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ARTICLE

Intoxicating effects of alcohol depend on acid-sensing ion channels

Gail I. S. Harmata[1,2,3,4,5,6], Aubrey C. Chan[1,2,3,6,7], Madison J. Merfeld[2,3,4,6], Rebecca J. Taugher-Hebl[2,3,6], Anjit K. Harijan[8], Jason B. Hardie[2,3,6], Rong Fan[2,3,6], Jeffrey D. Long[2,9], Grace Z. Wang[2,3,6], Brian J. Dlouhy[4], Amal K. Bera[8], Nandakumar S. Narayanan[1,3,10] and John A. Wemmie[1,2,3,4,6,11,12]✉

Persons at risk for developing alcohol use disorder (AUD) differ in their sensitivity to acute alcohol intoxication. Alcohol effects are complex and thought to depend on multiple mechanisms. Here, we explored whether acid-sensing ion channels (ASICs) might play a role. We tested ASIC function in transfected CHO cells and amygdala principal neurons, and found alcohol potentiated currents mediated by ASIC1A homomeric channels, but not ASIC1A/2 A heteromeric channels. Supporting a role for ASIC1A in the intoxicating effects of alcohol in vivo, we observed marked alcohol-induced changes on local field potentials in basolateral amygdala, which differed significantly in *Asic1a*$^{-/-}$ mice, particularly in the gamma, delta, and theta frequency ranges. Altered electrophysiological responses to alcohol in mice lacking ASIC1A, were accompanied by changes in multiple behavioral measures. Alcohol administration during amygdala-dependent fear conditioning dramatically diminished context and cue-evoked memory on subsequent days after the alcohol had cleared. There was a significant alcohol by genotype interaction. Context- and cue-evoked memory were notably worse in *Asic1a*$^{-/-}$ mice. We further examined acute stimulating and sedating effects of alcohol on locomotor activity, loss of righting reflex, and in an acute intoxication severity scale. We found loss of ASIC1A increased the stimulating effects of alcohol and reduced the sedating effects compared to wild-type mice, despite similar blood alcohol levels. Together these observations suggest a novel role for ASIC1A in the acute intoxicating effects of alcohol in mice. They further suggest that ASICs might contribute to intoxicating effects of alcohol and AUD in humans.

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INTRODUCTION

Alcohol use disorder (AUD) affects hundreds of millions of people worldwide [1]. Costs related to excessive alcohol use, including lost productivity and increased healthcare needs, total hundreds of billions of dollars per year in the U.S. alone [2]. For individuals, AUD causes numerous adverse health and social outcomes [3, 4]. Risk for AUD is not well understood but is thought to be reflected in an individual's acute response to the intoxicating effects of alcohol, with increased risk associated with reduced sedation and/or increased stimulation [5–8]. Thus, understanding the neurobiology of alcohol intoxication may provide critical insight into AUD.

Alcohol intoxication is thought to lie along a spectrum that depends on increasing blood alcohol level (BAL). This spectrum ranges from mild intoxication, characterized by euphoria and disinhibition, to severe intoxication which progresses from slowed reaction time to loss of coordination, impaired cognition, stupor, and even coma or death [9]. Alcohol readily interacts with proteins through hydrogen bonding and is known to bind to a diverse array of receptors and channels [10]. However, how alcohol exerts its varied intoxicating effects is complex and incompletely understood. A number of important molecular targets are well-established [11], and even more are likely to be discovered. Here, we investigated whether acid-sensing ion channels (ASICs) might play a role in alcohol's complex effects. No previous studies have tested if these channels contribute to the neurophysiological and behavioral effects of alcohol.

ASICs are cation channels of the degenerin/epithelial Na^+ channel (DEG/ENaC) family that are sensitive to extracellular pH [12, 13]. Extracellular acidosis produces a large inward cation current through ASICs with greater acidosis producing greater current [14]. ASICs are comprised of trimeric assemblies of subunits (e.g. ASIC1A, ASIC2A, and ASIC2B) into homo- and heterotrimeric complexes [12, 14–19]. Different subunit combinations can influence diverse channel properties including subcellular localization, kinetics, and pH sensitivity. In brain neurons,

[1]Interdisciplinary Graduate Program in Neuroscience, University of Iowa, Iowa City, Iowa, USA. [2]Department of Psychiatry, University of Iowa, Iowa City, IA, USA. [3]Iowa Neuroscience Institute, University of Iowa, Iowa City, IA, USA. [4]Department of Neurosurgery, University of Iowa, Iowa City, IA, USA. [5]Pharmacological Sciences Predoctoral Research Training Program, Department of Neuroscience and Pharmacology, University of Iowa, Iowa City, IA, USA. [6]Department of Veterans Affairs Medical Center, Iowa City, IA, USA. [7]Department of Internal Medicine, University of Iowa, Iowa City, IA, USA. [8]Department of Biotechnology, Bhupat and Jyoti Mehta School of Biosciences, Indian Institute of Technology Madras, Chennai, Tamil Nadu, India. [9]Department of Biostatistics, University of Iowa, Iowa City, IA, USA. [10]Department of Neurology, University of Iowa, Iowa City, IA, USA. [11]Department of Molecular Physiology and Biophysics, University of Iowa, Iowa City, IA, USA. [12]Roy J. Carver Chair of Psychiatry and Neuroscience, University of Iowa, Iowa City, IA, USA. ✉email: john-wemmie@uiowa.edu

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functioning, endogenous channels are thought to be comprised largely of ASIC1A homotrimers and ASIC1A/2A-containing heterotrimers [15, 18, 19]. The ASIC1A subunit is required for normal channel function within a physiologically relevant pH range (from below pH 7.4 to pH 5), as deleting ASIC1A eliminates currents evoked by acidosis in this range [20–24]. ASICs have been implicated in synaptic plasticity [20, 21, 23, 25] as well as in learning and memory [20, 23, 25–28]. Furthermore, ASIC1A influences the effects of other substances of abuse, including cocaine and morphine [20, 29]. Thus, ASIC1A is well-positioned to modify synaptic function and influence behavioral outcomes, and might therefore contribute to effects of alcohol. Here we conducted a series of experiments to test the novel hypothesis that ASIC1A plays a role in acute alcohol intoxication.

MATERIALS AND METHODS

CHO cell electrophysiology

Rat ASIC1A and ASIC2A were transiently expressed in Chinese Hamster Ovary-K1 (CHO) cells using rASIC1a-IRES2-DsRed plasmid (received from Francois Rugiero, University College London, UK) and pcDNA3.1-rASIC2A (received from Peter McNaughton, University of Cambridge, UK) with lipofectamine (Invitrogen, CA, USA). The holding potential was -60 mV for all cells. Acid-evoked current was assessed as previously described [30] with pH = 6.0 applied in the absence or presence of alcohol (10–100 mM). Acid and alcohol were applied using a custom-made gravity-driven fast perfusion system [30, 31]. One dose was tested per cell. Potentiation was calculated as ((current after alcohol – control current)/control current) * 100 percent. Percent potentiation by alcohol dose was analyzed by linear regression.

Mice

Both male and female *Asic1a*$^{+/+}$, *Asic1a*$^{-/-}$ [25], and *Asic2*$^{-/-}$ [32] mice were generated and bred in-house, and maintained on a congenic C57BL6/J background. Although effects of alcohol on these mice have never been previously tested, they have been extensively characterized and phenotyped in a number of physiological and behavioral assays, with relevant testing including fear conditioning, locomotion, locomotor stimulation by cocaine, and synaptic plasticity in amygdala and nucleus accumbens [20, 21, 25, 26, 28, 29, 32, 33]. Mice were housed on a 12-hour light-dark cycle with same-sex littermates in groups of 2–5, except for mice with chronic amygdala electrode implants, which were singly-housed. Brain activity and behaviors were tested in mice >10 weeks of age. Electrophysiological slice recordings were obtained at 8–12 weeks of age. Different mice were used for each behavioral assay except the intoxication scale and loss of righting reflex, in which the same mice were used with a one-week gap between experiments. All animal experiments were approved by the University of Iowa IACUC.

Basolateral amygdala (BLA) neuron electrophysiology

Coronal BLA slices (300 µm) were obtained and voltage-clamp recordings were made from visually identified principal neurons, using protocols and solutions as previously described [20]. Recordings were made with gluconate-based internal solution in the presence of 100 µM picrotoxin, 20 µM CNQX, and 50 µM d-APV. Acidic ACSF (buffered with 5 mM HEPES and 5 mM MES and titrated to pH 6.3 with NaOH) was applied from a recording pipette positioned ~10–30 µM from the cell body. Acidic ASCF containing no alcohol vs indicated alcohol dose were applied to each cell with a Femtojet 5247 (Eppendorf) 1–2 psi for 3 s. Three doses were tested: 5, 50, or 100 mM alcohol. Order of solution presentation was counterbalanced between cells. The holding potential was −70 mV. Alcohol (100 mM) did not change membrane resistance (*t*-test, $t\ (11) = 1.679$, $p = 0.121$).

Local field potential (LFP) recording

Mice were implanted with a 16-channel microelectrode array (MicroProbes, Gaithersburg, MD) targeting the basolateral amygdala (bregma + / −3.4 mm ML, −1.4 mm AP; −3.9 mm DV from brain surface). On day 1, mice were connected to the recording equipment (Plexon, Dallas, TX) and placed in a custom-built plexiglass chamber (approx. 20 cm × 20 cm × 33 cm tall) for acclimation and adjustment of recording parameters. On days 2 and 3, the mice were recorded for a 10-minute baseline and then for 30 min following intraperitoneal (i.p.) injection (0.0125 ml/g body weight) with 0.9% saline (day 2) or EtOH 1.5 g/kg (day 3). LFPs were recorded at 1000 Hz.

LFP data processing

Data were extracted from raw recordings using NeuroExplorer software (Plexon, Dallas, TX) and processed with MATLAB. LFP signal was averaged across 16 channels per mouse. Signal power was obtained by wavelet convolution across 30 frequency steps [34] and averaged across 60 s bins. Power data were normalized to a 6-minute window during the baseline period (minutes −8 to −2 prior to injection). Frequency bands were defined as delta (1–4 Hz), theta (5–8 Hz), alpha (9–12 Hz), beta (14–28 Hz), gamma (32–48 Hz), and high gamma (72–110 Hz). LFP frequency band power was analyzed with mixed effects models incorporating time, genotype, treatment, and their interactions as fixed effects, and mouse and treatment as random effects (R/RStudio).

Pavlovian fear conditioning

Mice were injected with saline (0 g/kg) or alcohol (0.25, 0.75, or 1.5 g/kg), returned to homecage for 5 min, then placed in fear conditioning chambers (Med Associates, VT). Fear conditioning was performed [35–39]. Briefly, on day 1 mice were habituated for 3 min followed by 5 tone (90 dB, 20 s)-footshock (0.75 mA, 1 s) pairings co-terminating, with an interstimulus interval of 120 s. On day 2, mice were placed back into the training context and freezing was assessed for 5 min. On day 3, freezing to conditioned stimulus (tone) was assessed in novel context. Two animals were excluded due to technical problems and 4 intra-group outliers were excluded (ROUT test). Freezing data was analyzed as a dose-response via linear regression (R/RStudio).

Open field test

Mice were injected with alcohol (0.25 to 2.5 g/kg) or saline (i.p.) and immediately placed into the center of the open field chamber (San Diego Instruments, San Diego, CA) [33, 40]. Activity was recorded by infrared beam breaks for 15 min. One intra-group outlier (ROUT test) was excluded. A best-fit regression model accounting for genotype and dose was identified by Akaike Information Criterion in the statistical software package R, and included a quadratic term for alcohol dose.

Blood alcohol levels (BALs)

Mice were injected with either high-dose alcohol (3.0 g/kg, i.p.), medium-dose alcohol (1.5 g/kg), or saline. Trunk blood was collected 6 min post-injection using K2 EDTA Microvette CB300 sampling tubes (Sarstedt Inc.). Plasma was analyzed using the Enzychrom kit (BioAssay Systems). The standard curve was calculated from alcohol-naïve mouse plasma spiked with known alcohol concentrations. BALs in alcohol-injected mice were compared by two-way ANOVA.

Intoxication scale

Mice were injected with alcohol (2.5, 3.0, and 3.5 g/kg, i.p.), placed into a plexiglass enclosure, and behavior was filmed for 30 min. Videos were scored by blinded observer. Total time in and initial latency to the following intoxication levels were quantified: (level 0) No observable effect on locomotion; (level 1) stumbling gait with upright posture, (level 2) stumbling gait/organized movements with frequent loss of posture (level 3) largely immobile with loss of posture, but with small movements, and (level 4) completely immobile and without maintained posture. These endpoints were previously used in similar sedation scale for rats [41, 42]. Mice were tested at each dose in a counterbalanced design with a 1-week interval between dosing. Total time spent in each level and latency to reach each level were analyzed by 2-way repeated measures ANOVAs, with planned *t*-test comparisons between genotypes.

Loss of righting reflex (LORR)

LORR duration following alcohol injection (3.5 g/kg i.p.) was assessed by placing mice supine in a V-shaped trough, modified from previous work [43]. Righting reflex was defined the ability to turn over 3 times in 30 s, or altogether resisting the supine position. Animals were tested every 5 min post-injection, and LORR duration was calculated as total time required to recover righting response. Mice had been previously tested for intoxication level, with a 1-week interval between assays. A *t*-test was used to compare LORR duration between genotypes.

Statistics

Data analysis was performed with Microsoft Excel, Graphpad Prism, R/RStudio [44, 45], and Mathworks MATLAB according to the needs of each experiment as described above. Third-party packages in R included the tidyverse [46], car [47], MASS [48], coin [49], lme4 [50], optimx [51], lmerTest [52], extrafont [53], and their dependencies. Details on all statistical tests can be found in Supplementary Table 1. In graphs, all error bars represent standard error of the mean (S.E.M.).

RESULTS

Effects of alcohol on ASIC-mediated acid-evoked current

To test if ASICs might mediate effects of alcohol, we examined whether ASIC function was directly impacted by alcohol. We tested effects of a range of alcohol concentrations (10–100 mM) on ASIC1A homomeric channels by transfecting the ASIC1A subunit into CHO cells, which do not endogenously express these channels or have acid-evoked currents [54–56]. In ASIC1A-transfected CHO cells, alcohol (100 mM) by itself induced no current at pH 7.4, suggesting it does not activate ASIC1A homomeric channels on its own, or any other ionotropic receptors in these cells (Supplementary Fig. 1). However, when combined with acidic pH (pH = 6.0), alcohol increased the amplitude of acid-evoked currents in a dose-dependent manner, with up to 34% potentiation at 100 mM of alcohol (Fig. 1A, B) (linear regression, effect of alcohol dose, $p < 0.0001$). We next tested whether alcohol similarly affected ASIC1A/2 A heteromeric channels expressed in CHO cells. Interestingly, alcohol had no effect on these channels when combined with extracellular pH 6.0 (Fig. 1C, D) (linear regression, $p = 0.7078$), suggesting alcohol may increase the function of ASIC1A homomeric channels but not of ASIC1A/2 A heteromeric channels.

To determine if endogenous ASICs in mouse brain neurons would be similarly affected by alcohol, we tested effects on basolateral amygdala (BLA) principal neurons. The BLA experiences physiological changes in response to alcohol exposure [57–63], is implicated in alcohol-related behaviors [64–66], abundantly expresses the ASIC1A subunit [15, 23, 26, 28], and is predicted to contain a substantial proportion of ASIC1A homomers [15]. Principal neurons were selected because they comprise the majority of neurons in the BLA [67] and have been found to have ASIC1A-dependent neuroplasticity [21]. As previously described [68], acidic pH (pH = 6.3) evoked large inward currents in *Asic1a*$^{+/+}$mice (Fig. 1E). Moreover, co-application of 100 mM alcohol significantly enhanced these currents (Fig. 1E, H) (*t*-test, $p = 0.0422$), while lower doses (5 and 50 mM) produced less potentiation with mean values consistent with those observed in CHO cells. We also tested *Asic1a*$^{-/-}$ mice and saw no acid-evoked current in either the presence or absence alcohol, suggesting the effects depend on ASIC1A (Fig. 1F). To isolate ASIC1A homomeric channels, we similarly tested BLA principal neurons from *Asic2*$^{-/-}$ mice, which lack both ASIC2A and ASIC2B subunits [32]. We again found that 100 mM alcohol potentiated the acid-evoked currents (Fig. 1G, H) (*t*-test, $p = 0.0415$). These results were consistent with the effects of alcohol in CHO cells, and suggest that alcohol can directly enhance activation of ASIC1A homomeric channels in BLA neurons. Interestingly, the normal expression of ASIC2 subunits in wild-type mice did not preclude these effects of alcohol, presumably because a major proportion of the acid-evoked current in BLA principal neurons is mediated by ASIC1A homomeric channels.

Effects of alcohol and ASIC1A on neural activity in the amygdala in vivo

The above-described effects of alcohol on ASIC1A function in amygdala neurons in vitro raised the possibility that alcohol would affect brain function in vivo in an ASIC1A-dependent manner. To test this hypothesis, we implanted microelectrode

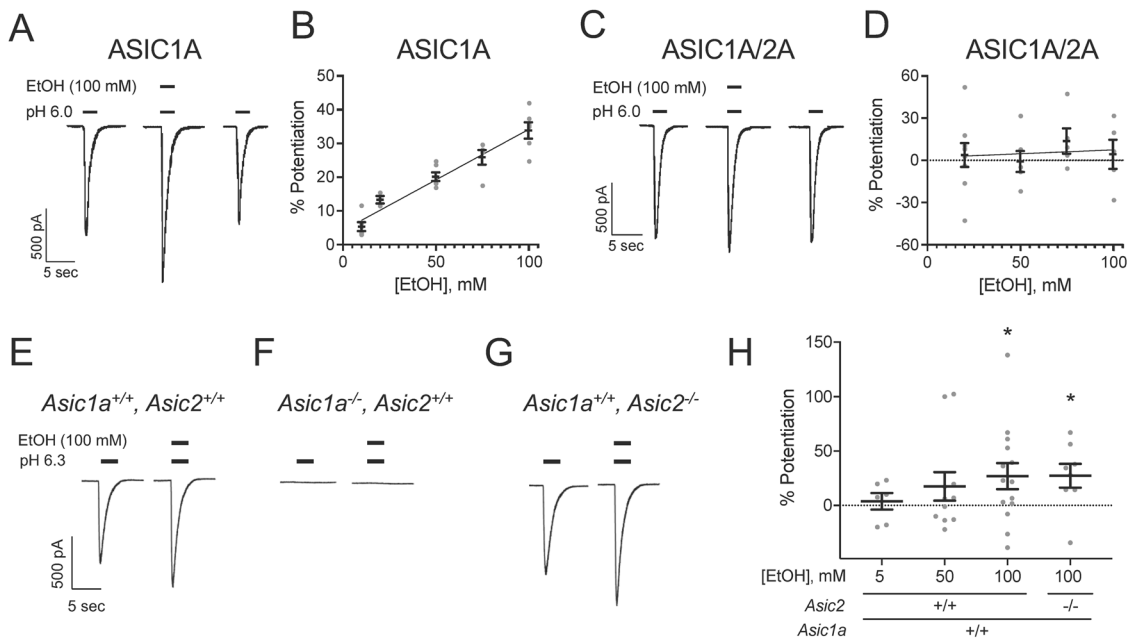

Fig. 1 Alcohol potentiates acid-evoked current in CHO cells and basolateral amygdala (BLA) principal neurons. A Representative traces of acid-evoked currents (pH = 6.0) in ASIC1A-expressing CHO cells, with and without alcohol (100 mM). **B** Increasing alcohol concentration enhanced acid-evoked current in a dose-dependent manner (effect of dose, $p < 0.0001$, $n = 3$–6). **C** Representative traces of acid-evoked currents (pH = 6.0) in ASIC1A/2A-expressing CHO cells, with and without alcohol (100 mM). **D** Alcohol did not dose-dependently potentiate acid-evoked current of ASIC1A/2 A heteromers (no effect of dose, $p = 0.7078$, $n = 5$–9). **E** Representative traces of acid-evoked currents (pH = 6.3) in BLA principal neurons of *Asic1a*$^{+/+}$ mice, with and without alcohol (100 mM). **F** Representative traces showing no acid-evoked currents (pH = 6.3) in BLA neurons of *Asic1a*$^{-/-}$ mice, with and without alcohol (100 mM). **G** Representative traces showing acid-evoked current in BLA neurons of *Asic2*$^{-/-}$ mice, with and without alcohol (100 mM). **H** Alcohol increased acid-evoked currents in BLA neurons of both *Asic1a*$^{+/+}$ (*t*-test, *$p = 0.0422$, $n = 14$) and *Asic2*$^{-/-}$ mice (*t*-test, *$p = 0.0415$, $n = 8$).

arrays into the basolateral amygdala of *Asic1a*$^{+/+}$ and *Asic1a*$^{-/-}$ mice. Local field potentials (LFPs) were recorded before and after saline or alcohol injections (Fig. 2A). We chose an alcohol dose (1.5 g/kg) previously found to alter LFPs in rodents [69–71] but not cause loss of consciousness. We computed power spectrograms normalized to pre-injection baseline (Fig. 2B–E) and compared specific frequency bands between groups (Fig. 2F–K). Compared to saline, alcohol had profound effects in both genotypes. In *Asic1a*$^{+/+}$ mice, alcohol suppressed power across multiple frequency bands, including delta, alpha, beta, gamma, and high gamma, while leaving theta relatively unaffected (Fig. 2F–K). In *Asic1a*$^{-/-}$ mice, alcohol also suppressed alpha, beta, gamma, and high gamma power. There were several genotype-dependent effects of alcohol. While alcohol suppressed delta in *Asic1a*$^{+/+}$ mice, in *Asic1a*$^{-/-}$ mice delta was largely unchanged (Fig. 2F) (genotype*treatment*time interaction, $p = 0.0268$). Alcohol also produced greater suppression of gamma and high gamma in *Asic1a*$^{-/-}$ mice compared to *Asic1a*$^{+/+}$ mice (Fig. 2J, K) (genotype*treatment interactions, $p < 0.0001$ and $p = 0.0454$). Most strikingly, alcohol transiently increased theta in the *Asic1a*$^{-/-}$ mice, but not in the *Asic1a*$^{+/+}$ mice (Fig. 2G) (genotype*treatment*time interaction, $p < 0.0001$). These results suggest alcohol exerts substantial effects on amygdala function in vivo, and that at least some of these effects depend on ASIC1A.

Effects of alcohol and ASIC1A on fear memory

To explore potential impacts of alcohol and ASICs on behavior, we next tested Pavlovian fear conditioning. This learning and memory task depends on the amygdala [72], and has been suggested to be sensitive to alcohol [36–39, 73] as well as ASIC1A disruption [26, 35]. *Asic1a*$^{+/+}$ and *Asic1a*$^{-/-}$ mice were injected with a range of alcohol doses (0.25, 0.75, or 1.5 g/kg) or saline (0 g/kg) on day 1, and trained to associate a previously neutral context and auditory cue with footshocks (Fig. 3A). Behavioral responses (freezing) during training and on subsequent test days were quantified (Fig. 3B–D, Supplementary Fig. 2). As previously reported, during training *Asic1a*$^{-/-}$ mice exhibited a marked deficit in freezing acquisition in the absence of alcohol [26, 33], which was further evident following alcohol injections (Fig. 3B) (linear regression, genotype effect, $p < 0.0001$). There was also an alcohol effect, with higher alcohol doses increasing freezing during acquisition (dose effect, $p < 0.0001$), which might be due to locomotor and/or sedating effects of alcohol, although prior to footshocks alcohol evoked little or no freezing by itself in either genotype (Supplementary Fig. 2A). The genotype by dose interaction during training was not significant ($p = 0.103$).

Context and auditory cue evoked memory were tested after injected alcohol had cleared, on days 2 and 3 (Fig. 3A). *Asic1a*$^{-/-}$ mice displayed significant deficits in both context and cue-evoked memory, as described previously [26, 33] (Fig. 3C, D, Supplementary Fig. 2C, D) (linear regression, genotype effect, $p < 0.0001$ for both tests). Interestingly, although alcohol exposure increased freezing during training, this exposure led to substantial memory impairment (less freezing) during testing to both context and auditory cues, with higher doses during training causing greater impairment during testing (dose effect $p < 0.0001$, both context and cue). Moreover, there were significant genotype by alcohol dose interactions (context testing $p < 0.0001$; cued testing $p = 0.0370$). These results are consistent with the amnesia-inducing effects of alcohol [62–66], and suggest alcohol differentially impaired memory in mice lacking ASIC1A (Fig. 3C, D). However, the large baseline effects of ASIC1A disruption make it challenging to discern the nature of these differential effects. Therefore, we next assessed the effects of alcohol on tasks in which ASIC1A disruption does not cause baseline differences.

Stimulating and sedating effects of alcohol and ASIC1A

ASIC1A disruption by itself does not alter locomotor activity [33], whereas alcohol induces prominent effects in a dose-dependent manner [74]. Thus, locomotor responses provide a practical advantage for testing behavioral interactions between alcohol and ASIC1A. We assessed locomotor activity for 15 min following injection with a range of eight different alcohol doses (0.0–2.5 g/kg, i.p.). The resulting dose-response data were fitted to a model (see Methods), shown as dashed curves in Fig. 4A (also see time courses, Supplementary Fig. 3). The two genotypes displayed similar levels of activity following saline injections. As alcohol dose increased from 0.25 to 1.5 g/kg, locomotor activity in both genotypes increased above saline levels, indicating that alcohol elicited locomotor stimulation. Higher alcohol doses (2.0–2.5 g/kg for wild-types and 2.5 g/kg for *Asic1a*$^{-/-}$ mice) suppressed activity below saline levels, suggesting sedation. Importantly, there was a significant dose by genotype interaction ($p = 0.0006$) driven by an upward and rightward shift in the *Asic1a*$^{-/-}$ mice, suggesting more stimulation and less sedation. Despite these differences in behavior, blood alcohol levels between genotypes were similar after alcohol injection in a separate cohort of mice (1.5 and 3.0 g/kg, i.p.) (Fig. 4B), suggesting the different locomotor responses to alcohol were unlikely due to different absorption or metabolism.

Because of the ASIC1A-dependent effects of alcohol dose in the open field, we wondered whether ASIC1A would affect other assessments of acute intoxication. To test the contribution of ASIC1A to alcohol-induced sedation, we assessed the loss of righting reflex, which is tested with higher alcohol doses than those used in the open field. *Asic1a*$^{-/-}$ mice recovered their righting reflex significantly faster than wild-type mice after an i.p. injection of 3.5 g/kg of alcohol (Fig. 4C) (*t*-test, $p = 0.0051$), suggesting that mice lacking ASIC1A were less severely obtunded by a high alcohol dose.

Because the sedating and intoxicating effects of alcohol tend to progress in severity from minor ataxia to loss of consciousness, we also classified behaviors along this progression using an alcohol intoxication scale spanning 30 min post-injection (Table 1). This scale allowed us to assess multiple alcohol doses with greater sensitivity than loss of righting reflex. We found that latency to reach each intoxication level and total time spent at each level depended highly on alcohol dose (Fig. 4D, E, Supplementary Fig. 4B). The lowest dose (2.5 g/kg, i.p.) caused mice to spend the majority of time in intoxication levels 1 and 2, characterized largely by ataxia, while the highest dose (3.5 g/kg, i.p.) caused mice to shift to intoxication levels 3 and 4, characterized by immobility and loss of consciousness. Overall, compared to wild-type mice, the *Asic1a*$^{-/-}$ mice spent more time at a lower level of intoxication (level 1), and less time at a higher level of intoxication (level 3) (repeated measures 2-way ANOVAs, genotype effects, $p = 0.0403$ and $p = 0.0012$ respectively). ASIC1A disruption also affected latency taking longer to reach intoxication levels 2 and 3, especially at the lowest alcohol dose (repeated measures 2-way ANOVAs, genotype effect for level 2, $p = 0.0024$; genotype by dose interaction for level 3, $p = 0.0480$). Taken together, the loss of righting reflex test and intoxication levels suggest that ASIC1A disruption shifts intoxication-related behavior towards the lower end of the spectrum, i.e., less severe intoxication.

DISCUSSION

Using multiple independent assays, these studies revealed diverse neural and behavioral effects of alcohol across a range of doses in wild-type mice that differed significantly in mice lacking ASIC1A. These results thus identify ASIC1A as a novel contributor to the complex molecular and behavioral actions of acute alcohol intoxication.

There are numerous ways by which ASIC1A function could affect alcohol intoxication. One previous study reported no direct

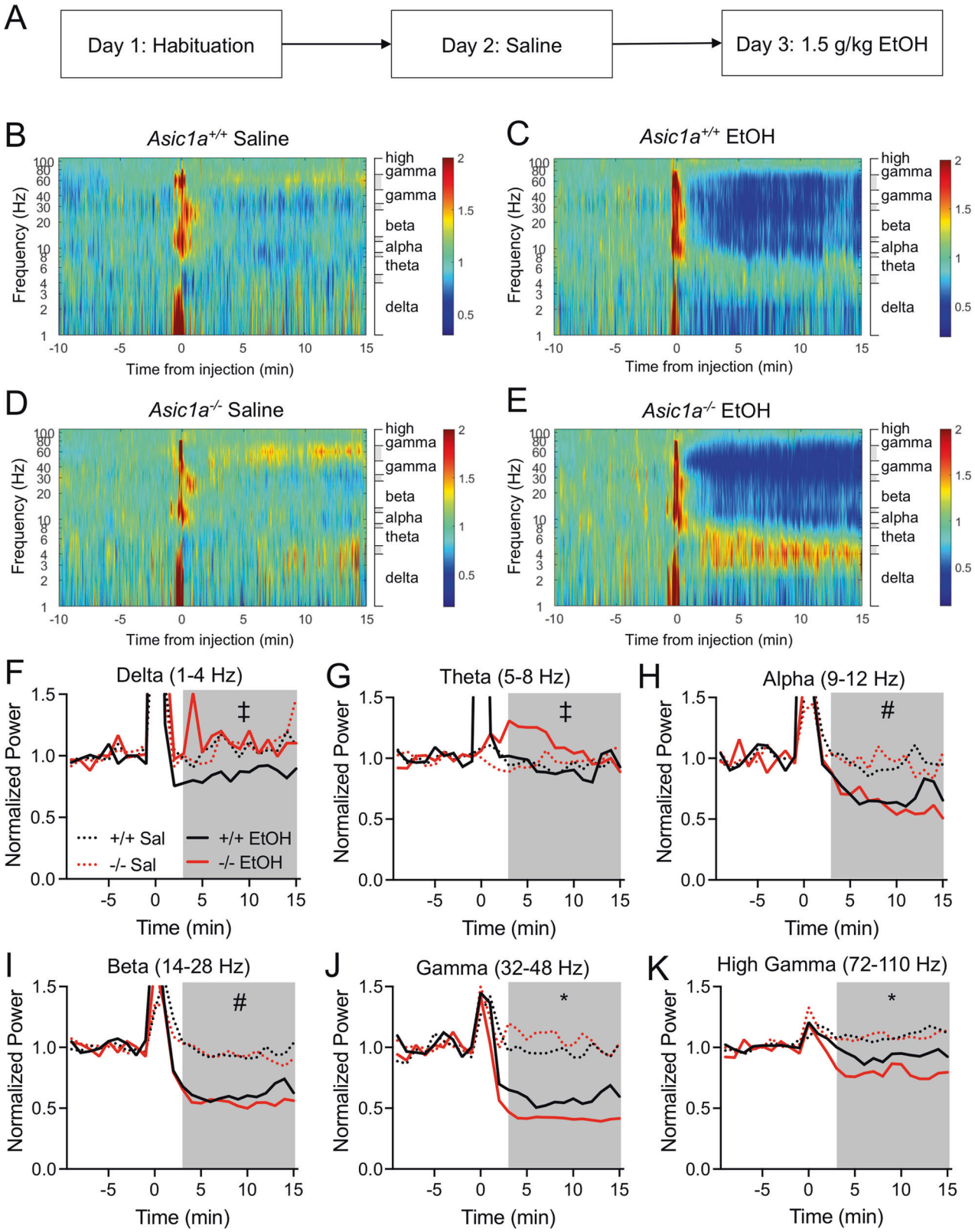

Fig. 2 ASIC1A disruption changes amygdala activity in the presence of alcohol. A Diagram of recording timeline ($n = 6$–7 mice per genotype). **B**–**E** Power spectrograms for four groups: (**B**) *Asic1a*$^{+/+}$ with saline, (**C**) *Asic1a*$^{+/+}$ with alcohol 1.5 g/kg (EtOH), (**D**) *Asic1a*$^{-/-}$ with saline, and (**E**) *Asic1a*$^{-/-}$ with alcohol. Data is normalized to the baseline period from −8 to −2 min (**F**–**J**) Normalized power over time across six frequency bands: (**F**) delta 1–4 Hz; (**G**) theta 5-8 Hz; (**H**) alpha 9–12 Hz; (**I**) beta 14–28 Hz; (**J**) gamma 32–48 Hz; and (**K**) high gamma 72–110 Hz. Dotted lines represent saline, solid lines represent EtOH. Black lines represent *Asic1a*$^{+/+}$ (+/+), and red lines indicate *Asic1a*$^{-/-}$ (−/−). Data from each frequency band were analyzed from minutes 2–15 post-injection in a mixed-effects model. Only the highest-order significant effect involving alcohol treatment is indicated. There was a time*genotype*treatment interaction (‡) for delta ($p = 0.0268$) and theta ($p < 0.0001$), a genotype*treatment interaction (*) for gamma ($p < 0.0001$) and high gamma ($p = 0.0454$), and a main effect of alcohol (#) for alpha ($p = 0.0008$) and beta ($p < 0.0001$).

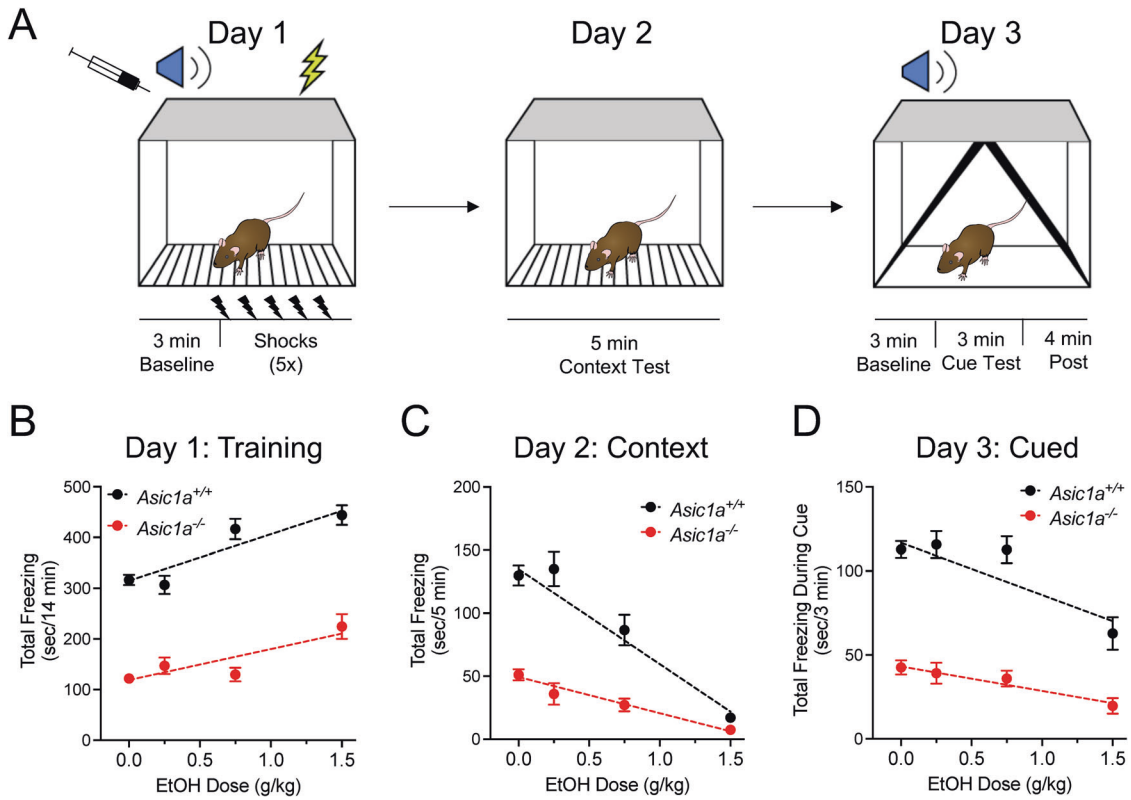

Fig. 3 Impairment of fear conditioning by alcohol and ASIC1A disruption. A Fear conditioning paradigm. Day 1, mice were injected with alcohol (EtOH) or saline and trained to associate context and auditory cue with aversive footshocks. Memory was subsequently tested in the absence of alcohol. Day 2, context-evoked freezing was tested. Day 3, auditory cue-evoked freezing was tested in a novel context. **B** During training (Day 1), there was a significant effect of both genotype ($p < 0.0001$, $n = 14–23$ per alcohol group, $n = 47–58$ per saline group) and alcohol dose ($p < 0.0001$) but no interaction ($p = 0.1031$). **C** During context testing (Day 2), there was a significant dose*genotype interaction ($p < 0.0001$). **D** During auditory cue-evoked testing (Day 3), there was a significant dose*genotype interaction ($p = 0.0370$).

effects of alcohol on ASICs, although that study used a different methodology that did not distinguish between ASIC1A homomers, ASIC1A/2 A heteromers, or other ASIC subunits [75]. In contrast, our results in CHO cells and BLA neurons suggest alcohol may enhance acid-induced activation of ASIC1A homomeric channels, which might contribute to alcohol intoxication. We speculate this effect of alcohol on channel function likely involves alcohol's ability to form hydrogen bonds [10]. Our results further suggest bonding may be specific to the ASIC1A subunit, and possibly a site formed between multiple ASIC1A subunits, given that ASIC1A/2 A heteromers were unaffected. Further studies will be required to pinpoint potential sites for such interactions.

Other possibilities for how ASICs affect alcohol intoxication are also conceivable. For example, alcohol might influence ASICs through its ability to induce metabolic acidosis [76–78]. Alternatively, alcohol increases neurotransmitter release from presynaptic vesicles, which are highly acidic and can transiently lower synaptic pH [21], and might thus facilitate ASIC activation at synapses [79, 80]. Additionally, ASIC1A may modulate previously established actions of alcohol on neurotransmitter systems, such as glutamatergic signaling. For example, alcohol preferentially inhibits NMDA receptors over AMPA receptors [81] and disrupting ASIC1A increases the AMPA/NMDA receptor ratio at glutamatergic synapses in multiple brain areas [20, 82]. Thus, molecular interactions between alcohol and ASIC1A could be multifold. Additional studies will be necessary to discern which potential mechanisms may be most important.

Consistent with such mechanistic possibilities, ASIC1A disruption substantially impacted alcohol-evoked changes in local field potentials in the amygdala in vivo. ASIC1A disruption altered alcohol responses in the gamma, delta, and theta frequency ranges. Gamma suppression was greater, delta suppression was reduced, and theta was exclusively potentiated in *Asic1a*$^{-/-}$ mice. These observations are largely consistent with previous studies linking these specific frequency bands with learning and memory and/or responses to emotional stimuli [83–93], and seem likely to contribute to the behavioral interactions observed here, particularly the fear conditioning effects. Although the relationship between local field potentials and neuronal activity is complex, these data suggest that alcohol and ASICs interact to influence brain activity.

Consistent with the above-described interactions between alcohol and ASICs on neural responses in vitro and in vivo, multiple behavioral outcomes here supported a critical role for ASIC1A in alcohol intoxication. The fear conditioning results suggest ASIC1A contributes to the amnestic effects of alcohol intoxication. However, the nature of this interaction is difficult to fully interpret because ASIC1A disruption by itself produced such dramatic effects on conditioned fear memory. Although, previous studies suggested that fear conditioning deficits in *Asic1a*$^{-/-}$ mice were not due to impaired shock sensitivity, an inability to freeze, impaired hearing, or altered locomotor activity [25, 26, 33].

The other interactions between alcohol and ASIC1A disruption were more straightforward to interpret, including effects on locomotion, loss of righting reflex, and intoxication severity scores. In each of these assays, *Asic1a*$^{-/-}$ mice displayed less sedation and more activation. Differences were due to more than just a shift in dose-response, because in the open field assay the alcohol-evoked hyperactivity displayed by *Asic1a*$^{-/-}$ mice was never reached by *Asic1a*$^{+/+}$ mice. Increased locomotor activity evoked by low-dose alcohol in mice likely parallels the excitement and disinhibition evoked in humans. Similarly, the hypoactivity, loss of

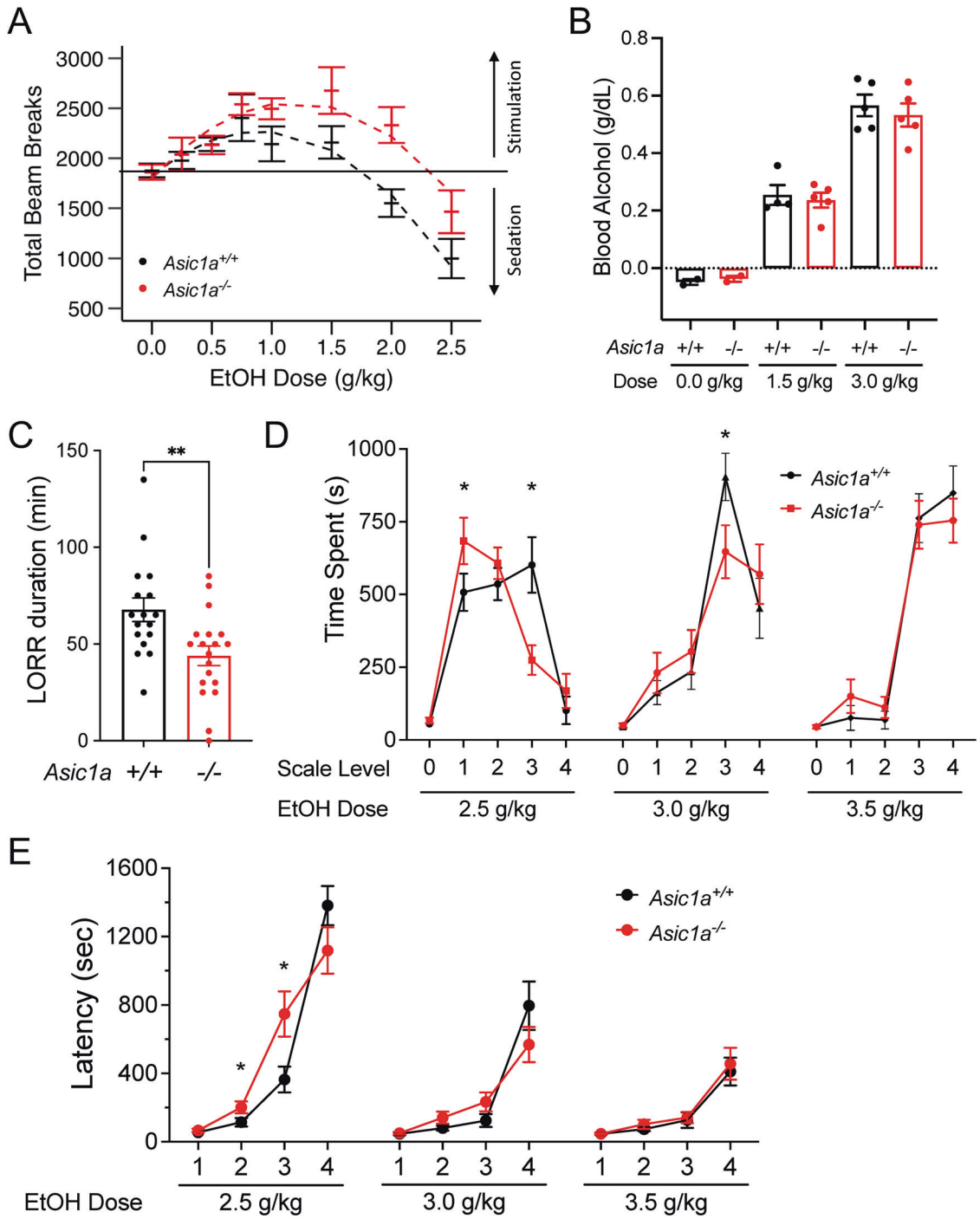

Fig. 4 ASIC1A disruption increases stimulating effects of alcohol and decreases sedating effects. A Biphasic, dose-dependent locomotor response to alcohol in the open field. A best-fit regression model revealed a dose*genotype interaction ($p = 0.0006$, $n = 6$–15 per group). **B** Blood alcohol level did not differ between genotypes at any dose tested, and there was no interaction with dose (2-way ANOVA, p-values > 0.05 for genotype effect and genotype*dose interaction, $n = 2$–7 per group). **C** Loss of righting reflex (LORR) duration following 3.5 g/kg EtOH i.p. was decreased in *Asic1a*$^{-/-}$ mice (t-test, $p = 0.0051$, $n = 17$–19 per group). **D** Total time at each level of intoxication (Table 1) across a range of alcohol doses. *Asic1a*$^{-/-}$ mice spent more time at a lower level of intoxication (level 1) (2-way repeated measures ANOVA, genotype effect, $p = 0.0403$, $n = 17$–19 per group), and less time at a higher able level of intoxication (level 3) (genotype effect, $p = 0.0012$). Significant planned comparisons are indicated with asterisks. **E** Latency to reach each level of intoxication across a range of alcohol doses. *Asic1a*$^{-/-}$ mice took longer to reach level 2 overall (genotype effect, $p = 0.0024$) and took longer to reach level 3 at the lowest dose (genotype*dose interaction, $p = 0.0480$). Significant planned comparisons are indicated with asterisks.

coordination, stupor, and loss of consciousness in mice at high doses closely resemble features of human intoxication. Because ASIC1A is widely expressed in brain, its effects on alcohol intoxication may result from channel action in a wide variety of brain sites. Pinpointing sites of ASIC1A action on specific behavioral effects of alcohol will require additional, and likely extensive, studies. Because blood alcohol levels did not differ between *Asic1a*$^{-/-}$ and *Asic1a*$^{+/+}$ mice, ASIC1A likely mediates

Table 1. Intoxication Scale Levels.

Level	Description
0	No observable effect on locomotion
1	Stumbling gait with upright posture
2	Stumbling gait and/or organized movements (e.g., grooming) with frequent loss of posture
3	Mostly immobile with loss of posture, but with small movements (e.g., paw twitches)
4	Completely immobile with loss of posture

these effects of alcohol rather than its absorption or clearance. In humans, genetic factors have been suggested to play a major role in acute responses to alcohol with an estimated heritability of 60% [94]. Importantly, people who report less sedation and/or more stimulation from alcohol are more likely to have a family history of AUD [5], a greater preference for alcohol [95], higher levels of alcohol consumption [96], as well as a higher risk of developing AUD [6–8]. This profile is remarkably similar to what we observed in *Asic1a*$^{-/-}$ mice, suggesting *ASICs* might contribute to AUD in humans.

In summary, this work identifies ASIC1A as a novel molecular contributor to the acute actions of alcohol. The results further suggest that disrupting ASIC1A in mice leads to phenotypes resembling human characteristics previously linked to AUD risk. Together these observations could have important clinical implications for people with genetic variations in *ASIC1A*, and may open new avenues for research into the mechanisms underlying AUD.

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AUTHOR CONTRIBUTIONS

GISH conceptualized experiments, acquired data, analyzed data, interpreted data, and wrote the manuscript. ACC conceptualized experiments, acquired data, analyzed data, and interpreted data. MJM conceptualized experiments and acquired data. RJT acquired data, analyzed data, interpreted data, and wrote the manuscript. AKH acquired and analyzed data. JBH acquired and analyzed data. RF acquired data. JDL was an essential contributor to advanced data analysis. GZW acquired data. BJD conceptualized experiments, provided funding, and interpreted data. AKB conceptualized experiments. NSN conceptualized experiments and advised on data analysis. JAW conceptualized experiments, interpreted data, provided funding, and wrote the manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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Correspondence and requests for materials should be addressed to John A. Wemmie.

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