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Ethanol Withdrawal Drives Anxiety-Related Behaviors by Reducing M-type Potassium Channel Activity in the Lateral Habenula

Seungwoo Kang[1,2], Jing Li[1,2], Wanhong Zuo[1,2], Rao Fu[1,2], Danielle Gregor[1,2], Kresimir Krnjevic[3], Alex Bekker[1] and Jiang-Hong Ye*[,1,2]

[1]Department of Anesthesiology, Rutgers, The State University of New Jersey, New Jersey Medical School, Newark, NJ, USA; [2]Department of Pharmacology, Physiology, and Neuroscience, Rutgers, The State University of New Jersey, New Jersey Medical School, Newark, NJ, USA; [3]Department of Physiology, McGill University, Montreal, Canada

Alcohol use disorders (AUDs) and anxiety disorders (ADs) are often seen concurrently, but their underlying cellular basis is unclear. For unclear reasons, the lateral habenula (LHb), a key brain region involved in the pathophysiology of ADs, becomes hyperactive after ethanol withdrawal. M-type K^+ channels (M-channels), important regulators of neuronal activity, are abundant in the LHb, yet little is known about their role in AUDs and associated ADs. We report here that in rats at 24 h withdrawal from systemic ethanol administration (either by intraperitoneal injection, 2 g/kg, twice/day, for 7 days; or intermittent drinking 20% ethanol in a two-bottle free choice protocol for 8 weeks), the basal firing rate and the excitability of LHb neurons in brain slices was higher, whereas the amplitude of medium afterhyperpolarization and M-type K^+ currents were smaller, when compared to ethanol naive rats. Concordantly, M-channel blocker (XE991)-induced increase in the spontaneous firing rate in LHb neurons was smaller. The protein expression of M-channel subunits, KCNQ2/3 in the LHb was also smaller. Moreover, anxiety levels (tested in open field, marble burying, and elevated plus maze) were higher, which were alleviated by LHb inhibition either chemogenetically or by local infusion of the M-channel opener, retigabine. Intra-LHb infusion of retigabine also reduced ethanol consumption and preference. These findings reveal an important role of LHb M-channels in the expression of AUDs and ADs, and suggest that the M-channels could be a potential therapeutic target for alcoholics.
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INTRODUCTION

Anxiety disorders (ADs), common symptoms of alcohol withdrawal, are important factors in the negative reinforcement leading to relapse (Driessen *et al*, 2001; Sinha, 2001; Wright *et al*, 1990). There is much interest in brain regions that drive anxiety in alcoholics. Most studies on alcohol-related anxiety in animal models focus on the amygdaloid structures (Gilpin *et al*, 2015; McBride, 2002; Pandey *et al*, 2003, 2006). Multidisciplinary work collectively suggests that the central amygdala is an integrative hub for ADs and alcohol use disorder (AUDs) (Gilpin *et al*, 2015). However, the cellular basis underlying the comorbidity of ADs and AUDs has not been completely uncovered. The lateral habenula (LHb) has received increasing attention recently because of its pivotal role in aversive behaviors (Proulx *et al*, 2014). Besides the well-known functions of the LHb such as the regulation of sleep and maternal behavior (Hikosaka, 2010), recent studies have shown that the LHb also acts as an important part in the reward circuit by providing 'negative value' signals to neuromodulator systems, particularly the dopaminergic and serotonergic systems (Proulx *et al*, 2014). The LHb relays information from the limbic forebrain to monoaminergic centers (Baldwin *et al*, 2011), controlling mood and emotions. Therefore, LHb disturbances have been implicated in the pathogenesis of psychiatric disorders such as depression and anxiety (Li *et al*, 2013; Zhao *et al*, 2015). Indeed, LHb hyperactivity is anxiogenic (Pobbe and Zangrossi, 2008). However, a role for the LHb in ADs associated with AUDs has not been demonstrated.

Since the transmission of neuronal signals relies critically on ion channels (Waxman and Zamponi, 2014), dysregulation of channel function in response to drug abuse may result in neuropathology. Over the past few decades, various K^+ channels have been identified as major sites of regulation in the homeostatic plasticity of intrinsic membrane excitability (Misonou, 2010). M-type voltage-gated K^+ channels (M-channels) have received particular attention because they tend to repolarize neurons and thus prevent repetitive firing (Delmas and Brown, 2005; Vervaeke *et al*, 2006).

*Correspondence: Professor J-H Ye, Department of Anesthesiology, Rutgers, The State University of New Jersey, New Jersey Medical School, 185 South Orange Avenue, Newark, NJ, 07103, USA, Tel: 973 972 1866, Fax: 973 972 0582, E-mail: ye@njms.rutgers.edu
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Accumulating evidence suggests that M-channels could be a target of alcohol's actions on neuronal function and behavior (Cavaliere *et al*, 2012; Knapp *et al*, 2014; Koyama *et al*, 2007; McGuier *et al*, 2015; Moore *et al*, 1990). A previous histological study had shown that the M-channel subunit KCNQ2 is abundantly expressed in the LHb (Castro *et al*, 2001). We therefore hypothesized that a reduction of LHb M-channel function may contribute to the anxiogenic effect of alcohol withdrawal, and tested this idea in experiments on rats, by combining electrophysiological, biochemical, chemogenetic, and behavioral approaches.

We found that during ethanol withdrawal, anxiety levels of rats and the activity of LHb neurons were increased, and M-channel expression was reduced. The elevated anxiety levels could be attenuated by selective inhibition of the LHb neurons with designer receptors exclusively activated by designer drugs (DREADDs) (Armbruster *et al*, 2007; Smith *et al*, 2016) or by the M-channel opener, retigabine. These findings suggest that M-channels play an important role in the pathology of ADs after alcohol withdrawal.

MATERIALS AND METHODS

In Vivo Systemic Administration of Ethanol

Male Sprague Dawley (SD) rats (4–5-week-old) were given intraperitoneal injections (i.p.) of ethanol (2 g/kg in 20% v/v), or an equivalent volume of saline twice a day for 7 days. Anxiety-related behaviors or neuronal properties were evaluated 24 h after the last injection. We selected this time point based on a previous observation of maximal anxiety-like behaviors in rats at 24 h of abstinence from repeated ethanol exposure (Gibula-Bruzda *et al*, 2015). Each behavior was tested in a separate group.

Intermittent Access to 20% Ethanol Two-Bottle Free Choice Drinking (IA2BC)

We measured ethanol intake in the Long Evans rat, a strain commonly employed for voluntary ethanol consumption, in the IA2BC drinking paradigm (Li *et al*, 2011b; Simms *et al*, 2008; Wise, 1973). Detailed IA2BC paradigm is provided in Supplementary Materials and Methods.

Stereotaxic Surgery and Microinjections

Stereotaxic surgery and histological verification were performed as described (Li *et al*, 2016; Zuo *et al*, 2015). The details are provided in Supplementary Materials and Methods.

Intra-LHb Chemogenetic Virus Injection and Clozapine-n-oxide (CNO) Treatment

We introduced the engineered human muscarinic receptor, either the inhibitory hm4D or the excitatory hm3Dq by injecting AAV5-CaMKIIa-hm4D-mCherry, AAV5-CaMKIIa-hm3Dq-mCherry, or control AAV5-CaMKIIa-eGFP (titers of 10^{12}–10^{13} vg/ml, UNC Vector Core, Chapel Hill, NC) bilaterally into the LHb (AP −3.4 mm, ML ±0.73 mm, DV −4.8 mm) of SD rats at P32-35 weighing 100–120 g. A volume of 350 nl per side was delivered at a rate of 70 nl min^{-1}. Ethanol injections were started 2-weeks later. At 24 h after the last ethanol injection, CNO (5 mg/kg, dissolved in 0.5% DMSO v/v saline, i.p.) was given 30 min before the behavioral test (Smith *et al*, 2016). Ethanol naive rats were injected with saline and received the same handling as ethanol-treated rats.

Measurement of Anxiety-like Behaviors

The anxiety-like behaviors were measured by elevated plus maze test (EPM), Marble burying test (MBT), and Open field test (OPT). The detailed conditions of each test are described in Supplementary Materials and Methods.

Brain Slice Preparation and Electrophysiology

The brain slices preparation and electrophysiology were performed according to previously described criteria (Zuo *et al*, 2015)(Supplementary Materials and Methods).

Western Blotting, Immunofluorescence, and Antibodies

The procedures of western blotting and immunofluorescence and all antibodies used in the present study are described in Supplementary Materials and Methods.

Measurements of Blood Ethanol Concentration

Blood samples were acquired from the lateral tail vein of rats 2 h after LHb infusion of aCSF or retigabine and the ethanol concentration was measured as described (Carnicella *et al*, 2009; Li *et al*, 2011b; Simms *et al*, 2008).

Intermittent Access to Sucrose Using a Two-Bottle Choice Drinking Protocol

A separate group of rats implanted with cannulae in the LHb, were trained to drink sucrose under intermittent access to 2% sucrose by the two-bottle choice procedure, 7 days after the surgery, as described (Li *et al*, 2011b).

Drugs

We purchased common salts and apamin from Sigma Aldrich (St. Louis, MO, USA), retigabine from Alomone (Jerusalem, Israel), XE991 from Tocris (Bristol, UK), and ethanol from Pharmco Products Inc (Brookfield, CT). CNO was from NIDA Drug supply program (NIH, Bethesda, MD).

Data Analysis and Statistics

We measured the mean frequency of spontaneous firing over the last 3-min of 5-min periods of recording and calculated drug-induced changes by normalizing the data to the preceding 3-min of baseline firing. Statistical analyses were performed using Prism (Graphpad, La Jolla, CA). All compiled data are presented as mean ± SEM. Statistical significance was assessed using paired or unpaired *t*-tests, and one- or two-way ANOVA with *post hoc* multiple-comparisons, when appropriate. Values of $p<0.05$ were considered significant.

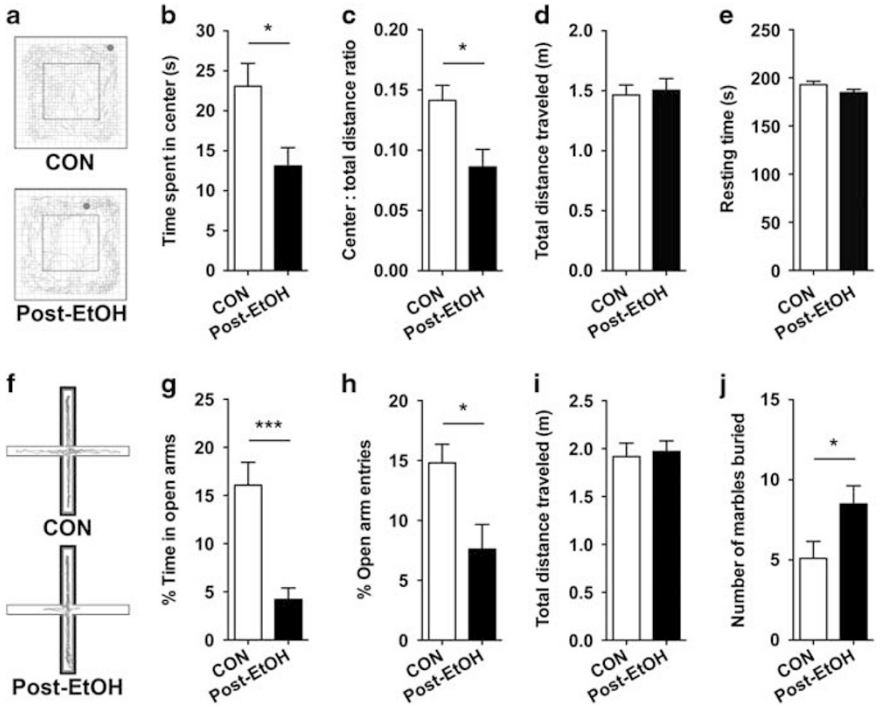


Figure 1 Anxiety-like behaviors in rats withdrawn from systemic administration of ethanol. (a–e) Open field data. (a) Representative traces show time spent in center of chamber by rats at 24 h withdrawal from repeated systemic ethanol administration (Post-EtOH) are shorter than that by ethanol-naive rats (CON). (b–e) Mean (± SEM) data ($N_{rat} = 10$/group): (b) mean times spent in center (Unpaired *t*-test, t = 2.711), (c) proportion of total distance travelled at center, (d) total distances traveled, and (e) resting time. Unpaired *t*-test: $*p < 0.05$, $**p < 0.01$. (f–j) Elevated plus maze data: (f) Representative traces show Post-EtOH rats spend much less time in open arms than do CON rats. (g–i) Summary data: (g) time spent in open arms; (h) entries into open arms, and (i) total distance traveled by CON and Post-EtOH rats. Unpaired *t*-test: $*p < 0.05$. $N_{rat} = 10$/group. (j) Number of marbles buried in 30-min marble-burying anxiety test. Unpaired *t*-test: $*p < 0.05$, $***p < 0.001$. $N_{rat} = 10$/group.

RESULTS

Anxiety-like Behaviors in Juvenile Rats after Discontinuing Ethanol Administration

Anxiety-like behaviors were examined 24 h after the last injection of ethanol (2 g/kg, i.p. twice per day for 7d, Post-EtOH rat) or saline (CON rats). In the OPT, while the total distance traveled and resting time were similar (Figure 1a, d and e), the distance traveled and time spent in the center of the open field were significantly shorter for Post-EtOH rats than CON rats (Figure 1a–c), suggesting increased anxiety levels. Correspondingly, the time spent in the open arms and the entries into open arms of the EPM were significantly reduced for Post-EtOH rats compared to CON rats (Figure 1f–i). Likewise, Post-EtOH rats buried significantly more marbles than did CON rats in the MBT (Figure 1j).

Increased Activity of LHb Neurons in Slices from Juvenile Rats after Discontinuing *In Vivo* Ethanol Administration

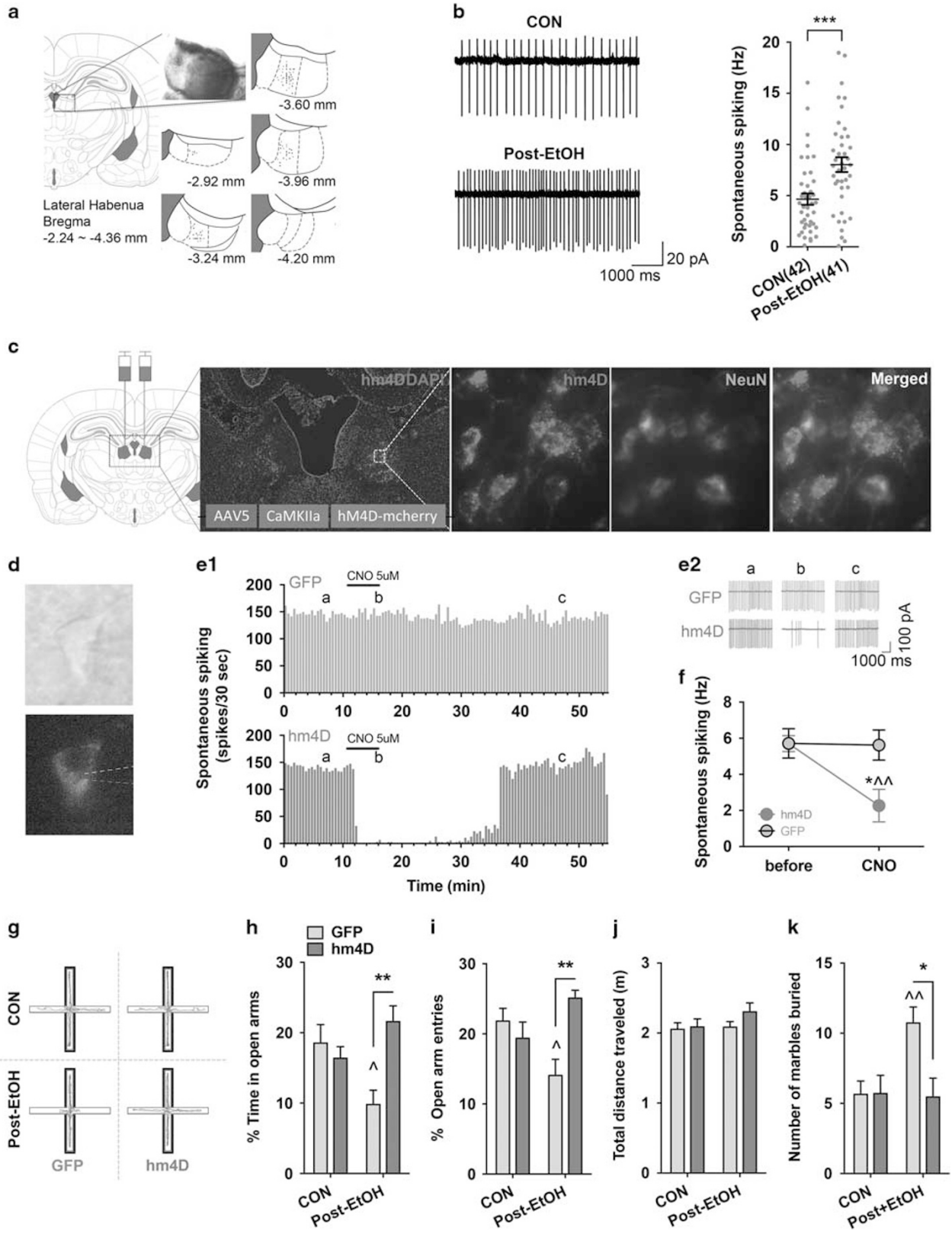
To assess the role of the LHb in the anxiety-like behaviors seen after ethanol withdrawal, we first measured the spontaneous activity of neurons in the LHb, particularly those in the medial region (Figure 2a), where neurons project mainly to the dorsal raphe (Proulx *et al*, 2014), a brain area that controls generalized anxiety in rats (Sena *et al*, 2003; Spiacci *et al*, 2012). The basal firing rate of LHb neurons in slices from Post-EtOH rats was significantly higher than that of CON rats (Figure 2a and b).

Chemogenetic Inhibition of LHb Neurons Alleviates Anxiety-like Behaviors of Juvenile Rats

To further assess the role of the LHb in anxiety-like behaviors after ethanol withdrawal, we recorded from LHb neurons in slices from rats infected three weeks earlier with either an AAV5 carrying hm4D inhibitory muscarinic receptors or eGFP control virus bilaterally in the LHb (Figure 2c and d). Bath application of CNO (5 uM) significantly reduced the spontaneous firing of LHb neurons in slices infected with hm4D but not those infected with eGFP (Figure 2e and f). Importantly, systemic administration of CNO (5 mg/kg, i.p.) 24 h after the last injection of ethanol (2 g/kg, i.p. twice per day for 7 days) significantly alleviated the anxiety-like behaviors in the EPM (Figure 2g–j) and in the MBT of rats infected with hm4D but not in those infected with eGFP (Figure 2k). Rats injected with AAV-eGFP did not show any difference in baseline levels in the EPM and the MBT compared to those of CON rats without viral injection (effects of virus; % time in open arms, $F_{1,46} = 3.302$, $p = 0.0757$; number of marbles buried, $F_{1,38} = 1.886$, $p = 0.1777$), and both groups of rats showed anxiety-like behaviors after repeated ethanol injection, regardless of viral injection (effects of EtOH; % time in open arms, $F_{1,46} = 20.55$, $p < 0.0001$; number of marbles buried, $F_{1,38} = 16.49$, $p = 0.0002$; Figures 1g, j and 2h, k).

Chemogenetic Activation of LHb Neurons Initiates Anxiety-like Behaviors in Ethanol Naive Rats

To ascertain the role of the LHb in the initiation of anxiety-like behaviors, we mimicked the ethanol-withdrawn states of LHb neurons by bilaterally transducing LHb neurons with AAV5 that carried the chemogenetic receptor, hm3Dq, under the control of the CaMKIIa promoter. Hm3Dq is a modified muscarinic receptor 3 that effectively induces firing of neurons in response to the ligand, CNO (Alexander *et al*,



2009). As expected, bath application of CNO increased the spontaneous firing rate of the LHb neurons in slices from rats infected with hm3Dq but not those infected with eGFP (Supplementary Figure S1A and B). Moreover, activation of LHb neurons by systemic administration of CNO (5 mg/kg, i.p.) significantly decreased the time spent in the open arms of the EPM (Supplementary Figure S1C–F) and the number of buried marbles in the MBT (Supplementary Figure S1G).

Changes in Intrinsic Properties of LHb Neurons in Slices from Juvenile Rats Withdrawn from Systemic Ethanol Exposure

To investigate the mechanism of LHb hyperactivity in ethanol-withdrawn rats, we injected a series of incremental depolarizing current pulses into LHb neurons. Such currents evoked more firing in LHb neurons from Post-EtOH rats than those from CON rats (Figure 3a–c), suggesting that ethanol withdrawal increases the intrinsic excitability of LHb neurons.

Changes in action potentials may result from altering voltage-gated Na^+ and/or voltage-gated K^+ currents (Kourrich *et al*, 2015). Increased excitability could result from increased Na^+ channel function (Halter *et al*, 1995; Zhang *et al*, 1998). However, this is unlikely a major factor since action potential thresholds and peak amplitudes were similar in LHb neurons from CON and Post-EtOH rats (Figure 3b, d, e and i). We therefore examined the possible contribution of K^+ channels. Resting membrane potentials (RMP) and action potential durations did not differ between LHb neurons from CON and Post-EtOH rats (Figure 3b, f and h), suggesting no change in inwardly rectifying K^+ current. Conversely, close examination of the K^+ channel-mediated afterhyperpolarization revealed that whereas its earliest component, the fast after hyperpolarization (fAHP) was unchanged (Figure 3j), the second one (medium afterhyperpolarization, mAHP) was significantly smaller in LHb neurons of Post-EtOH rats (Figure 3k), resulting in shorter inter-spike interval in Post-EtOH rats than in CON rats (Figure 3g).

Involvement of M-current in Ethanol-Induced Adaptation of LHb Neurons of Juvenile Rats

The mAHP is generated mainly by small conductance Ca^{2+}-activated K^+ channels (SK channels) and M-type K^+ channels (M-channels) (Gu *et al*, 2005; Kang *et al*, 2014). Since KCNQ2, a subtype of the M-channel, is abundant in the LHb (Castro *et al*, 2001), we tested the effects of XE991, a KCNQ-channel blocker. As expected, XE991 (20 μM) substantially accelerated the firing of LHb neurons in slices from CON rats, confirming the existence of functional M-channels (Figure 3l and n). Importantly, XE991's effect was significantly weaker in the LHb neurons of Post-EtOH rats than in LHb neurons of CON rats, consistent with a reduction of M-channel function in LHb neurons of Post-EtOH rats (Figure 3l, n and p).

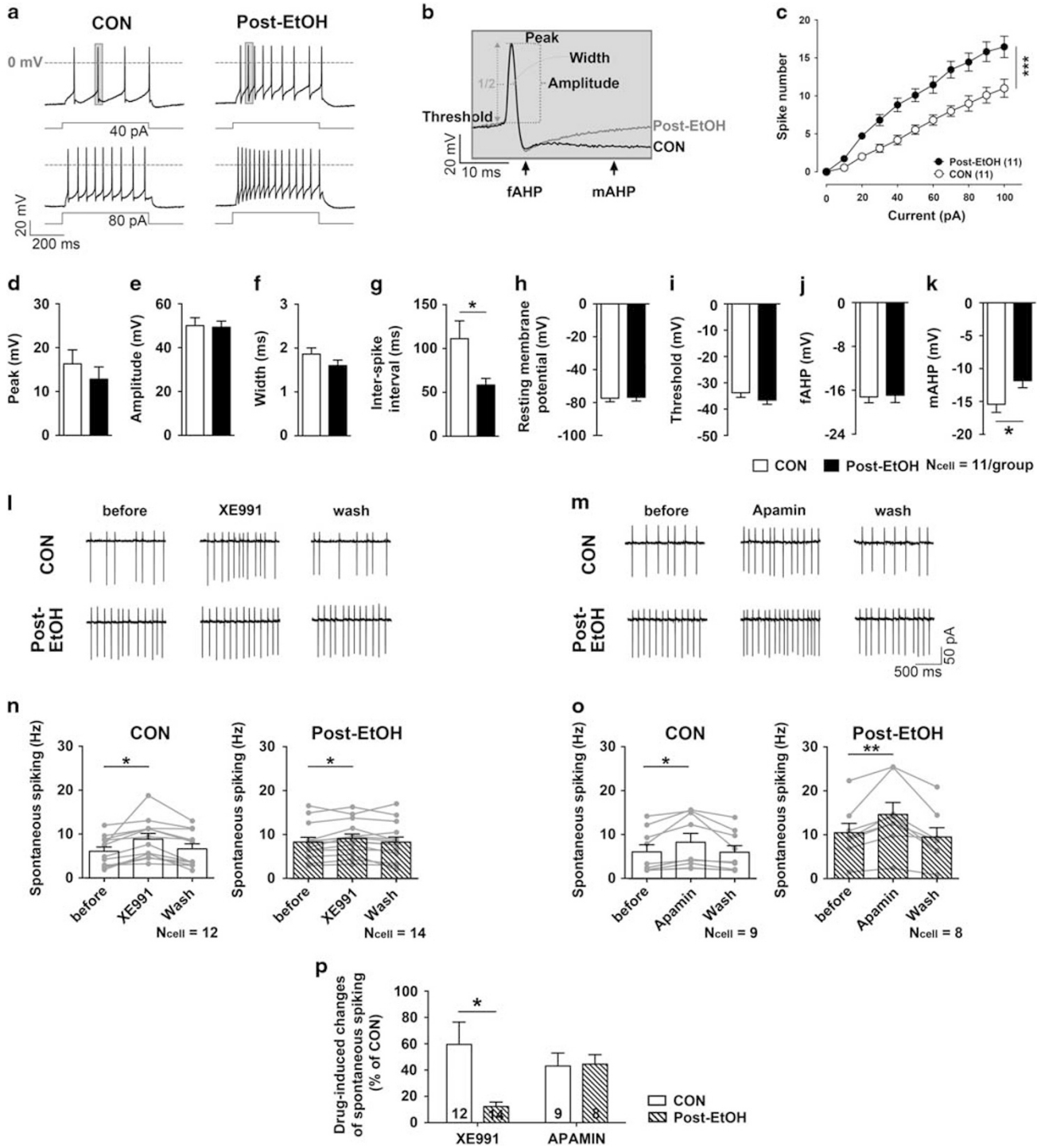
The SK type of K^+ channel that contributes to the mAHP is also present in the LHb although at low levels (Pedarzani *et al*, 2000; Vielhaber *et al*, 2004). Bath application of the SK channel blocker, apamin (100 nM), significantly accelerated firing of LHb neurons (Figure 3m and o) similarly in both the CON and Post-EtOH rats (Figure 3p). These results suggest that the hyperactivity of LHb neurons in Post-EtOH rats is mediated by the reduction of M-channel function. This possibility is further supported by the observation that although XE991 (20 μM) significantly reduced mAHP (Supplementary Figure S2A and E) and increased the number of spikes evoked by a depolarizing pulse in LHb neurons from CON rats (Supplementary Figure S2A and C), it had no significant effect on LHb neurons from Post-EtOH rats (Supplementary Figure S2B, D and E).

To measure M-current directly (Supplementary Figure S3), we injected 10 mV hyperpolarizing steps from −30 to −60 mV at a holding potential of −20 mV. The slow inward relaxation—reflecting the closing of the voltage-dependent M-current—was largest during the step to −40 mV (left panel in Figure 4a and open circles in Figure 4b). The much smaller inward relaxations in LHb neurons of Post-EtOH rats (Figure 4a and b) indicate a correspondingly weaker M-channel activity. Since the total cell capacitance was similar for cells from both groups of rats, the difference in M-current is unlikely to be due to a difference in cell size (Figure 4c).

Figure 2 Withdrawal from systemic ethanol administration increases activity of LHb neurons in brain slices and chemogenetic inhibition of the LHb attenuates anxiety-like behaviors. (a) Schematic of location of recorded neurons. (b) Representative traces and summary data show increased spontaneous firing of LHb neurons after ethanol withdrawal. Unpaired *t*-test: ***$p<0.001$. Numbers of neurons are indicated. (c) hm4D-mcherry expression in LHb neurons after viral vector injection and immunofluorescence of the neuronal marker NeuN. Strong signal of hm4DmCherry is overlapped with NeuN. (d) CCD camera captured IR (upper) and ET-DSRed filtered fluorescence (bottom) image of the LHb neuron after viral injection for loose cell-attached patch-clamping recording. (e, f) Bath applied CNO (5 μM) sharply reduced the firing rate of LHb neurons infected with AAV-CaMKIIa-hm4D-mCherry viruses (hm4D), but not those with control AAV5-CaMKIIa-eGFP (GFP). Two-way ANOVA, for Drug treatment $F_{1,14}=20.14$, $p=0.0005$, for interaction $F_{1,14}=18.03$, $p=0.0008$, Bonferroni's *post hoc* test: * $p<0.05$ *vs* hmD4 before, ^^$p<0.01$ *vs* GFP CNO. $N_{cell}=8$/group. (g–k) Elevated plus maze data: (g) representative traces show that after systemic CNO injection (5 mg/kg, i.p.), Post-EtOH rats but not the CON rats, infected with hm4D spend more time in open arms than those infected with eGFP. (h–i) Summary of times spent in open arms (for GFP/hm4D expression $F_{1,46}=4.184$, $p=0.0466$, for interaction $F_{1,46}=8.795$, $p=0.0048$, *post hoc* test: ^$p<0.05$ *vs* CON GFP, **$p<0.01$ *vs* Post-EtOH GFP, H), entries into open arms (for GFP/hm4D expression $F_{1,46}=4.443$, $p=0.0405$, for interaction $F_{1,46}=10.93$, $p=0.0018$, *post hoc* test: ^$p<0.05$ *vs* CON GFP, **$p<0.01$ *vs* Post-EtOH GFP, I) and total distance traveled (for GFP/hm4D expression $F_{1,46}=1.679$, $p=0.2015$, for interaction $F_{1,46}=0.9011$, $p=0.3474$, *post hoc* test: all $p>0.05$, J) by rats infected with GFP (grey bars) or hm4D (dark bars), as revealed by two-way ANOVA, Bonferroni's *post hoc* test. $N_{rat}=16$(CON GFP), $=8$(CON hm4D), $=14$(Post-EtOH GFP), $=12$(Post-EtOH hm4D). (k) In 30-min MBTs performed after CNO injections, substantially fewer marbles were buried by hm4D-infected rats than by eGFP-infected rats during ethanol withdrawal. Two-way ANOVA, for drug treatment $F_{1,34}=5.371$, $p=0.0266$, for interaction $F_{1,34}=5.611$, $p=0.0237$, Bonferroni's *post hoc* test: ^^$p<0.01$ *vs* CON GFP, *$p<0.05$ *vs* Post-EtOH GFP. $N_{rat}=13$(CON GFP), 8(CON hm4D), $=9$(Post-EtOH GFP), $=8$(Post-EtOH hm4D).

Down-Regulation of M-Channel Expression in the LHb of Ethanol-Withdrawn Juvenile Rats

Previous studies have identified that the heterotetramer channel complex KCNQ2/3 belonging to KCNQ family (Kv7) is the main molecular correlates of the native M-current (Shah *et al*, 2002; Wang *et al*, 1998) and have shown the prominent subcellular localization at somata, axon initial segments, and the nodes of KCNQ2 and KCNQ3 subunits (Klinger *et al*, 2011; Trimmer, 2015). The M-channel subunits KCNQ2 (Castro *et al*, 2001) and KCNQ3 are abundantly expressed in the LHb (Supplementary Figure S4). Specifically, the immunoreactivities for KCNQ2 and KCNQ3 were observed in the somata of glutamatergic neurons in the LHb and appeared more pronounced in the membrane. KCNQ gene expression is correlated with M-current density and neuronal excitability (Mucha *et al*, 2010). To determine whether M-channel expression in the LHb is changed by withdrawal from chronic ethanol exposure, we quantified M-channel



expression in the LHb in Western blots. Both KCNQ2 and KCNQ3 subunits in LHb were significantly reduced in tissue from Post-EtOH compared to tissue from CON rats (Figure 4d and e). These results suggest that the observed smaller M-currents could result from the lower expression of KCNQ2 and KCNQ3 in the LHb after ethanol withdrawal.

Activation of LHb M-Channels Reduces Anxiety-like Behaviors in Ethanol-Withdrawn Rats

To determine whether LHb M-channels contribute to the anxiety-like behaviors seen after ethanol withdrawal, we first examined the effect of the M-channel activator, retigabine, on LHb neuronal activity. As expected, bath application of retigabine dose-dependently reduced the spontaneous firing of LHb neurons in slices from both CON rats and Post-EtOH rats; and the reduction was significantly weaker in LHb neurons from Post-EtOH rats (Figure 5a and b), although retigabine at higher doses (10 and 30 μM) substantially decreased LHb activities of both groups of rats. Specifically, retigabine (30 μM) sharply decreased LHb firing in slices from both CON (to $4.8 \pm 2.5\%$ of baseline, $n = 5$, paired t-test, $t = 37.57$, $p < 0.0001$) and Post-EtOH rats (to $10.6 \pm 8.0\%$ of baseline, $n = 5$, paired t-test, $t = 11.16$, $p = 0.0004$) (Figure 5b). Importantly, intra-LHb infusion of retigabine at

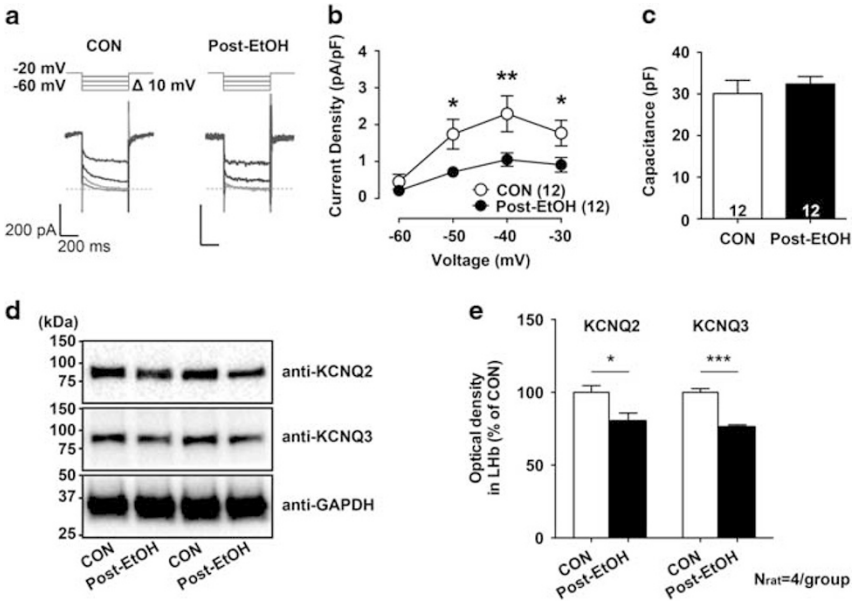


Figure 4 Reduction of M-current and expression of M-channels in LHb neurons in ethanol-withdrawn rats. (a) Sample traces show reduced inward current relaxations in neurons from ethanol-withdrawn rats (Post-EtOH) compared to neurons from ethanol-naive rats (CON). At a holding potential of −20 mV, ongoing M-currents were deactivated by 500-ms repolarizing steps from − 30 to − 60 mV. (b) Density of M-currents is much lower in Post-EtOH rats than in CON rats. Two-way ANOVA, for CON/EtOH treatment, $F_{1,22} = 5.705$, $p = 0.025$, for interaction, $F_{3,66} = 3.619$, $p = 0.017$, Bonferroni's *post hoc* test: $^{*}p < 0.05$, $^{**}p < 0.01$. $N_{cell} = 12$/group. (c) Pooled results show no difference in total cell capacitance between LHb neurons from ethanol-withdrawn and naive rats. Unpaired t-test, $t = 0.6206$, $p = 0.5413$. $N_{cell} = 12$/group. (d,e) Examples (d) and pooled results (e) of western blots show reduced KCNQ2 and KCNQ3 expression in the LHb from ethanol-withdrawn rats compared to CON rats. Note an even greater loss of KCNQ3 expression in the Post-EtOH rats. Unpaired t-test: for KCNQ2 $t = 2.754$, $^{*}p < 0.05$, for KCNQ3 $t = 8.287$, $^{***}p < 0.001$. $N_{rat} = 4$/group.

Figure 3 Increased excitability and reduced M-channel blocker (XE991)-induced increase in the firing rate LHb neurons in slices of rats withdrawn from systemic ethanol administration. (a) Current injections elicit significantly more spikes in LHb neurons of ethanol-withdrawn rats (Post-EtOH, right) than naive rats (CON, left). (b) Superimposed action potentials reveal loss of second phase of afterhyperpolarization (AHP) in Post-EtOH rats. (c) Number of spikes evoked over a wide range of current pulses was consistently higher in Post-EtOH rats than CON rats. Two-way ANOVA, for CON/EtOH $F_{1,20} = 18.58$, $p = 0.0003$, for interaction $F_{10,200} = 5.808$, $p < 0.0001$. $N_{cell} = 11$/group. (d–k) Electrical properties of LHb neurons in CON and Post-EtOH rats: (d) membrane potential at peak of spike (Unpaired t-test, $t = 0.8259$, $p = 0.4186$), (e) spike amplitude (Unpaired t-test, $t = 0.1581$, $p = 0.8760$), (f) spike width (at the ½ point from the peak to the threshold potential, Unpaired t-test, $t = 1.406$, $p = 0.1750$), (g) inter-spike time (Unpaired t-test, $t = 2.442$, $p = 0.0240$), (h) RMP (Unpaired t-test, $t = 0.1922$, $p = 0.8495$), (i) spike threshold (Unpaired t-test, $t = 1.191$, $p = 0.2477$), (j) amplitude of fast AHPs (Unpaired t-test, $t = 0.1663$, $p = 0.8696$), and (k) medium AHPs (Unpaired t-test, $t = 2.205$, $p = 0.0393$) (With the threshold as a baseline, fAHP and mAHP were measured as the lowest point 2 5 ms and 20–40 ms after the peak of the action potential, respectively.). $^{*}p < 0.05$. $N_{cell} = 11$/group. (l, n, p) Examples of firing recorded in cell-attached mode (l) and summary data (n) show that the accelerated LHb firings induced by the M-channel blocker XE991 (20 μM) (One-way ANOVA, $F_{2,22} = 10.51$, $p = 0.0006$, Bonferroni's *post hoc* test: $^{***}p < 0.0001$) is reduced in LHb neurons from rats 24 h after end of ethanol administration (Post-EtOH). Unpaired t-test: $t = 2.947$, $p = 0.007$ (p). (m, o, p) Examples of firing (m) and summary data (o) show that the SK channel blocker apamin (100 nM) causes a similar acceleration of firing in LHb neurons from CON rats and Post-EtOH rats (for CON group, One-way ANOVA, $F_{2,16} = 6.982$, $p = 0.0066$, Bonferroni's *post hoc* test: $^{*}p < 0.05$/ for Post-EtOH group, One-way ANOVA, $F_{2,14} = 14.44$, $p = 0.0004$, Bonferroni's *post hoc* test: $^{*}p < 0.01$/ for CON *vs* Post-EtOH, Unpaired t-test, $t = 0.1098$, $p = 0.9140$). (p) Histogram summarizing these results. Cell numbers are indicated.

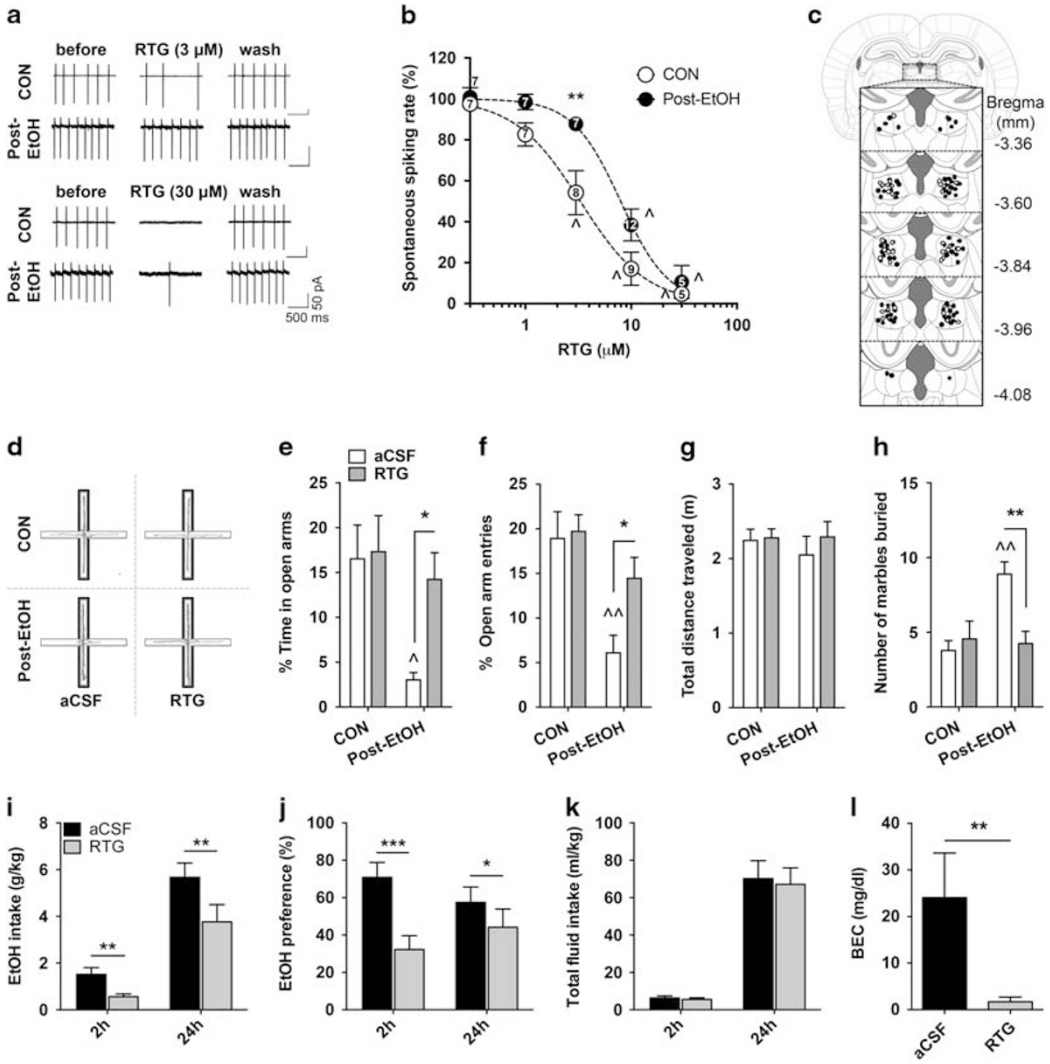


Figure 5 Intra-LHb retigabine reduces anxiety-like behaviors and voluntary ethanol intake. (a–b) Current traces recorded in cell-attached mode (a) and pooled data (b) show that retigabine (RTG)-induced reduction of spontaneous firing was blunted in LHb neurons of Post-EtOH rats compared with ethanol naive rats (CON). Two-way ANOVA, $F_{1,83} = 13.19$, $p = 0.0005$, Bonferroni's *post hoc* test: $^{**}p < 0.01$ between CON and Post-EtOH. $^{\wedge}p < 0.0001$ *vs* baseline. Numbers in the circles represent *n* values. (c) Schematic of injection sites in LHb: filled and blank circles are respectively locations for ethanol i.p. injected rats and rats undergoing the intermittent access 2-bottle free choice drinking (IA2BC) procedure. (d–h) LHb infusion of retigabine (RTG, 10 ng/side in 200 nl) rescues the anxiety-like behaviors of Post-EtOH rats in EPM test (d–g) and MBT (h). (d) Typical traces in EPM test. (e–g), Intra-LHb infusion of retigabine made Post-EtOH rats spent longer time in the open arms (for drug treatment $F_{1,37} = 5.216$, $p = 0.0282$, *post hoc* test: $^{\wedge}p < 0.05$ *vs* CON aCSF, $^{*}p < 0.05$ *vs* Post-EtOH aCSF) and entered the open arm more often (for drug treatment $F_{1,37} = 4.254$, $p = 0.0462$, *post hoc* test: $^{\wedge\wedge} < 0.01$ *vs* CON aCSF, $^{*}p < 0.05$ *vs* Post-EtOH aCSF), but does not significantly change the total distance traveled (for drug treatment, $F_{1,37} = 0.4211$, $p = 0.5204$), as revealed by two-way ANOVA followed Bonferroni's *post hoc* test. In the naive (CON) rats, intra-LHb retigabine did not show any significant difference in the time spent in open arms and numbers of open arm entries, and total distance traveled. All *post hoc* tests: $p > 0.05$. $N_{rat} = 10$(CON aCSF), 9(CON RTG), 11(Post-EtOH aCSF), 11(Post-EtOH RTG). (h) Intra-LHb retigabine also significantly reduced the number of marbles buried in 30-min by Post-EtOH rats. Retigabine did not significantly change the number of marbles buried by CON rats. Two-way ANOVA, for drug treatment $F_{1,31} = 4.58$, $p = 0.0403$, Bonferroni's *post hoc* test: $^{\wedge\wedge} < 0.01$ *vs* CON aCSF, $^{**} < 0.05$ *vs* Post-EtOH aCSF. $N_{rat} = 9$(CON aCSF), 9(CON RTG), 9(Post-EtOH aCSF), 8(Post-EtOH RTG). (i–k) Retigabine (10 ng/side in 200 nl, intra-LHb) decreased ethanol intake (Paired *t*-test, for 2 h $t = 4.547$, $p = 0.0014$, for 24 h, $t = 3.778$, $p = 0.0044$, i) and preference (Paired *t*-test, for 2 h $t = 5.930$, $p = 0.0002$, for 24 h $t = 2.484$, $p = 0.0348$, j) without altering total fluid intake at 2 and 24 h (Paired *t*-test, for 2 h $t = 0.8003$, $p = 0.4441$, for 24 h $t = 0.5367$, $p = 0.6045$, k) in rats trained to drink in the intermittent access 20% ethanol two-bottle choice (IA2BC) paradigm. (l) BECs (mg/dl) after 2 h of voluntary ethanol consumption in IA2BC procedure. Paired *t*-test: $t = 5.93$, $^{**}p < 0.01$. $N_{rat} = 10$/group.

a dose that changes ethanol intake in rats (McGuier *et al*, 2015), had significant anxiolytic effects on Post-EtOH rats: these animals spent a significantly longer time in, and entered more frequently into the open arms of the EPM, and reduced the numbers of marbles buried in the MBT, compared to the CON rats (Figure 5c–h).

Activation of LHb M-Channels Reduces Voluntary Alcohol Consumption and Anxiety-like Behaviors of Adult Rats

Having discovered that LHb M-channels play a critical role in anxiety-related behaviors after ethanol withdrawal, we hypothesized that this mechanism may contribute to the

drinking behaviors. We therefore trained rats to drink in the intermittent access 20% ethanol two-bottle free choice paradigm (IA2BC rats). In keeping with previous reports (Li *et al*, 2011b; Simms *et al*, 2008), we observed ethanol consumption escalated from 2.5 ± 0.5 g/kg/24 h in the first week to 6.1 ± 0.9 g/kg/24 h in the 8th week ($n = 12$, paired *t*-test, $t = 3.08$, $p = 0.015$). We conducted the following experiments on IA2BC rats that were drinking ethanol for 8–10 weeks when ethanol intake reached a high and stable level. In line with the observation on juvenile rats described above, and on adult rats (Li *et al*, 2016), the basal firing rate of LHb neurons in slices obtained at 24 h withdrawal from IA2BC rats was significantly higher than CON rats (CON: 4.4 ± 0.5 Hz, $n = 26$ *vs* CIEVD: 6.9 ± 0.5 Hz, $n = 25$, Unpaired *t*-test, $t = 3.539$, $p = 0.0009$). Moreover, XE991-induced acceleration of LHb firing was significantly weaker in slices obtained at 24 h withdrawal from IA2BC rats compared with CON rats (Supplementary Figure S5A–C).

At 24 h after withdrawal, these rats showed pronounced anxiety-like behaviors, which were significantly attenuated by LHb infusion of retigabine (Supplementary Figure S5D–G). Retigabine infusion also sharply reduced ethanol intake (paired *t*-test, $t = 4.547$, $p = 0.0014$) and preference (paired *t*-test, $t = 5.930$, $p = 0.0002$): this effect lasted for 24 h (paired *t*-test, $t = 3.778$, $p = 0.0044$ for EtOH intake and $t = 2.484$, $p = 0.0348$ for EtOH preference; Figure 5i–k), but disappeared by 48 h (not shown). Accordingly, retigabine significantly reduced the blood ethanol level (BEC) measured 2 h after the access to the ethanol bottles (Paired *t*-test, $t = 5.93$, $p = 0.0002$; Figure 5l). To determine whether the effect of retigabine was LHb specific, we infused the same amount of retigabine into the mediodorsal thalamic nuclei or the paraventricular nucleus of thalamus, which are adjacent to the habenula, and failed to detect a significant change in ethanol intake and in anxiety-like behaviors in the EPM (Supplementary Figure S6A–F). Moreover, intra-LHb infusions of retigabine did not significantly alter sucrose intake (Supplementary Figure S6G–H).

DISCUSSION

We report here evidence of apparent anxiety-like behaviors in rats at 24 h withdrawal from repeated systemic administration of alcohol. In addition, while the LHb neuronal excitability is increased, the M-current and the expression of the M-channel subunit KCNQ2/3 are reduced. Importantly, chemogenetic inhibition of LHb neurons or intra-LHb administration of the M-channel activator retigabine alleviates the anxiety-like behaviors and reduces alcohol intake. These findings suggest that M-channels in the LHb play a significant role in anxiety-related behaviors after ethanol withdrawal.

In this study, we first described apparent anxiety-like behaviors in rats withdrawn from *in vivo* systemic ethanol administration, as shown by the reduced time spent in the center of an open field, and in the open arms of a maze, as well as the increased number of buried marbles. Since the total distance traveled was not different, ethanol withdrawal did not noticeably affect locomotor activity. These anxiety-like behaviors were observed in rats 24 h after the end of systemic administration of ethanol (passive or voluntary), and in both juvenile and adult rats. Our data are thus consistent with previous reports of the anxiogenic effects of ethanol on rodents (Baldwin *et al*, 1991; Knapp *et al*, 2005). Notably, the MBT measures not only anxiety value (Njung'e and Handley, 1991; Zhao-Shea *et al*, 2015), but also obsessive compulsive disorder (OCD) (Albelda and Joel, 2012). As OCD commonly occurs alongside drug and alcohol addiction (De Ridder *et al*, 2016; Karg *et al*, 2012), our MBT data thus suggest an important role of the LHb in OCD.

Although the LHb has been linked with anxiety-related behaviors (Chan *et al*, 2016; Dolzani *et al*, 2016; Pobbe and Zangrossi, 2008; Shelton *et al*, 2016), its role in the context of ethanol withdrawal was unknown. We found that LHb neurons in ethanol-withdrawn (Post-EtOH) rats had a significantly higher spontaneous firing rate (Li *et al*, 2016) and excitability, suggesting a possible contribution of LHb hyperactivity to the increased anxiety levels. This possibility was supported by our data showing that selective inhibition of LHb neurons by chemogenetic or pharmacological approaches mitigated elevated anxiety-like phenotypes associated with ethanol withdrawal, and that selective activation of LHb neurons can induce anxiety-like phenotypes. Searching for the underlying cellular and molecular mechanisms, we found that the LHb neurons of Post-EtOH rats had a smaller mAHP, compared to CON rats, and that the M-channel blocker XE991 significantly increased the firing rate of LHb neurons in CON but not Post-EtOH rats, suggesting that M-channel dysregulation may contribute to the observed increased excitability of LHb neurons in Post-EtOH rats. This possibility was supported by the observation that in the Post-EtOH rats, both M-current and KCNQ2/3 expression was reduced, and that the M-channel activator retigabine reduced LHb neuron firing. These data support a link between LHb activity and anxiety-like behaviors.

Previous studies have associated M-channels with ethanol intake (Knapp *et al*, 2014; McGuier *et al*, 2015), and have proposed M-channels as a target of ethanol's actions on neuronal function. These studies showed that acute ethanol inhibits M-currents in human embryonic kidney cells expressing KCNQ2/3 (Cavaliere *et al*, 2012), in rat VTA dopamine neurons (Koyama *et al*, 2007), and in rat hippocampal pyramidal neurons (Moore *et al*, 1990); and chronic ethanol exposure downregulates KCNQ2 expression in synaptoneurosomes of mice (Most *et al*, 2015). In addition, M-channels regulate tolerance and memory impairments induced by acute ethanol in Drosophila (Cavaliere *et al*, 2012). Consistent with these findings, we showed that intra-LHb infused retigabine attenuates anxiety levels and reduces ethanol intake and preference. Our data of the anxiolytic effect of retigabine are consistent with previous reports (Hansen *et al*, 2008; Korsgaard *et al*, 2005). Thus, retigabine is a potential therapeutic option for alcoholics. This possibility is supported by recent reports that either systemic administration, or infusion of retigabine into the nucleus accumbens significantly reduces ethanol intake in rats (Knapp *et al*, 2014; McGuier *et al*, 2015). Notably, since retigabine has been approved by the US food and drug administration for epilepsy treatment, clinical trials could be started relatively quickly. However, caution must be exercised since M-channels have been associated with cognitive functions (Millichap and Cooper, 2012).

The observed reduction of M-channel protein expression during ethanol withdrawal may be caused by changes in the activity of transcriptional factors that suppress the corresponding gene expression. The transcription factor SP1 and transcription repressor REST (repressor element 1-silencing transcription factor) have been identified as common mechanisms of regulating KCNQ2 and KCNQ3 expression (Mucha *et al*, 2010; Rose *et al*, 2011). Indeed, the activity of the transcription factor that suppresses KCNQ2 expression is enhanced after ethanol exposure. Ethanol increases REST expression levels or enhances the REST binding activity to its binding site, RE-1, in an ethanol concentration-dependent manner (Cai *et al*, 2011; Ishii *et al*, 2008; Tateno *et al*, 2006). Transcriptional regulation via transcription repressors, including REST, has been suggested as a therapeutic option for anxiety and depression because REST activity may be a common mechanism underlying the pathophysiology of anxiety and depression (Albert and Fiori, 2014). This may also explain why enhanced LHb neuronal excitability contributes to depression (Li *et al*, 2011a, 2013). Given the role of LHb in depression-linked behaviors (Lecca *et al*, 2014; Li *et al*, 2013) and the connection between anxiety and depression (Pini *et al*, 1997), it will be interesting to investigate the role of LHb M-channel in depression.

Although we did not identify the cell type we recorded from in the current study, they most likely are glutamatergic since 95% of LHb neurons are glutamatergic (Meye *et al*, 2013; Suzuki *et al*, 2012; Weiss and Veh, 2011). As mentioned, the LHb consists of the lateral and the medial parts, each having different connectivity. Whereas the lateral part projects mainly to DA neurons in the VTA and substantial nigra, indirectly via the rostromedial tegmental nucleus (Aizawa *et al*, 2013; Proulx *et al*, 2014), the medial part projects mainly to the serotonergic system (dorsal and median raphe nuclei, DR and MR) (Proulx *et al*, 2014). The DR is the largest cluster of ascending serotonergic projections in the rat brain (Ferron *et al*, 1982), which participates in mediating anxiety- and depression-related behaviors (Dolzani *et al*, 2016; Graeff *et al*, 1996; Teissier *et al*, 2015). Conversely, the VTA also has been known as a pivotal area to modulate anxiety and reward motivation when animals are exposed to a known anxiety-causing environment (Tovote *et al*, 2015). The functional significance of the various sub-connections from the LHb during ethanol withdrawal remains unclear. Future studies thus are needed to clarify which circuit (the LHb-DR/MR or the LHb-VTA) is more heavily affected by ethanol exposure and withdrawal.

Interestingly, a previous rat study found increased alcohol consumption after LHb lesion (Haack *et al*, 2014), in contrast to the reduced alcohol intake following retigabine inhibition of the LHb observed in the current study. The mechanisms underlying the apparent disparity are unclear. However, the conditions of these two experiments are very different. One major difference is the time when retigabine or the lesion was applied. In Haack's study, the LHb was lesioned 1 week before the animal was trained to drink alcohol. LHb lesions increased the rate of escalation of intake, leading to higher consumption levels. By contrast, in our current study, retigabine was applied to the LHb of rats that have been drinking ethanol for two months, when the animals probably became dependent on alcohol (Fu *et al*, 2015; Li *et al*, 2011b). Our data of LHb inhibition leading to reduced drinking is in line with our recent finding that high frequency deep brain stimulation, which inhibits the LHb, leads to reduced ethanol drinking (Li *et al*, 2016). One limitation of the current study is that although our data support a link between increased LHb activity and anxiety-like behaviors, direct evidence for anxiety contributing to increased alcohol intake is still lacking.

In summary, we provide here several lines of new information about the role of LHb M-channels in the anxiogenic effect of ethanol withdrawal. The increased anxiety-like behaviors of ethanol-withdrawn rats were paralleled by increased excitability of LHb neurons, and reduced M-current and M-channel expression. Activation of M-channels attenuates both ethanol drinking and anxiety-like behaviors. These findings thus identify downregulation of M-channels in LHb as an anxiogenic mechanism, and suggest an important role of the M-channels in ethanol-induced neuronal adaptation. Thus, M-channels could be a promising therapeutic target for alcoholics.

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AUTHOR CONTRIBUTIONS

SK conceived and designed all molecular and behavioral experiments. SK, JL, and WZ performed stereotaxic surgery and behavioral experiments. SK performed electrophysiology. SK and JL performed western blotting. SK and RF performed immunofluorescence. SK and JY performed statistical analysis, prepared figures and wrote the manuscript. All authors contributed to reviewing and editing the manuscript.

REFERENCES

Aizawa H, Cui W, Tanaka K, Okamoto H (2013). Hyperactivation of the habenula as a link between depression and sleep disturbance. *Front Hum Neurosci* **7**: 826.

Albelda N, Joel D (2012). Current animal models of obsessive compulsive disorder: an update. *Neuroscience* **211**: 83–106.

Albert PR, Fiori LM (2014). Transcriptional dys-regulation in anxiety and major depression: 5-HT1A gene promoter architecture as a therapeutic opportunity. *Curr Pharm Des* **20**: 3738–3750.

Alexander GM, Rogan SC, Abbas AI, Armbruster BN, Pei Y, Allen JA *et al* (2009). Remote control of neuronal activity in transgenic mice expressing evolved G protein-coupled receptors. *Neuron* **63**: 27–39.

Armbruster BN, Li X, Pausch MH, Herlitze S, Roth BL (2007). Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. *Proc Natl Acad Sci USA* **104**: 5163–5168.

Baldwin HA, Rassnick S, Rivier J, Koob GF, Britton KT (1991). CRF antagonist reverses the 'anxiogenic' response to ethanol withdrawal in the rat. *Psychopharmacology* **103**: 227–232.

Baldwin PR, Alanis R, Salas R (2011). The role of the habenula in nicotine addiction. *J Addict Res Ther* **Suppl 1**: 002.

Cai L, Bian M, Liu M, Sheng Z, Suo H, Wang Z *et al* (2011). Ethanol-induced neurodegeneration in NRSF/REST neuronal conditional knockout mice. *Neuroscience* **181**: 196–205.

Carnicella S, Amamoto R, Ron D (2009). Excessive alcohol consumption is blocked by glial cell line-derived neurotrophic factor. *Alcohol* **43**: 35–43.

Castro PA, Cooper EC, Lowenstein DH, Baraban SC (2001). Hippocampal heterotopia lack functional Kv4.2 potassium channels in the methylazoxymethanol model of cortical malformations and epilepsy. *J Neurosci* **21**: 6626–6634.

Cavaliere S, Gillespie JM, Hodge JJ (2012). KCNQ channels show conserved ethanol block and function in ethanol behaviour. *PLoS ONE* **7**: e50279.

Chan J, Ni Y, Zhang P, Chen Y, Zhang J (2016). D1-like dopamine receptor dysfunction in the lateral habenula nucleus increased anxiety-like behavior in rat. *Neuroscience* **340**: 542–550.

De Ridder D, Leong SL, Manning P, Vanneste S, Glue P (2016). Anterior cingulate implant for obsessive-compulsive disorder. *World Neurosurg* **97**: 754.e7–754.e16.

Delmas P, Brown DA (2005). Pathways modulating neural KCNQ/M (Kv7) potassium channels. *Nat Rev Neurosci* **6**: 850–862.

Dolzani SD, Baratta MV, Amat J, Agster KL, Saddoris MP, Watkins LR *et al* (2016). Activation of a Habenulo-Raphe circuit is critical for the behavioral and neurochemical consequences of uncontrollable stress in the male rat. *eNeuro* **3**: ENEURO.0229-16.2016.

Driessen M, Meier S, Hill A, Wetterling T, Lange W, Junghanns K (2001). The course of anxiety, depression and drinking behaviours after completed detoxification in alcoholics with and without comorbid anxiety and depressive disorders. *Alcohol Alcohol* **36**: 249–255.

Ferron A, Descarries L, Reader TA (1982). Altered neuronal responsiveness to biogenic amines in rat cerebral cortex after serotonin denervation or depletion. *Brain Res* **231**: 93–108.

Fu R, Gregor D, Peng Z, Li J, Bekker A, Ye J (2015). Chronic intermittent voluntary alcohol drinking induces hyperalgesia in Sprague-Dawley rats. *Int J Physiol Pathophysiol Pharmacol* **7**: 136–144.

Gibula-Bruzda E, Marszalek-Grabska M, Witkowska E, Izdebski J, Kotlinska JH (2015). Enkephalin analog, cyclo[N(epsilon),N(beta)-carbonyl-D-Lys(2),Dap(5)] enkephalinamide (cUENK6), inhibits the ethanol withdrawal-induced anxiety-like behavior in rats. *Alcohol* **49**: 229–236.

Gilpin NW, Herman MA, Roberto M (2015). The central amygdala as an integrative hub for anxiety and alcohol use disorders. *Biol Psychiatry* **77**: 859–869.

Graeff FG, Guimaraes FS, De Andrade TG, Deakin JF (1996). Role of 5-HT in stress, anxiety, and depression. *Pharmacol Biochem Behav* **54**: 129–141.

Gu N, Vervaeke K, Hu H, Storm JF (2005). Kv7/KCNQ/M and HCN/h, but not KCa2/SK channels, contribute to the somatic medium after-hyperpolarization and excitability control in CA1 hippocampal pyramidal cells. *J Physiol* **566**(Pt 3): 689–715.

Haack AK, Sheth C, Schwager AL, Sinclair MS, Tandon S, Taha SA (2014). Lesions of the lateral habenula increase voluntary ethanol consumption and operant self-administration, block yohimbine-induced reinstatement of ethanol seeking, and attenuate ethanol-induced conditioned taste aversion. *PLoS ONE* **9**: e92701.

Halter JA, Carp JS, Wolpaw JR (1995). Operantly conditioned motoneuron plasticity: possible role of sodium channels. *J Neurophysiol* **73**: 867–871.

Hansen HH, Waroux O, Seutin V, Jentsch TJ, Aznar S, Mikkelsen JD (2008). Kv7 channels: interaction with dopaminergic and serotonergic neurotransmission in the CNS. *J Physiol* **586**: 1823–1832.

Hikosaka O (2010). The habenula: from stress evasion to value-based decision-making. *Nat Rev Neurosci* **11**: 503–513.

Ishii T, Hashimoto E, Ukai W, Tateno M, Yoshinaga T, Saito S *et al* (2008). Lithium-induced suppression of transcription repressor NRSF/REST: effects on the dysfunction of neuronal differentiation by ethanol. *Eur J Pharmacol* **593**: 36–43.

Kang S, Xu M, Cooper EC, Hoshi N (2014). Channel-anchored protein kinase CK2 and protein phosphatase 1 reciprocally regulate KCNQ2-containing M-channels via phosphorylation of calmodulin. *J Biol Chem* **289**: 11536–11544.

Karg RS, Bose J, Batts KR, Forman-Hoffman VL, Liao D, Hirsch E *et al* (2012). Past year mental disorders among adults in the United States: results from the 2008–2012 Mental Health Surveillance Study. In: *CBHSQ Data Review*, Rockville, MD, pp 1–19.

Klinger F, Gould G, Boehm S, Shapiro MS (2011). Distribution of M-channel subunits KCNQ2 and KCNQ3 in rat hippocampus. *Neuroimage* **58**: 761–769.

Knapp CM, O'Malley M, Datta S, Ciraulo DA (2014). The Kv7 potassium channel activator retigabine decreases alcohol consumption in rats. *Am J Drug Alcohol Abuse* **40**: 244–250.

Knapp DJ, Overstreet DH, Breese GR (2005). Modulation of ethanol withdrawal-induced anxiety-like behavior during later withdrawals by treatment of early withdrawals with benzodiazepine/gamma-aminobutyric acid ligands. *Alcohol Clin Exp Res* **29**: 553–563.

Korsgaard MP, Hartz BP, Brown WD, Ahring PK, Strobaek D, Mirza NR (2005). Anxiolytic effects of Maxipost (BMS-204352) and retigabine via activation of neuronal Kv7 channels. *J Pharmacol Exp Ther* **314**: 282–292.

Kourrich S, Calu DJ, Bonci A (2015). Intrinsic plasticity: an emerging player in addiction. *Nat Rev Neurosci* **16**: 173–184.

Koyama S, Brodie MS, Appel SB (2007). Ethanol inhibition of m-current and ethanol-induced direct excitation of ventral tegmental area dopamine neurons. *J Neurophysiol* **97**: 1977–1985.

Lecca S, Meye FJ, Mameli M (2014). The lateral habenula in addiction and depression: an anatomical, synaptic and behavioral overview. *Eur J Neurosci* **39**: 1170–1178.

Li B, Piriz J, Mirrione M, Chung C, Proulx CD, Schulz D *et al* (2011a). Synaptic potentiation onto habenula neurons in the learned helplessness model of depression. *Nature* **470**: 535–539.

Li J, Bian W, Dave V, Ye JH (2011b). Blockade of GABA(A) receptors in the paraventricular nucleus of the hypothalamus attenuates voluntary ethanol intake and activates the hypothalamic-pituitary-adrenocortical axis. *Addict Biol* **16**: 600–614.

Li J, Zuo W, Fu R, Xie G, Kaur A, Bekker A *et al* (2016). High frequency electrical stimulation of lateral habenula reduces voluntary ethanol consumption in rats. *Int J Neuropsychopharmacol* **19**: pyw050.

Li K, Zhou T, Liao L, Yang Z, Wong C, Henn F *et al* (2013). betaCaMKII in lateral habenula mediates core symptoms of depression. *Science* **341**: 1016–1020.

McBride WJ (2002). Central nucleus of the amygdala and the effects of alcohol and alcohol-drinking behavior in rodents. *Pharmacol Biochem Behav* **71**: 509–515.

McGuier NS, Griffin WC 3rd, Gass JT, Padula AE, Chesler EJ, Mulholland PJ (2015). Kv7 channels in the nucleus accumbens are altered by chronic drinking and are targets for reducing alcohol consumption. *Addict Biol* **21**: 1097–1112.

Meye FJ, Lecca S, Valentinova K, Mameli M (2013). Synaptic and cellular profile of neurons in the lateral habenula. *Front Hum Neurosci* **7**: 860.

Millichap JJ, Cooper EC (2012). KCNQ2 potassium channel epileptic encephalopathy syndrome: divorce of an electro-mechanical couple? *Epilepsy Curr* **12**: 150–152.

Misonou H (2010). Homeostatic regulation of neuronal excitability by K(+) channels in normal and diseased brains. *Neuroscientist* **16**: 51–64.

Moore SD, Madamba SG, Siggins GR (1990). Ethanol diminishes a voltage-dependent K+ current, the M-current, in CA1 hippocampal pyramidal neurons *in vitro*. *Brain Res* **516**: 222–228.

Most D, Ferguson L, Blednov Y, Mayfield RD, Harris RA (2015). The synaptoneurosome transcriptome: a model for profiling the emolecular effects of alcohol. *Pharmacogenomics J* **15**: 177–188.

Mucha M, Ooi L, Linley JE, Mordaka P, Dalle C, Robertson B *et al* (2010). Transcriptional control of KCNQ channel genes and the regulation of neuronal excitability. *J Neurosci* **30**: 13235–13245.

Njung'e K, Handley SL (1991). Evaluation of marble-burying behavior as a model of anxiety. *Pharmacol Biochem Behav* **38**: 63–67.

Pandey SC, Roy A, Zhang H (2003). The decreased phosphorylation of cyclic adenosine monophosphate (cAMP) response element binding (CREB) protein in the central amygdala acts as a molecular substrate for anxiety related to ethanol withdrawal in rats. *Alcohol Clin Exp Res* **27**: 396–409.

Pandey SC, Zhang H, Roy A, Misra K (2006). Central and medial amygdaloid brain-derived neurotrophic factor signaling plays a critical role in alcohol-drinking and anxiety-like behaviors. *J Neurosci* **26**: 8320–8331.

Pedarzani P, Kulik A, Muller M, Ballanyi K, Stocker M (2000). Molecular determinants of Ca2+-dependent K+ channel function in rat dorsal vagal neurones. *J Physiol* **527**(Pt 2): 283–290.

Pini S, Cassano GB, Simonini E, Savino M, Russo A, Montgomery SA (1997). Prevalence of anxiety disorders comorbidity in bipolar depression, unipolar depression and dysthymia. *J Affect Disord* **42**: 145–153.

Pobbe RL, Zangrossi H Jr (2008). Involvement of the lateral habenula in the regulation of generalized anxiety- and panic-related defensive responses in rats. *Life Sci* **82**: 1256–1261.

Proulx CD, Hikosaka O, Malinow R (2014). Reward processing by the lateral habenula in normal and depressive behaviors. *Nat Neurosci* **17**: 1146–1152.

Rose K, Ooi L, Dalle C, Robertson B, Wood IC, Gamper N (2011). Transcriptional repression of the M channel subunit Kv7.2 in chronic nerve injury. *Pain* **152**: 742–754.

Sena LM, Bueno C, Pobbe RL, Andrade TG, Zangrossi H Jr, Viana MB (2003). The dorsal raphe nucleus exerts opposed control on generalized anxiety and panic-related defensive responses in rats. *Behav Brain Res* **142**: 125–133.

Shah M, Mistry M, Marsh SJ, Brown DA, Delmas P (2002). Molecular correlates of the M-current in cultured rat hippocampal neurons. *J Physiol* **544**(Pt 1): 29–37.

Shelton K, Bogyo K, Schick T, Ettenberg A (2016). Pharmacological modulation of lateral habenular dopamine D2 receptors alters the anxiogenic response to cocaine in a runway model of drug self-administration. *Behav Brain Res* **310**: 42–50.

Simms JA, Steensland P, Medina B, Abernathy KE, Chandler LJ, Wise R *et al* (2008). Intermittent access to 20% ethanol induces high ethanol consumption in Long-Evans and Wistar rats. *Alcohol Clin Exp Res* **32**: 1816–1823.

Sinha R (2001). How does stress increase risk of drug abuse and relapse? *Psychopharmacology* **158**: 343–359.

Smith KS, Bucci DJ, Luikart BW, Mahler SV (2016). DREADDS: use and application in behavioral neuroscience. *Behav Neurosci* **130**: 137–155.

Spiacci A Jr, Coimbra NC, Zangrossi H Jr (2012). Differential involvement of dorsal raphe subnuclei in the regulation of anxiety- and panic-related defensive behaviors. *Neuroscience* **227**: 350–360.

Suzuki A, Ishida Y, Aizawa S, Sakata I, Tsutsui C, Mondal A *et al* (2012). Molecular identification of GHS-R and GPR38 in Suncus murinus. *Peptides* **36**: 29–38.

Tateno M, Ukai W, Hashimoto E, Ikeda H, Saito T (2006). Implication of increased NRSF/REST binding activity in the mechanism of ethanol inhibition of neuronal differentiation. *J Neural Transm* **113**: 283–293.

Teissier A, Chemiakine A, Inbar B, Bagchi S, Ray RS, Palmiter RD *et al* (2015). Activity of Raphe serotonergic neurons controls emotional behaviors. *Cell Rep* **13**: 1965–1976.

Tovote P, Fadok JP, Luthi A (2015). Neuronal circuits for fear and anxiety. *Nat Rev Neurosci* **16**: 317–331.

Trimmer JS (2015). Subcellular localization of K+ channels in mammalian brain neurons: remarkable precision in the midst of extraordinary complexity. *Neuron* **85**: 238–256.

Vervaeke K, Gu N, Agdestein C, Hu H, Storm JF (2006). Kv7/KCNQ/M-channels in rat glutamatergic hippocampal axons and their role in regulation of excitability and transmitter release. *J Physiol* **576**(Pt 1): 235–256.

Vielhaber S, Feistner H, Weis J, Kreuder J, Sailer M, Schroder JM *et al* (2004). Primary carnitine deficiency: adult onset lipid storage myopathy with a mild clinical course. *J Clin Neurosci* **11**: 919–924.

Wang HS, Pan Z, Shi W, Brown BS, Wymore RS, Cohen IS *et al* (1998). KCNQ2 and KCNQ3 potassium channel subunits: molecular correlates of the M-channel. *Science* **282**: 1890–1893.

Waxman SG, Zamponi GW (2014). Regulating excitability of peripheral afferents: emerging ion channel targets. *Nat Neurosci* **17**: 153–163.

Weiss T, Veh RW (2011). Morphological and electrophysiological characteristics of neurons within identified subnuclei of the lateral habenula in rat brain slices. *Neuroscience* **172**: 74–93.

Wise RA (1973). Voluntary ethanol intake in rats following exposure to ethanol on various schedules. *Psychopharmacologia* **29**: 203–210.

Wright S, Sternberg H, Bjornskov EK, Stephenson DT, Kushner PD (1990). Family of human neuronal external surface epitopes defined by Torpedo monoclonal antibodies. *J Neurosci Res* **25**: 486–502.

Zhang XF, Hu XT, White FJ (1998). Whole-cell plasticity in cocaine withdrawal: reduced sodium currents in nucleus accumbens neurons. *J Neurosci* **18**: 488–498.

Zhao H, Zhang BL, Yang SJ, Rusak B (2015). The role of lateral habenula-dorsal raphe nucleus circuits in higher brain functions and psychiatric illness. *Behav Brain Res* **277**: 89–98.

Zhao-Shea R, DeGroot SR, Liu L, Vallaster M, Pang X, Su Q *et al* (2015). Increased CRF signalling in a ventral tegmental area-interpeduncular nucleus-medial habenula circuit induces anxiety during nicotine withdrawal. *Nat Commun* **6**: 6770.

Zuo W, Fu R, Hopf FW, Xie G, Krnjevic K, Li J *et al* (2015). Ethanol drives aversive conditioning through dopamine 1 receptor and glutamate receptor-mediated activation of lateral habenula neurons. *Addict Biol* **22**: 103–116.

Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)