KCNK13 potassium channels in the ventral tegmental area of rats are important for excitation of VTA neurons by ethanol

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Abstract

Background: Alcohol excites neurons of the ventral tegmental area (VTA) and the release of dopamine from these neurons is a key event in ethanol reward and reinforcement. Many mechanisms for ethanol actions on neurons of the VTA have been proposed, but antagonists generally do not eliminate ethanol-induced excitation of VTA neurons. We have previously demonstrated that the ion channel KCNK13 plays an important role in ethanol excitation of mouse VTA neurons. Here, we elaborate on that finding and further assess the importance of KCNK13 in rats.

Methods: Rats (Sprague-Dawley and Fisher 344) were used in these studies. In addition to single unit electrophysiology in brain slices, we used quantitative PCR and immunohistochemistry to discern the effects of ethanol and the brain slice preparation method on the expression levels of *Kcnk13* and KCNK13.

Results: Interestingly, immunohistochemistry demonstrated that the levels of KCNK13 were significantly reduced during procedures normally used to prepare brain slices for electrophysiology, such that there is a reduction of about 75% in KCNK13 protein at the time that electrophysiological recordings would normally be made. Extracellular recordings demonstrated that ethanol excitation of VTA neurons was reduced after knockdown of *Kcnk13* using a small interfering RNA (siRNA) delivered via the recording micropipette. Real-time PCR demonstrated that expression of *Kcnk13* was altered in a time-dependent manner after alcohol withdrawal.

Conclusions: KCNK13 plays an important role in ethanol stimulation of rat VTA neurons, and KCNK13 is dynamically regulated by cell damage, by ethanol exposure, and during withdrawal. KCNK13 is a novel alcohol-sensitive protein and further investigation of this channel may offer new avenues for the development of agents useful in altering the rewarding effect of alcohol.

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Supplementary Data:
Additional information (Figure S1) is available in the Supplementary Materials section.

Introduction

Dopaminergic (DA) neurons of the ventral tegmental area (VTA) are important mediators of alcohol reward and reinforcement (Koob and Volkow, 2010). The mechanism of activation of VTA neurons by alcohol has been explored, with a number of possible neurotransmitters and ion channels being implicated in ethanol-induced excitation (Morikawa and Morrisett, 2010). Evidence that acutely dissociated VTA neurons are activated by ethanol suggests that a key factor for alcohol-induced excitation of these neurons is a molecular target located on the VTA neurons themselves (Brodie et al., 1999). Similarly, ethanol excitation of VTA neurons still occurs when $GABA_A$, $GABA_B$, NMDA, AMPA, metabotropic glutamate, muscarinic and nicotinic cholinergic receptors are blocked (Nimitvilai et al., 2016), again suggesting that the primary target of alcohol is neither pre-synaptic terminals nor receptors of these neurotransmitters. One possibility for the target of alcohol action is an ion channel on the membrane of the VTA neuron. Although evidence indicates that several ion channels (Okamoto et al., 2006, Rivera-Meza et al., 2014, Herman et al., 2015) are affected by ethanol in the VTA, blocking those channels fails to prevent alcohol effects in VTA neurons (McDaid et al., 2008, Koyama et al., 2007, Appel et al., 2003).

Leak potassium channels participate in maintaining the resting membrane potential and control of neuronal excitability. These potassium channels are constitutively open and maintain the negative resting membrane potential (Goldstein et al., 2001). There are at least 15 different two-pore potassium channels having a variety of pharmacological and physiological profiles. Some two-pore potassium channels are activated by volatile anesthetics and others are inhibited (Enyedi and Czirjak, 2010). The Tandem-pore Halothane-Inhibited potassium (THIK) channels, KCNK12 and KCNK13, differ from other leak channels in that they are inhibited by isoflurane, halothane, gadolinium, and by high extracellular calcium concentration (Enyedi and Czirjak, 2010). There are only a few reports assessing the function of THIK channels in the central nervous system. We demonstrated that KCNK13 channels in mouse VTA neurons are important for ethanol excitation (You et al., 2019). It is important to examine rat VTA neurons as well as mouse, as there has been speculation regarding differences between rat and mouse in some studies of alcohol in VTA neurons (Okamoto et al., 2006). Ethanol inhibition of KCNK13 in the VTA is behaviorally important, as downregulation of this protein altered alcohol drinking (You et al., 2019). Here, we provide evidence that KCNK13 is important for ethanol excitation of rat VTA neurons, that *Kcnk13* gene expression is regulated by alcohol, and that the expression of the channel protein is downregulated as a result of the typical method for brain slice preparation. Our results indicate that KCNK13 may be an important target of ethanol action on VTA neurons.

Materials and Methods

Animals

Male rats (Fisher 344) were purchased from Envigo (Indianapolis, IN). Rats used in the acute studies were 90–120 grams on arrival; male Sprague-Dawley rats treated with Lieber-DeCarli diet were 250 gm on arrival. Sprague-Dawley rats were used for the alcohol withdrawal studies in this report because of their characterization in the Lieber-DeCarli diet model in our laboratory and that of the Center for Alcohol Research in Epigenetics (You et al., 2018a, You et al., 2018b). We do not compare Fisher 344 and Sprague-Dawley data directly.

For some of the studies included as supplemental data, male C57BL/6J mice (C57) were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were 4–5 weeks old on arrival and used for siRNA electrophysiology experiments within two weeks of arrival.

All rats and mice were treated in compliance with the NIH *Guide for the Care and Use of Laboratory Animals.* All experimental methods in this study were approved by the Animal Care Committee of the University of Illinois at Chicago.

Lieber-DeCarli Diet: For the results shown in Figure 6 below, Sprague-Dawley rats (270–290 gm) were given access to ethanol in a Lieber-DeCarli diet protocol. Chronic ethanol administration of oral Lieber-DeCarli ethanol-containing diet feeding was performed as described previously (27). Male adult Sprague–Dawley rats were single cage housed, randomly assignment to three groups were used: control diet fed (C) and 24h (n=4) or 72h (n=16) withdrawal from ethanol diet (W).

Rats were given Lieber-DeCarli diet (Bio-Serv, Inc., Frenchtown, NJ, USA) as their only source of both food and fluid. Diet was provided daily between 5:00 and 6:00 p.m, right before the beginning of the dark cycle of the housing facility. Control and ethanol diet rats were pair-fed. For the first three days, all rats received control diet. Then the pair-fed control group (C) continued to receive the control liquid diet for the entire length of treatment. Rats in the ethanol-fed group (W) were gradually introduced to ethanol over a 7-day period (concentrations increased daily from day 1 to day 7; ethanol concentrations were 1.8%, 3.2%, 4.5%, 5.4%, 6.3%, 7.2%, 8.1% v/v, respectively) and then maintained on 9% (v/v) ethanol-containing Lieber-DeCarli liquid diet for 15 days. The rats in the withdrawal group (W) were then withdrawn from ethanol for either 24 hours before sacrifice or 72 hours before sacrifice. Tissue taken from these areas containing the VTA were then used for mRNA expression.

Electrophysiology

The brain slice preparation technique was similar to that described previously (Brodie et al., 1990, Nimitvilai et al., 2016). The rats were anesthetized with isoflurane and immediately subjected to decapitation. After removing the parietal and occipital plates of the skull, chilled cutting solution was poured over the cortex to begin chill the brain; the brain was then rapidly removed from the cranium. The brain was placed cortex down on a chilled platform and ice-cold cutting solution was poured over the whole brain, which was then

roughly dissected to a smaller block to remove the cerebral cortices and tissue posterior to the rostral pons and anterior to the optic chiasm. This block of tissue was mounted to the chuck of the vibratome (Campden 7000smz, Lafayette Instruments, Lafayette, IN) and submerged into chilled (4° C) cutting solution in the vibratome. Coronal sections (400 μm) of the VTA were cut in chilled cutting solution and then immediately placed in the recording chamber in which artificial cerebrospinal fluid (aCSF) was flowing continuously (2 ml/min; 35° C). Standard aCSF was used in these experiments (in mM: NaCl 126, KCl 2.5, NaH_2PO_4 1.24, $CaCl_2$ 2.4, $MgSO_4$ 1.3, $NaHCO_3$ 26, glucose 11). The composition of the cutting solution was (in mM): KCl 2.5, $CaCl_2$ 2.4, $MgSO_4$ 1.3, $NaHCO_3$ 26, glucose 11, and sucrose 220. All solutions were saturated with 95% O_2/ 5% CO_2 (pH=7.4).

Recording electrodes were positioned in the VTA under a dissecting microscope using a precision micromanipulator (Siskiyou Corporation, Grants Pass, OR, USA). Neurons within the lateral VTA had electrophysiological criteria for DA neurons (Lacey et al., 1989, Mueller and Brodie, 1989, Brodie et al., 1988). Not all VTA neurons in this study were tested but every neuron tested with baclofen (0.1–1 μM) was inhibited; sensitivity to baclofen is characteristic of DA neurons but not GABAergic neurons in the VTA (Margolis et al., 2012).

Except for siRNA, drugs were infused into the aCSF using calibrated pumps from stock solutions. Most of the salts and ethanol (95%; 190 proof) were purchased from Sigma (St. Louis, MO). Isoflurane was purchased from Henry Schein Animal Health (Dublin, OH).

Extracellular recording electrodes (1.5 mm diameter glass tubing with filament, resistance 2–4 MΩ (Sutter Instruments, Novato, CA); tip diameter approximately 0.5 – 0.7 μm) were filled with 0.9% NaCl. A high-gain extracellular amplifier (x-Cell, FHC, Inc., Bowdoin, ME) and an IBM-PC-based data acquisition system (ADInstruments, Inc.) were used to collect firing rate data. Drug effects were calculated as change in firing rate compared to pre-drug baseline using one-minute averages. The change in firing rate is expressed as a percentage of the initial firing rate to control for changes in firing rate that could occur over time.

For siRNA delivery, siRNA targeting *Kcnk13* and *Kcnk12* was purchased from Dharmacon (ON-TARGETplus siRNA). As the sequence of *Kcnk12* is related to but different from that of *Kcnk13*, si*Kcnk12* was used as control siRNA. It should be noted that in experiments in which siRNA was included in the recording micropipette, the amplitude of the action potential signal was used as an index of distance from the electrode tip to the neuron being recorded, and the amplitude of the action potential signal was maximized (2–4 mV) in order to insure that the siRNA was delivered as efficiently as possible to the neuron.

Immunofluorescent staining of brain sections

Rats were randomly assigned to one of three groups. One set of rats were sacrificed using isoflurane followed by perfusion with chilled saline and 4% paraformaldehyde (PFA); subsequently, VTA brain slices (250 μm) were made. The second set of rats were sacrificed using isoflurane; brain slices (250 μm) were made from the brains of these rats as for electrophysiology, and then immersed into 4% PFA for fixation. The third set treated the same as the second set, except that the brain slices were incubated in aCSF for 1 hours

before fixation with PFA. Brain tissue was processed for immunohistochemistry as described previously (Kharazia et al., 2003). Primary antibodies for TH and KCNK13 detection (Fig 2) were mouse anti-TH (EMD Millipore, Burlington, MA, catalog number MAB318) and rabbit anti-KCNK13 (Novus, Littleton, CO, catalog number NBP2–41132). Images of KCNK13 expression in VTA (KCNK13 and TH) were obtained using a Zeiss LSM 710 confocal microscope (Carl Zeiss, Thornwood, NY). Intensity of immunofluorescence was quantified using the ImageJ software (National Institutes of Health).

Quantitative real-time PCR (qPCR)

For validation of siRNA knockdown of gene expression, rats were sacrificed using isoflurane; brain slices containing VTA were made same as for electrophysiology. Slices then were incubated in aCSF containing siRNA (targeting *Kcnk13*, *Kcnk12 or non-targeting control siRNA,* Dharmacon ON-TARGETplus siRNA) for 2 hours before shock freezing for measurement of mRNA expression. For chronic ethanol studies, rats were sacrificed either 24 or 72 hours after alcohol withdrawal from a 15 day Lieber-DeCarli diet regimen (You et al., 2018b). The VTA was dissected on ice using RNase-free conditions. RNA was isolated from VTA tissue using the RNeasy mini kit (Qiagen, Valencia, CA) and subjected to first strand cDNA synthesis using reverse transcriptase (Thermo Fisher, Waltham, MA). Quantitative real-time PCR was used to determine the mRNA level of *Kcnk13* or *Kcnk12.* Relative expression was calculated using the delta Cq method. The expression of *Gapdh* was used as a reference for the expression levels of *Kcnk13* and *Kcnk12*.

Statistical Analysis

Origin (Originlab, Northampton, MA) or GraphPad Prism version 6.05 (GraphPad Software, Inc., La Jolla, CA) were used for statistical analysis. Mean data are shown as mean ± S.E.M. Differences in ethanol response (Figure 1B) and mRNA expression (Figures 3, 4, 6) were assessed with unpaired t tests (between subjects). One-way ANOVA was used to assess significance of levels of immunoreactivity (Figure 2). Two-way ANOVAs were used to compare concentration-responses between groups (Figures 1A, 1C, 5, S1). As comparisons were made between immunofluorescence under different conditions, a one-way ANOVA was used to establish statistical significance for differences between the groups. Bonferroni post-hoc tests were used for multiple means comparison testing for ANOVAs as appropriate.

Results

Ethanol excitation in the VTA is reduced by high extracellular calcium or gadolinium, and is mimicked by isoflurane.

While there are no specific antagonists for most two-pore potassium channels, several non-selective agents can interfere with these ion channels, including external gadolinium and volatile anesthetics like halothane and isoflurane (Enyedi and Czirjak, 2010, Goldstein et al., 2001), and high external calcium ion (Ca^{++}) (Goldstein et al., 2001, Rajan et al., 2000). Some of these conditions, including high external calcium ion concentration, reduce ethanol excitation in mouse VTA neurons (You et al., 2019), pointing to the possibility that a specific leak potassium channel might underlie ethanol-induced excitation in the VTA of rats

as well. In initial experiments with rat VTA neurons, we increased the extracellular Ca^{++} concentration from 2.4 mM to a total of 10 mM. We began with normal aCSF and increased the calcium concentration for each neuronal response recorded; we did not randomize the regular calcium/high calcium administration. We cannot rule out an order-dependence of the calcium effect, although we have never observed an order-dependent effect of ethanol administration. The high calcium condition blocked ethanol-induced excitation (Figure 1A). Neurons in this experiment initially were spontaneously active at a rate of 1.51 ± 0.3 Hz; in high extracellular Ca^{++} medium, ethanol excitation reverted to a concentration-dependent inhibition (two-way repeated measures ANOVA, for the effect of calcium $F_{1,3}=11.43$, P<0.05, n=4 cells from 3 rats); this is similar to the action of high external calcium concentration on ethanol responses in mice (You et al., 2019).

Gadolinium ion inhibits KCNK13 channels (Rajan et al., 2001). We tested the excitatory effect of 120 mM ethanol in the presence of gadolinium (100 μM). The initial firing rate was 1.58 ± 0.3 Hz. Ethanol was tested, and then the same concentrations were tested in the presence of gadolinium (100 μM). In the presence of gadolinium there was a reduction of the excitatory effect of 120 mM ethanol; in the absence of gadolinium, 120 mM ethanol increased the firing rate by $47.2 \pm 12.2\%$, and in the presence of 100 μM gadolinium, 120 mM ethanol decreased the firing rate by 15.6% (paired t-test, DF=7, t-statistic 2.804, P<0.05, n=8 cells from 4 rats) (Figure 1B).

Since volatile anesthetics can affect tandem-pore potassium channels, we also assessed how isoflurane affected spontaneous neuronal firing rate of rat VTA neurons. Initial firing rate of neurons in this experiment was 1.58 ± 0.21 Hz. Isoflurane (200 – 600 μM) mimicked the excitatory effect of ethanol in a concentration-dependent manner (one-way repeated measures ANOVA, $F_{5,10}=44.47$, P<0.001 n=8 cells from 4 rats) (Figure 1C). Some leak potassium channels are activated by volatile anesthetics (which should lead to hyperpolarization and inhibition) but both THIK channels (KCNK12 and KCNK13) are blocked by isoflurane (which should lead to depolarization and excitation), so we focused subsequent experiments on KCNK12 and KCNK13. It should be noted that all rats were anesthetized briefly with isoflurane prior to sacrifice, but as the excitatory effects of isoflurane were reversible with washout, and all slices were incubated in the recording chamber in drug-free aCSF for one hour before recording, the acute effect of the isoflurane anesthesia is likely to be minimal in these experiments.

KCNK13 immunoreactivity is downregulated by brain slice preparation procedure

For VTA neurons to reach the same level of ethanol excitation, higher concentrations of ethanol are needed in vitro (Brodie et al., 1990) compared to blood ethanol concentrations of ethanol achieved in vivo (Gessa et al., 1985). It has been shown that leak potassium channels are downregulated following axotomy (Ungless et al., 2002, Tulleuda et al., 2011, Acosta et al., 2014), and as there is neuronal damage that is a natural consequence of the in vitro brain slicing procedure, it is possible that a leak channel like KCNK13 is downregulated prior to electrophysiological recordings of VTA neurons under standard brain slicing methods. To examine this possibility, immunohistochemistry was performed in brain slices under three different conditions: with fixation in vivo perfusion, as would normally be performed for

immunohistochemistry, and with fixation at two time points after making brain slices suitable for electrophysiology: Immediately after cutting the slice, and after one hour incubation in aCSF, which is the earliest time at which electrophysiological recordings would normally be performed. Brain slices were assessed for KCNK13 immunoreactivity as well as TH immunoreactivity. TH immunoreactivity was assessed as a control for general tissue deterioration and loss of dopamine neurons. The results of this experiment are shown in Figure 2. Figure 2A illustrates typical immunoreactivity of brain slices as observed by confocal microscope. There was a significant decrease in TH immunoreactivity after one-hour incubation in aCSF compared to the other two conditions (one-way ANOVA, $F_{2,51}$ = 6.72, P<.005, n=18) (Figure 2B). In contrast, there was a decrease in KCNK13 immunoreactivity in both conditions in which fixation occurred in vitro, and the brain slice incubated for one hour in aCSF prior to fixation had significantly less KCNK13 immunoreactivity than the other two conditions (one-way ANOVA, $F_{2,51}$ = 37.19, P<.001, n=18) (Figure 2C). When controlling for possible tissue deterioration by expressing KCNK13 immunoreactivity as a percentage of TH immunoreactivity, there was a significant time-dependent decrease in KCNK13 reactivity (one-way ANOVA, $F_{2,51}$ = 15.04, P<.001, n=18) (Figure 2D). In fact, the level of KCNK13 immunoreactivity at the time that electrophysiological recordings would be made was less than 25% of the level in the tissue subjected to fixation in vivo.

si*Kcnk13* exposure downregulates Kcnk13 mRNA

As there is no selective antagonist of KCNK13, we tested whether siRNA could downregulate *Kcnk13* mRNA in brain slices containing the VTA. We incubated VTA slices with siRNA that targeted *Kcnk13* for two hours. The results of these experiments are shown in Figure 3. We observed that incubation with si*Kcnk13* produced a decrease in expression of *Kcnk13* mRNA of 30.8%, to 0.721 ± 0.07 compared to 1.04 ± 0.11 in slices incubated with a scrambled control siRNA (two-sample t-test, t=2.41, P < 0.05, n=6 samples from 6 rats per group).

As stated above in the Introduction, KCNK13 and KCNK12 are closely related potassium channels. In a separate experiment, we compared the effects of si*Kcnk13* and si*Kcnk12* on the expression of *Kcnk12* and *Kcnk13*. We incubated VTA slices with siRNA that targeted either *Kcnk12* or *Kcnk13* for two hours. The results of qPCR on these slices is shown in Figure 4. Expression of *Kcnk13* was reduced by si*Kcnk13*-exposure (n=12 from 12 rats) but not by si*Kcnk12*-exposure (n=13 from 13 rats) compared to the si*Control* group (n=14 from 14 rats) (one way ANOVA, $F_{2,36}$ = 7.77, P<0.002) (Figure 4A). *Kcnk12* expression was reduced by si*Kcnk12* (n=12 from 12 rats) compared the si*Kcnk13*-exposed group (n=13 from 13 rats) but not compared to the si*Control*-exposed group (n=12 from 12 rats) (one way ANOVA, $F_{2,34}$ = 3.28, P<0.05) (Figure 4B). These experiments demonstrate that downregulation by siRNA was specific to the mRNA for each gene.

si*Kcnk13* decreases ethanol excitation of rat VTA neurons

We used exposure to si*Kcnk13* to specifically affect KCNK13 channels to determine their contribution to ethanol-induced excitation. si*Kcnk13* (or si*Kcnk12* as a control) was included in the electrode filling solution, and ethanol responses were assessed at the

beginning of the recording (as control condition since at this point the effects of the siRNA would be minimal) and two hours after the initiation of the recording to study the effect of *Kcnk13* downregulation. We used si*Kcnk12* as a control instead of a scramble control as *Kcnk12* is closely related to *Kcnk13* and is a more rigorous control than a scramble. The initial firing rate of the neurons exposed to si*Kcnk13* was 1.51 ± 0.27 Hz, and that of those exposed to si*Kcnk12* was 1.52 ± 0.21 Hz. Inclusion of si*Kcnk13* produced no significant change in baseline firing rate (at 2 h, the firing rate was 1.60 ± 0.24; paired t-test t-statistic = 0.715, DF=7, P>0.05) a significant time-dependent reduction of the excitatory effect of ethanol (Two-way RM ANOVA, $F_{1,7}$=6.58 P<0.05, n=8 cells from 7 rats) (Figure 5A), whereas the inclusion of si*Kcnk12* has no significant effect on ethanol excitation of VTA neurons (Two-way RM ANOVA, $F_{1,6}$=0.002 P>0.05, n=7 cells from 5 rats) (Figure 5B). Using this same method, similar results were obtained with mouse VTA neurons (Supplementary Data), consistent with our previously reported reduction of ethanol excitation of VTA neurons treated in vivo with shRNA targeting *Kcnk13* (You et al., 2019).

Expression of Kcnk13 is altered by in vivo ethanol exposure

In order to assess whether *Kcnk13* expression was altered in response to ethanol exposure, we performed a number of qPCR experiments. We examined the expression levels of *Kcnk13* mRNA during withdrawal from chronic ethanol treatment. These results are illustrated in Figure 6. During withdrawal from Lieber-DeCarli diet, there is a time-dependent change in *Kcnk13* expression. At 24 h withdrawal from Lieber-DeCarli diet, there was an increase in expression of *Kcnk*13 mRNA; control expression level of *Kcnk13* was 1.02 ± 0.11, and 24 after withdrawal from ethanol diet, the expression level was 1.61 ± 0.22 (two-sample t-test, DF=7, t-statistic = −2.54, P<0.05; n=5 samples from 5 rats/group). Interestingly, when measured 72 h after withdrawal, *Kcnk*13 expression was significantly decreased; control expression level of *Kcnk13* was 1.04 ± 0.07, and 72 after withdrawal from ethanol diet, the expression level was $0.79 \pm 0.0.04$ (two-sample t-test, DF=30, t-statistic = 2.95, P<0.01; n=16 samples from 16 rats per group).

Discussion

Using pharmacological and molecular methods, we report that KCNK13 channels play a key role in ethanol excitation of VTA neurons of rats. In regard to some controversy and speculation about the similarity of mechanism of alcohol-induced excitation of VTA neurons of rats versus mice (for example, (Okamoto et al., 2006)), it is important to establish that KCNK13 is also important for ethanol responses in rat VTA neurons. The response of KCNK13 of VTA neurons to ethanol is likely to be behaviorally relevant, as *Kcnk13* knockdown in the VTA increased ethanol consumption in mice (You et al., 2019). Among the cellular elements involved in alcohol effects in the VTA (You et al., 2018a), in this report and in our previously published work (You et al., 2019), we show that KCNK13 channels are coded by ethanol-sensitive genes (as shown by the responsiveness of *Kcnk13* to ethanol treatment) and appear to be important for ethanol-induced excitation of VTA neurons.

Leak potassium channels have been shown to be downregulated following cellular trauma such as axotomy. Leak potassium current has been reported to be reduced after axotomy in

Aplysia (Ungless et al., 2002). Axotomy induces downregulation of the specific leak potassium channels TREK2 (in rat nociceptors) (Acosta et al., 2014) and TRASK (Tulleuda et al., 2011). The decrease in KCNK13 immunoreactivity that we observed (Figure 2 and 3) is consistent with the observations made for these other leak potassium channels, as axotomy occurs during brain slice preparation. Concentrations of ethanol needed to activate DA-like VTA neurons in rodent brain slices (20–120 mM, although lower concentrations under some conditions can increase activity (Avegno et al., 2016, Mrejeru et al., 2015)) are higher than those achieved during ethanol intake by humans (e.g., 0.08 g/dl alcohol concentration is about 17mM). Similarly, lower ethanol concentrations evoke dopamine release as measured with *in vivo* microdialysis (Vena and Gonzales, 2015) or *in vivo* voltammetry (Signs et al., 1987, Jones et al., 2006). The results shown in Figure 2 indicates that there is a relatively rapid and profound decrease in KCNK13 immunoreactivity subsequent to standard brain slice preparation procedures. In fact, the level of KCNK13 that would be present at the beginning of a brain slice recording could be decreased by 75% compared to the level present in the intact animal. As a 22.2% decrease in *Kcnk13* mRNA and a 15.1% decrease in KCNK13 immunoreactivity produced a 50% decrease in ethanol excitation in the mouse VTA (You et al., 2019), a 75% decrease in a major target of alcohol such as KCNK13 may explain the lower ethanol efficacy in brain slice experiments. Although it may be surprising that additional downregulation with siRNA (Figure 5) could affect the response to ethanol subsequent to a decrease of 75% from the native levels, this observation indicates the importance of KCNK13 in the response of VTA neurons to ethanol. We also noted that siRNA reduction of *Kcnk13* over two hours did not alter the baseline firing rate; an increase in baseline firing might be expected with the downregulation of a leak potassium channel like KCNK13. There may be numerous reasons for the lack of effect on baseline firing, including compensation by upregulation of other leak potassium channels during the KCNK13 downregulation. We speculate that the importance of leak potassium channels in regulating the firing rate of these neurons could induce this compensation in the event of downregulation of KCNK13. Studies of the mechanisms of the downregulation of KCNK13 would be useful in elucidating the regulation of this ion channel by axotomy or other trauma.

Isoflurane produced excitation similar to ethanol (Figure 1C). KCNK13 channels are inhibited by anesthetics like halothane and isoflurane, so this observation is consistent with the presence of these channels on VTA neurons. Isoflurane is non-specific in that it also activates some two-pore channels, like the TREK channel (Pavel et al., 2020). However, in the presence of isoflurane, it is likely that the effects of ethanol on KCNK13 would be occluded; studies of the VTA in intact, anesthetized preparations may not be suitable for studying this action of ethanol, and other actions of ethanol may appear to predominate (e.g., effects on GABA versus dopamine neurons of the VTA (Gallegos et al., 1999)). It is also possible that the exposure to isoflurane immediately prior to sacrifice could alter KCNK13 expression, but the brief duration of exposure before sacrifice, and the time between sacrifice and the first recording (at least one hour) should minimize the consequences of that isoflurane exposure. Likewise, exposure to ethanol in the recording chamber at time 0 could alter expression of KCNK13 within the two-hour time, and that could compete with the siKcnk13 reduction in function. Additional careful experiments will be needed to rule out

this possibility, but it is clear that siKcnk13 reduces both Kcnk13 mRNA and ethanol excitation of VTA neurons.

In addition to the involvement in the electrophysiological response to ethanol, *Kcnk13* expression is altered by in vivo ethanol exposure. Acute ethanol administration increased the expression of *Kcnk13* mRNA after four hours in mice rather than one hour (You et al., 2019). This increase in *Kcnk13* mRNA expression was correlated with a significant increase in the effect of ethanol to excite VTA neurons (You et al., 2019). The increase in mRNA expression points to the dynamic regulation of *Kcnk13* levels, and the cellular importance of this ion channel in VTA neurons. In the present study, we demonstrated a change in *Kcnk13* mRNA correlated with ethanol withdrawal. At 24 hours after removal of alcohol-containing Lieber-DeCarli diet, there was a significant increase in *Kcnk13* mRNA, similar to levels we observed after acute ethanol treatment in mice. At 72 hours of withdrawal, there was a significant decrease in *Kcnk13* levels. As a decrease in *Kcnk13* mRNA was correlated with an increase in alcohol intake in mice (You et al., 2019), it is tempting to speculate that decreased expression of *Kcnk13* in the VTA during protracted withdrawal may be one factor in alcohol seeking behavior. Studies designed to elucidate the mechanisms of the dynamic regulation of KCNK13 could yield useful information about novel means to reduce alcohol seeking during protracted withdrawal.

Numerous studies have implicated other neurotransmitters and ion channels that participate in ethanol-induced excitation. Ethanol affects M-current (Koyama et al., 2007), h-current (Okamoto et al., 2006, Brodie and Appel, 1998) and barium-sensitive potassium conductances (McDaid et al., 2008, Herman et al., 2015). KCNK13-mediated excitation appears to be important for excitation of VTA neurons. A variety of targets of alcohol action could alter membrane properties (such as membrane resistance) and influence excitation mediated by KCNK13 blockade, including ion channels (Okamoto et al., 2006, McDaid et al., 2008, Herman et al., 2015), and neurotransmitter receptors (Larsson et al., 2002, Ericson et al., 2008, Steffensen et al., 2009, Theile et al., 2011, Steffensen et al., 2000). Blocking these receptors and ion channels does not block ethanol-induced excitation of VTA neurons (please see (Nimitvilai et al., 2016)), whereas treatments that block KCNK13 (e.g., gadolinium (Figure 1A) or high extracellular Ca^{++} (Figure 1B; (You et al., 2019))), can block ethanol-induced excitation of VTA neurons, suggesting that KCNK13 is a key target of ethanol in the VTA. KCNK13 deserves in-depth studies as it has a crucial role in ethanol stimulation of VTA neurons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

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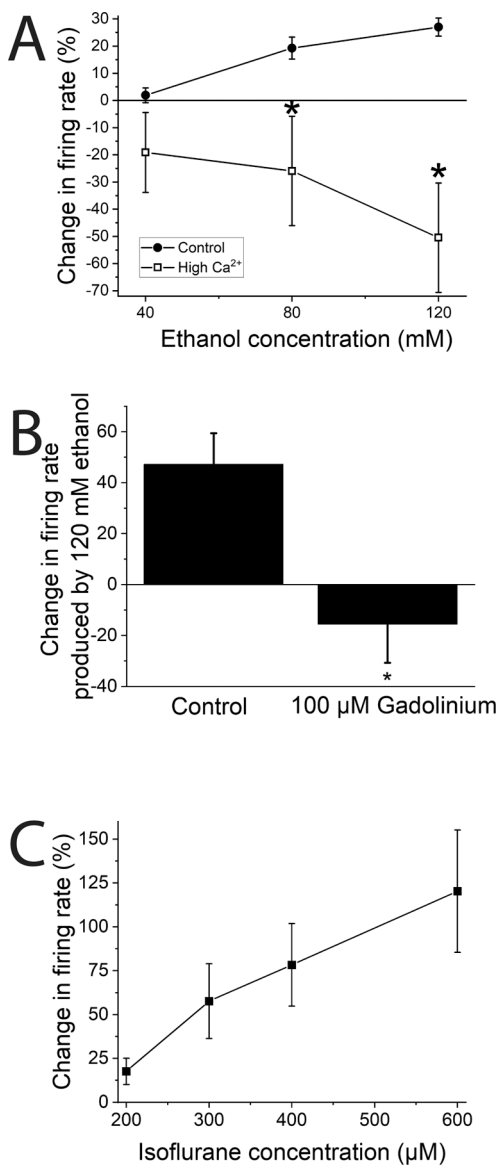
Figure 1.
Pharmacological antagonism of KCNK13 and ethanol excitation of rat VTA neurons.
A. Ethanol excitation is reduced by high extracellular calcium concentration. Increased external calcium concentration (final total external calcium: 7.5–12.5 mM) blocked ethanol the excitatory effect of ethanol on VTA neurons. (two-way ANOVA, $F_{1,3} = 11.43$; $p<0.05$; means comparison with Bonferroni test, $*$ = $P < 0.05$; n=4 cells from 3 rats).
B. Gadolinium reduces ethanol excitation of rat VTA neurons. The excitatory effect of ethanol (120 mM) was reduced by 100 μM gadolinium. (paired-sample t-test, DF=7, t-statistic = 2.80, $*$=$P<0.05$; n=8 cells from 4 rats).
C. Isoflurane excites VTA neurons. Isoflurane (200–600 μM) increased firing rate of VTA neurons in a concentration-dependent manner. (one way repeated measures ANOVA, $F_{3,6} = 8.27$; $p<0.02$; n=8 cells from 4 rats)
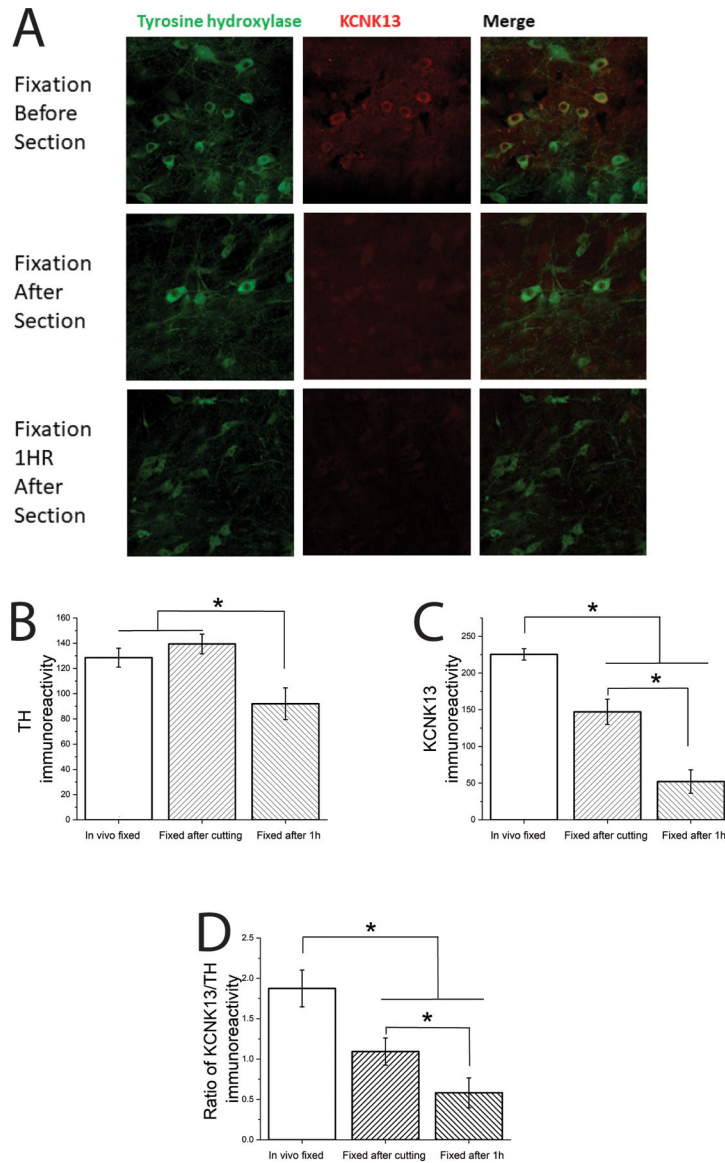
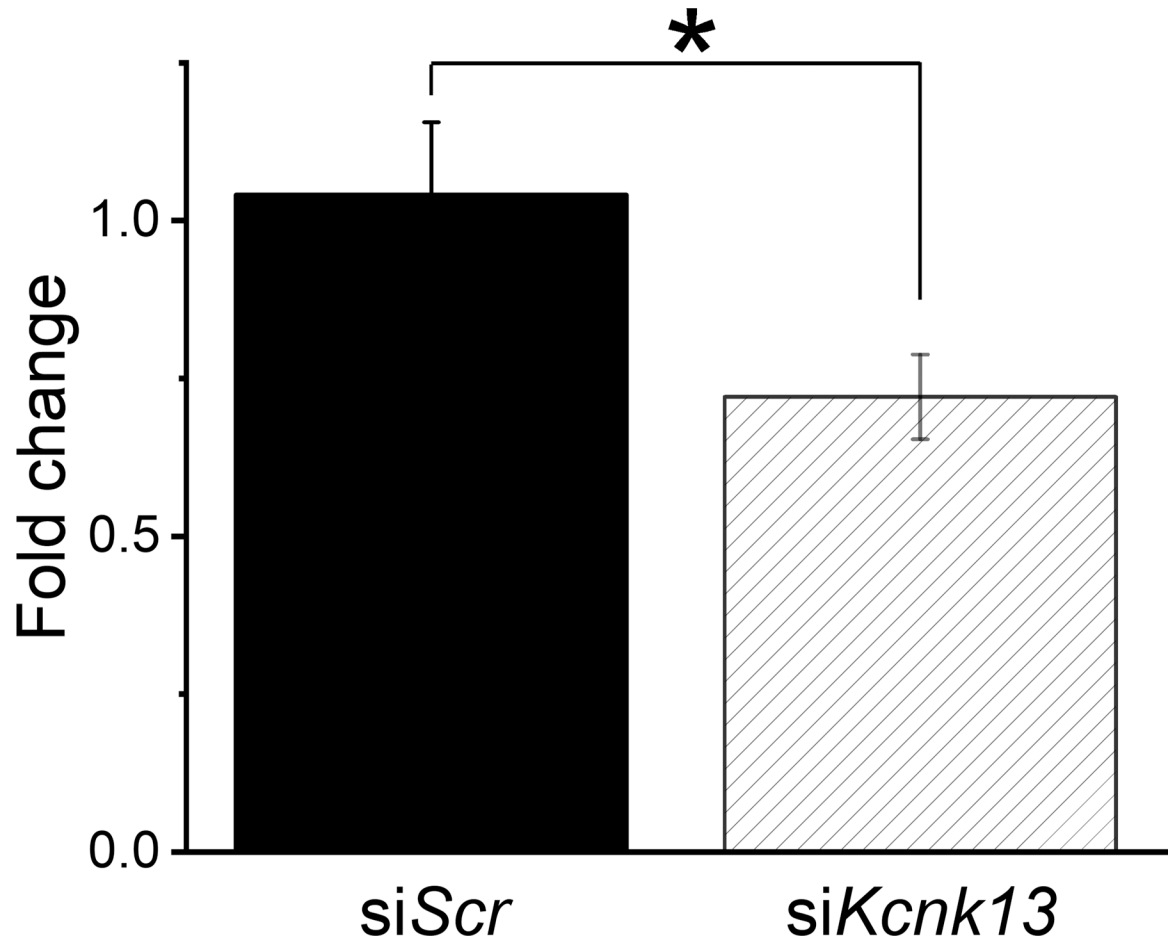**Figure 2.**
Brain slice preparation and incubation in aCSF reduces KCNK13 immunoreactivity

A. Immunohistochemistry: KCNK13 and TH in VTA slices VTA slices (250 μm thick) were processed three different ways: After in vivo perfusion with paraformaldehyde (**Fixation before section**), *in vitro* fixation immediately after brain slices were prepared as for electrophysiology (**Fixation after section**), and *in vitro* fixation after brain slices were prepared as for electrophysiology and incubated for 1 hour as we would before electrophysiological recording (**Fixation 1 HR after section**)

B. TH immunoreactivity was significantly reduced after 1 hour incubation in aCSF compared to the other two conditions (one-way ANOVA, $F_{2,51} = 6.72$, P<0.005, means comparison with Bonferroni test *=P<0.05, n=18 (6 per group));

C. KCNK13 immunoreactivity was significantly reduced in a time-dependent manner over the three conditions (one-way ANOVA, $F_{2,51} = 37.19$, P<0.001, means comparison with Bonferroni test *=P<0.05, n=18 (6 per group));

D. When calculated as a ratio of TH to KCNK13 immunoreactivity, there was a significant time-dependent reduction in KCNK13 (one-way ANOVA, $F_{2,51} = 15.04$, P<0.001, means comparison with Bonferroni test *=P<0.05, n=18 (6 per group)), suggesting that the reduction in KCNK13 was not a function of a general reduction in immunoreactivity over time.
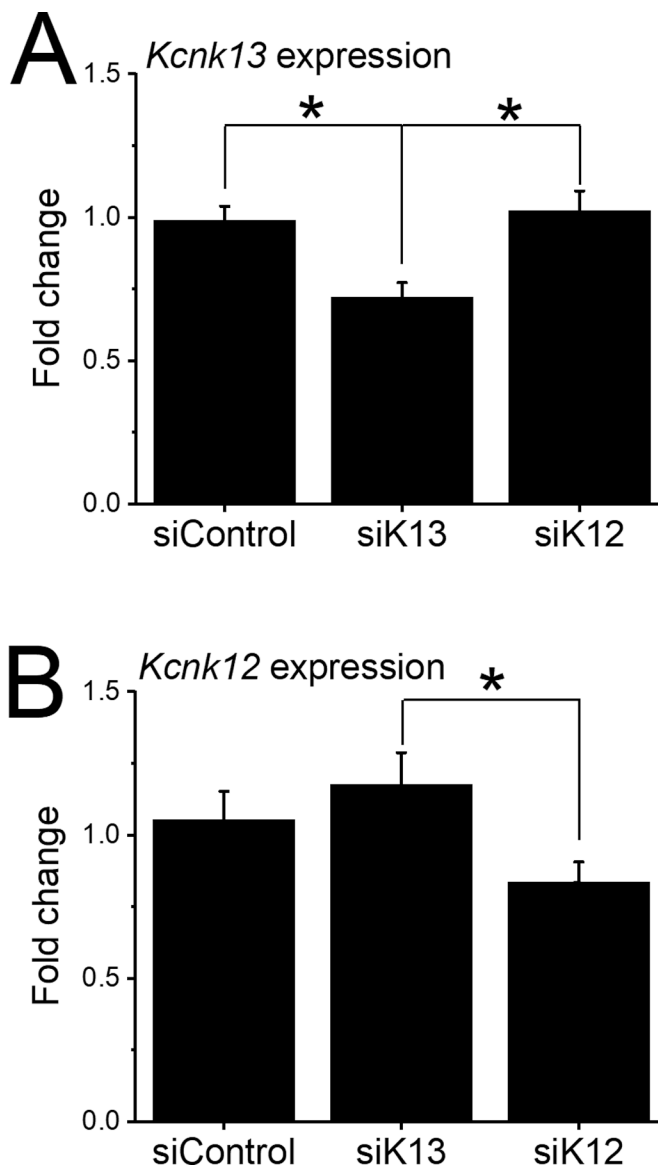
Figure 3.
Incubation for two hours with siRNA targeting *Kcnk13* reduces *Kcnk13* mRNA
Incubation of coronal brain slices of the VTA (400 μm thick) with siRNA targeting *Kcnk13*
reduced the levels of *Kcnk13* mRNA. The si*Kcnk13* reduced the levels of *Kcnk13* by 30.8%,
from 1.04 ± 0.11 to 0.721 ± 0.07 (two-sample t-test, t=2.41, * = P<0.05, n=6 per group).

Figure 4.
Selectivity of two-hour incubation with siRNA targeting *Kcnk12* and *Kcnk13*.
Comparison was made in the effect of incubation of coronal brain slices of the VTA (400 μm thick) with siRNA targeting *Kcnk12, Kcnk13* or scrambled *siRNA (siScr)*.

A. si*Kcnk13* or siScr incubation: Incubation with si*Kcnk13* significantly reduced the level of *Kcnk13* (n=12) compared to si*Scr* (n=14) and to *Kcnk12* (n=13) *Kcnk13* levels (one-way ANOVA, $F_{2,36}$=7.77, P<0.01; means comparison with Bonferroni test, * = P<0.05 for significance).

B. si*Kcnk12* or si*Scr* incubation: Incubation with si*Kcnk12* significantly reduced the level of *Kcnk12* (n=12) compared to *Kcnk13* (n=13) but not to si*Scr*; there was no significant difference between *Kcnk13* and *Scr* groups (one-way ANOVA, $F_{2,34}$=3.28, * = P<0.05; means comparison with Bonferroni test, P<0.05 for significance).
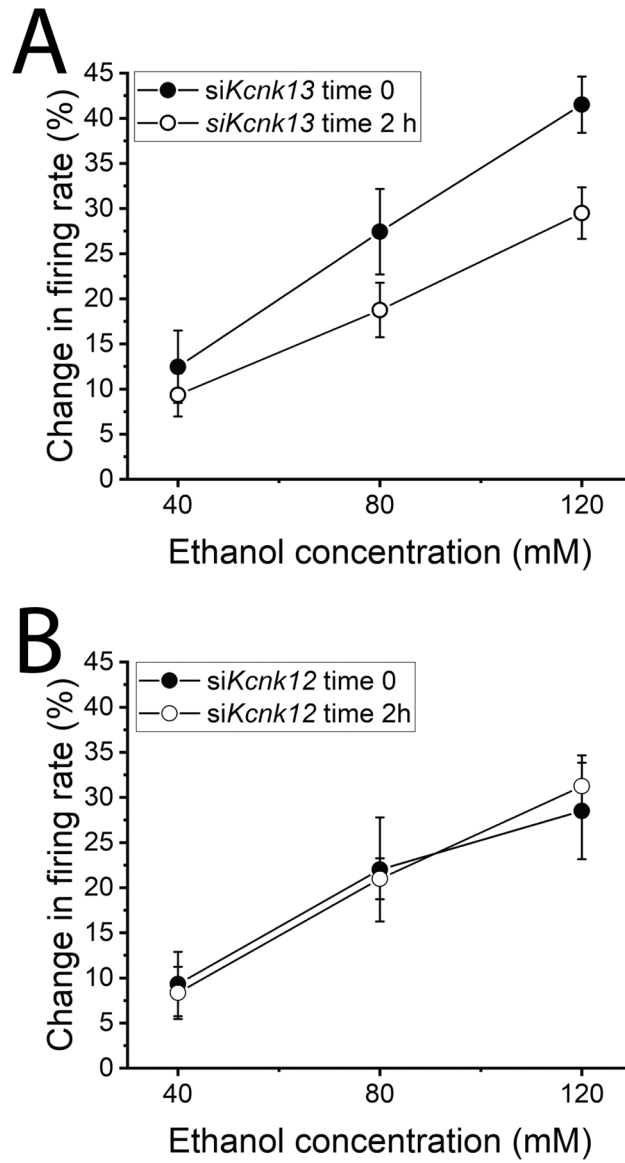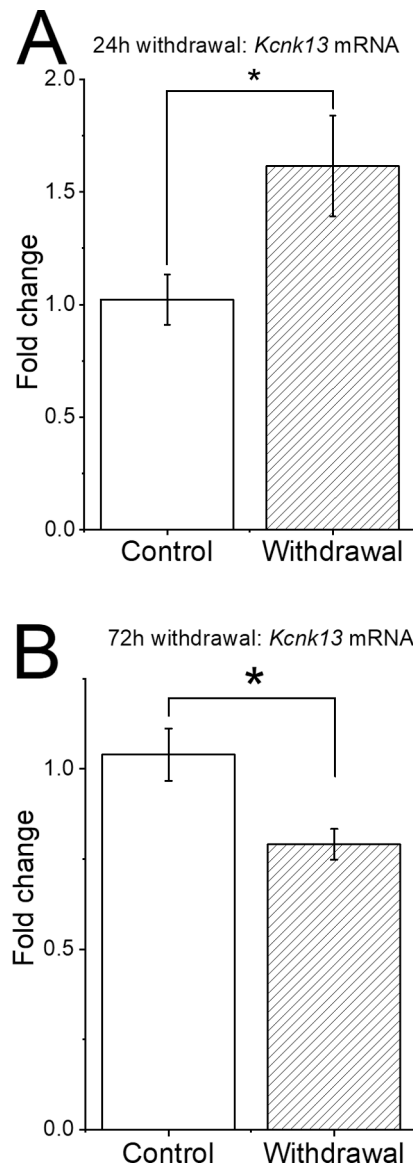
Figure 5.
Electrophysiology: si*Kcnk13,* not si*Kcnk12*, reduced ethanol excitation of rat VTA neurons
A. si*Kcnk13 was i*ncluded in the recording pipette. Change in firing rate of VTA neurons produced by ethanol early in the recording and at least 2 hours after initiation of the recording. The response to ethanol was significantly reduced by the inclusion of si*Kcnk13* in the recording pipette. (Two-way RM ANOVA, $F_{1,7}=6.58$ P<0.05, n=8)
B. si*Kcnk12 i*ncluded in the recording pipette. Change in firing rate of VTA neurons produced by ethanol early in the recording and at least 2 hours after initiation of the recording. The response to ethanol was not significantly reduced by the inclusion of si*Kcnk12* in the recording pipette. (Two-way RM ANOVA, $F_{1,6}=0.002$ P>0.05, n=7)

Figure 6.
Withdrawal from Lieber-DeCarli diet produces time-dependent changes in *Kcnk13* expression in the VTA of Sprague-Dawley rats.

A. After 24-hour withdrawal from a 15-day course of alcohol intake via Lieber-DeCarli diet, there was a significant *increase* in *Kcnk13* expression compared to the VTA of rats given control diet. * = P< 0.05.

B. After 72-hour withdrawal from a 15-day course of alcohol intake via Lieber-DeCarli diet, there was a significant *decrease* of *Kcnk13* expression compared to the VTA of rats given control diet. * = P< 0.01.