

# Nf1 Regulates Alcohol Dependence-Associated Excessive Drinking and Gamma-Aminobutyric Acid Release in the Central Amygdala in Mice and Is Associated with Alcohol Dependence in Humans

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## ABSTRACT

**BACKGROUND:** The neurofibromatosis type 1 (Nf1) gene encodes a GTPase activating protein that negatively regulates small GTPases of the Ras family.

**METHODS:** We assessed alcohol-related behaviors including alcohol sensitivity, dependent and nondependent drinking, and basal and alcohol-induced gamma-aminobutyric acid (GABA) release in the central nucleus of the amygdala (CeA) in Nf1 heterozygous null mice (Nf1<sup>+/-</sup>). We also investigated the associations of NF1 polymorphisms with alcohol dependence risk and severity in humans.

**RESULTS:** Nf1<sup>+/-</sup> mice do not differ from wild-type mice in nondependent drinking, such as 24-hour, 2-bottle choice drinking in the dark binge drinking or limited access 2-bottle choice. However, Nf1<sup>+/-</sup> mice failed to escalate alcohol drinking following chronic intermittent ethanol vapor exposure (CIE) to induce dependence. Alcohol acutely increases GABA release in the CeA and alcohol dependence is characterized by increased baseline GABA release in CeA. Interestingly, GABA release in Nf1<sup>+/-</sup> mice is greater at baseline than wild-type mice, is not elevated by induction of dependence by CIE, and failed to show alcohol-induced facilitation both before and after CIE. Additionally, we observed that multiple variants in the human NF1 gene are associated with a quantitative measure of alcohol dependence in both African Americans and European Americans.

**CONCLUSIONS:** In this translational investigation, we found that Nf1 activity regulates excessive drinking and basal and ethanol-stimulated GABA release in the mouse central amygdala. We also found that genetic variation in NF1 may confer an inherent susceptibility to the transition from nondependent to dependent drinking in humans.

**Keywords:** Alcohol dependence, Amygdala, Electrophysiology, GABA, Genetic association, Presynaptic mechanisms

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NF1, the causative gene of neurofibromatosis type 1 in humans, encodes a large multifunctional protein with Ras GTPase activating protein activity termed neurofibromin. Neurofibromin acts as a negative regulator for the Ras family of small G proteins and the Ras-extracellular signal-regulated kinase (ERK) signal transduction pathway (1). Evidence supports a role for the Ras-ERK pathway in the effects of ethanol and in the long-lasting neuroadaptive changes induced by ethanol dependence (2–7). In particular, the activity of the Ras-ERK pathway is regulated by ethanol in multiple brain regions (6–9) and is involved in the increased drinking associated with withdrawal in dependent mice in the chronic intermittent ethanol vapor (CIE) paradigm (9). Previous studies also implicated Nf1 in presynaptic gamma-aminobutyric acid (GABA) release (10,11), which is a target of the effects of ethanol in the central nucleus of the amygdala (CeA) (12,13).

Here, we show that Nf1 heterozygous null (Nf1<sup>+/-</sup>) mice do not show increased drinking in the CIE paradigm, while not differing from control mice in nondependent ethanol drinking behavior. Baseline GABA release in the CeA was higher in Nf1<sup>+/-</sup> than in wild-type (WT) mice and was not increased by acute ethanol exposure. CIE also failed to increase baseline GABA release in Nf1<sup>+/-</sup> as it did in WT mice. As increased presynaptic GABA release in the CeA is associated with increased drinking in animals with a history of dependence (13), the high basal GABAergic inhibition in the CeA of Nf1<sup>+/-</sup> mice and the failure of CIE to increase baseline GABA release in the CeA of Nf1<sup>+/-</sup> mice are consistent with their resistance to CIE-induced increased drinking. Further, we found that multiple NF1 variants are associated with a quantitative measure of alcohol dependence in both African Americans (AAs) and European Americans (EAs). Taken together, these

results suggest that NF1 regulates alcohol consumption and may be an important genetic factor in the transition from nondependent to dependent drinking.

## METHODS AND MATERIALS

### Mice

Nf1<sup>+/-</sup> mice established by Tyler Jacks, Ph.D. (Massachusetts Institute of Technology) were obtained from Alcino Silva, Ph.D. (University of California Los Angeles) and were backcrossed to C57BL/6J mice for more than eight generations.

### Twenty-Four Hour, Two-Bottle Choice Paradigm

WT and Nf1<sup>+/-</sup> mice were single housed and presented with two 50 mL conical tubes filled with water and allowed to habituate for 1 week. After the habituation period, one of the two bottles was replaced with an ethanol solution in escalating concentrations (3, 6, 9, 12, 15, and 20% vol/vol) starting on Monday of each week. Both bottles were weighed daily Monday through Friday, and mice were weighed each Wednesday. Bottle positions were switched daily to avoid side preferences. Both 50 mL bottles were replaced with a single 500 mL water bottle over the weekend.

### Drinking in the Dark Paradigm

Drinking in the dark (DID) involves three daily drinking sessions of 2 hours and a fourth session of 4 hours (14,15). At the beginning of each session, the water bottle is replaced with a bottle containing 20% (vol/vol) ethanol for 2 hours in the home cage starting 3 hours into the dark phase.

### Chronic Intermittent Ethanol Vapor Exposure

The present paradigm is based on previous studies by the group of Becker and Lopez (16) with minor modifications (17). Briefly, WT and Nf1<sup>+/-</sup> mice were trained to drink ethanol (20% vol/vol) or water in the two-bottle choice (2BC) paradigm with limited access (2 hours/day) starting 3 hours into the dark phase for 15 days. Each bout of ethanol vapor exposure consisted of 16 hours per day for 4 days. Before exposure to ethanol vapor, mice were injected intraperitoneally with a solution of 1.5 g/kg ethanol and 68.1 mg/kg pyrazole and immediately placed into ethanol vapor chambers (La Jolla Alcohol Research, La Jolla, California). Tail blood sampling for blood ethanol level determination was carried out daily. Target blood alcohol levels were 150 to 200 mg%. Seventy-two hours following removal from the chambers, mice received access to water versus 20% ethanol for 2 hours and again over the next 4 days. The following week, mice were re-exposed to the ethanol vapor and again tested for 2BC drinking for 5 days. Three vapor bouts followed by 2BC were carried out. Mice were weighed every 4 to 6 days throughout the 2BC sessions and daily during the vapor exposure bouts. Food and water were available ad libitum and mice were group housed except during the ethanol drinking sessions.

### Electrophysiological Methods

**Brain Slice Electrophysiological Recordings.** Coronal slices (300 μm) were obtained from naive (nonethanol-exposed) mice, as described in Bajo *et al.* (18) and Herman *et al.* (19). Neurons were visualized using infrared differential interference

contrast optics and an EXi Aqua camera (QImaging, Surrey, British Columbia, Canada) at 60× magnification. Whole-cell voltage-clamp recordings ( $V_{\text{hold}} = -60$  mV) were made with patch pipettes (4 MΩ to 6 MΩ; Warner Instruments, Hamden, Connecticut) coupled to a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, California), low-pass filtered at 2 kHz to 5 kHz, digitized (Digidata 1440A; Molecular Devices), and stored on a computer using pClamp 10 software (Axon Instruments). The intracellular solution for voltage-clamp recordings was composed of (in mmol/L): potassium chloride 145; ethylene glycol tetraacetic acid 5; magnesium chloride 5; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid 10; sodium-adenosine triphosphate 2; sodium-guanosine triphosphate .2. GABA A receptor currents were isolated with the glutamate receptor blockers 6,7-dinitroquinoxaline-2,3-dione (20 μmol/L) and DL-2-amino-5-phosphonovaleate (50 μmol/L) and the GABA B receptor antagonist CGP55845A (1 μmol/L). Frequency, amplitude and decay of inhibitory postsynaptic currents (IPSCs) were analyzed and visually confirmed using semi-automated, threshold-based mini detection software (Mini Analysis, Synaptosoft Inc., Decatur, Georgia). Data are presented as mean ± standard error. In all cases,  $p < .05$  was the criterion for statistical significance. An expanded version of the methods is presented in Supplement 1.

**Drugs and Chemicals.** 6,7-dinitroquinoxaline-2,3-dione (10 μmol/L), DL-2-amino-5-phosphonovaleate (50 μmol/L), and CGP 55845A (1 μmol/L) were from Tocris Bioscience (Bristol, United Kingdom). Tetrodotoxin (1 μmol/L) was from Sigma (St. Louis, Missouri).

### Human Genetic Study Methods

**Subject Ascertainment and Classification.** The sample consisted of both small families and unrelated cases and control subjects ascertained in two independent studies. In the Yale-Penn sample, individuals were recruited from the community and from substance abuse treatment centers at four US clinical sites. All subjects were recruited for studies of the genetics of drug (opioid or cocaine) or alcohol dependence (20). The sample consisted of small nuclear families originally collected for linkage studies and unrelated individuals. Subjects gave written informed consent as approved by the institutional review board at each site, and certificates of confidentiality were obtained from National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism (see Supplement 1 for additional details). All subjects were interviewed using an electronic version of the Semi-Structured Assessment for Drug Dependence and Alcoholism (21) to derive diagnoses for lifetime dependence on several substances including alcohol according to DSM-IV criteria. Subjects who met DSM-IV criteria for alcohol abuse but not for alcohol dependence were excluded from further analyses. Additional details of this sample, including results of a genome-wide association studies of alcohol and cocaine dependence, were published previously (20,22).

The second dataset included publically available genome-wide association study data from the Study of Addiction: Genetics and Environment ([http://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study\\_id=phs000092.v1.p1](http://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000092.v1.p1)).

Genotyping, data cleaning, population assignment, imputation, and statistical analysis are described in Supplement 1.

## RESULTS

### Nf1 Regulates Dependence-Induced Increases in Drinking

Nf1<sup>+/-</sup> and WT mice were trained to consume ethanol in a 24-hour, 2BC paradigm at increasing concentrations of ethanol (Figure 1A). Ethanol intake of Nf1<sup>+/-</sup> and WT mice increased similarly with increasing ethanol concentrations and did not differ between the two genotypes (Figure 1A). Ethanol intake of Nf1<sup>+/-</sup> and WT mice also did not differ in the DID binge drinking paradigm (Figure 1B) or in the limited access 2BC baseline preceding CIE (Figure 2A–C), although a nonsignificant trend toward lower ethanol consumption was seen in Nf1<sup>+/-</sup> mice ( $t_{19} = 1.658, p = .1138$ ) both in DID and in the limited access 2BC preceding CIE ( $t_{15} = 1.511, p = .1516$ ). Following exposure to alcohol vapors to induce dependence in the CIE paradigm, WT mice progressively increased their ethanol drinking and their intake was significantly higher than their prevapor baseline levels starting after the first bout of vapor (Figure 2B). Conversely, Nf1<sup>+/-</sup> mice did not significantly increase ethanol drinking after the CIE procedure and their alcohol intake was significantly lower than that of WT mice by repeated measures analysis of variance (Figure 2B). Nf1<sup>+/-</sup> mice also did not differ in the duration of loss of righting reflex or kinetics of alcohol elimination after a bolus of alcohol (3.5 g/kg intraperitoneal; Figure S1 in Supplement 1). Additionally, Nf1<sup>+/-</sup> mice drank equal amounts of graded solutions of saccharin or quinine as WT mice, showing that taste function was unaffected by this Nf1 mutation (Figure S2 in Supplement 1). Thus, Nf1<sup>+/-</sup> mice do not differ from WT mice in nondependent drinking behavior, including 24-hour 2BC, the DID binge drinking paradigm, and limited access 2BC at baseline. However, unlike WT mice, they did not increase their drinking after vapor exposure in the CIE procedure to induce dependence.

### Nf1 Regulates GABA Release in the Central Amygdala

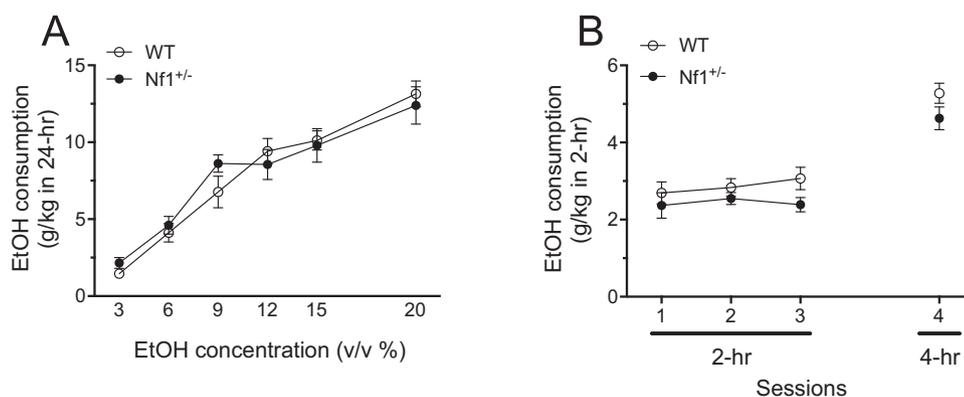
Spontaneous IPSCs (sIPSCs) were measured using whole-cell voltage-clamp recordings in neurons from the medial CeA of WT and Nf1<sup>+/-</sup> mice. A total of 31 CeA neurons from six mice (2 to 5 neurons/mouse) were examined. Baseline membrane

properties were similar between WT and Nf1<sup>+/-</sup> mice with the exception of resting membrane potential, which was significantly lower in Nf1<sup>+/-</sup> mice reflecting a more hyperpolarized resting state (Figure 3A). Neurons from Nf1<sup>+/-</sup> mice displayed an average sIPSC frequency of  $3.4 \pm .6$  Hz ( $n = 12$ ; Figure 3B lower trace and 3C), which was not significantly different from the sIPSC frequency in neurons from WT mice ( $2.8 \pm .8$  Hz,  $n = 9$ ; Figure 3B upper trace and 3C). However, when sIPSCs were separated by amplitude using a previously described method (11), closer examination with a focus on amplitude revealed that neurons from Nf1<sup>+/-</sup> mice had a significantly greater frequency of sIPSCs in low-amplitude bins (20–29 pA and 30–39 pA) as compared with WT mice (Figure 3D). In contrast to the differences in sIPSC frequency, there was no significant difference in sIPSC amplitude or decay between WT and Nf1<sup>+/-</sup> mice.

Miniature IPSCs (mIPSCs) were assessed following superfusion of the sodium (Na<sup>+</sup>) channel blocker tetrodotoxin (1  $\mu$ mol/L). Similar to what was observed in sIPSCs, CeA neurons from Nf1<sup>+/-</sup> mice showed a trend for increased mIPSC frequency as compared with WT mice ( $3.0 \pm .6$  Hz,  $n = 14$  and  $2.1 \pm .4$  Hz,  $n = 13$ , respectively; Figure 4A,B); however, the difference was not statistically significant. When mIPSCs were separated by amplitude using the same previously described method (11), neurons from Nf1<sup>+/-</sup> mice displayed a significantly greater frequency of mIPSCs in low-amplitude bins (20–29 pA) as compared with WT mice (Figure 4C). There was no significant difference in mIPSC amplitude or decay between WT and Nf1<sup>+/-</sup> mice. Collectively, these data suggest that Nf1 in the CeA regulates phasic GABA release.

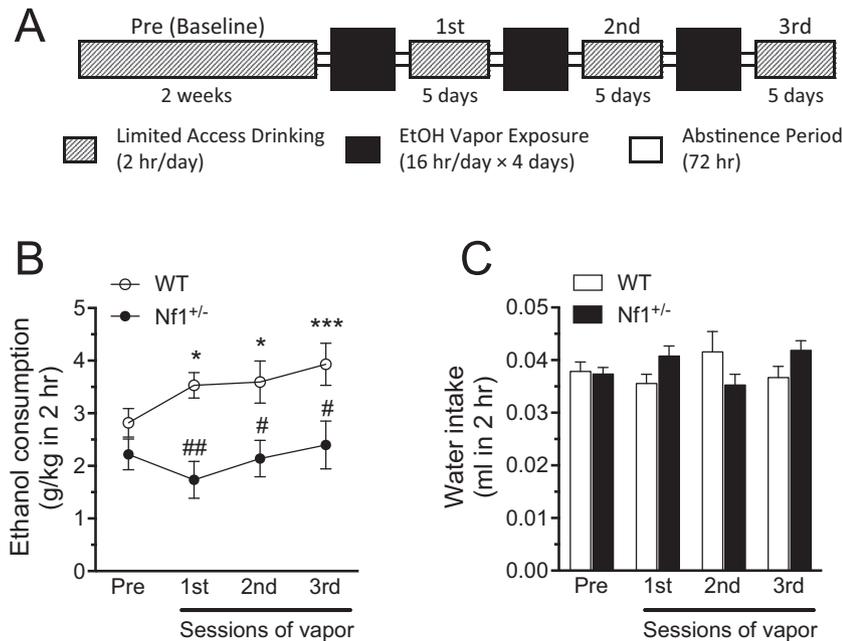
### The Ethanol-Induced Increase in GABA Release Is Lost in Nf1<sup>+/-</sup> Mice

As augmented GABA release is one of the major actions of ethanol in the CeA (12) and may contribute to the development of ethanol dependence (13), we assessed the effect of acute ethanol application on CeA neurons from WT and Nf1<sup>+/-</sup> mice. In WT mice, acute ethanol (44 mmol/L) significantly increased mIPSC frequency but not amplitude in 8 of 10 CeA neurons (Figure 5A,C). However, in Nf1<sup>+/-</sup> mice, acute ethanol did not significantly change mIPSC frequency or amplitude in



**Figure 1.** Alcohol intake by Nf1 heterozygous null mice (Nf1<sup>+/-</sup>) did not differ from wild-type (WT) mice in a 24-hour, 2-bottle choice paradigm or in the drinking in the dark paradigm. (A) In the 24-hour paradigm, both WT and Nf1<sup>+/-</sup> mice consumed progressively greater amounts of ethanol (EtOH) at increasing alcohol concentrations (3% to 20% vol/vol). Significant main effects of ethanol dose ( $F_{5,95} = 90.23, p < .0001$ ), but not genotype or the interaction of ethanol dose and genotype, were revealed by repeated measures two-way analysis of variance. (B) Both WT and Nf1<sup>+/-</sup> mice consumed ethanol to a similar degree in the drinking in

the dark paradigm. There was no significant difference between genotypes (WT and Nf1<sup>+/-</sup>) in 2-hour (days 1–3) and 4-hour (day 4) sessions.



**Figure 2.** Nf1 heterozygous null mice (Nf1<sup>+/-</sup>) did not show increased alcohol intake after vapor exposure to induce dependence. **(A)** Schematic diagram of chronic intermittent ethanol vapor exposure. **(B)** Nf1<sup>+/-</sup> mice consumed significantly less ethanol in limited access 2-hour drinking sessions after vapor treatments compared with wild-type (WT) mice. The vapor treatments induced a significant increase in ethanol intake in WT mice but not in Nf1<sup>+/-</sup> mice. Significant main effects of genotype ( $F_{1,15} = 9.995$ ,  $p = .007$ ) and session ( $F_{3,45} = 3.787$ ,  $p = .017$ ) and the interaction of genotype and session ( $F_{3,45} = 3.105$ ,  $p = .036$ ) were revealed by repeated measures two-way analysis of variance. Post hoc analysis confirmed that WT mice consumed more alcohol than Nf1<sup>+/-</sup> mice after each alcohol vapor bout. \* $p < .05$ ; \*\*\* $p < .001$  vs. before vapor (WT mice only); # $p < .05$ ; ## $p < .01$  Nf1<sup>+/-</sup> vs. WT mice at the same point (Bonferroni post hoc test). **(C)** Water intake did not change through the experiment and was not different between WT and Nf1<sup>+/-</sup> mice.

12 of 12 CeA neurons (Figure 5B,C). When mIPSCs from neurons in both groups were subjected to the same amplitude-based analysis described previously, a larger proportion of the ethanol-induced increase in mIPSC frequency in WT mice occurred at lower amplitude mIPSCs. There was no significant difference by bin in the raw mIPSC data in either group (likely due to variability in baseline frequency), but when the mIPSC frequency was normalized to control/baseline, there was a significant difference in effect between the WT and Nf1<sup>+/-</sup> mice in the lower amplitude bins (20–29 pA and 30–39 pA; Figure 5D). These data suggest that the lack of increased mIPSC frequency with ethanol in Nf1<sup>+/-</sup> mice was due to the partial loss of Nf1, which resulted in an increased baseline frequency at these lower amplitudes and loss of the ability to further increase GABA release with ethanol. This implicates Nf1 in the ethanol-induced increase in GABA release in the CeA and suggests that alterations in endogenous Nf1 levels could contribute to differential vulnerability to the development of alcohol dependence.

### Chronic Intermittent Ethanol Does Not Alter Baseline GABA Release in Nf1<sup>+/-</sup> Mice

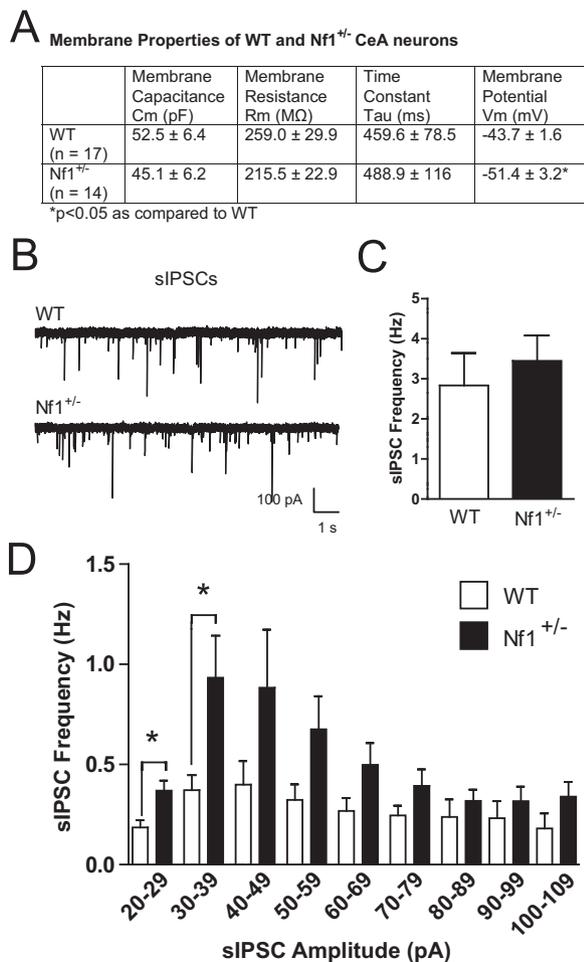
To determine whether Nf1 is involved in the changes in inhibitory transmission in the CeA following CIE, sIPSCs and mIPSCs were measured in CeA neurons from air control WT, CIE WT, and CIE Nf1<sup>+/-</sup> mice. A total of 40 neurons from nine mice (2 to 6 neurons/mouse) were examined. Neurons from CIE WT mice displayed an average sIPSC frequency of  $3.0 \pm .7$  Hz, which was significantly greater than the average sIPSC frequency observed in neurons from air control WT mice ( $1.1 \pm .2$  Hz;  $p < .05$ ,  $n = 11$  [CIE WT] and  $n = 14$  [air control WT]; Figure 6A,B). Neurons from naive Nf1<sup>+/-</sup> mice displayed an average sIPSC frequency of  $3.4 \pm .6$  Hz ( $n = 12$ ), which was not significantly different from the sIPSC frequency in neurons from CIE Nf1<sup>+/-</sup> mice ( $2.9 \pm .7$  Hz,  $n = 16$ ; Figure 6A,B).

Amplitude-based analysis revealed that the increase in baseline sIPSC frequency in CIE WT and CIE Nf1<sup>+/-</sup> mice occurred primarily in low-amplitude sIPSCs (20–29, 30–39, 40–49, and 50–59 pA;  $p < .05$ ; Figure 6C).

Similar to sIPSCs, baseline mIPSC frequency was significantly greater in CeA neurons from CIE WT mice than in neurons from air control WT mice, and there was no significant difference in baseline mIPSC frequency in neurons from CIE Nf1<sup>+/-</sup> mice as compared with neurons from naive Nf1<sup>+/-</sup> mice, although mIPSC frequency in naive Nf1<sup>+/-</sup> and CIE Nf1<sup>+/-</sup> mice was greater than in both air control WT and CIE WT mice. Neurons from CIE WT mice displayed an average mIPSC frequency of  $1.3 \pm .1$  Hz, which was significantly greater than the average mIPSC frequency observed in neurons from air control WT mice ( $.7 \pm .1$  Hz;  $p < .05$ ,  $n = 9$  [CIE WT] and  $n = 14$  [air control WT]; Figure 7A,B). Neurons from naive Nf1<sup>+/-</sup> mice displayed an average mIPSC frequency of  $2.8 \pm .8$  Hz ( $n = 9$ ), which was not significantly different from the mIPSC frequency in neurons from CIE Nf1<sup>+/-</sup> mice ( $2.8 \pm .6$  Hz,  $n = 15$ ; Figure 7A,B). Amplitude-based analysis revealed that the increase in mIPSC frequency in CIE WT and CIE Nf1<sup>+/-</sup> mice occurred in low-amplitude sIPSCs (20–29, 30–39, 40–49, and 50–59 pA;  $p < .05$ ; Figure 7C). These data suggest that Nf1 is involved in the increase in baseline inhibitory transmission in the CeA that is associated with the postdependent state and may contribute to the behavioral consequences of alcohol dependence. However, it is also possible that partial genetic deletion of Nf1 elevates baseline inhibitory transmission to such an extent that any further increase by CIE is masked.

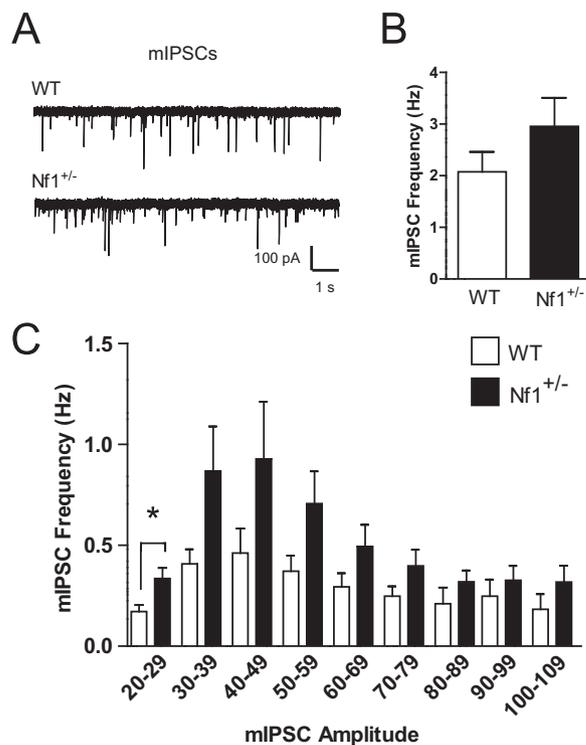
### CIE Does Not Alter the Effect of Acute Ethanol on GABA Release in CIE WT or CIE Nf1<sup>+/-</sup> Mice

We next determined whether CIE altered the effects of acute ethanol on GABA release. In air control WT mice, acute



**Figure 3.** Nf1<sup>+/-</sup> mice display greater baseline spontaneous inhibitory neurotransmission in central nucleus of the amygdala (CeA) neurons than wild-type (WT) mice. **(A)** Summary of baseline membrane properties of neurons from WT (n = 17) and Nf1<sup>+/-</sup> mice (n = 14), \*p < .05 as compared with WT mice. **(B)** Representative whole-cell voltage-clamp recordings of spontaneous inhibitory postsynaptic currents (sIPSCs) in CeA neurons from WT mice (upper trace) and Nf1<sup>+/-</sup> mice (lower trace). **(C)** Summary of the mean sIPSC frequency in CeA neurons from WT mice (white bar) and Nf1<sup>+/-</sup> mice (black bar). **(D)** Summary of the mean sIPSC frequency of individual sIPSC amplitude bins in CeA neurons from Nf1<sup>+/-</sup> mice (black bars) as compared with CeA neurons from WT mice (white bars); \*p < .05 by unpaired *t* test, n = 9 (WT) and n = 12 (Nf1<sup>+/-</sup>).

ethanol (44 mmol/L) significantly increased mIPSC frequency in 9 of 12 CeA neurons to 155.8 ± 9.9% of control/baseline (Figure 7E). In CIE WT mice, acute ethanol also significantly increased mIPSC frequency in 8 of 13 neurons to 142.8 ± 7.2% of control/baseline, which was not statistically significant from air control WT mice (Figure 7D,E). In CIE Nf1<sup>+/-</sup> mice, acute ethanol did not significantly change mIPSC frequency in 13 of 13 CeA neurons, which was not significantly different from naive Nf1<sup>+/-</sup> mice (Figure 7E). Consistent with previous observations, the ethanol-induced increase in mIPSC frequency in air control and CIE WT mice occurred primarily in low-amplitude sIPSCs (20–29 pA and 30–39 pA; p < .05; Figure 7F). Collectively, these data implicate Nf1 in the

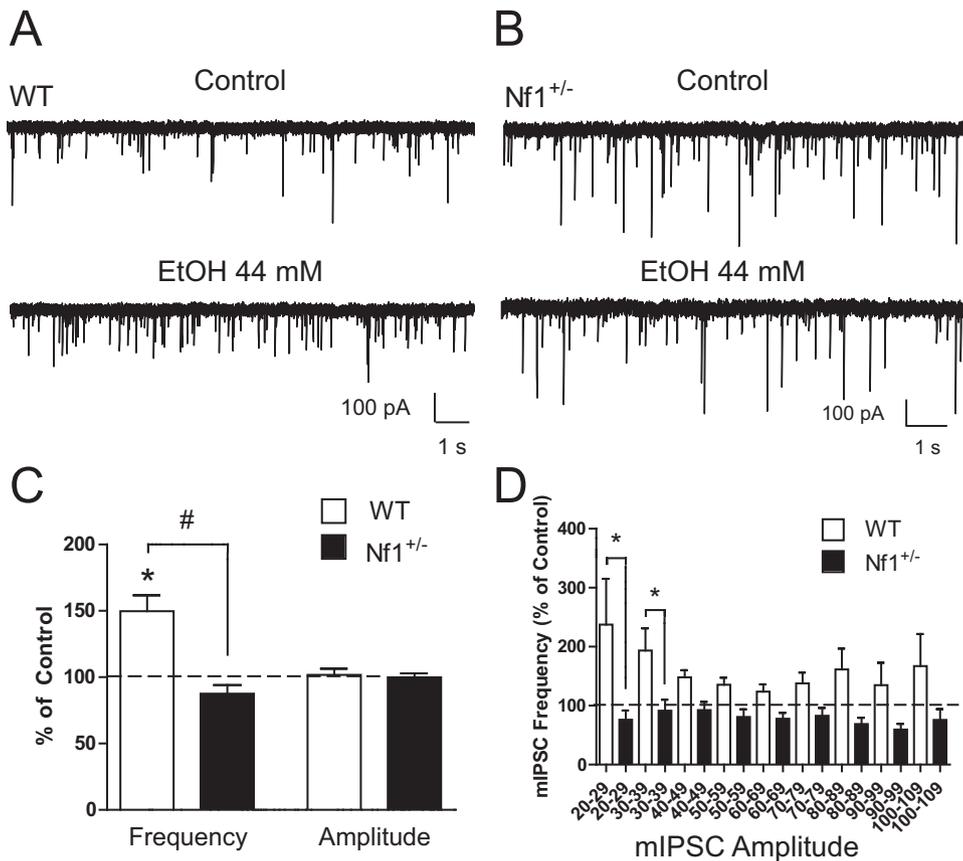


**Figure 4.** Nf1<sup>+/-</sup> mice display greater baseline miniature inhibitory neurotransmission in central nucleus of the amygdala (CeA) neurons than wild-type (WT) mice. **(A)** Representative whole-cell voltage-clamp recordings of miniature inhibitory postsynaptic currents (mIPSCs) in CeA neurons from a WT mouse (upper trace) and a Nf1<sup>+/-</sup> mouse (lower trace). **(B)** Summary of the mean mIPSC frequency in CeA neurons from WT mice (white bar) and Nf1<sup>+/-</sup> mice (black bar). **(C)** Summary of the mean mIPSC frequency of individual spontaneous inhibitory postsynaptic current amplitude bins in CeA neurons from Nf1<sup>+/-</sup> mice (black bars) as compared with CeA neurons from WT mice (white bars); \*p < .05 by unpaired *t* test, n = 13 (WT) and n = 14 (Nf1<sup>+/-</sup>).

increase in GABA release produced by ethanol in both naive and chronic ethanol exposed rodent models.

### NF1 Variants Are Associated with Alcohol Dependence Risk and Severity in Humans

As Nf1 appeared to be involved in the effects of acute and chronic ethanol in the mouse, we tested 560 well-imputed single nucleotide polymorphisms (SNPs) of the human *NF1* gene (equivalent to 54 independent tests) with minor allele frequencies of at least 3% in a sample of 4577 African Americans and 303 such SNPs (32 independent tests) in a sample of 4709 European Americans for association with two alcohol traits: 1) DSM-IV alcohol dependence with all control subjects having been exposed to alcohol; and 2) the DSM-IV symptom count (a measure of severity). We also evaluated association at the gene level. In AAs, 18 SNPs were significantly associated with symptom count after correcting for the number of independent tests (minimum p = .0004 for rs115215764; Table 1 and Table S1 in Supplement 1). Although no single SNP was significant after multiple correction in EAs, 220 SNPs were associated with the dependence trait at p < .05 (Table S2 in Supplement 1), which yielded a



**Figure 5.** *Nf1*<sup>+/-</sup> mice do not display the ethanol-induced increase in inhibitory neurotransmission observed in wild-type (WT) mice. **(A)** Representative whole-cell voltage-clamp recording from a WT central nucleus of the amygdala (CeA) neuron before (Control) and during superfusion of ethanol (EtOH) (44 mmol/L). **(B)** Representative whole-cell voltage-clamp recording from an *Nf1*<sup>+/-</sup> CeA neuron before (Control) and during superfusion of ethanol (44 mmol/L). **(C)** Summary of the change in mean miniature inhibitory postsynaptic current (mIPSC) frequency and amplitude produced by ethanol superfusion; \**p* < .05 by one-sample *t* test for independent significance, #*p* < .05 by unpaired *t* test; *n* = 8 (WT) and *n* = 12 (*Nf1*<sup>+/-</sup>). **(D)** Summary of the change in mean mIPSC frequency by amplitude of mIPSC produced by ethanol superfusion in CeA neurons from *Nf1*<sup>+/-</sup> mice (black bars) as compared with CeA neurons from WT mice (white bars); \**p* < .05 by unpaired *t* test, *n* = 8 (WT) and *n* = 12 (*Nf1*<sup>+/-</sup>).

significant gene-based test result (*p* = .03) for *NF1*. This finding is consistent with the high linkage disequilibrium across the entire gene in EAs, which is also evident in an African-ancestry population (Figure S3 in Supplement 1). The tight linkage disequilibrium in the gene makes the identification of any specific causal variants difficult without functional studies. The association of *NF1* polymorphisms with alcohol dependence risk and severity in humans, the lack of increased drinking in *Nf1*<sup>+/-</sup> mice after exposure to ethanol vapors sufficient to increase drinking in WT mice, and dysregulation of GABA release in the CeA (a key mechanism in alcohol reinforcement and dependence) implicate *NF1* in the transition to dependent drinking.

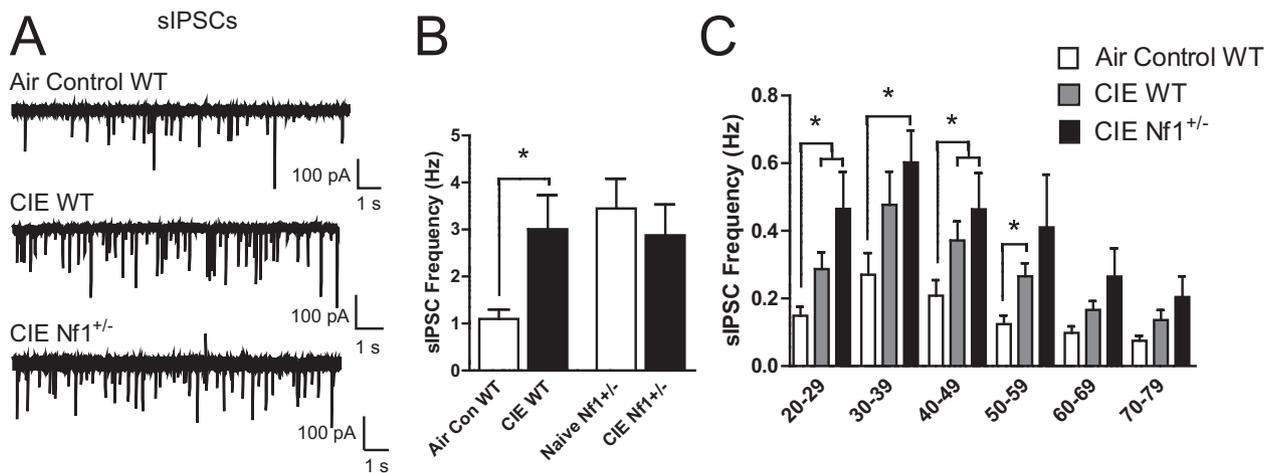
## DISCUSSION

It has been hypothesized that alcohol ingestion is driven by multiple sources of reinforcement that change with the individual's transition from social drinking to alcohol abuse and dependence on alcohol. Neuroadaptive changes within the brain reward and stress systems that include the CeA are key to this transition. In particular, electrophysiological studies have revealed that acute alcohol facilitates spontaneous and evoked GABAergic transmission in the CeA via presynaptic and postsynaptic mechanisms and that induction of dependence is characterized by augmented GABAergic transmission due to enhanced GABA release (18).

Rodents exposed to chronic intermittent alcohol vapor to the point of dependence escalate their alcohol intake during repeated withdrawal periods and show increased alcohol drinking (4,16,23–25). While in this paradigm, alcohol vapor is passively administered to induce dependence; numerous studies have demonstrated that it has predictive validity for alcohol dependence (26,27).

In the present study, we observed that *Nf1*<sup>+/-</sup> mice did not increase alcohol intake after CIE, in contrast to WT mice. While a limitation of the present model is their generalized *Nf1* deficiency, the alcohol-related phenotype of *Nf1*<sup>+/-</sup> mice was rather specific. In fact, *Nf1*<sup>+/-</sup> mice and WT mice do not significantly differ in nondependent drinking behavior, including in the 24-hour 2BC, limited access 2BC, and the DID binge drinking paradigm, but only in the transition to escalated alcohol drinking brought about by a history of vapor exposure to induce dependence.

In *Nf1*<sup>+/-</sup> mice, we also observed augmented baseline presynaptic GABA release in the CeA, which was not further increased by the application of acute alcohol or CIE exposure, unlike in CeA neurons from WT mice. This increase was selectively in lower amplitude GABA currents, consistent with increased vesicular release of GABA (at low quantal size), which has also been reported in the medial prefrontal cortex of *Nf1*<sup>+/-</sup> mice (11). In contrast to the medial prefrontal cortex, the increased vesicular release in the CeA is action potential independent, as it was observed in both sIPSCs and mIPSCs,



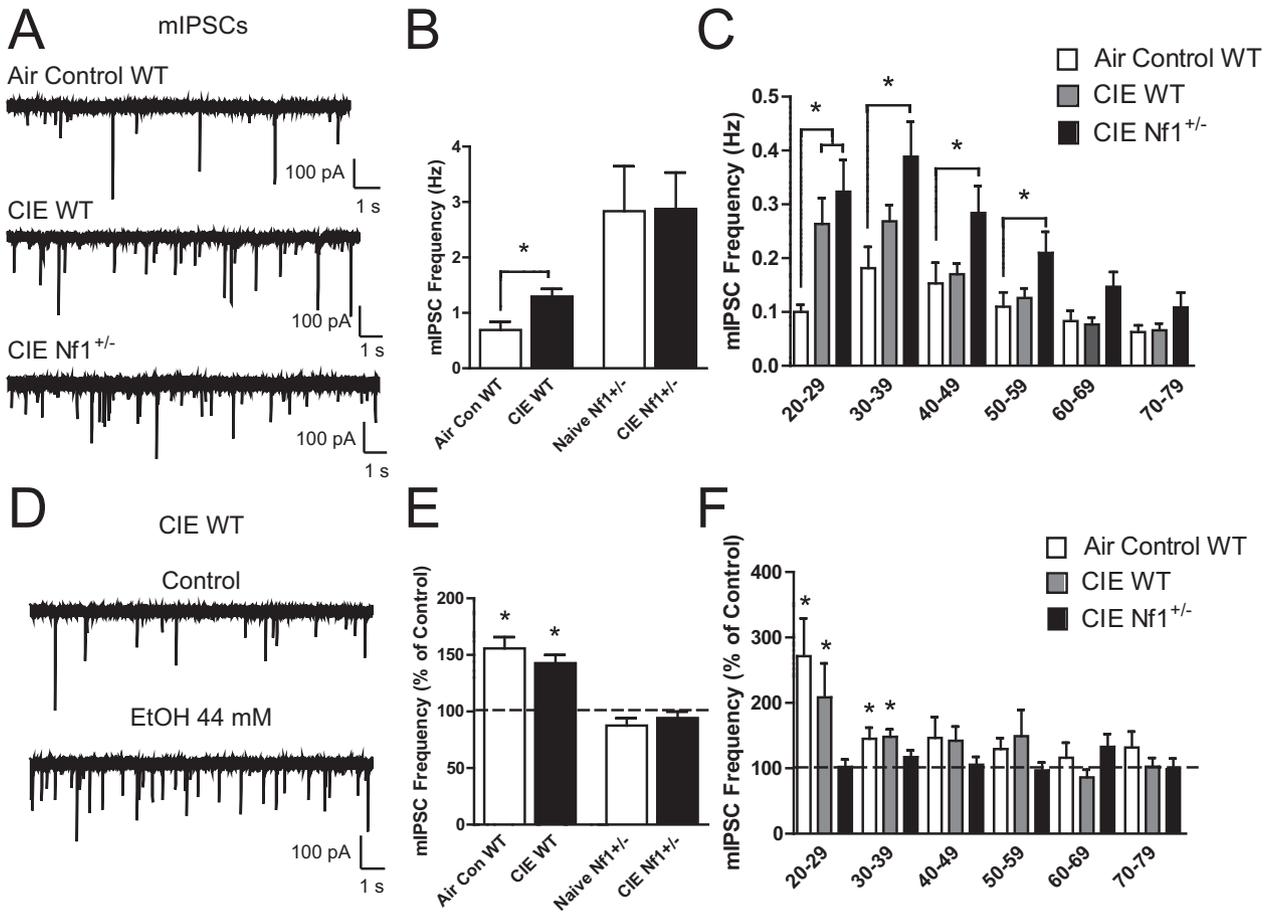
**Figure 6.** Chronic intermittent ethanol vapor (CIE) wild-type (WT) mice display greater baseline spontaneous inhibitory neurotransmission in central nucleus of the amygdala (CeA) neurons than air control WT mice but Nf1<sup>+/-</sup> mice show no effect of CIE on baseline spontaneous inhibitory neurotransmission. **(A)** Representative whole-cell voltage-clamp recordings of spontaneous inhibitory postsynaptic currents (sIPSCs) in CeA neurons from an air control WT mouse (upper trace), a CIE WT mouse (middle trace), and a CIE Nf1<sup>+/-</sup> mouse (lower trace). **(B)** Summary of the mean sIPSC frequency in CeA neurons from air control (Con) WT mice and naive Nf1<sup>+/-</sup> mice (white bars) and CIE WT and CIE Nf1<sup>+/-</sup> mice (black bars)  $p < .05$  by unpaired *t* test,  $n = 14$  (air control WT),  $n = 11$  (CIE WT),  $n = 12$  (naive Nf1<sup>+/-</sup>), and  $n = 16$  (CIE Nf1<sup>+/-</sup>). **(C)** Summary of the mean sIPSC frequency of individual sIPSC amplitude bins in CeA neurons from air control WT mice (white bars), CIE WT mice (gray bars), and CIE Nf1<sup>+/-</sup> mice (black bars);  $p < .05$  by unpaired *t* test,  $n = 14$  (air control WT),  $n = 11$  (CIE WT),  $n = 12$  (naive Nf1<sup>+/-</sup>), and  $n = 16$  (CIE Nf1<sup>+/-</sup>).

suggesting that the increased vesicular release is due to actions on the soma and/or the presynaptic terminal of local interneurons. In addition, acute ethanol increased the frequency of mIPSCs in CeA neurons from both naive and CIE WT mice, specifically increasing low-amplitude GABA currents. Lack of further increases in GABA release in CeA neurons from Nf1<sup>+/-</sup> mice by application of alcohol indicates that under drug-stimulated conditions, Nf1 facilitates action potential independent vesicular GABA release in both naive and CIE conditions. These findings are reminiscent of PKC $\epsilon$ <sup>-/-</sup> mice, which also show increased GABAergic tone due to enhanced GABA release in the CeA that is not further augmented by alcohol (18). Alcohol-regulated presynaptic terminals in the CeA originate from diverse sources that include, among others, the bed nucleus of the stria terminalis, ventral tegmental area, basolateral amygdala, and nucleus accumbens as well as intrinsic fibers (28). Thus, it is possible that while changes in Nf1 following CIE have functional consequences in the CeA due to presynaptic actions, cellular changes in Nf1 may occur in specific neuronal populations in other brain regions. Together, the data presented here are consistent with a role for Nf1 in the ethanol-induced increase in GABA release in alcohol reinforcement and suggest that Nf1 is critically involved in alcohol's actions in the CeA and in the transition to increased alcohol drinking in mice with a history of dependence in the CIE paradigm.

Nf1 regulates the activity of the small G proteins K-Ras and H-Ras, which have been previously implicated in alcohol drinking and dependence (8,9). A polymorphism in the Ras-specific guanine-nucleotide releasing factor 2, which is involved in Ras activation, has also been associated with alcohol abuse (29).

The present results suggest that the basal level of Nf1 is key in gating the transition to dependence. In this regard, we previously observed that the K-Ras-ERK pathway is regulated by alcohol administration (6,7) and also found that K-Ras<sup>+/-</sup> mice—similar to Nf1<sup>+/-</sup> mice—did not show increased alcohol drinking after exposure to alcohol vapor in the CIE model (9). The latter, together with the present results, suggest that both abnormally high and low K-Ras activity interferes with the development of increased alcohol drinking in the CIE paradigm. Similarly, both increased and reduced levels of Ras activity have been shown to be detrimental to learning. In fact, both K-Ras (30) and Nf1 (31) deficiencies impair cognitive function. Additionally, mice lacking the alternatively spliced exon 23a of Nf1—inclusion of which reduces the Ras-GTPase activating activity of Nf1 (32)—show learning impairments reminiscent of Nf1<sup>+/-</sup> mice (31). The latter findings, together with the present results, show that the level and dynamic regulation of K-Ras activity needs to be tightly regulated for K-Ras-mediated forms of plasticity, including increased alcohol drinking in the CIE paradigm (9).

The CIE paradigm of increased drinking in the setting of alcohol dependence is an established animal model of alcohol dependence (26,27), which has helped to identify candidate genes for alcohol effects and dependence (33,34). We used the paradigm to investigate whether genetic variations in NF1 may confer a vulnerability to the effects of ethanol and susceptibility to the emergence of dependent drinking in humans. The human genetics data presented are derived from a relatively large sample that is very well characterized in terms of both genotypes and phenotypes and was analyzed using state-of-the-art statistical methods to maximize power and limit common sources of bias. We found significant associations using single-SNP tests in AAs and a gene-based test in



**Figure 7.** Chronic intermittent ethanol vapor (CIE) wild-type (WT) mice display greater baseline miniature inhibitory neurotransmission in central nucleus of the amygdala (CeA) neurons than air control WT mice but Nf1<sup>+/-</sup> mice show no effect of CIE on baseline miniature inhibitory neurotransmission. **(A)** Representative whole-cell voltage-clamp recordings of miniature inhibitory postsynaptic currents (mIPSCs) in CeA neurons from an air control WT mouse (upper trace), a CIE WT mouse (middle trace), and a CIE Nf1<sup>+/-</sup> mouse (lower trace). **(B)** Summary of the mean mIPSC frequency in CeA neurons from air control (Con) WT mice and naive Nf1<sup>+/-</sup> mice (white bars) and CIE WT and CIE Nf1<sup>+/-</sup> mice (black bars) \**p* < .05 by unpaired *t* test, *n* = 14 (air control WT), *n* = 9 (CIE WT), *n* = 9 (naive Nf1<sup>+/-</sup>), and *n* = 15 (CIE Nf1<sup>+/-</sup>). **(C)** Summary of the mean mIPSC frequency of individual spontaneous inhibitory postsynaptic current amplitude bins in CeA neurons from air control WT mice (white bars), CIE WT mice (gray bars), and CIE Nf1<sup>+/-</sup> mice (black bars); \**p* < .05 by unpaired *t* test, *n* = 14 (air control WT), *n* = 11 (CIE WT), *n* = 12 (naive Nf1<sup>+/-</sup>), and *n* = 16 (CIE Nf1<sup>+/-</sup>). **(D)** Representative whole-cell voltage-clamp recording from a CIE WT CeA neuron before (Control) and during superfusion of ethanol (EtOH) (44 mmol/L). **(E)** Summary of the change in mean mIPSC frequency produced by ethanol in air control WT (*n* = 9), CIE WT (*n* = 8), naive Nf1<sup>+/-</sup> (*n* = 12), and CIE Nf1<sup>+/-</sup> (*n* = 13); \**p* < .05 by one sample *t* test. **(F)** Summary of the change in mean mIPSC frequency by amplitude of mIPSC produced by ethanol in CeA neurons from air control WT mice (white bars), CIE WT mice (gray bars), and CIE Nf1<sup>+/-</sup> mice (black bars); \**p* < .05 by one-sample *t* test, *n* = 9 (air control WT), *n* = 8 (CIE WT), *n* = 12 (naive Nf1<sup>+/-</sup>), and *n* = 13 (CIE Nf1<sup>+/-</sup>).

EAs. That the findings were obtained in both populations argues against their being false positive. Further studies are needed to identify the causal variants, which also may be population specific.

Together, these findings indicate that Nf1 activity regulates excessive drinking and spontaneous and ethanol-stimulated GABA release in mice and that genetic variation in Nf1 is associated with a susceptibility to the transition from non-dependent to dependent drinking in EAs and with severity of dependence in AAs. These data support a role for Nf1 regulation as a molecular mechanism for the transition to excessive drinking associated with alcohol dependence and the development of new therapeutic interventions to limit excessive alcohol drinking.

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**Table 1. DSM-IV Alcohol Dependence Symptom Count Association Results in African Americans for NF1 SNPs Significant After Multiple Test Correction**

BP	SNP	Type	Alleles (Effect/Ref)	MAF	<i>p</i> Value	Dir
29482458	rs115215764	INT	TC	.07	4.16E-04	++
29552466	rs17880276	INT	AG	.07	4.20E-04	++
29537463	rs111434970	INT	TC	.09	4.92E-04	++
29572925	rs17883349	INT	TC	.07	5.63E-04	++
29578251	rs112855251	INT	TC	.07	5.73E-04	++
29612657	rs113376659	INT	AG	.07	5.86E-04	++
29700816	rs17879205	INT	GA	.07	5.87E-04	++
29696951	rs74999855	INT	AT	.07	5.87E-04	++
29696785	rs16972218	INT	GA	.07	5.88E-04	++
29586586	rs8079598	INT	AC	.07	5.93E-04	++
29621415	rs77568419	INT	AC	.07	5.96E-04	++
29688189	rs17882683	INT	TC	.07	5.96E-04	++
29679861	rs17882112	INT	AG	.07	6.06E-04	++
29507762	rs17883335	INT	CA	.13	6.32E-04	++
29650308	rs111841173	INT	AG	.07	6.47E-04	++
29635594	rs16963584	INT	TC	.07	7.37E-04	++
29575803	rs17878249	INT	GA	.11	7.96E-04	++
29629499	rs113692076	INT	TG	.08	8.93E-04	++

In each case the minor allele was associated with an increased symptom count.

BP, SNP position in base pairs; Dir, direction of the effect with respect to the effect allele in the discovery (first symbol) and replication (second symbol) samples; INT, intronic; MAF, minor allele frequency; Ref, referent; SNP, single nucleotide polymorphism.

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