in the closed arm of the maze. Anymaze

video tracking software was used to assess the number of entries and time spent in the open and closed arms in a testing period of 5 min.

Ethanol drinking paradigm.

Two-Bottle Choice 24 h Intermittent Access: Prior to the start of drinking, mice were acclimated to single housing for a week and assigned a specific location in the free-standing rack for the duration of the test. Mice were given access to two bottles of water for 24 h and on the next day, bottles were replaced with ones containing either 20% ethanol or water. This pattern of access to ethanol was alternated every 24 h with 3 weekly drinking sessions starting on Monday, Wednesday and Friday. The placing of the ethanol bottle was alternated each ethanol drinking session to control for side preferences. Every day one empty cage was set up with 1 water and 1 ethanol bottle to monitor whether there was loss of fluid due to changes in room conditions or spillage. This was negligible and not corrected for. Fluid consumed was recorded in ml and ethanol consumption in g/Kg was calculated using mice weights recorded every Monday.

Statistical Analysis

Data is shown as mean $\pm$ standard error of the mean and statistical analyses were performed using Graphpad prism 8 (Graphpad Software Inc., La Jolla, CA). SPSS data analysis software (IBM Corp, Armonk, NY; version 24) was used, when indicated, to adjust for data covariance and simultaneously assess the interaction of the variables (sex, genotype and time). Repeated measures ANOVA, Student's t-test were used to evaluate differences between groups, the test used for each data set is indicated in the results section. For data sets that did not pass a Gaussian distribution test, non-parametric Mann-Whitney and Wilcoxon signed rank tests were performed as indicated.

Results

NMDAR-mediated currents are less sensitive to acute ethanol in A825W mice

Results from cell culture studies demonstrate that recombinant NMDARs containing the A825W GluN2A subunit are less sensitive to ethanol than wild-type GluN2A NMDARs (Salous, Ren et al. 2009, Smothers and Woodward 2016). In this study, we extend these findings to mice engineered to globally express the GluN2A (A825W) subunit. Importantly, baseline parameters of NMDAR-mediated currents, namely rise-time and decay-time were not affected by genotype in mPFC neurons (females, rise-time: WT 4.4 ± 0.33 ms, $n=8$; A825W 5.7 ± 0.97 ms, $n=12$; $p>0.05$; decay-time: WT 32.17 ± 2.53 ms, A825W 29.71 ± 2.54 ms, $p>0.05$; males, rise-time: WT 5.78 ± 0.77 ms, A825W 4.78 ± 0.16 ms, $p>0.05$; decay-time: WT 42.46 ± 2.59 ms, A825W 41.70 ± 4.06 ms, $p>0.05$; by Student's t-test) and in cerebellar Purkinje neurons (females, rise-time: WT 2.18 ± 0.36 ms, A825W 2.06 ± 0.35 ms, $p>0.05$; decay-time: WT 12.31 ± 1.13 ms, A825W 9.14 ± 1.36 ms, $p>0.05$; males, rise-time: WT 1.5 ± 0.18 ms, A825W 1.22 ± 0.19 ms, $p>0.05$; decay-time: WT 11.04 ± 1.2 ms, A825W 10.17 ± 1.56 ms, $p>0.05$; by Student's t-test). As shown in Figure 2, bath application of 44 mM ethanol (equivalent to a blood ethanol concentration of 200 mg/dL) significantly inhibited NMDAR-mediated synaptic currents evoked in mPFC neurons from WT mice (Figure 2A males, 11.82 ± 1.86 % decrease from baseline amplitude, $t=6.356$,

df=7, $p < 0.05$; Figure 2C females, $11.26 \pm 4\%$ decrease from baseline, $t=2.812$, df=7, $p < 0.05$, by one sample t test). However, in slices from A825W animals, 44 mM ethanol had no significant effect on NMDAR-mediated currents (males, $-0.52 \pm 3.32\%$ change from baseline, $t=0.1576$, df=5, $p > 0.05$; females, $4.2 \pm 4.1\%$ decrease from baseline, $t=1.022$, df=10, $p > 0.05$; by one sample t test). In cerebellar Purkinje neurons from male WT mice, NMDAR-mediated currents elicited via climbing fiber stimulation were significantly inhibited by 44 mM ethanol ($29.13 \pm 6.75\%$ decrease from baseline amplitude, $t=4.316$, df=8, $p < 0.05$, Figure 2B). NMDAR-mediated currents in Purkinje neurons from male A825W mice were not inhibited by 44 mM ethanol ($3.25 \pm 4.03\%$ decrease from baseline for males $t=0.8061$, df=5, $p > 0.05$; by one sample-t test). In female WT mice, cerebellar NMDAR-EPSCs showed similar sensitivity to 44 mM EtOH as their male counterparts ($29.46 \pm 6.14\%$ decrease from baseline for WT females, $t=4.796$, df=6, $p < 0.05$, by one sample t test, Figure 2D). NMDAR-EPSCs from female A825W mice showed a small ($5.77 \pm 1.95\%$), but significant inhibition by ethanol ($t=2.958$, df=5, $p > 0.05$ by one sample t-test) as compared to baseline recordings and the magnitude of this inhibition was statistically different from that measured in WT female mice (Student's t-test $p < 0.05$).

Sedative/hypnotic effects of ethanol in male and female A825W mice

The sedative/hypnotic effects of ethanol were tested by measuring the time to lose and regain the righting reflex following an i.p. injection of 3.5 g/kg ethanol. The latency to the loss of righting reflex (LORR) was not different between male WT and A825W mice (WT: 94.92 ± 4.76 s; A825W: 99.25 ± 5.26 s, $t=0.6117$, df=23, $p > 0.05$ by Student's t-test, Figure 3A). However, LORR duration was significantly decreased in A825W male mice when compared to WT (WT: 136.2 ± 14.62 min; A825W: 89.77 ± 8.4 min, $t=2.806$, df=23, $p < 0.05$ by Student's t-test, Figure 3B). In contrast, females showed no genotypic difference in the latency to LORR (WT: 80 ± 3.42 s; A825W: 85.50 ± 3.37 s, $t=1.146$, df=18, $p > 0.05$ by Student's t-test, Figure 3D) or its duration (WT: 154.3 ± 21.28 min; A825W: 151.2 ± 20.82 min, $t=0.1029$, df=19, $p > 0.05$ by Student's t-test, Figure 3E). Ethanol metabolism, as measured by the significant decrease in blood ethanol concentration (BEC) values over time (males: $F(DFn,DFd): F(2,375,52.25)$, $p < 0.05$; females: $F(4,84)=4.858$, $p < 0.05$; by two-way repeated measures ANOVA), was not affected by the A825W mutation in either male or female mice (males: $p > 0.05$, females $p > 0.05$, by two-way ANOVA, repeated measures, effect of time $\times$ genotype; for times post-injection= 30,60,90,180,240 min, in both male and females, $p > 0.05$ by sidak's multiple comparison test; Figures 3C and 3F). However, following injection of ethanol, BEC values in female WT and A825W were slightly but significantly lower than those measured in male mice (A825W: time post-injection, 30 min, 60 min: $p < 0.05$; WT: time post-injection 120 min: $p < 0.05$, Figure 3C vs Figure 3F by Mixed-effects model analysis, effect of sex, SPSS data analysis software).

Alcohol impairment of motor coordination is reduced in male A825W mice

The motor incoordinating effects of ethanol were tested using a fixed speed (5 rpm) rotarod. In WT mice, an i.p. injection of 2.5 g/kg ethanol produced profound ataxia evidenced by their inability to remain on the rotarod. The time course for the latency to fall was significantly different between male WT and male A825W ($F(12, 216) = 2.291$, $p < 0.05$ by two-way ANOVA, Figure 4A), with A825W male mice showing a faster recovery from

ethanol-induced motor incoordination. On average, male WT mice recovered to baseline motor performance approximately 100 minutes after the ethanol injection (7 out of 10 mice had recovered to 100% of baseline performance); from this time point onwards the average latency to fall was not significantly different from 60 seconds -the entire duration of the trial- ($t=1.887$, $df=9$, $p>0.05$ by one sample t-test, Figure 4A WT). In contrast, while male A825W mice also showed loss of rotarod performance immediately following ethanol injection, the average latency to fall was already statistically undistinguishable from 60 seconds at 60 minutes after alcohol injections, with 6 of 10 mice staying on for the duration of the trial ($t=2.090$, $df=9$, $p>0.05$ by one sample t-test, Figure 4A, A825W). In female mice, the time to recovery of rotarod performance following the ethanol injection was not significantly different from that of male WT mice ($F(1,16)=0.9718$, $p>0.05$ by two-way ANOVA, Figure 4A WT data vs Figure 4B WT data) and was not affected by the A825W mutation ($F(12, 192) = 0.3691$, $p<0.05$ by two-way ANOVA, Figure 4B).

The locomotor effects of ethanol are altered in male A825W mice

Alcohol has biphasic effects on locomotor activity with low doses producing stimulation reflected in rodents as an increase in exploratory behavior and as psychomotor activation in humans. At higher doses, alcohol reduces locomotion and produces sedation in humans (Hendler, Ramchandani et al. 2013). Following an i.p. injection of saline, open field activity, measured as total distance traveled, was not different between WT and A825W for male or female mice (males: $p>0.05$ by Mann-Whitney test, $n=19-20$; females: $p>0.05$, Student's t-test, $n=9-10$; Figures 5A and 5C). Total distance travelled after alcohol injections was normalized to each subject's distance travelled after saline injection and expressed as percent of saline. Averaged normalized values were then compared to 100 using the one sample t-test for each individual dose. Subsequently, Kruskal-Wallis test was used to compare locomotion across doses and genotype (Figures 5B and 5D). When challenged with a low dose of alcohol (0.75 g/kg), male WT mice showed a significant increase in total distance travelled as compared to activity following saline (130.5 ± 8.13 % of baseline, $t=3.750$, $df=19$, $p<0.05$ by one sample t-test; Figure 5B). This same dose had no effect on locomotion in male A825W mice (109.5 ± 9.30 % of baseline, $t=0.9729$, $df=18$, $p>0.05$ by One sample t-test, Figure 5B). Following 1.5 g/kg ethanol, there was no significant difference in the total distance travelled by WT male mice as compared to saline (89.24 ± 8.19 % of baseline, $t=1.314$, $df=17$, $p>0.05$ by one sample t-test) while locomotor activity in A825W male mice was significantly increased by this ethanol dose (130.4 ± 11.51 % of baseline, $t=2.642$, $df=17$, $p<0.05$ by One sample t-test). Additionally, the normalized distance travelled following the 1.5g/kg ethanol dose was significantly different between WT and A825W male mice ($p<0.05$ by Kruskal-Wallis test). The highest ethanol dose tested, 2 g/kg, significantly decreased the total distance travelled in WT male mice (76.30 ± 10.15 % of baseline, $t=2.336$, $df=19$, $p<0.05$ by one sample t-test) but had no significant effect in on A825W mice (85.58 ± 11.42 % of baseline, $t=1.263$, $df=18$, $p=0.16$, by one sample t-test; Figure 5B).

In contrast to the results found with male mice, there was no genotypic difference in the effect of ethanol on locomotor activity in female mice (Figure 5D). In WT females, the lowest ethanol dose did not increase the total distance travelled (134.7 ± 16.81 % of

baseline, $t=2.065$, $df=7$, $p>0.05$ by one sample t-test). Nonetheless, ethanol significantly increased the total distance travelled in female A825W mice (142.9 ± 14.87 % of baseline, $t=2.886$, $df=9$, $p<0.05$ by one sample t-test), and this change in locomotion did not differ from that in WT females ($p>0.05$, by Kruskal-Wallis test). At 1.5 g/kg, ethanol did not significantly change the average total distance travelled by female WT or A825W mice (WT: 98.96 ± 16.37 % of baseline, $t=0.06348$, $df=8$, $p>0.05$; A825W: 119.5 ± 14.46 % of baseline, $t=1.350$, $df=9$, $p>0.05$ by one sample t-test). Similar to WT male mice, 2g/kg ethanol significantly decreased the total distance travelled by female WT and A825W mice (WT: 62.94 ± 9.94 % of baseline, $t=3.730$, $df=8$, $p<0.05$; A825W: 70.68 ± 8.18 % of baseline, $t=3.585$, $df=9$, $p<0.05$ by one sample t-test).

The A825W mutation does not alter the anxiolytic effect of ethanol

The anxiolytic effects of ethanol were tested in male and female WT and A825W mice using the elevated zero-maze. Each mouse received either saline or ethanol and was tested only once on the zero-maze to prevent habituation. As expected, following a 1.25 g/kg ethanol injection, WT male and female mice spent significantly more time in the open areas of the maze compared to those injected with saline (WT males: 14 ± 6.8 % of total time after saline vs 44 ± 5.1 % of total time after 1.25 g/kg ethanol, $Z=2.47$; WT females: 18.72 ± 2.23 % of total time after saline vs 45.89 ± 5.02 % of total time after 1.25 g/kg ethanol $Z=2.28$, $p<0.05$; by Kruskal-Wallis test; Figures 6A and 6C). Similarly, male and female A825W mice injected with 1.25 g/kg ethanol spent significantly more time in the open areas of the maze when compared to saline injected animals (A825W males: 16 ± 4 % of total time after saline vs 48 ± 5.8 % of total time after 1.25g/kg ethanol, $Z=5.28$, A825W females: 17.50 ± 2.5 % of total time after saline vs 42.74 ± 9.36 % of total time after 1.25g/kg ethanol, $Z=2.19$, $p<0.05$; by Kruskal-Wallis test, Figures 6A and 6C). The total distance travelled following the 1.25 g/kg ethanol injection for both WT and A825W male and female mice (Figures 6B and 6D) when compared to saline was not significantly different (WT male: $Z=0.96$, $p>0.05$; A825W male: $Z=1.92$, $p>0.05$, Figure 6B: WT female: $Z=0.99$, $p>0.05$; A825W female: $Z=1.65$, $p>0.05$; by Kruskal-Wallis test) likely due to the between-subjects design of the anxiety testing.

The A825W mutation does not affect ethanol consumption

To examine whether expression of ethanol-insensitive GluN2A-containing NMDARs influences voluntary ethanol consumption, we examined drinking using an intermittent access model. Both male and female A825W mice consumed 20% alcohol at similar levels to their WT counterparts (males: $p>0.05$, $F(1, 10) = 0.58$; females: $p>0.05$, $F(1, 11) = 1.031$, two-way ANOVA; Figures 7A and 7C). Alcohol preference was not affected by the A825W mutation in male or female mice (males: $p=0.06$, $F(1, 10) = 4.38$; females: $p=0.98$, $F(1, 11) = 0.0006731$, two-way ANOVA; Figures 7B and 7D). In line with previous reports, female WT and A825W mice showed significantly higher levels of alcohol consumption compared to their male counterparts ($p<0.0001$, three-way ANOVA mixed-effects analysis, comparison by sex, $F(1, 252) = 412.0$). Similarly, alcohol preference was significantly higher in female mice as compared to males ($p<0.0001$, three-way ANOVA mixed-effects analysis, comparison by sex, $F(1, 252) = 161.4$).

Discussion

Previous studies using recombinant expression systems showed that GluN2A(A825W) containing NMDARs are markedly less sensitive to ethanol inhibition than wild-type receptors (Honse, Ren et al. 2004, Xu, Smothers et al. 2015, Smothers and Woodward 2016). In the present study using GluN2A(A825W) mice, we show for the first time that this effect extends to neuronal NMDARs in two brain regions where the GluN2A subunit is expressed (Piochon, Irinopoulou et al. 2007, McQuail, Beas et al. 2016). Moreover, we characterize the alterations in alcohol-induced behaviors in these mutant mice and show that male, but not female GluN2A(A825W) mice are less sensitive to the sedative and motor-incoordinating effects of ethanol and have altered responses to the locomotor effects of ethanol. In contrast, there was no effect of the GluN2A(A825W) mutation on ethanol-induced anxiolysis or voluntary drinking in either male or female mice. These findings add to the growing literature describing the actions of ethanol in mice engineered to express ethanol-resistant ion channels.

Although the A825W mutation produced a similar reduction in the ethanol inhibition of NMDAR-mediated currents in slices from male and female mice, only male mice showed genotypic-dependent differences in ethanol-related behaviors. The mechanisms underlying this dichotomy are currently unknown but could reflect sex-dependent differences in brain areas involved in the measured behaviors and sexually dimorphic expression of NMDAR subunits in these circuits. For example, female rats have been shown to express higher levels of GluN1 and GluN2B in areas of hippocampus and cortex (Wang, Ma et al. 2015) and it is known that differential levels of estrogens and androgens influence NMDAR number, affinity, subunit composition and synaptic localization (Smith and McMahon 2006). In addition, estradiol has been shown to increase GluN2B subunit mRNA, the number of GluN2B binding sites, and the synaptic localization of GluN2B-containing receptors (Cyr, Thibault et al. 2001, Waters, Mazid et al. 2019). NMDAR-mediated dopamine homeostasis in the PFC is oppositely tuned in females when compared to males, and this is also thought to result from genomic androgen action on cortical neurons (Locklear, Cohen et al. 2016). These findings suggest that expression or function of GluN2A containing NMDARs might differ between male and female mice in brain regions underlying behaviors (motor effects, sedation) affected by the A825W mutation. This possibility, while intriguing, is at odds with findings from the present study showing that NMDAR EPSCs evoked in cerebellar Purkinje neurons, a presumed site of the motor-impairing actions of ethanol, showed a similar loss of sensitivity to ethanol in both male and female A825W mice. This could simply reflect the fact that not all cerebellar regions were tested but also highlights the need for comprehensive studies using subunit-dependent antagonists to carefully map the contribution of GluN2 subunits to NMDAR-evoked responses across key ethanol-sensitive brain regions in male and female mice.

It is also important to note that the sex-dependent effects of the A825W mutation on ethanol-induced behaviors observed in the present study are not restricted to NMDARs (Crabbe, Phillips et al. 2006). For example, previous studies in mice with modified GABA_A ρ and glycine receptors also reported sex-specific differences on ethanol-induced behaviors (Blednov, Benavidez et al. 2012, Blednov, Benavidez et al. 2014). In mice expressing

ethanol-resistant mutations in the glycine receptor $\alpha 1$ subunit, voluntary alcohol consumption was decreased in females, but not in males; while duration of LORR and condition taste aversion was decreased in male, but not female mutant mice (Blednov, Benavidez et al. 2012). In mutant mice null for the $\rho 1$ subunit of the GABA_A receptor, ethanol intake was reduced in males but not in females; and only males showed potentiation of ethanol-induced sedation (Blednov, Benavidez et al. 2014). These findings, taken together with those of the present study, reinforce the importance of including both sexes in studies of ethanol action on mutant mice.

To date, only a few studies have used genetically modified mice to examine the role of NMDARs in ethanol action and drinking. These include those that used animals lacking the entire GluN2A subunit (Boyce-Rustay and Holmes 2006) and previous studies from our laboratory with mice expressing the ethanol-insensitive GluN1(F639A) subunit (den Hartog, Beckley et al. 2013, den Hartog, Gilstrap et al. 2017). Overall, results from these studies show some similarities but also important differences. Unlike GluN2A (A825W) mice, ethanol-naïve GluN2A KO mice show poor performance on an accelerated rotarod, balance beam, and wire-hang test (Boyce-Rustay and Holmes 2006). Mice expressing GluN2A subunits lacking the intracellular C terminus also display impaired motor coordination in rotarod and balance beam tests (Sprengel, Suchanek et al. 1998). The sensitivity to ethanol-induced ataxia in GluN2A KO mice was affected in a task-dependent manner with motor behaviors such as balance beam, wire hang test, elevated plus maze and the accelerated rotarod (although at only the 2g/kg dose of ethanol) displaying increased sensitivity to ethanol with no change in the ethanol sensitivity of grip strength or open field behavior (Boyce-Rustay and Holmes 2006). In the present study, male GluN2A(A825W) mice showed less persistent impairment on the rotarod task following a dose of 2.5 g/kg ethanol evidenced by a quicker recovery to baseline performance. Male GluN1(F639A) mice also showed faster recovery of motor coordination following the same dose of ethanol (den Hartog, Beckley et al. 2013). These effects were mirrored by a rightward shift in the locomotor enhancing effects of ethanol in both GluN2A(A825W) and GluN1(F639A) mice (den Hartog, Beckley et al. 2013). GluN2A KO mice showed no change in the locomotor stimulating effects of ethanol (Boyce-Rustay and Holmes 2006). Together, these results suggest that the ethanol sensitivity of GluN2A containing NMDARs may be an important factor that contributes to the motor-incoordinating effects of ethanol.

In addition to changes to rotarod performance, male A825W mice had reduced duration of LORR compared to wild-type mice following a sedative dose of ethanol. This finding is opposite to that from GluN2A KO and GluN1(F639A) mice that both showed normal duration of ethanol-induced sedation (Boyce-Rustay and Holmes 2005, Boyce-Rustay and Holmes 2006, den Hartog, Beckley et al. 2013). While the locus for the acute sedative effects of alcohol is not precisely known, previous studies showed a genetic correlation between LORR and inhibition of cerebellar Purkinje neuron discharge in response to acute ethanol administration in various inbred strains of mice. The firing rate of Purkinje neurons from long-sleep mice displayed greater inhibition during local application of ethanol as compared to neurons in short-sleep mouse strains (Spuhler, Hoffer et al. 1982, Johnson, Hoffer et al. 1985). GluN2A-containing NMDARs are expressed in the adult cerebellum (Sakimura, Kutsuwada et al. 1995) and there is evidence that these receptors modulate

Purkinje neuron activity. For example, NMDAR activation inhibits cerebellar Purkinje neuron spiking via activation of molecular layer interneurons that provide inhibitory control over cerebellar output (Liu, Zhao et al. 2014, Liu, Lan et al. 2016). In addition, NMDARs at climbing fiber to Purkinje neuron synapses modulate the waveform of complex spikes. Altered ethanol-modulation of Purkinje neuron output by cerebellar GluN2A-containing NMDARs is a plausible mechanism by which reduced ethanol sensitivity in male GluN2A (A825W) mice could reduce the duration of LORR.

Although a role for the GluN2A subunit in anxiety has been suggested, as GluN2A KO mice show reduced baseline levels of anxiety and depression-related behaviors (Boyce-Rustay and Holmes 2006); the anxiolytic effect of acutely administered alcohol was not affected in A825W male and female mice. Limbic structures believed to mediate emotional processing, such as the amygdala, also express the ethanol-sensitive GluN2B subunit (Lopez de Armentia and Sah 2003, Roberto, Schweitzer et al. 2004), that are implicated in acute alcohol-induced anxiolytic responses (Pandey, Ugale et al. 2008, Moonat, Sakharkar et al. 2011). Previously, we showed that the anxiolytic effect of ethanol was blunted in GluN1(F639A) mice (den Hartog, Beckley et al. 2013) suggesting that reduced ethanol inhibition of GluN2B containing receptors in the amygdala of those mice mediated this effect although this remains to be directly tested. We note that a previous attempt to generate mice expressing the ethanol-resistant GluN2B(G826W) subunit was unsuccessful due to lack of viable offspring (JJW; unpublished observation).

With respect to drinking, high resolution genome screening studies have identified quantitative trait loci linked to alcohol preference in alcohol preferring rodents and humans within chromosome 16, where the *Grin2a* gene resides, and have postulated that the GluN2A subunit is a plausible candidate for influencing alcohol consumption and dependence (Carr, Habegger et al. 2003, Schumann, Johann et al. 2008, Lo, Lossie et al. 2016, Colville, Iancu et al. 2017). Moreover, it has been suggested that the loss of GluN2A subunit-containing NMDARs impairs the ability to form or express learned reward-related responses to ethanol (Boyce-Rustay and Holmes 2006). GluN2A deletion does not affect alcohol consumption in non-dependent animals but these mice do not show the dependence-induced escalation in drinking observed in wild-type animals (Boyce-Rustay and Holmes 2006, Jury, Radke et al. 2018). In the present study, we saw no change in drinking by GluN2A(A825W) mice using an intermittent access drinking protocol that, while generating relatively high levels of consumption, is not considered a dependence model. Interestingly, studies with rats have reported an increase in alcohol intake over time in the two-bottle choice 24-hour intermittent-access paradigm (Simms, Steensland et al. 2008, Bito-Onon, Simms et al. 2011, Mill, Bito-Onon et al. 2013). However, this phenomenon is less consistent in mice with increases being reported in some studies (Melendez 2011, Hwa, Nathanson et al. 2015). but not others (Crabbe, Harkness et al. 2012) including previous studies from our laboratory (den Hartog, Beckley et al. 2013, den Hartog, Zamudio-Bulcock et al. 2016). Together, these findings suggest that ethanol-sensitive GluN2A-containing NMDARs are not critical for regulating voluntary alcohol consumption in non-dependent animals. In a previous study, we reported that GluN1(F639A) mice showed altered patterns of ethanol consumption depending on the drinking model used (den Hartog, Beckley et al. 2013). Mutant F639A mice showed reduced ethanol drinking compared to wild-type mice in

the 2-hr limited access, 2-bottle choice assay and an increase over control mice when alcohol was available every other day. While interesting, it is important to note that the amount of ethanol consumed by GluN1(F639A) mice and their wild-type littermates in that study was substantially lower than that observed in the present study and others where C57BL/6J mice are used. This may reflect the mixed genetic background of the GluN1(F639A) mice that were generated on a 129 background and then back-crossed for two generations with C57BL/6J mice. Isogenic 129 and hybrid B6 × 129 mice have been reported to show reduced consumption and preference for ethanol although the magnitude of this effect depends on the 129 sub-strain used (Lim, Zou et al. 2012).

In conclusion, the results of this study suggest that in male mice, ethanol inhibition of GluN2A containing NMDARs contributes to the locomotor and sedative effects of ethanol while having little effect on ethanol drinking or ethanol-induced anxiolysis. The similarity of these changes to those observed in mice engineered to express other ion channels with reduced ethanol sensitivity (e.g. glycine, GABA_A) is an important reminder of the many targets of ethanol in the central nervous system and the polygenic nature of alcohol addiction. Future studies employing mice expressing multiple ethanol-insensitive targets could be a valuable approach for generating a better understanding of the critical factors that control an individual's sensitivity to ethanol.

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Figure 1. *Grin2a* gene targeting approach.

A) Gene targeting approach was used to replace the alanine codon for position 825 in exon 11 of *Grin2a* with a tryptophan codon and to insert a floxed neomycin cassette into intron 11. B) Following Cre-mediated excision of the floxed neo cassette, the knock-in allele was generated. C) PCR/restriction fragment polymorphism strategy to genotype A825W knock-in mice. Inset shows gel bands corresponding to homozygous, heterozygous and wild-type GluN2A(A825W) mice.

Figure 2. NMDA-mediated synaptic currents in male and female A825W mice are less sensitive to acute ethanol.

Exemplar traces of electrically evoked NMDA-mediated currents recorded in mPFC (A; n=8-6 cells from 5 and 3 mice, respectively; C; n=8-11 cells from 4 and 5 mice, respectively) and cerebellar Purkinje neurons (B; n=9-6 cells from 5 and 3 mice, respectively; D; n=7-6 cells from 3 and 4 mice, respectively) under baseline (black) and during bath application of 44 mM EtOH (red). Summary plots show ethanol inhibition as percent of control (mean ± SEM). Symbols: (*); value significantly different from control, $p < 0.05$ (#); significant genotype effect, $p < 0.05$.

Figure 3. Sedative/hypnotic effects of ethanol are reduced in male but not female A825W mice. Latency to loss of righting reflex (LORR) in male (A; n=12-13) and female mice (D; n=10) following an i.p. injection of 3.5 g/kg ethanol. There was no effect of genotype (males: $p=0.07$, unpaired t-test; females $p=0.26$, Mann-Whitney test) on LORR. Duration of LORR in male (B) and female (E) mice. (WT vs A825 comparison: males, * $p=0.01$; females: $p=0.92$, unpaired t-tests). Blood ethanol concentration (BEC) in male (C; n=12) and female (F; n=12) mice after i.p. injection of 3.5 g/Kg ethanol. No genotype-induced effect changes were found (males: $p=0.64$; females: $p=0.80$, Two-way ANOVA).

Figure 4. Alcohol induced impairment of motor coordination is reduced in male but not female A825W mice.

Graphs show latency of WT (black) and A825 (red) male (A; n=10) and female (B; n=8-10) mice to fall from the rotarod (velocity, 5 rpm) following an i.p. injection of 2.5 g/kg ethanol. Effect of genotype in males: ** $p=0.0092$, Two-way ANOVA. No main effect of genotype in females: $p=0.97$, Two-way ANOVA.

Figure 5. Ethanol-induced changes in locomotor activity is altered in male but not female A825W mice.

(A, C) Summary plots show total distance travelled after a saline injection in male (A) and female (C) WT (open black bar) and A825W (open red bar) mice. (B, D) Summary plots show total distance travelled after i.p. injection of different doses of ethanol in male (B; n=18-20) and female (D; n=8-10) WT (open black bar) and A825W (open red bar) mice. Symbol: (*), value significantly different from baseline saline injection; $p < 0.05$. (\$), value significantly different from WT.

Figure 6. Ethanol-induced anxiolysis is similar between male and female WT and A825W mice. Summary plots show percent time spent in the open arms of the zero maze (A, C) and total distance travelled (B, D) in male (A, B; n=5) and female (C, D; n=4-5) WT (open black bar) and A825W (open red bar) mice. Each mouse received a single injection of either saline or ethanol. Symbol: (*); value significantly different from saline control, $p < 0.05$.

Figure 7. Intermittent Access Ethanol drinking is not altered in A825W mice.

Mice were presented with 2-bottles containing either water or ethanol (20%, v/v) every other day and allowed 24 hr access. On non-alcohol days, both bottles contained water. Summary plots show per session ethanol consumption (A, C) and ethanol preference relative to water (B, D) in male (A, B; $n=5-7$) and female (C, D; $n=6-7$) WT (black) and A825W (red) mice. Note higher ethanol consumption and preference in female compared to male groups (Three-way Anova, mixed effect analysis, sex $p<0.0001$). Data are mean $\pm$ SEM.