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Nf1 regulates alcohol dependence-associated excessive drinking and GABA 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 non-dependent drinking, and basal and alcohol-induced GABA release in the central nucleus of the amygdala (CeA) in Nf1 heterozygous null mice (Nf1^{+/-}). We also investigated the associations of Nf1 polymorphisms with alcohol dependence risk and severity in humans.

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Results—Nf1^{+/-} mice do not differ from wild-type (WT) mice in non-dependent drinking, such as 24-hr, 2-bottle choice (2BC), drinking in the dark binge drinking, or limited access 2BC. However, Nf1^{+/-} 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^{+/-} mice is greater at baseline than WT 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 non-dependent to dependent drinking in humans.

Keywords

Amygdala; GABA; Alcohol dependence; Electrophysiology; Genetic Association; Presynaptic Mechanisms

Introduction

NF1, the causative gene of neurofibromatosis type 1 in humans, encodes a large multifunctional protein with Ras GTPase Activating Protein (GAP) activity termed neurofibromin. Neurofibromin acts as a negative regulator for the Ras family of small G proteins and the Ras-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 GABA release (10, 11), which is a target of the effects of ethanol in the CeA (12, 13). Here, we show that Nf1 heterozygous null (Nf1^{+/-}) mice do not show increased drinking in the CIE paradigm while not differing from control mice in non-dependent ethanol drinking behavior. Baseline GABA release in the CeA was higher in Nf1^{+/-} than in wild-type (WT) mice and was not increased by acute ethanol exposure. CIE also failed to increase baseline GABA release in Nf1^{+/-} 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^{+/-} mice and the failure of CIE to increase baseline GABA release in the CeA of Nf1^{+/-} 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 non-dependent to dependent drinking.

Materials and Methods

Mice

Nf1^{+/-} mice established by Dr. Tyler Jacks (Massachusetts Institute of Technology) were obtained from Dr. Alcino Silva (University of California Los Angeles) and were backcrossed to C57BL/6J mice for more than 8 generations.

Twenty-four hour, two-bottle choice (2BC) paradigm

WT and Nf1^{+/-} 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% v/v) 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 (DID) paradigm

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

Chronic intermittent ethanol vapor exposure (CIE)

The present paradigm is based on previous studies by the group of Howard Becker (16) with minor modifications (17). Briefly, WT and Nf1^{+/-} mice were trained to drink ethanol (20% v/v) or water in the 2BC paradigm with limited access (2 hr/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 (IP) 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, CA). Tail blood sampling for blood ethanol level (BAL) determination was carried out daily. Target blood alcohol levels were 150-200 mg%. Seventy-two hours following removal from the chambers, mice received access to water vs. 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 4-6 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 μ m) were obtained from naive (non-ethanol-exposed) mice, as described (18, 19). Neurons were visualized using infrared differential interference contrast (IR-DIC) optics and an EXi Aqua camera (QImaging) at 60 $\times$ magnification. Whole-cell voltage-clamp recordings ($V_{\text{hold}} = -60\text{mV}$) were made with patch pipettes (4-6 M Ω ; Warner Instruments)

coupled to a Multiclamp 700B amplifier (Molecular Devices), low-pass filtered at 2-5 kHz, digitized (Digidata 1440A; Axon Instruments), and stored on a computer using pClamp 10 software (Axon Instruments). The intracellular solution for voltage-clamp recordings was composed of (in mM): KCl 145; EGTA 5; MgCl₂ 5; HEPES 10; Na-ATP 2; Na-GTP 0.2. GABA_A receptor currents, were isolated with the glutamate receptor blockers 6,7-dinitroquinoxaline-2,3-dione DNQX, 20 μM and DL-2-amino-5-phosphonovalerate AP-5, 50 μM and the GABA_B receptor antagonist CGP55845A (1 μM). 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.). Data are presented as mean ± standard error. In all cases, p<0.05 was the criterion for statistical significance. An expanded version of the methods is presented in the online supplemental information.

Drugs and Chemicals

DNQX (6,7-dinitroquinoxaline-2,3-dione, 10 μM), AP-5 (DL-2-amino-5-phosphonovalerate, 50 μM) and CGP 55845A (1 μM) were from Tocris Bioscience. Tetrodotoxin (TTX; 1 μM) was from Sigma.

Human Genetic Study Methods

Subject Ascertainment and Classification

The sample consisted of both small families and unrelated cases and controls ascertained in two independent studies. In the Yale-Penn sample, individuals were recruited from the community and from substance abuse treatment centers at four U.S. 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 (SNFs) 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 (NIDA) and National Institute on Alcohol Abuse and Alcoholism (NIAAA) (see supplemental materials for additional details). All subjects were interviewed using an electronic version of the Semi-Structured Assessment for Drug Dependence and Alcoholism (SSADDA) version 6.1 (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 study of alcohol dependence were published previously (20).

The second dataset included publically available GWAS data from the Study of Addiction: Genetics and Environment (SAGE) (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 the supplemental methods on line.

Results

Nf1 regulates dependence-induced increases in drinking

Nf1^{+/-} and WT mice were trained to consume ethanol in a 24-hr, 2-bottle choice (2BC) paradigm at increasing concentrations of ethanol (Fig. 1A). Ethanol intake of Nf1^{+/-} and WT mice increased similarly with increasing ethanol concentrations and did not differ between the two genotypes (Fig. 1A). Ethanol intake of Nf1^{+/-} and WT mice also did not differ in the drinking in the dark (DID) binge drinking paradigm (Fig. 1B) or in the limited access 2BC baseline preceding CIE (Fig. 2A-C), although a non-significant trend toward lower ethanol consumption was seen in Nf1^{+/-} mice ($t(19) = 1.658$, $P = 0.1138$) both in DID and in the limited access 2BC preceding CIE ($t(15) = 1.511$, $P = 0.1516$). 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 pre-vapor baseline levels starting after the first bout of vapor (Fig. 2B). Conversely, Nf1^{+/-} 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 ANOVA (Fig. 2B). Nf1^{+/-} mice also did not differ in the duration of loss of righting reflex (LORR) or kinetics of alcohol elimination after a bolus of alcohol (3.5g/kg IP, Supplemental Fig. S1). Additionally, Nf1^{+/-} mice drank equal amounts of graded solutions of saccharin or quinine as WT mice, showing that taste function was unaffected by this Nf1 mutation (Supplemental Fig. S2). Thus, Nf1^{+/-} mice do not differ from WT mice in non-dependent drinking behavior, including 24-hr 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 central amygdala (CeA) of WT and Nf1^{+/-} mice. A total of 31 CeA neurons from 6 mice (2-5 neurons/mouse) were examined. Baseline membrane properties were similar between WT and Nf1^{+/-} mice with the exception of resting membrane potential, which was significantly lower in Nf1^{+/-} mice reflecting a more hyperpolarized resting state (Fig. 3A). Neurons from Nf1^{+/-} mice displayed an average sIPSC frequency of 3.4 ± 0.6 Hz ($n = 12$; Fig. 3B, lower trace and 3C) which was not significantly different from the sIPSC frequency in neurons from WT mice (2.8 ± 0.8 Hz, $n = 9$; Fig. 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^{+/-} mice had a significantly greater frequency of sIPSCs in low-amplitude bins (20-29 and 30-39 pA) as compared to WT mice (Fig. 3D). In contrast to the differences in sIPSC frequency, there was no significant difference in sIPSC amplitude or decay between WT and Nf1^{+/-} mice.

Miniature IPSCs (mIPSCs) were assessed following superfusion of the Na⁺ channel blocker tetrodotoxin (1 μ M). Similar to what was observed in sIPSCs, CeA neurons from Nf1^{+/-} mice showed a trend for increased mIPSC frequency as compared to WT mice (3.0 ± 0.6 Hz, $n = 14$ and 2.1 ± 0.4 Hz, $n = 13$, respectively; Fig 4A and 4B), however the difference was

not statistically significant. When mIPSCs were separated by amplitude using the same previously-described method (11), neurons from $Nf1^{+/-}$ mice displayed a significantly greater frequency of mIPSCs in low-amplitude bins (20-29 pA) as compared to WT mice (Fig. 4C). There was no significant difference in mIPSC amplitude or decay between WT and $Nf1^{+/-}$ mice. Collectively these data suggest that $Nf1$ in the CeA regulates phasic GABA release.

The ethanol-induced increase in GABA release is lost in $Nf1^{+/-}$ 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 $Nf1^{+/-}$ mice. In WT mice, acute ethanol (EtOH, 44 mM) significantly increased mIPSC frequency but not amplitude in 8/10 CeA neurons (Figure 5A and 5C). However, in $Nf1^{+/-}$ mice, acute ethanol did not significantly change mIPSC frequency or amplitude in 12/12 CeA neurons (Figure 5B and 5C). 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, there was a significant difference in effect between the WT and $Nf1^{+/-}$ 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 $Nf1^{+/-}$ mice is 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 (CIE) does not alter baseline GABA release in $Nf1^{+/-}$ 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 $Nf1^{+/-}$ mice. A total of 40 neurons from 9 mice (2-6 neurons/mouse) were examined. Neurons from CIE WT mice displayed an average sIPSC frequency of 3.0 ± 0.7 Hz, which was significantly greater than the average sIPSC frequency observed in neurons from air control WT mice (1.1 ± 0.2 Hz; $*p < 0.05$, $n = 11$ (CIE WT) and $n = 14$ (air control WT); Fig. 6A and 6B). Neurons from naive $Nf1^{+/-}$ mice displayed an average sIPSC frequency of 3.4 ± 0.6 Hz ($n = 12$), which was not significantly different from the sIPSC frequency in neurons from CIE $Nf1^{+/-}$ mice (2.9 ± 0.7 Hz, $n = 16$; Fig. 6A and 6B). Amplitude-based analysis revealed that the increase in baseline sIPSC frequency in CIE WT and CIE $Nf1^{+/-}$ mice occurred primarily in low-amplitude sIPSCs (20-29, 30-39, 40-49 and 50-59 pA; $*p < 0.05$; 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 $Nf1^{+/-}$ mice as compared to neurons from naive $Nf1^{+/-}$ mice, although mIPSC frequency in naive $Nf1^{+/-}$ and CIE $Nf1^{+/-}$

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 ± 0.1 Hz, which was significantly greater than the average mIPSC frequency observed in neurons from air control WT mice (0.7 ± 0.1 Hz; $*p < 0.05$, $n = 9$ (CIE WT) and $n = 14$ (air control WT); Fig. 7A and 7B). Neurons from naive $Nf1^{+/-}$ mice displayed an average mIPSC frequency of 2.8 ± 0.8 Hz ($n = 9$) which was not significantly different from the mIPSC frequency in neurons from CIE $Nf1^{+/-}$ mice (2.8 ± 0.6 Hz, $n = 15$; Figure 7A and 7B). Amplitude-based analysis revealed that the increase in mIPSC frequency in CIE WT and CIE $Nf1^{+/-}$ mice occurred in low-amplitude sIPSCs (20-29, 30-39, 40-49 and 50-59 pA; $*p < 0.05$; Figure 7C). These data suggest that *Nf1* is involved in the increase in baseline inhibitory transmission in the CeA that is associated with the post-dependent 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 $Nf1^{+/-}$ mice

We next determined whether CIE altered the effects of acute ethanol on GABA release. In air control WT mice, acute ethanol (EtOH, 44 mM) significantly increased mIPSC frequency in 9/12 CeA neurons to 155.8 ± 9.9 % of control (Figure 7E). In CIE WT mice, acute ethanol also significantly increased mIPSC frequency in 8/13 neurons to 142.8 ± 7.2 % of control, which was not statistically significant from air control WT mice (Figure 7D and 7E). In CIE $Nf1^{+/-}$ mice, acute ethanol did not significantly change mIPSC frequency in 13/13 CeA neurons, which was not significantly different from naive $Nf1^{+/-}$ mice (Fig. 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 and 30-39 pA; $*p < 0.05$; Figure 7F). Collectively, these data implicate *Nf1* in the 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 SNPs of the human *NF1* gene (equivalent to 54 independent tests) with minor allele frequencies of at least 3% in a sample of 4,577 African Americans (AAs) and 303 such SNPs (32 independent tests) in a sample of 4,709 European Americans (EAs) for association with two alcohol traits: 1) DSM-IV alcohol dependence with all controls 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 $P = 0.0004$ for rs115215764, see Table 1 and Supplemental Table S1). Although no single SNP was significant after multiple correction in EAs, 220 SNPs were associated with the dependence trait at $P < 0.05$ (Supplemental Table S2), which yielded a significant gene-based test result ($P = 0.03$) for *NF1*. This finding is consistent with the high linkage disequilibrium (LD) across the entire gene in EAs, which is also evident in an African-ancestry population (Supplemental Figure S3). The tight LD 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*^{+/-} 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 pre- 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, 29-31). While in this paradigm alcohol vapor is passively administered to induce dependence, numerous studies have demonstrated that it has predictive validity for alcohol dependence (32, 33).

In the present study, we observed that *Nf1*^{+/-} 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*^{+/-} mice was rather specific. In fact, *Nf1*^{+/-} mice and WT mice do not significantly differ in non-dependent drinking behavior, including in the 24-hr, 2-bottle choice (2BC), limited access 2BC, and the drinking in the dark (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*^{+/-} 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 (mPFC) of *Nf1*^{+/-} mice (11). In contrast to the mPFC, the increased vesicular release in the CeA is action potential independent as it was observed in both sIPSCs and mIPSCs, 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*^{+/-} 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 *PKCε*^{-/-} 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 which include, among others, the bed nucleus of the stria terminalis, ventral tegmental area, basolateral amygdala,

nucleus accumbens as well as intrinsic fibers (34). 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 (RASGRF2), which is involved in RAS activation, has also been associated with alcohol abuse (35).

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^{+/-} mice - similar to Nf1^{+/-} 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 (36) and Nf1 (37) 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 (38) - show learning impairments reminiscent of Nf1^{+/-} mice (37). 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 (32, 33), which has helped to identify candidate genes for alcohol effects and dependence (39, 40). 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 is 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 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Alcohol intake by *Nf1* heterozygous null mice (*Nf1*^{+/-}) did not differ from wild-type (WT) mice in a 24-h, 2-bottle choice (2BC) paradigm or in the drinking in the dark (DID) paradigm. **A)** In the 24-h paradigm, both WT and *Nf1*^{+/-} mice consumed progressively greater amounts of ethanol at increasing alcohol concentrations (3-20% v/v). 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 ANOVA. **B)** Both WT and *Nf1*^{+/-} mice consumed ethanol to a similar degree in the DID. There was no significant difference between genotypes (WT and *Nf1*^{+/-}) in 2-hr (day 1-3) and 4-hr (day 4) sessions.

Figure 2.

Nf1 heterozygous null mice (Nf1^{+/-}) did not show increased alcohol intake after vapor exposure to induce dependence. **A)** Schematic diagram of CIE. **B)** Nf1^{+/-} mice consumed significantly less ethanol in limited access 2-hour drinking sessions after vapor treatments, compared with WT mice. The vapor treatments induced a significant increase in ethanol intake in WT mice, but not in Nf1^{+/-} 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 ANOVA. Post-hoc analysis confirmed that WT mice consumed more alcohol than Nf1^{+/-} mice after each alcohol vapor bout. * $p < .05$; *** $p < .001$ versus before vapor (WT mice only), # $p < .05$; ## $p < .01$ Nf1^{+/-} versus 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^{+/-} mice.

A Membrane Properties of WT and Nf1^{+/-} CeA neurons

	Membrane Capacitance Cm (pF)	Membrane Resistance Rm (MΩ)	Time Constant Tau (ms)	Membrane Potential Vm (mV)
WT (n = 17)	52.5 ± 6.4	259.0 ± 29.9	459.6 ± 78.5	-43.7 ± 1.6
Nf1 ^{+/-} (n = 14)	45.1 ± 6.2	215.5 ± 22.9	488.9 ± 116	-51.4 ± 3.2*

*p<0.05 as compared to WT

B

C

D

Figure 3. Nf1^{+/-} mice display greater baseline spontaneous inhibitory neurotransmission in CeA neurons than wild-type mice. **A.** Summary of baseline membrane properties of neurons from WT (n = 17) and Nf1^{+/-} mice (n = 14), *p < .05 as compared to WT mice. **B.** Representative whole-cell voltage-clamp recordings of sIPSCs in CeA neurons from WT mice (upper trace) and Nf1^{+/-} mice (lower trace). **C.** Summary of the mean sIPSC frequency in CeA neurons from WT mice (white bar) and Nf1^{+/-} mice (black bar). **D.** Summary of the mean sIPSC frequency of individual sIPSC amplitude bins in CeA neurons from Nf1^{+/-} mice (black bars)

as compared to CeA neurons from WT mice (white bars); $*p < .05$ by unpaired t -test, $n = 9$ (WT) and $n = 12$ (Nf1^{+/-})

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Figure 4.

Nf1^{+/-} mice display greater baseline miniature inhibitory neurotransmission in CeA neurons than wild-type mice. **A.** Representative whole-cell voltage-clamp recordings of mIPSCs in CeA neurons from a WT mouse (upper trace) and a Nf1^{+/-} mouse (lower trace). **B.** Summary of the mean mIPSC frequency in CeA neurons from WT mice (white bar) and Nf1^{+/-} mice (black bar). **C.** Summary of the mean mIPSC frequency of individual sIPSC amplitude bins in CeA neurons from Nf1^{+/-} mice (black bars) as compared to CeA neurons from WT mice (white bars); * $p < .05$ by unpaired t -test, $n = 13$ (WT) and $n = 14$ (Nf1^{+/-}).

Figure 5.

Nf1^{+/-} mice do not display the ethanol-induced increase in inhibitory neurotransmission observed in wild-type mice. **A.** Representative whole-cell voltage-clamp recording from a WT CeA neuron before (Control) and during superfusion of ethanol (EtOH, 44 mM). **B.** Representative whole-cell voltage-clamp recording from an Nf1^{+/-} CeA neuron before (Control) and during superfusion of ethanol (EtOH, 44 mM). **C.** Summary of the change in mean 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^{+/-}). **D.** Summary of the change in mean mIPSC frequency by amplitude of mIPSC produced by ethanol superfusion in CeA neurons from Nf1^{+/-} mice (black bars) as compared to CeA neurons from WT mice (white bars); **p* < .05 by unpaired *t*-test, *n* = 8 (WT) and *n* = 12 (Nf1^{+/-}).

Figure 6.

CIE WT mice display greater baseline spontaneous inhibitory neurotransmission in CeA neurons than air control WT mice but Nf1^{+/-} mice show no effect of CIE on baseline spontaneous inhibitory neurotransmission. **A.** Representative whole-cell voltage-clamp recordings of sIPSCs in CeA neurons from an air control WT mouse (upper trace), a CIE WT mouse (middle trace), and a CIE Nf1^{+/-} mouse (lower trace). **B.** Summary of the mean sIPSC frequency in CeA neurons from air control WT mice and naive Nf1^{+/-} mice (white bars) and CIE WT and CIE Nf1^{+/-} mice (black bars) **p* < .05 by unpaired *t*-test, *n* = 14 (air control WT), *n* = 11 (CIE WT), *n* = 12 (naive Nf1^{+/-}) and *n* = 16 (CIE Nf1^{+/-}). **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^{+/-} mice (black bars); **p* < .05 by unpaired *t*-test, *n* = 14 (air control WT), *n* = 11 (CIE WT), *n* = 12 (naive Nf1^{+/-}) and *n* = 16 (CIE Nf1^{+/-}).

Figure 7.

CIE WT mice display greater baseline miniature inhibitory neurotransmission in CeA neurons than air control WT mice but Nf1^{+/-} mice show no effect of CIE on baseline miniature inhibitory neurotransmission. **A.** Representative whole-cell voltage-clamp recordings of mIPSCs in CeA neurons from an air control WT mouse (upper trace), a CIE WT mouse (middle trace), and a CIE Nf1^{+/-} mouse (lower trace). **B.** Summary of the mean mIPSC frequency in CeA neurons from air control WT mice and naive Nf1^{+/-} mice (white bars) and CIE WT and CIE Nf1^{+/-} mice (black bars) **p* < .05 by unpaired *t*-test, *n* = 14 (air control WT), *n* = 9 (CIE WT), *n* = 9 (naive Nf1^{+/-}) and *n* = 15 (CIE Nf1^{+/-}). **C.** Summary of the mean mIPSC frequency of individual sIPSC amplitude bins in CeA neurons from air control WT mice (white bars), CIE WT mice (gray bars) and CIE Nf1^{+/-} mice (black bars); **p* < .05 by unpaired *t*-test, *n* = 14 (air control WT), *n* = 11 (CIE WT), *n* = 12 (naive Nf1^{+/-}) and *n* = 16 (CIE Nf1^{+/-}). **D.** Representative whole-cell voltage-clamp recording from a CIE WT CeA neuron before (Control) and during superfusion of ethanol (EtOH, 44 mM). **E.** Summary of the change in mean mIPSC frequency produced by ethanol in air control WT (*n* = 9), CIE WT (*n* = 8), naive Nf1^{+/-} (*n* = 12) and CIE Nf1^{+/-} (*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^{+/-} mice (black bars); * $p < .05$ by one-sample t -test, $n = 9$ (air control WT), $n = 8$ (CIE WT), $n = 12$ (naive Nf1^{+/-}), and $n = 13$ (CIE Nf1^{+/-}).

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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	P-value	Dir
29482458	rs115215764	INT	TC	0.07	4.16E-C04	++
29552466	rs17880276	INT	AG	0.07	4.20E-C04	++
29537463	rs111434970	INT	TC	0.09	4.92E-C04	++
29572925	rs17883349	INT	TC	0.07	5.63E-C04	++
29578251	rs112855251	INT	TC	0.07	5.73E-C04	++
29612657	rs113376659	INT	AG	0.07	5.86E-C04	++
29700816	rs17879205	INT	GA	0.07	5.87E-C04	++
29696951	rs74999855	INT	AT	0.07	5.87E-C04	++
29696785	rs16972218	INT	GA	0.07	5.88E-C04	++
29586586	rs8079598	INT	AC	0.07	5.93E-C04	++
29621415	rs77568419	INT	AC	0.07	5.96E-C04	++
29688189	rs17882683	INT	TC	0.07	5.96E-C04	++
29679861	rs17882112	INT	AG	0.07	6.06E-C04	++
29507762	rs17883335	INT	CA	0.13	6.32E-C04	++
29650308	rs111841173	INT	AG	0.07	6.47E-C04	++
29635594	rs16963584	INT	TC	0.07	7.37E-C04	++
29575803	rs17878249	INT	GA	0.11	7.96E-C04	++
29629499	rs113692076	INT	TG	0.08	8.93E-C04	++

BP = SNP position in base pairs; INT = intronic; MAF = minor allele frequency; Dir = direction of the effect with respect to the effect allele in the discovery (first symbol) and replication (second symbol) samples. In each case the minor allele was associated with an increased symptom count.