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Dependence-induced ethanol drinking and GABA neurotransmission are altered in *Alk* deficient mice

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Abstract

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase that is expressed in the brain and implicated in alcohol abuse in humans and behavioral responses to ethanol in mice. Previous studies have shown an association of human *ALK* with acute responses to alcohol and alcohol dependence. In addition, *Alk* knockout (*Alk* −/−) mice consume more ethanol in a binge-drinking test and show increased sensitivity to ethanol sedation. However, the function of ALK in excessive drinking following the establishment of ethanol dependence has not been examined. In this study, we tested *Alk* −/− mice for dependence-induced drinking using the chronic intermittent ethanol-two bottle choice drinking (CIE-2BC) protocol. We found that *Alk* −/− mice initially consume more ethanol prior to CIE exposure, but do not escalate ethanol consumption after exposure, suggesting that ALK may promote the escalation of drinking after ethanol dependence. To determine the mechanism(s) responsible for this behavioral phenotype we used an electrophysiological approach to examine GABA neurotransmission in the central nucleus of the amygdala (CeA), a brain region that regulates alcohol consumption and shows increased GABA signaling after chronic ethanol exposure. GABA transmission in ethanol-naïve *Alk* −/− mice was enhanced at baseline and potentiated in response to acute ethanol application when compared to wild-type (*Alk* +/+) mice. Moreover, basal GABA transmission was not elevated by CIE exposure in *Alk* −/− mice as it was in *Alk* +/+ mice. These data suggest that ALK plays a role in dependence-induced drinking and the regulation of presynaptic GABA release in the CeA.

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Keywords

addiction; ALK; amygdala; chronic intermittent ethanol; GABA; synaptic

1. Introduction

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase that acts as an oncogene in various human cancers, including anaplastic large cell lymphoma (for which it is named), neuroblastoma, and lung cancer (Hallberg and Palmer, 2013). ALK is expressed in the developing and adult mammalian nervous system (Iwahara et al., 1997; Vernersson et al., 2006) and *Alk* knockout (*Alk* −/−) mice display behavioral phenotypes related to psychiatric disorders such as depression and anxiety. For instance, *Alk* −/− mice show less immobility in the tail suspension and Porsolt swim tests, increased novel object recognition, enhanced retention of spatial memory, and reduced anxiety in the elevated zero maze (Bilsland et al., 2008; Weiss et al., 2012). ALK also regulates behaviors related to alcohol abuse. *Alk* −/− mice are more sensitive to ethanol-induced sedation and consume more ethanol in a limited-access drinking test (Lasek et al., 2011), phenotypes consistent with ALK modulating acute responses to alcohol. In humans, polymorphisms in *ALK* are associated with acute ethanol-induced body sway and subjective measures of ethanol-induced euphoria (Lasek et al., 2011). Interestingly, a meta-analysis of genome-wide association studies has found that polymorphisms in human *ALK* are also associated with alcohol dependence (Wang et al., 2011). However, *Alk* −/− mice have not been investigated for their role in behaviors related to chronic ethanol exposure or dependence.

Here, we tested *Alk* −/− mice for dependence-induced ethanol consumption using the chronic intermittent ethanol-two bottle choice ethanol consumption (CIE-2BC) protocol. In this paradigm, animals exhibit increased alcohol intake in a limited-access ethanol/water choice after chronic passive exposure to ethanol vapor. This procedure has been successfully used in rats (Vendruscolo and Roberts, 2014) and C57BL/6 mice (Becker and Lopez, 2004; Finn et al., 2007; Griffin et al., 2009) to model the motivational aspects of ethanol dependence and excessive ethanol drinking associated with the addicted state. We found that *Alk* −/− mice drink more ethanol prior to CIE, but do not escalate ethanol consumption after CIE in comparison to *Alk* +/+ mice.

As the central nucleus of the amygdala (CeA) promotes excessive ethanol consumption, particularly in ethanol-dependent animals (Koob et al., 2014), and *Alk* −/− mice drink more ethanol than *Alk* +/+ mice and do not escalate drinking after CIE, we hypothesized that the physiology of CeA neurons might be altered in *Alk* −/− mice. Specifically, the CeA is critically involved in the development of the negative emotional state that drives dependence-induced alcohol consumption (Koob and Volkow, 2010), and basal CeA GABA neurotransmission is augmented by acute ethanol and in ethanol-dependent animals (Roberto et al., 2012; Roberto et al., 2004). Since GABA neurotransmission in the CeA appears to play a critical role in ethanol intake (Hyytia and Koob, 1995; Roberts et al., 1996), we examined GABA neurotransmission in ethanol-naïve, ethanol non-dependent, and ethanol-dependent *Alk* −/− and +/+ mice using whole-cell recordings from CeA slices. We

observed increased basal GABA neurotransmission in ethanol-naïve *Alk* −/− mice. In addition, CIE treatment did not further augment GABA transmission in *Alk* −/− mice as it did in *Alk* +/+ mice. These alterations in GABA release are consistent with the ethanol consumption phenotypes observed in *Alk* −/− mice prior and subsequent to chronic ethanol exposure. Our results suggest that ALK regulates GABA release in the CeA and provide a novel link between ALK, GABA neurotransmission, and excessive ethanol consumption.

2. Material and methods

2.1 Experimental animals

Alk −/− mice have been described (Lasek et al., 2011) and were previously backcrossed to C57BL/6J mice for 4 generations. Heterozygote breeding was used to produce *Alk* +/+ and homozygous *Alk* knockout (−/−) littermates for this study. For the CIE-2BC experiments, 71 mice were used, with 67 completing the study. Mice were tested starting at 11–13 weeks of age in 3 cohorts. Cohort 1 (n = 35) consisted of 11 male *Alk* +/+, 11 male *Alk* −/−, 6 female *Alk* +/+, and 7 female *Alk* −/− mice. Cohort 2 (n = 19) consisted of 6 male *Alk* +/+, 7 male *Alk* −/−, 2 female *Alk* +/+, and 4 female *Alk* −/− mice. Finally, Cohort 3 consisted of 9 female *Alk* +/+ and 8 female *Alk* −/− mice. Two *Alk* −/− mice from each of cohorts 2 & 3 that were in the ethanol vapor exposure groups were removed from the study due to ill health. For the electrophysiology experiments, we used 13 male *Alk* +/+ and 11 male *Alk* −/− mice. Mice were housed in groups of 2–4 except during the daily 2 h 2BC testing of ethanol consumption. Mice had access to food and water *ad libitum*. Efforts were taken to minimize suffering and to reduce the number of animals used. Lights were on a 12 h light/dark cycle with lights off at 8 am. All experimental approaches were approved by The Scripps Research Institute (TSRI) Institutional Animal Care & Use Committee and performed according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

2.2 CIE-2BC procedure

For the first 15 days of testing (5 days per week for 3 weeks), 30 min before the lights went off (7:30 am), mice were individually housed for 2 h with access to 2 drinking tubes, one containing 15% ethanol and the other containing water. Ethanol and water consumption after the 2 h period were recorded. Following this baseline period of drinking, mice were divided by genotype and sex, based on equal ethanol and water consumption, into 2 balanced treatment groups that were exposed to intermittent ethanol vapor or air in identical chambers. Chambers consisted of standard plastic mouse-sized shoebox cages containing up to 4 mice per chamber (La Jolla Alcohol Research Inc., La Jolla, CA, USA). Ethanol vapor was created by dripping 95% ethanol (Pharmco-AAPER, Brookfield, CT, USA) into a 2 l Erlenmeyer vacuum flask kept at 50°C on a warming tray. Air was blown over the bottom of the flask at a rate of 11 l/min. Concentrations of ethanol vapor were adjusted by varying the rate at which the ethanol was pumped into the flask, which in turn, was based on the blood alcohol levels of the mice. Ethanol vapor was independently introduced into each sealed chamber through a stainless steel manifold. Air was pumped into identical chambers for controls. The ethanol vapor group was injected with 1.75 g/kg ethanol and 68.1 mg/kg pyrazole (an alcohol dehydrogenase inhibitor) and placed in the chambers to receive

intermittent vapor for 4 days (16 h vapor on, 8 h off). The control group was injected with 68.1 mg/kg pyrazole in 0.9% saline and placed in chambers delivering air for the same periods as the ethanol vapor group and received 2BC testing at the same time as the vapor group. Following each 16 h bout of ethanol vapor exposure, mice were removed and on the 2nd and 4th day, tail blood was sampled for blood alcohol levels. Blood was collected in capillary tubes and emptied into 1.5 ml centrifuge tubes containing evaporated heparin and kept on ice. Samples were centrifuged and plasma was decanted into fresh 1.5 ml centrifuge tubes. The plasma was injected into an oxygen-rate alcohol analyzer (Analox Instruments, Lunenburg, MA, USA) for blood alcohol determination. Five pairs of ethanol standards (0.5 – 3.0 mg/ml) were analyzed prior to the samples. Target blood alcohol levels were 200–250 mg%. Following the fourth day of exposure, mice were allowed 72 h of undisturbed time in their home cages. The mice were then given 5 days of access to 2 bottles containing 15% ethanol or water for 2 h to measure ethanol drinking and preference. The 4 days of vapor or air exposure and 5 days of 2 bottle choice testing were repeated for a total of 4 rounds, designated CIE1-CIE4.

2.3 Electrophysiology

CeA slices were prepared as previously described (Roberto et al., 2010) from male *Alk* +/+ and *Alk* −/− mice (25–30g). We chose to do these experiments in male mice since we did not find any sex differences in ethanol consumption after CIE (see Results). Mice from behavioral testing (after 4 rounds of CIE-2BC) were used for electrophysiology and were either exposed to air and subjected to 2BC ethanol consumption, or were exposed to ethanol vapor and subjected to 2BC ethanol consumption. Additional groups of ethanol naïve mice were used to measure baseline GABA transmission. Mice were anesthetized with isoflurane (3%) and decapitated. Brains were rapidly removed and placed into oxygenated (95% O_2 and 5% CO_2; pH 7.3) ice-cold high-sucrose solution containing (in mM): sucrose 206; KCl 2.5; NaH_2PO_4 1.2; $MgCl_2$ 7; $CaCl_2$ 0.5; $NaHCO_3$ 26; glucose 5; HEPES 5. Transverse slices 300 μm thick were cut on a Vibratome (Leica VT1000S, Leica Microsystems, Buffalo Grove, IL, USA) and transferred into oxygenated artificial cerebrospinal fluid (ACSF) of the following composition (in mM): NaCl, 130; KCl, 3.5; NaH_2PO_4, 1.25; $MgSO_4$·7H2O, 1.5; $CaCl_2$, 2.0; $NaHCO_3$, 24; glucose, 10. Slices were incubated for 30 min at 35–37°C and then kept at room temperature for the remainder of the experiment. Individual slices were transferred to a recording chamber mounted on the stage of an upright microscope (Olympus BX50WI, Tokyo, Japan) for each experiment. Recordings were performed in continuously oxygenated ACSF perfused at a rate of 2–3 ml/min, 1–8 h after slice preparation. Drugs were added to the ACSF from stock solutions to obtain known concentrations in the superfusate.

Recordings were performed in the whole cell configuration from 48 neurons in the medial subdivision of the CeA using voltage clamp mode. Neurons were visualized using infrared differential interference contrast (IR-DIC) optics and a CCD camera (EXi Aqua and ROLERA-XR, QImaging, Surrey, BC, Canada). Recordings were performed in gap-free acquisition mode (sample rate 10 kHz) and low-pass filtered (10kHz), using a Multiclamp 700B amplifier, Digidata 1440A and pClamp 10 software (Molecular Devices, Sunnyvale, CA, USA). Patch pipettes (impedance range 3–7MΩ) were pulled from borosilicate glass

(Warner Instruments, Hamden, CT, USA) and filled with KCl internal solution (in mM): KCl 145; EGTA 5; $MgCl_2$ 5; HEPES 10; Na-ATP 2; Na-GTP 0.2. Pharmacologically-isolated $GABA_A$ receptor-mediated spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in the presence of 6,7-dinitroquinoxaline-2,3-dione (DNQX, 20 μM, from Tocris, Ellisville, MO, USA), DL-2-amino-5-phosphonovalerate (AP-5, 30 μM, from Sigma) and CGP 55845A (1 μM, from Sigma) to block AMPA, NMDA and $GABA_B$ receptors. Cells were held at −60 mV. Experiments with a series resistance >15 MΩ or a >20% change in series resistance, as monitored with a 10 mV pulse, were excluded from the final data set. The sIPSC frequency, amplitude and kinetics were analyzed with Mini Analysis software (Synaptosoft Inc., Fort Lee, NJ, USA), with sIPSCs <5 pA excluded from the final data set and the average sIPSC measures derived from a minimum time interval of 3 minutes. Ethanol was purchased from Remet (La Miranda, CA, USA).

2.4 Data analysis and statistics

For the CIE-2BC experiments, a four-way ANOVA was performed for CIE episodes (CIE1-4) and treatments (air or vapor) with between subjects factors of genotype, sex and treatment, and the within-subjects factor of CIE episode. Data was further analyzed using two-way ANOVA for each genotype and CIE episode with the between subjects factors of treatment and sex using Prism 6.0g software for Mac (GraphPad, San Diego, CA, USA). For the electrophysiology experiments, MiniAnalysis 5.1 software was used for data analysis and Prism 5.0 software (GraphPad) was used for statistical analyses. The electrophysiology results were evaluated with cumulative probability analysis, and significance determined with the Kolmogorov-Smirnov nonparametric two-sample test. T-test analyses were used for individual means comparisons, and within-subject one-way repeated measures ANOVAs were used to compare sIPSCs within a group. When appropriate, the Bonferroni *post hoc* comparisons were used to assess significance between treatments. Data are presented as the mean ± SEM.

3. Results

3.1 Alk −/− mice do not escalate ethanol consumption after CIE

We previously discovered that *Alk* −/− mice consume more ethanol in a limited-access ethanol consumption test (Lasek et al., 2011). To determine if *Alk* −/− mice exhibit altered ethanol consumption after chronic ethanol exposure, we tested them for 2BC ethanol consumption after CIE. Prior to CIE (baseline ethanol consumption), *Alk* −/− mice consumed 63% more ethanol than *Alk* +/+ controls (two-way ANOVA, genotype effect: $F_{1,63} = 4.7$, p = 0.03), consistent with our previous results. Female mice drank slightly more than male mice; however, the effect of sex and the genotype by sex interaction were not significant. Fig. 1 shows the CIE-2BC data with the male and female data combined within each genotype. Analysis of the data across all 4 CIE exposures, using a four-way repeated measures ANOVA, indicated that there were significant effects of genotype ($F_{1,59} = 4.9$, p = 0.03), treatment ($F_{1,59} = 4.8$, p = 0.03) and CIE episode ($F_{3,177} = 6.0$, p = 0.0007). Again, although female mice of both genotypes drank slightly more ethanol than males, there were no significant sex effects. We next performed two-way ANOVAs for the 2BC drinking after each CIE episode for *Alk* +/+ and *Alk* −/− mice separately, with the between subject factors

of treatment and sex. In *Alk* +/+ mice, there were no effects of sex even when examining these unitary tests. However, there were significant effects of treatment in CIE2 (116% increase in ethanol vapor-exposed compared to air control, $F_{1,30} = 4.6$, p = 0.04), CIE3 (140% increase in ethanol vapor-exposed compared to air control, $F_{1,30} = 6.2$, p = 0.02), and CIE4 (125% increase in ethanol vapor-exposed compared to air control, $F_{1,30} = 6.5$, p = 0.02), demonstrating that *Alk* +/+ mice escalated their ethanol consumption after the second round of ethanol vapor exposure (Fig. 1A). For *Alk* −/− mice, there was a significant effect of sex in CIE2 ($F_{1,29} = 5.3$, p = 0.03), with female mice drinking more than males. However, there were no significant effects of treatment or a treatment by sex interaction in any of the ethanol consumption tests, indicating that *Alk* −/− mice do not escalate ethanol drinking after repeated intermittent ethanol vapor exposure (Fig. 1B).

3.2 Increased basal GABA transmission in the CeA of Alk −/− mice

The CeA is implicated in excessive ethanol consumption, particularly in ethanol-dependent animals (Koob et al., 2014). Since *Alk* −/− mice drink more ethanol than *Alk* +/+ mice and do not escalate drinking after CIE, we hypothesized that the physiology of CeA neurons might be altered in *Alk* −/− mice. To test this, we recorded from medial CeA neurons using intracellular voltage-clamp in the whole-cell configuration and pharmacologically isolated $GABA_A$-mediated spontaneous inhibitory postsynaptic currents (sIPSCs). Basal GABAergic activity was significantly increased from 0.49 ± 0.05 Hz in *Alk* +/+ CeA neurons (n = 8) to 0.62 ± 0.05 Hz in *Alk* −/− CeA neurons (n = 8), representing an overall increase of 27% (Fig. 2, p < 0.05). The average sIPSC amplitude and rise and decay times were not significantly different in neurons from *Alk* +/+ compared to *Alk* −/− mice (Fig. 2). The higher occurrence of spontaneous GABA activity in the CeA of *Alk* −/− mice indicates that *Alk* deletion leads to increased inhibitory transmission in the CeA. Since increased sIPSC frequency is indicative of a higher probability of GABA release (De Koninck and Mody, 1994), the increased frequency of sIPSCs in CeA neurons from *Alk* −/− mice suggests that the effect occurs at a presynaptic site to alter GABA release, rather than an alteration of $GABA_A$ receptor function.

3.3 Enhancement of GABA transmission by acute ethanol is amplified in Alk −/− mice

We next examined the effect of acute ethanol treatment on GABA transmission in *Alk* +/+ and *Alk* −/− mice by superfusing 44 mM ethanol onto CeA slices. This concentration of ethanol has been shown to elicit an optimum enhancement of GABAergic transmission in the CeA (Roberto et al., 2003). Application of ethanol for 8–10 minutes onto CeA neurons from *Alk* +/+ mice increased the sIPSC frequency from 0.49 ± 0.05 Hz to 0.69 ± 0.10 Hz (Fig. 3, n = 8). CeA neurons from *Alk* −/− mice also displayed an increase of sIPSCs from 0.62 ± 0.05 Hz in control condition to 1.01 ± 0.08 Hz upon superfusion of ethanol (n = 8). A two-way ANOVA demonstrated that there were significant main effects of ethanol, genotype, and a significant ethanol by genotype interaction (ethanol: $F_{1, 14} = 58.51$, p < 0.0001; genotype: $F_{1, 14} = 5.49$, p = 0.034; ethanol by genotype interaction: $F_{1, 14} = 5.74$, p = 0.031). Ethanol caused a significantly greater enhancement of sIPSC frequency in neurons from *Alk* −/− mice compared to neurons from *Alk* +/+ mice (Fig. 3C, p < 0.05, 63 ± 8% increase in *Alk* −/− vs. 41 ± 7% increase in *Alk* +/+ neurons). The sIPSC amplitude was not significantly affected by ethanol in CeA neurons from *Alk* +/+ (101 ± 3%; n = 8) or *Alk* −/

− (101 ± 4%; n = 8) mice (Fig. 3C), indicative of a selective effect of ethanol on GABA release in both genotypes. In addition, sIPSC kinetics (rise and decay) were not significantly altered (data not shown). After washout of ethanol for 10–20 minutes, sIPSC frequency returned to pre-ethanol levels in neurons from both *Alk* +/+ and *Alk* −/− mice (0.48 ± 0.05 Hz for *Alk* +/+ and 0.67 ±0.06 Hz for *Alk* −/−; data not shown). Together, these data indicate that homozygous *Alk* deletion enhances GABA transmission in CeA neurons in response to acute ethanol to a greater extent than in neurons expressing normal levels of *Alk*.

3.4 CIE augments basal GABA transmission in neurons from Alk +/+ but not Alk −/− mice

Since *Alk* −/− mice do not escalate ethanol drinking after CIE, we next tested for differences in GABA transmission after CIE in CeA neurons from *Alk* +/+ and *Alk* −/− mice. *Alk* +/+ ethanol-naïve mice (control) and mice that were exposed to air and underwent 2BC showed sIPSC frequencies that were not significantly different (0.49 ± 0.05 Hz and 0.51 ± 0.05 Hz respectively, n = 8; Fig. 4A), indicating that 2BC ethanol consumption did not effect basal GABA transmission. However, in *Alk* +/+ mice that were chronically exposed to ethanol vapor, the basal sIPSC frequency was 0.66 ± 0.06 Hz (n = 9; Fig. 4A), a 35% ($p < 0.05$) and 29% ($p < 0.05$) augmentation compared to ethanol-naïve and air-exposed mice, respectively. The sIPSC amplitudes were comparable in neurons from all three groups of *Alk* +/+ mice (control, 48 ± 7 pA, n = 8; air, 52 ± 6 pA, n = 8; ethanol vapor, 46 ± 5 pA, n = 9). In contrast to *Alk* +/+ mice, CIE treatment did not change basal GABAergic transmission in CeA neurons from *Alk* −/− mice (Fig. 4B). Note that the higher sIPSC frequency obtained in *Alk* +/+ neurons upon CIE treatment (Fig. 4A, right column) is comparable to the sIPSC frequency obtained in control *Alk* −/− neurons (Fig. 4B), suggesting that *Alk* deletion elicits an effect similar to CIE treatment. Finally, as in *Alk* +/+ mice, the sIPSC amplitude and kinetics remained unchanged after CIE in neurons from *Alk* −/− mice (data not shown).

3.5 CIE does not modify the effect of acute ethanol on GABA transmission

After assessing the influence of CIE on basal GABAergic transmission, we next determined if CIE would affect the response to acute ethanol in *Alk* −/− mice. In *Alk* +/+ mice, superfusion of 44 mM ethanol increased sIPSC frequency by 41 ± 7% in neurons from ethanol-naïve mice (n = 8), 38% ± 7 in neurons from mice exposed to air-2BC (n = 7), and 36 ± 6% in neurons from mice exposed to CIE-2BC (n = 8) (Fig. 5A, no significant differences). After washout of ethanol for 10–20 minutes, sIPSC frequencies in all groups returned to control levels (data not shown). In addition, sIPSC amplitudes were not affected by ethanol in all groups (data not shown). We observed a similar pattern in neurons obtained from *Alk* −/− mice. Although application of acute ethanol elicited a larger effect on GABAergic transmission overall in CeA neurons from *Alk* −/− compared to *Alk* +/+ mice (as in Fig. 3), CIE did not alter the extent of the increase in sIPSC frequency by acute ethanol. Superfusion of 44 mM ethanol onto CeA slices from *Alk* −/− mice augmented sIPSC frequency by 63 ± 8% in ethanol-naïve neurons (n = 8), 62 ± 9% in neurons from mice exposed to air (n = 6), and 52 ± 9% in neurons from mice exposed to ethanol vapor (Fig. 5B, n = 8). After ethanol washout, sIPSC frequencies returned to control levels in all groups and the amplitude and kinetics of sIPSCs were not affected by ethanol (data not shown). These results indicate that the effect of acute ethanol remains unchanged after CIE

and that chronic ethanol exposure does not induce tolerance in neurons from *Alk* +/+ or *Alk* −/− mice.

4. Discussion

In this study we provide evidence that ALK regulates alcohol consumption and GABA neurotransmission in the CeA. To our knowledge, this is the first study that shows that ALK may regulate dependence-induced drinking and GABA neurotransmission. Our key findings are that *Alk* −/− mice initially consume more ethanol and have increased basal and ethanol-stimulated GABA release in the CeA when compared to *Alk* +/+ mice. However, after chronic ethanol exposure, *Alk* +/+ mice escalate their ethanol consumption, whereas *Alk* −/− mice do not. Moreover, basal GABA release in *Alk* −/− mice is not enhanced by CIE as it is in *Alk* +/+ mice. Thus, it appears that eliminating ALK creates a “dependence-like” phenotype, such that the knockout mice are predisposed to consume more ethanol as a result of increased GABA neurotransmission in the CeA.

We previously found that *Alk* −/− mice consume more ethanol in a binge-like, limited-access intermittent drinking procedure in which mice were given a single bottle of 20% ethanol for 4 h per day, 3 days per week (Lasek et al., 2011). Here, we reproduced the drinking phenotype prior to chronic ethanol exposure (baseline drinking) in a 2-hour, 2BC procedure in which mice were given a choice between water and 15% ethanol for 5 consecutive days per week. The increased ethanol consumption observed in *Alk* −/− mice may be due to increased basal GABA neurotransmission, since GABA signaling in the CeA clearly affects ethanol intake (Hyytia and Koob, 1995; Roberts et al., 1996). CeA neurons in *Alk* −/− mice also respond more robustly to acute ethanol stimulation of GABA release, and this might contribute to the increased drinking behavior in these mice.

It should be noted that increased ethanol consumption exhibited by *Alk* −/− mice prior to ethanol exposure might be due to developmental compensation, since these mice lack *Alk* during embryogenesis. We recently treated adult mice with small-molecule ALK inhibitors and found decreased ethanol consumption in a limited access binge-like drinking test (Dutton et al., 2016). Since we observed the opposite phenotype in *Alk* −/− mice, we suspect that there is compensation occurring. *Alk* −/− mice have increased striatal levels of phosphorylated mitogen-activated protein kinase kinase (MEK, or MAP2K), a signaling molecule in the Ras/extracellular signal-regulated kinase (ERK) pathway (Lasek et al., 2011), although it is well established that ALK activates the Ras/ERK pathway (Hallberg and Palmer, 2013). In fact, we have found that acute ethanol treatment increases ALK-dependent ERK phosphorylation in a neuroblastoma cell line, and that treatment of C57BL/6J mice with an ALK inhibitor reduces ethanol-stimulated ERK phosphorylation in the CeA (He et al., 2015). Alteration of the Ras/ERK signaling pathway in *Alk* −/− mice could account for the increased ethanol consumption observed in these mice. Ras/ERK signaling clearly plays a role in alcohol consumption, although the relationship is complicated by the fact that activation and inhibition of this pathway can have the same phenotype (Agoglia et al., 2015; Ben Hamida et al., 2012; Faccidomo et al., 2009; Mulligan et al., 2006; Stacey et al., 2012). This is similar to the learning and memory literature, in

which deficits or increases in Ras/ERK signaling are detrimental to learning and memory (Fasano and Brambilla, 2011).

In addition to our observation that ethanol-naïve *Alk* −/− mice consume more ethanol than control mice, we found that *Alk* −/− mice do not escalate ethanol consumption after CIE, in contrast to *Alk* +/+ mice. There are two possible explanations for this effect. The most parsimonious explanation is that the high levels of drinking exhibited by *Alk* −/− mice are already at a maximum, such that it is difficult to see a further increase in ethanol consumption during the two-hour drinking test (*i.e.* a ceiling effect). The other possibility is that ALK plays an active role in promoting dependence-induced drinking. We tested *Alk* mRNA expression levels by quantitative real-time PCR in a small group of wild-type mice (n = 3) exposed to CIE and found that *Alk* mRNA increases by approximately 47% in the CeA when compared to control mice treated with air ($p = 0.057$, data not shown). This result suggests that *Alk* expression is regulated by ethanol and may contribute to the escalation of ethanol consumption during the development of ethanol dependence. The fact that we do not see an escalation in ethanol consumption after CIE in *Alk* −/− mice is consistent with increased *Alk* expression after CIE promoting excessive drinking. Future studies will address this possibility by treating mice with small-molecule ALK inhibitors or by using RNA interference to determine if this can reduce escalation of alcohol consumption and GABA neurotransmission induced by chronic ethanol exposure. This experiment would overcome problems with possible developmental compensation in *Alk* −/− mice.

Interestingly, two other members of the Ras/ERK signaling pathway, neurofibromin (encoded by the *Nf1* gene) and K-Ras, have been shown to modulate the escalation of ethanol consumption after chronic ethanol exposure. Heterozygous *Nf1* (+/−) and homozygous K-Ras (−/−) mice do not escalate ethanol consumption after CIE (Repunte-Canonigo et al., 2015; Repunte-Canonigo et al., 2010), similar to what we observed in *Alk* −/− mice. Neurofibromin is a Ras GTPase activating protein and a negative regulator of Ras/ERK signaling. *Nf1* +/− mice have higher levels of phosphorylated ERK in the brain and basal GABA release (Cui et al., 2008; Repunte-Canonigo et al., 2015), also similar to what we found in *Alk* −/− mice. In *Drosophila*, genetic or pharmacological inhibition of *Alk* rescues the increased ERK activation and associative learning deficits observed in *Nf1* mutants (Gouzi et al., 2011), suggesting that ALK and NF1 function in the same intracellular signaling pathway. These results, combined with the human genetic association studies that implicate the *NF1* and *ALK* genes in alcohol dependence (Repunte-Canonigo et al., 2015; Wang et al., 2011), support the possibility that ALK may play an active role in dependence-induced drinking through regulation of the Ras/ERK signaling pathway.

In addition to the ethanol consumption phenotypes, we observed increased basal and acute ethanol-stimulated GABA release in the CeA of *Alk* −/− mice, indicating that ALK regulates GABA release. How might this occur? Again, one possibility is through the Ras/ERK pathway. ERK phosphorylates synapsin I, a critical component for vesicular neurotransmitter release (Jovanovic et al., 1996), and neurofibromin regulates GABA release in inhibitory neurons in an ERK-dependent manner in the hippocampus (Cui et al., 2008). We hypothesize that ALK functions in GABA neurons in the CeA to regulate GABA release through phosphorylation of synapsin I via the Ras/ERK pathway. Future studies will test this

hypothesis using ALK inhibitors and examine in detail the signaling pathways downstream of ALK that regulate GABA neurotransmission.

CIE has been shown to increase GABA neurotransmission in rats and mice (Repunte-Canonigo et al., 2015; Repunte-Canonigo et al., 2010; Roberto et al., 2004). Although ethanol-naïve *Alk* −/− mice display increased GABA release, GABA neurotransmission after CIE is not augmented in *Alk* −/− mice as it is in *Alk* +/+ mice. This phenotype may be the result of a ceiling effect, which we cannot rule out at this point. However, *Alk* −/− mice still show acute ethanol-stimulation of GABA release after CIE, similar to *Alk* +/+ mice, demonstrating that GABA release can still be enhanced despite the high basal levels observed in *Alk* −/− mice and perhaps arguing against a ceiling effect. To more conclusively demonstrate a causal relationship between ALK and the enhancement of GABA neurotransmission after CIE, further experiments are necessary using ALK inhibitors.

Together, our data implicate ALK in excessive drinking, potentially contributing to the transition to dependence-induced drinking. ALK expression or kinase activity might be modified by chronic ethanol exposure, leading to changes in signaling cascades downstream of ALK that regulate the development of altered GABA neurotransmission observed after CIE. ALK-dependent processes are likely not the only processes regulating GABA release and subsequent increases in drinking in mice, but may play a modulatory role. Increased GABA neurotransmission in the CeA after CIE would promote the negative affective state that drives cycles of excessive alcohol drinking. Future studies will examine in more detail the molecular mechanisms through which ALK signals to regulate GABA release and ethanol consumption.

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Abbreviations

ALK	anaplastic lymphoma kinase
CeA	central nucleus of the amygdala
CIE	chronic intermittent ethanol
ERK	extracellular signal-regulated kinase
GABA	gamma aminobutyric acid
sIPSCs	spontaneous inhibitory post-synaptic currents
2BC	two bottle choice

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- Mice deficient in the *Alk* gene consume more ethanol than controls.
- *Alk* deficient mice exhibit enhanced basal GABA release in the amygdala.
- Ethanol-potentiated GABA release is higher in *Alk* deficient mice.
- *Alk* deficient mice do not escalate ethanol drinking after chronic ethanol exposure.
- GABA release does not increase after chronic ethanol exposure in *Alk* deficient mice.

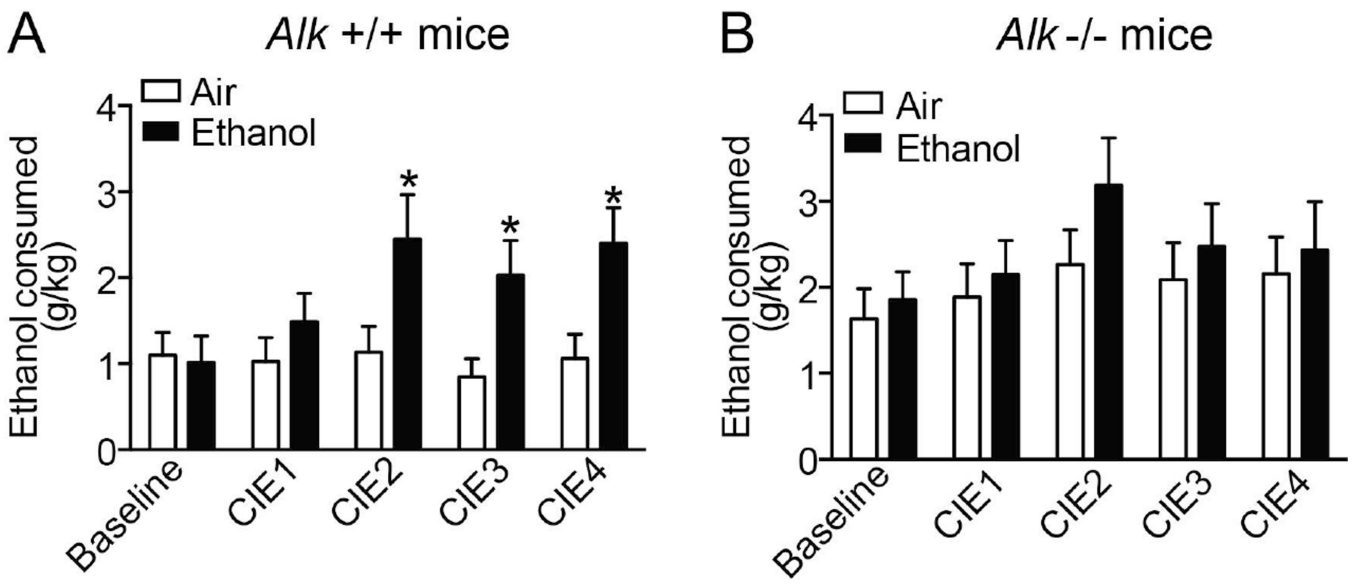

Fig. 1.

Alk −/− mice do not escalate ethanol consumption after CIE. *Alk* +/+ (**A**) and *Alk* −/− (**B**) mice were subjected to 4 rounds of CIE-2BC (CIE1-4) with air (as a control) or ethanol vapor exposure. Ethanol consumption was measured before the CIE protocol (baseline) and after each round of CIE and is expressed as g ethanol consumed per kg body weight. Data from male and female mice were combined within each genotype since no significant sex differences were observed. *Alk* +/+ mice escalated ethanol drinking after the second round of CIE, while *Alk* −/− mice did not. Data are expressed as mean ± SEM, *$p < 0.05$.

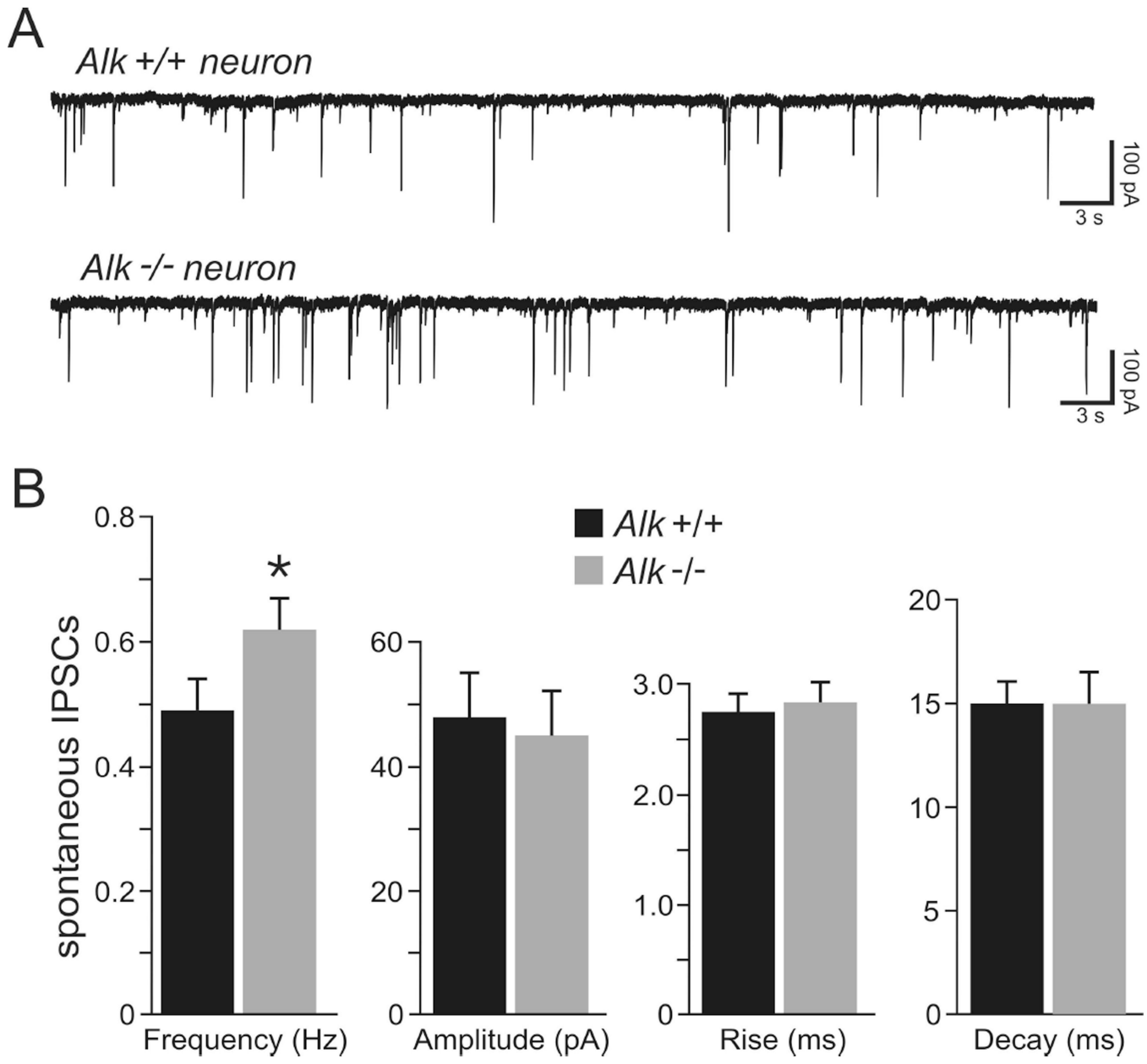

Fig. 2.

Basal spontaneous GABA transmission is higher in neurons from *Alk* −/− mice. (**A**) Representative whole-cell current recordings of sIPSCs in CeA neurons obtained from an *Alk* +/+ (top trace) and an *Alk* −/− (bottom trace) mouse. A higher frequency of sIPSCs was observed in neurons from *Alk* −/− mice compared to neurons from *Alk* +/+ animals. (**B**) Mean sIPSC frequency in CeA neurons from *Alk* +/+ and *Alk* −/− mice, showing an increase in sIPSC frequency in *Alk* −/− mice. Mean sIPSC amplitude, rise time, and decay time in *Alk* +/+ and *Alk* −/− mice are also shown, showing no significant differences between *Alk* +/+ and *Alk* −/− mice in these measures. Data are expressed as mean ± SEM, *$p < 0.05$.

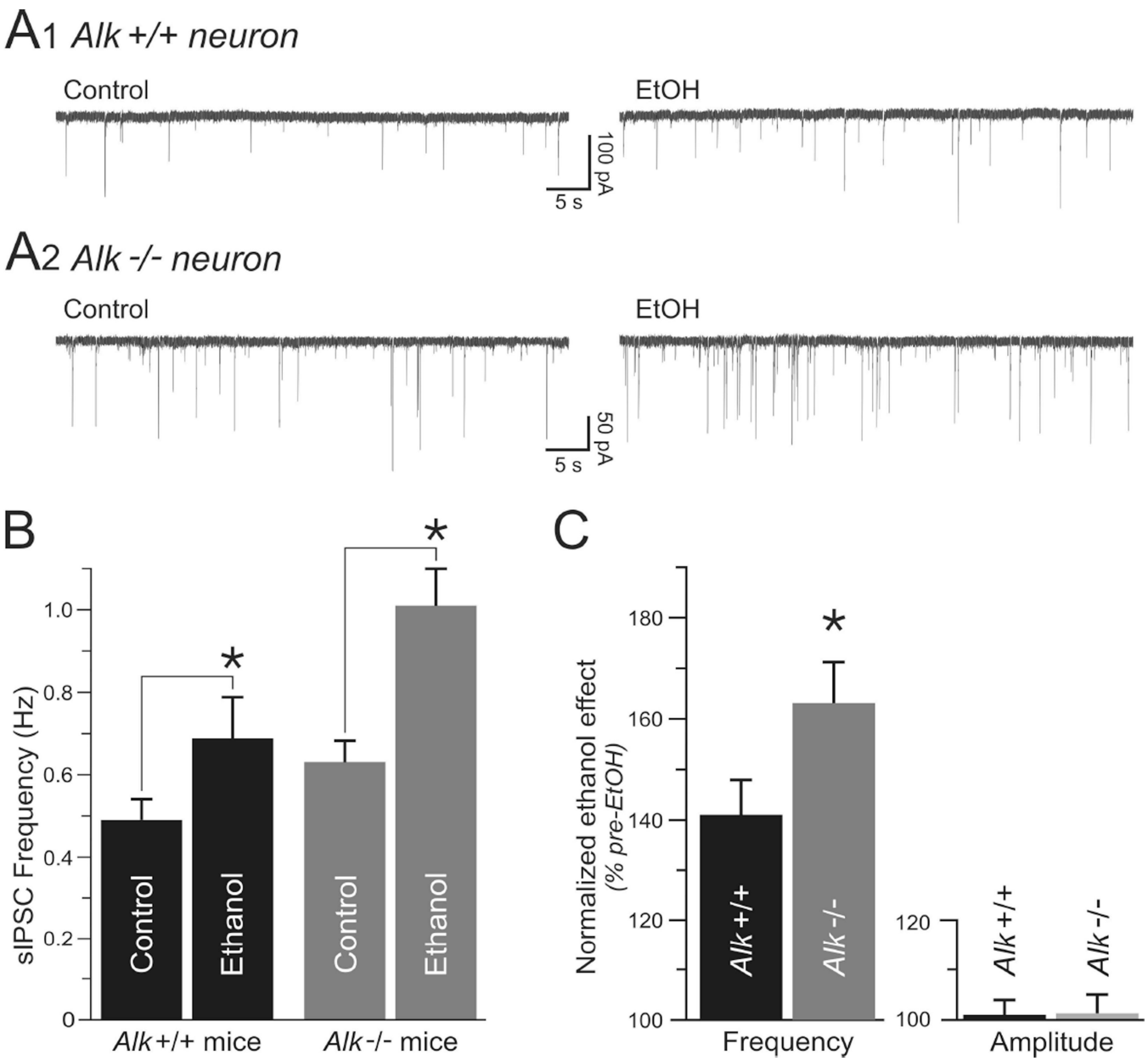

Fig. 3.

Enhancement of GABA transmission by acute ethanol is amplified in *Alk* −/− mice. (**A**) Representative whole-cell current recordings of sIPSCs in CeA neurons from an *Alk* +/+ (top traces) and an *Alk* −/− (bottom traces) mouse in the absence of ethanol (control, left traces) and after exposure of slices to 44 mM ethanol (ethanol, right traces). Ethanol significantly increased sIPSC frequency. (**B**) Mean sIPSC frequency in CeA neurons from *Alk* +/+ and *Alk* −/− mice in the absence and presence of ethanol. (**C**) Normalized effect of ethanol on sIPSC frequency in *Alk* +/+ and *Alk* −/− mice, expressed as the percentage of pre-ethanol sIPSC frequency. The increase in sIPSC frequency by acute ethanol application was more pronounced in *Alk* −/− mice. Mean sIPSC amplitude in *Alk* +/+ and *Alk* −/− mice

is also shown, showing no significant difference between genotypes. Data are expressed as mean ± SEM, *$p < 0.05$.

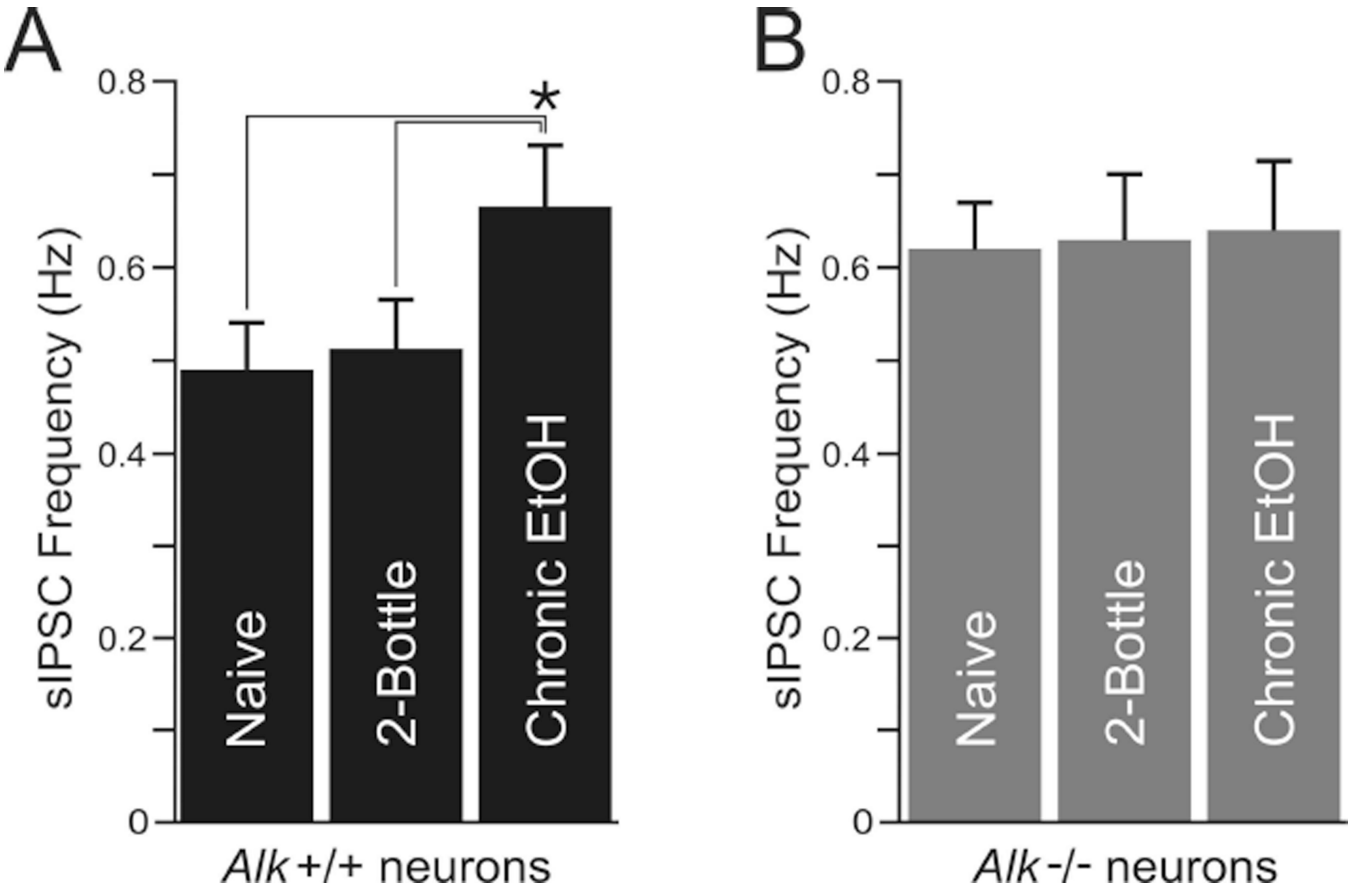

Fig. 4.

CIE augments GABA transmission in neurons from *Alk* +/+ but not *Alk* −/− mice. Mean sIPSC frequency in CeA neurons from *Alk* +/+ (**A**) and *Alk* −/− (**B**) mice that were ethanol-naïve, underwent air exposure and 2BC (2-bottle), or CIE-2BC (chronic EtOH). *Alk* +/+ mice showed increased sIPSC frequency after CIE, whereas *Alk* −/− mice did not. Note that the sIPSC frequency in *Alk* −/− mice was higher than *Alk* +/+ mice under all conditions and compared to the sIPSC frequency in ethanol-vapor treated *Alk* +/+ mice. Data are expressed as the mean ± SEM. *$p < 0.05$.

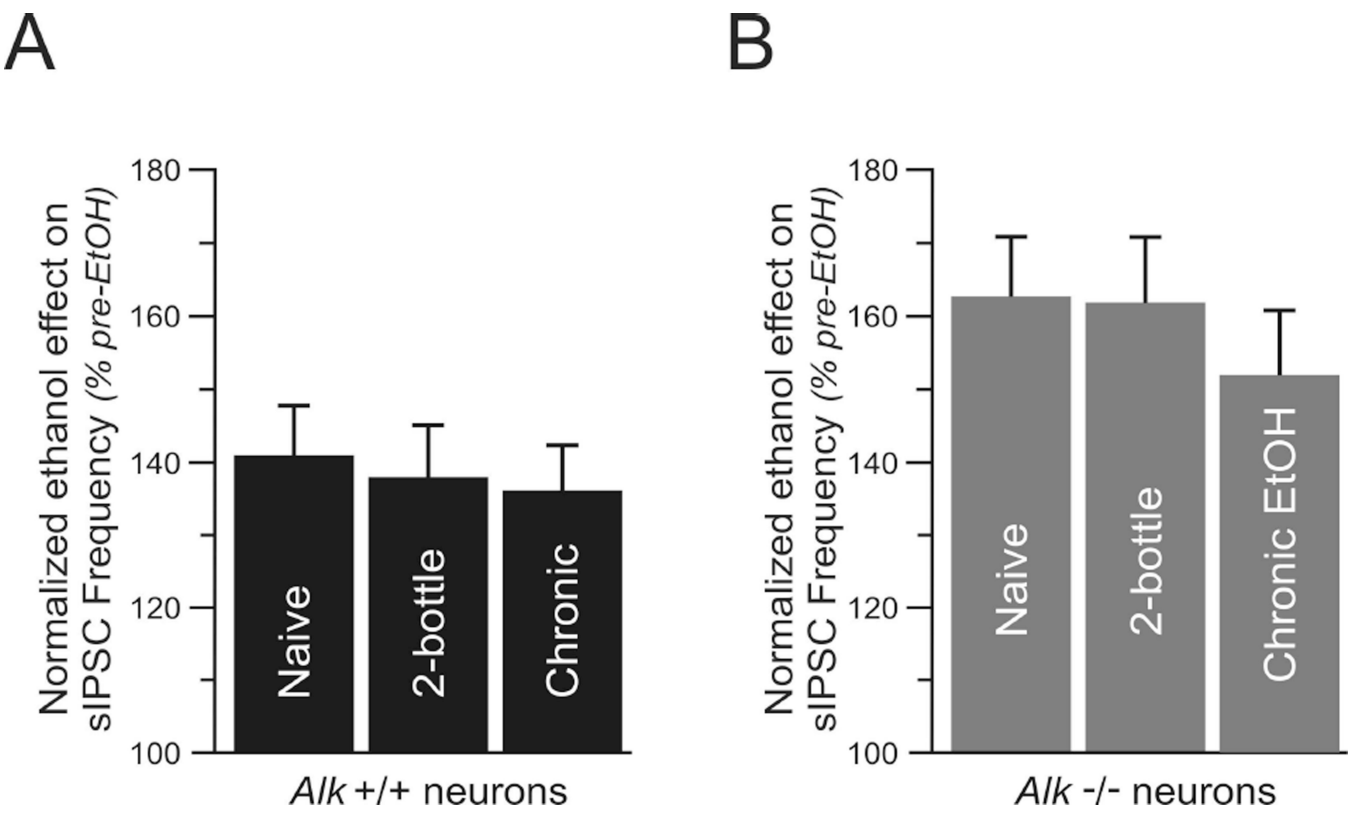

Fig. 5.

CIE does not modify the effect of acute ethanol on GABA transmission. Normalized sIPSC frequency in neurons from *Alk* +/+ (**A**) and *Alk* −/− (**B**) mice. CeA slices were treated with 44 mM ethanol. Data shows the normalized ethanol effect, expressed as the percentage increase in sIPSC frequency prior to ethanol treatment. Comparisons are made between neurons from ethanol-naïve mice, mice that underwent air exposure and 2BC (2-bottle), and mice that received CIE-2BC (chronic EtOH). CIE did not alter the ability of acute ethanol to increase sIPSC frequency in CeA neurons from *Alk* +/+ and *Alk* −/− mice. Note that the acute effect of ethanol was greater in neurons from *Alk* −/− compared to *Alk* +/+ mice. Data are expressed as the mean ± SEM.