Overexpression of Hyperpolarization-Activated Cyclic Nucleotide-Gated Channels into the Ventral Tegmental Area Increases the Rewarding Effects of Ethanol in UChB Drinking Rats

Mario Rivera-Meza, María Elena Quintanilla, Diego Bustamante, Ricardo Delgado, Marianne Buscaglia, and Mario Herrera-Marschitz

Background: A number of studies have shown that ethanol (EtOH) activates dopamine neurocircuitries and is self-administered into the ventral tegmental area (VTA) of the rat brain. In vitro and in silico studies have showed that hyperpolarization-activated cyclic nucleotide-gated (HCN) ionic channels on VTA dopamine neurons may constitute a molecular target of EtOH; however, there is no in vivo evidence supporting this assumption.

Methods: Wistar-derived University of Chile Drinking (UChB) rats were microinjected into the VTA with a lentiviral vector coding for rat HCN-2 ionic channel or a control vector. Four days after vector administration, daily voluntary EtOH intake was assessed for 30 days under a free-access paradigm to 5% EtOH and water. After EtOH consumption studies, the effect of HCN-2 overexpression was also assessed on EtOH-induced conditioned place preference (CPP); EtOH-induced locomotion, and EtOH-induced dopamine release in the nucleus accumbens (NAcc).

Results: Rats microinjected with the HCN-2 coding vector into the VTA showed (i) a ~2-fold increase in their voluntary EtOH intake compared to control animals, (ii) lentiviral-HCN-2-treated animals also showed an increased CPP to EtOH (~3-fold), (iii) a significant higher locomotor activity (~2-fold), and (iv) increased dopamine release in NAcc upon systemic administration of EtOH (~2-fold).

Conclusions: Overexpression of HCN-2 ionic channel in the VTA of rats results in an increase in voluntary EtOH intake, EtOH-induced CPP, locomotor activity, and dopamine release in NAcc, suggesting that HCN levels in the VTA are relevant for the rewarding properties of EtOH.

Key Words: Ethanol, HCN Channels, UChB Rats, VTA, Dopamine.

THE VENTRAL TEGMENTAL area (VTA) of the rat brain contains dopaminergic neurons projecting axons to the ipsilateral nucleus accumbens (NAcc), amygdala, and prefrontal cortex (Ungerstedt, 1971). It is well established that ethanol (EtOH) and other drugs of abuse, which produce reward and dependence (e.g., cocaine, amphetamine, nicotine), activate this system increasing dopamine release in mesolimbic regions (see Zocchi et al., 2003). Studies performed in rats have showed that upon systemic administration of EtOH, there is an increase in dopamine release in

DOI: 10.1111/acer.12344

the NAcc (Imperato and Di Chiara, 1986; Quintanilla et al., 2007). Recent studies have also shown that rats genetically selected for EtOH intake self-administer EtOH or its metabolites via a guide cannula implanted into the VTA (Rodd et al., 2004, 2005), demonstrating the relevance of the activation of the mesolimbic system in the rewarding effects of EtOH.

Dopamine neurons are known to show spontaneous firing, characterized by a low-frequency discharge, which is independent of driving synaptic inputs (see Grace and Bunney, 1983; Overton and Clark, 1997). This autonomous activity is supported by an inward cation current, denominated I_h, which is activated by membrane hyperpolarization (Neuhoff et al., 2002; Seutin et al., 2001). This cationic depolarizing current, originally described in cardiac pacemaker cells (Brown and Difrancesco, 1980), sets the membrane potential to more positive voltages, near the threshold of activation of T-calcium channels, leading to a sustained firing of the neurons (see Biel et al., 2009).

The ion channels underlying I_h current were discovered about a decade ago (Ludwig et al., 1998) and were termed hyperpolarization-activated cyclic nucleotide-gated (HCN) ionic channels due to their dual mode of gating. HCN channels are characterized by 3 major properties: (i) channel acti-

vation by membrane hyperpolarization potentials, (ii) facilitation of channel activation by the binding of intracellular cAMP to a cyclic nucleotide-binding domain (CNBD), and (iii) unspecific inward permeation to Na^+ and K^+ (Altomare et al., 2001). HCN channels are tetramers (homo or heterotetramers) whose subunits are encoded by 4 genes (HCN1-4) that are widely expressed in the heart and the central nervous system (CNS). Studies performed in rat brains have showed that the 4 subunits of HCN channels are expressed with different distribution patterns (Monteggia et al., 2000; Notomi and Shigemoto, 2004). While both the HCN2 and HCN1 subunits are expressed in NAcc, the HCN2 subunit is the most expressed subunit in the VTA (Notomi and Shigemoto, 2004).

There is experimental evidence supporting the hypothesis that HCN channels on dopamine neurons constitute a molecular target for EtOH. Using mice VTA brain slices, Brodie and Appel (1998) showed that EtOH increased the firing rate of VTA dopamine neurons and also changed the shape of the spontaneous action potential, in agreement with an enhanced I_h current. Recent in vitro studies have further showed that EtOH increases I_h current and the firing rate of dopamine neurons, effects blocked by HCN antagonists, such as ZD7288 or cesium chloride (McDaid et al., 2008; Okamoto et al., 2006). Although performed on cardiac cells, a recent in vitro electrophysiological study showed that pharmacological concentrations (~20 μM) of EtOH elicited a reversible activation of human HCN channels and that the activating effect of EtOH was markedly attenuated by the HCN blocker ivabradine (Chen et al., 2012).

A computational modeling study of dopamine neurons in vivo suggests that EtOH, via HCN channels, changes the firing patterns from a basal pacemaker to a bursting pattern, increasing dopamine release in terminal areas (Migliore et al., 2008). Mice showing high EtOH-induced locomotor activity also showed an increased density of HCN channels on dopamine neurons compared to mice selected for low locomotor response to EtOH, indicating a possible role of HCN channels on the stimulant effects of EtOH (Beckstead and Phillips, 2009).

Migliore and colleagues (2008) proposed that the effect of EtOH on HCN ionic channels represents an important cellular mechanism mediating its pharmacological effects. However, there is no in vivo evidence supporting this assumption. In the present study, we tested the hypothesis that overexpression of HCN ionic channels into the VTA increases the rewarding and stimulating properties of EtOH in rats. Using a lentiviral vector coding the rat HCN2 cDNA, the HCN2 channel subunit was overexpressed in the VTA of alcohol-preferring rats for studying its effect on the rewarding and stimulant properties of EtOH, measuring (i) voluntary EtOH intake, (ii) EtOH-induced conditioned place preference (CPP), (iii) EtOH-induced locomotion, and (iv) EtOH-induced NAcc dopamine release.

MATERIALS AND METHODS

Cloning of the Rat HCN Channel Subunit 2

The Rat HCN Channel Subunit 2 (rHCN2) cDNA (GenBank NM_053684.1), including 5′ *Eco* RI and 3′ *Bam* HI restriction sites, was ordered to Genscript Corp. (Piscataway, NJ). The rHCN2 cDNA was cloned downstream the CMV promoter in the *Eco* RI and *Bam* HI sites of the pAAV-MCS plasmid (Stratagene, Cedar Creek, TX) and named pAAV-rHCN2.

In Vitro Expression of rHCN2

Human embryonic kidney (HEK-293T) cells were used to express the rHCN2 cDNA. HEK-293T cells (ATCC CRL-11268) were obtained from American Type Cell Collection (Manassas, VA) and grown in Dulbecco's modified Eagle medium containing 4.5 mg/ml of glucose, 2 mg/ml of sodium bicarbonate, 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin. The culture medium was supplemented with 10% fetal bovine serum. HEK-293T cells were plated on 6-well plates at 1×10^6 cells/well and transfected with a mixture of 7.2 μg of polyethylenimine and 2 μg of either pAAV-rHCN2 or control plasmid pAAV-GFP. Forty-eight hours after transfection, the cells were analyzed by Western blots and electrophysiology.

Western Blot Analysis

Transfected cells or VTA samples were homogenized in 300 μl of RIPA buffer (150 mM NaCl, 1% Tritón X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) by 2 cycles of sonication. Protein concentration was determined using the Pierce BCA Protein Assay (Thermo Scientific, Rockford, IL). Samples (20 to 40 μg of protein) were subjected to electrophoresis in denaturing 9% polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane and subjected to immunoblot analysis with mouse monoclonal antibodies against HCN2 (N71/37; UC Davis/NIH NeuroMab Facility, Davis CA) or beta-actin (Sigma, St. Louis, MO). Bands were detected by chemoluminescence generated by horseradish peroxidase coupled to a goat anti-mouse secondary antibody (sc-2005; Santa Cruz Biotechnology, Santa Cruz, CA) and the SuperSignal West Pico Chemiluminescent Substrate from Thermo Scientific.

Electrophysiological Studies

After 48 hours of transfection with pAAV-rHCN2, HEK-293T cells were detached with trypsin and resuspended in phosphate-buffered saline (PBS). The cells were visualized in a NIKON (Tokyo, Japan) inverted microscope equipped with phase contrast and Hofmann optics. Currents were recorded at room temperature through whole-cell patch clamp technique performed with 4 MΩ resistance electrodes equipped with an Axon 1D Amplifier (Molecular Devices Corp., Sunnyvale, CA). The internal solution contained (mM) KCl 120, CaCl$_2$ 1, EGTA 2, Hepes 4, NaCl 5, ATP 2, and the external solution contained (mM) NaCl 72.5, KCl 61.3, Na$_2$HPO$_4$ 4, NaH$_2$PO$_4$ 0.7, CaCl$_2$ 0.5, HEPES 2, ATP 1. The rHCN2 subunit current was activated by a 2-second hyperpolarizing pulse to -120 mV from a holding potential of -60 mV. Data were analyzed by a customized program pCLAMP 8.2 (Molecular Devices).

Generation of Lentiviral Vectors

The rHCN2 cDNA was cloned downstream of the EF1α promoter in the Xba*I* and BamH*I* sites of the lentiviral plasmid pHIV-GFP (Addgene plasmid 21373; Cambridge, MA) enabling bicistronic expression with a GFP reporter (Welm et al., 2008). This

plasmid was named pHIV-rHCN2-GFP. Lentiviral vectors were generated in HEK-293T cells by co-transfection with pHIV-rHCN2-GFP and packaging plasmids pΔ8.9 and pVSV-G. Control lentiviral vectors were generated using pHIV-GFP and the same packaging vectors. After 48 hours of transfection, viruses were concentrated from cell culture supernatant with 5% polyethylene glycol (PEG-8000; Sigma). After centrifugation to precipitate viral particles, they were resuspended in PBS. Titer of lentiviruses was estimated in HEK-293T cells by measuring of GFP expression using flow cytometry and expressed as transfer units per mL (TU/mL).

Animals

Wistar-derived rats of the University of Chile Drinking (UChB) lineage were used in the in vivo experiments. This line has been bred selectively for high alcohol preference over 80 generations (Mardones and Segovia-Riquelme, 1983; Quintanilla et al., 2006). Eighteen alcohol-naïve female UChB rats weighing between 200 and 250 g (~18 weeks old) were housed in individual cages in a temperature- and humidity-controlled room under a 12-hour light-dark cycle, with free access to water and food. Experimental protocols were approved by the Institutional Animal Experimentation Ethics Board (CBA#0375 FMUCH).

Intracerebral Administration of Lentiviral Vectors and Voluntary EtOH Intake

Animals were anesthetized with a mixture of air and isoflurane and placed in a stereotaxic frame according to the atlas of Paxinos and Watson (1986). The brain was exposed, and a 2-μL Hamilton syringe filled with lentiviral vectors (LV-rHCN2-GFP or control LV-GFP) was inserted into the left VTA (coordinates: B-5.6; L-0.5; V-7.4, from Bregma and dura, respectively), and 2 μl of the corresponding vector (7.5 $\times$ 10^4 TU/μL) was infused at 0.2 μl/min. After recovering, animals were transferred to individual cages at the animal station, with access to water and food ad libitum. Four days after surgery, the rats were exposed to 24 hours a day drinking paradigm, consisting of free availability to 2 bottles, 1 containing 5% (v/v) EtOH and the other containing water during 30 days. To amplify the effect of overexpressing HCN2 levels into the VTA on the EtOH intake, the spontaneous EtOH consumption of the animals was lowered by reducing the concentration of EtOH offered to 5%, rather than 10% standard concentration (Karahanian et al., 2011). EtOH consumption was determined on a daily basis and expressed as g EtOH/kg body weight/d. Following drinking determinations, the same group of rats was used for testing EtOH-induced (i) CPP, (ii) locomotor activity, and (iii) in vivo microdialysis.

Conditioned Place Preference

Four days after the voluntary EtOH consumption paradigm (38 days after lentiviral administration), an EtOH-induced CPP test was performed in the same group of rats. The CPP apparatus consisted of a rectangular box with 2-end compartments (one, black walls-smooth floor; and the other, white walls-gridded floor) and a middle passageway (gray walls-smooth floor). Removable guillotine doors separated the middle area from the end compartments. A first preconditioning session was conducted for assessing unconditioned chamber preference. The rats were placed in the passageway leading to the conditioning chambers for 15 min. The time each rat spent in each chamber was recorded and the non-preferred side of the apparatus for each individual animal was determined. During the conditioning phase, guillotine doors were closed to confine animals in 1 side of the apparatus. During 10 days, rats were administered with EtOH (0.5 g/kg, intraperitoneally ([i.p.]) and 10 minutes later placed in the less preferred compartment for 15 minutes. On alternate days, the rats were administered saline and placed in the preferred compartment. The postconditioning phase started 24 hours after the last conditioning trial. The postconditioning phase consisted of a 15-minute choice test with no injection, while the rats freely moved through the passageway between the 2 chambers. The time spent by each rat in each compartment was recorded during 15-minute trials. Data are expressed as percentage of total time spent in the EtOH-paired chamber.

Locomotor Activity

Four days after concluding EtOH-induced CPP studies (54 days after lentiviral administration), an EtOH-induced locomotor activity test was performed in the same group of rats. The open-field apparatus consisted of a chamber (38 $\times$ 38 $\times$ 38 cm) painted black and floor marked with lines each 9.5 cm forming a 4 $\times$ 4 grid. Animals received 3 daily 15-minute habituation sessions, prior to drug tests. In the first session, rats treated with the lentivectors LV-rHCN2-GFP or LV-GFP were administered a dose of 0.5 g EtOH/kg (i.p.), and 10 minutes later, each rat was individually placed in the center of the open field for recording horizontal locomotion for 15 minutes. An activity unit (AU) represents the complete crossing from one square to another. Twenty-four hours later, a second session was performed, but using saline as a treatment.

In Vivo Microdialysis

Approximately 2 months after intracerebral administration of lentiviral vectors, the animals were anesthetized (air and isoflurane mixture) and placed in a stereotaxic frame with the skull oriented according to the atlas of Paxinos and Watson (1986). Rats were implanted with a microdialysis probe (dialysis length, 2 mm; diameter, 0.6 mm; cutoff, 6kD; model 9.14.2; AgnTho's AB, Lidingö, Sweden) into the left NAcc (A+1.7, L−0.7, V−8.2). Microdialysis probes were connected to a perfusion CMA/100 pump (CMA/Microdialysis AB, Stockholm, Sweden) and perfused with Ringer solution (pH ~ 7) at a rate of 2 μl/min. Samples (60 μl) were collected every 30 minutes and assayed for dopamine by high-performance liquid chromatography coupled to electrochemical detection as previously described (Bustamante et al., 2002). The identification and quantification of substances was achieved by comparison with standard solutions prepared similarly to samples. Peak integration was performed with an ad-hoc analogous–digital card and CSW software (Pronexus, Stockholm, Sweden). Immediately after baseline sampling (four 30-minute samples), rats were treated with 20% (v/v) EtOH (in saline), to give a dose of 1.0 g of EtOH/kg body weight (i.p.). Four additional 30-minute samples were collected and analyzed for dopamine as described above.

Statistical Analysis

Data are expressed as means $\pm$ SE. Statistical differences are analyzed by Student's t-test or 2-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. A level of $p < 0.05$ is considered for statistical significance.

RESULTS

Cloning of Rat HCN2 cDNA, Generation of Lentiviral Vectors, and Stereotaxic Administration

The functionality of the rHCN2 cDNA was studied in HEK-293T cells, because this cell line does not express the

HCN2 gene (Zhang et al., 2009). HEK-293T cells were transfected with the plasmid vectors pAAV-rHCN2 or pAAV-GFP as control of transfection. The Western blot analysis of HEK-293T cells transfected with pAAV-rHCN2 and pAAV-GFP showed that HCN2 protein expression was only detected in cells transfected with pAAV-rHCN2 (Fig. 1*A*). The apparent molecular weight of the protein codified by the rHCN2 cDNA was of approximately 100 KDa; in agreement with previous reports about the size of the rat HCN2 protein. The capacity of the protein encoded by the rHCN2 cDNA to form a functional ionic channel was studied in mammalian cells. Thus, transfected HEK-293T cells were analyzed by whole-cell patch clamp technique. It was found that pAAV-rHCN2 transfected cells generated an inward current in response to a hyperpolarizing pulse applied to the cell (Fig. 1*B*). Control mock transfected cells show a small leak current upon application of a hyperpolarizing pulse.

Once confirmed the functionality of the cDNA for rat HCN2, we generated 2 lentiviral vectors: (i) a vector coding for rat HCN2 and a GFP reporter (LV-rHCN2-GFP), and (ii) a control vector coding for the GFP reporter only (LV-GFP) (Fig. 1*C*). The correct expression of the vectors was confirmed by infecting HEK-293T cells. Using immunofluorescence, it was found that the rHCN2 channel was expressed, showing a membrane-associated signal distribution, co-localizing with the GFP reporter (Fig. 1*C*). These results indicate that the LV-rHCN2-GFP vector was correctly assembled and expressed efficiently both genes.

We injected the active LV-rHCN2-GFP or control LV-GFP lentivectors into the posterior left VTA of alcohol-preferring UChB (naive to EtOH consumption) rats by stereotaxic surgery. To confirm whether lentiviral vectors effectively transduced and expressed the encoded genes into the VTA, a Western blot analysis of HCN2 levels in the VTA of treated animals was performed at approximately 8 weeks after vector administration (Fig. 1*D*, insert). The quantification of the immunoblot analysis (Fig. 1*D*) showed that HCN2 levels in the VTA of animals transduced with LV-rHCN2-GFP were ~2-fold higher than those in animals that received the LV-GFP control vector (1.90 ± 0.49 vs. 0.97 ± 0.23 arbitrary units; t-test: $t = 1.86$, df = 12, $p < 0.05$).

Effect of VTA rHCN2 Overexpression on Voluntary EtOH Intake

Figure 2*A* shows that animals receiving a single intracerebral administration of LV-rHCN2-GFP significantly increased their voluntary EtOH intake, 2-way ANOVA; $F(1, 59) = 103.8$, $p < 0.001$, compared to rats treated with the LV-GFP control lentivector. Figure 2*B* shows the average EtOH consumption during the 30-day period of EtOH access. The mean voluntary EtOH intake by the rats treated with LV-rHCN2-GFP was ~2-fold higher compared to the control group treated with LV-GFP (3.29 ± 0.15 vs.

2.01 ± 0.11 g   EtOH/kg/d; t-test: $t = 6.7$,   df = 58, $p < 0.001$).

Effect of VTA rHCN2 Overexpression on EtOH-Induced CPP

Figure 3 shows that rats treated with LV-rHCN2-GFP or LV-GFP lentivectors did not present any significant differences in the time spent in the less preferred compartment before conditioning (21.28 ± 4.08 vs. $18.98 \pm 3.56\%$ of time; t-test: $t = -2.3$, df = 16, $p = 0.34$, white bars). On alternate days, animals received 5 conditioning sessions on which EtOH (0.5 g/kg, i.p.) was administered and placed in the less preferred compartment for 15 minutes. The postconditioning session showed that rats treated with the LV-rHCN2-GFP vector developed a marked CPP, expressed as a ~3-fold increase in the time spent in the alcohol-paired compartment compared to the preconditioning value (53.83 ± 7.84 vs. $18.98 \pm 3.56\%$ of time; t-test: $t = -4.05$, df = 16, $p < 0.001$). The postconditioning time spent in the EtOH-paired compartment by control rats (LV-GFP) showed a slight but nonsignificant CPP increase (32.20 ± 6.06 vs. $21.28 \pm 4.08\%$ of time; t-test: $t = -1.49$, df = 16, $p = 0.07$).

Effect of VTA rHCN2 Overexpression on EtOH-Induced Locomotor Activity

Figure 4 shows the EtOH—(0.5 g/kg, i.p.) or saline-induced locomotor activity (3 successive 5-minute intervals after administration of EtOH or saline) of rats treated with LV-rHCN2-GFP or LV-GFP lentivectors. Figure 4*A* shows that rats treated with the control vector LV-GFP did not show any change in locomotor activity after EtOH administration compared to saline (2-way ANOVA; drug treatment effect: N.S.). Figure 4*B* shows that rats treated with LV-rHCN2-GFP vector and a dose of EtOH displayed an overall significant increase in locomotor activity compared to that produced by saline administration, 2-way ANOVA; drug treatment effect: $F(1, 53) = 8.007$, $p < 0.01$. A post hoc Bonferroni's test showed a significant difference only in the first 5-minute interval (47.9 ± 5.1 vs. 31.0 ± 3.9 AU; $p < 0.001$). No significant differences in locomotor activity were observed in the subsequent second and third 5-minute intervals. A 2-way ANOVA analysis (viral treatment × time interval) of the EtOH-induced locomotor activity data showed a significant overall increase in the animals treated with LV-rHCN2-GFP lentivector versus the control animals treated with LV-GFP vector, 2-way ANOVA; viral treatment effect: $F(1, 53) = 4.245$, $p < 0.05$.

Effect of VTA rHCN2 Overexpression on EtOH-Induced Dopamine Release in NAcc

The marked increase in the rewarding and stimulating effects of EtOH showed by the animals treated with
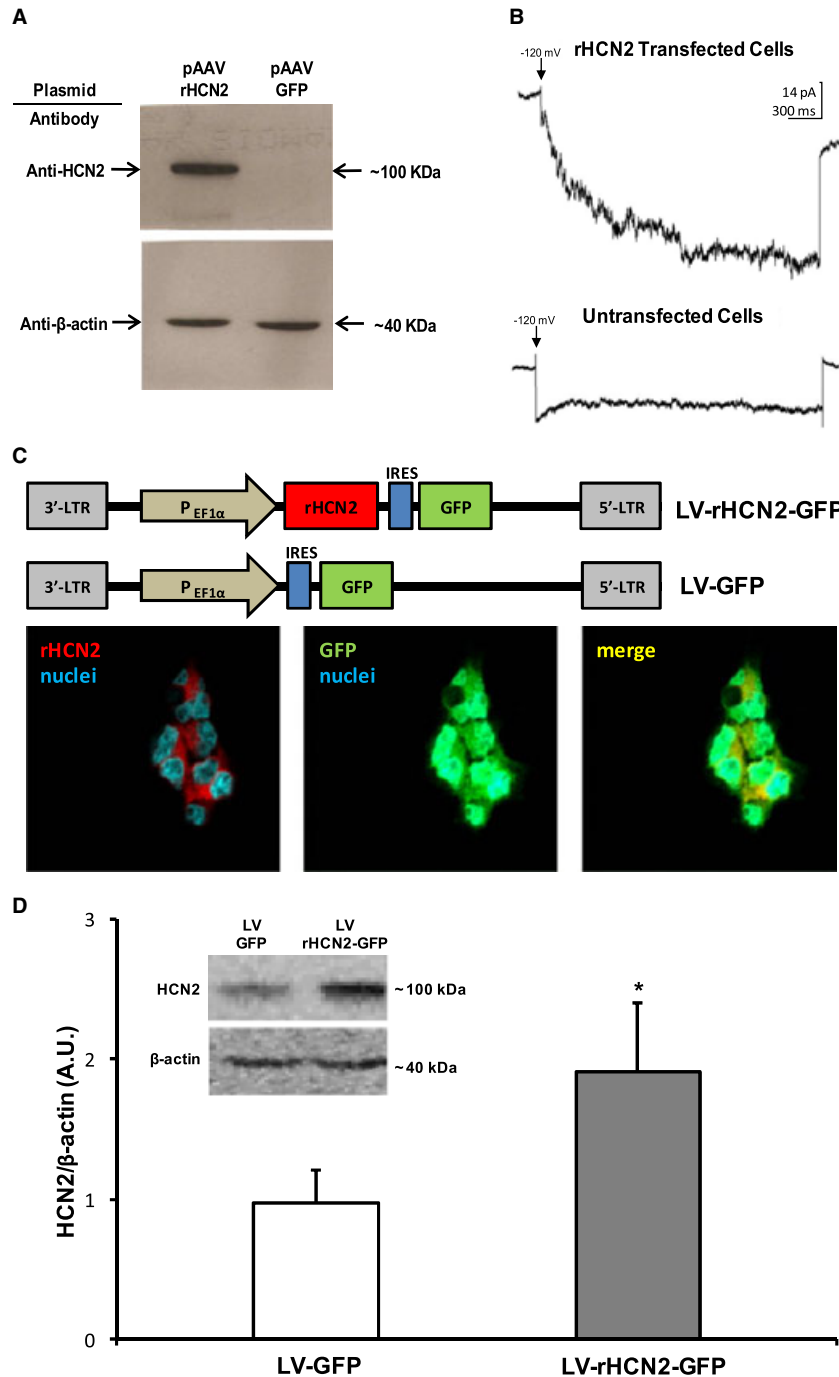
Fig. 1. In vitro expression of rat HCN2 cDNA, generation of lentiviral vectors, and stereotaxic administration into the VTA of UChB rats. (**A**) Western blot analysis of HCN2 subunit in HEK-293T cells transfected with the plasmids coding for rat HCN2 (pAAV-rHCN2) or a corresponding control (pAAV-GFP). (**B**) Whole-cell patch clamp analysis of HEK-293T cells transfected with pAAV-rHCN2 plasmid. Currents were elicited by applying a 2-second hyperpolarizing pulse to the cell. Control untransfected cells show only a minor leak current upon application of a hyperpolarizing pulse. (**C**) Diagrammatic representation of the lentiviral vectors used in the study. In the LV-rHCN2-GFP vector, the cDNA of the rat HCN2 subunit (rHCN2) and the GFP reporter were incorporated in a bicistronic expression cassette controlled by the ubiquitous promoter of the elongation factor 1α (P$_{EF1\alpha}$). The control vector LV-GFP codifies only for the GFP reporter. 3'-LTR, 5'-LTR. The correct expression of these vectors was confirmed by infection of HEK-293T cells. After 72 hours, the infected cells were analyzed by fluorescence immunohistochemistry. Microphotographs show a group of cells transduced with the LV-rHCN2-GFP vector, labeling the channel (red), GFP (green), and the nucleus (DAPI; blue). (**D**) Western blot analysis of HCN2 subunit levels in the VTA of rats stereotaxically injected with 1.5 $\times$ 10^5 TU/2 μL of either LV-rHCN2-GFP ($n = 6$) or LV-GFP ($n = 7$) lentiviral vectors. Bars represent means $\pm$ SEM of relative expression (HCN2/β-actin); *$p < 0.05$ for LV-rHCN2-GFP vs. LV-GFP group; Student's t-test. HCN2, hyperpolarization-activated cyclic nucleotide-gated 2; IRES, internal ribosome entry site; LTR, long terminal repeats; UChB, University of Chile Drinking; VTA, ventral tegmental area.
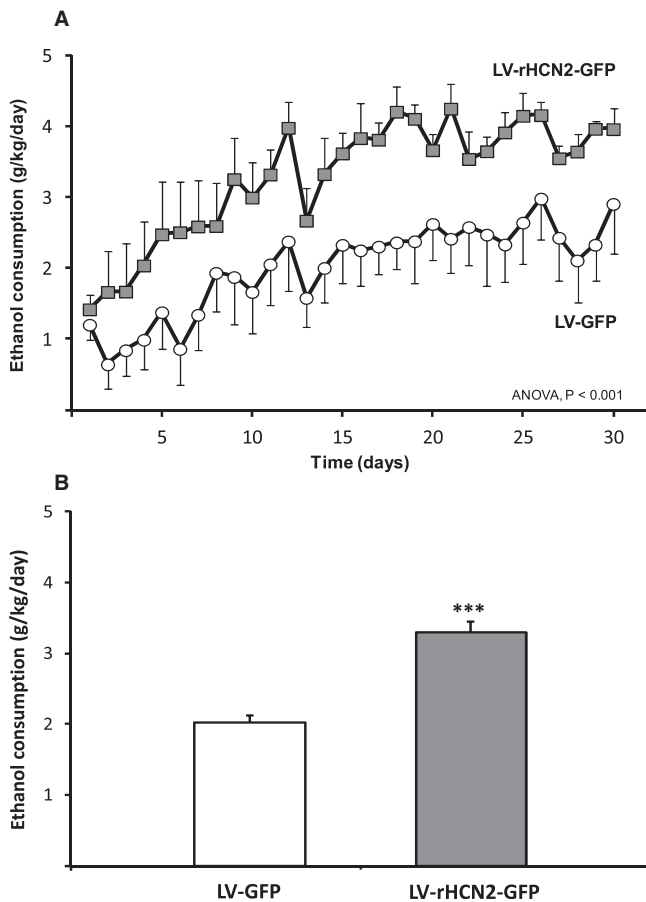
A

B

Fig. 2. HCN2 overexpression increases voluntary ethanol (EtOH) intake in UChB rats. Eighteen alcohol-naïve rats from the drinking UChB line were administered into the VTA with 1.5×10^5 TU of either LV-rHCN2-GFP or LV-GFP lentiviral vectors. Ninety-six hours after the administration of the vectors (Day 1), the rats were allowed to free access of 5% EtOH and water for 30 days. (**A**) Data represent daily EtOH intake (g/kg/d) (means $\pm$ SE; $n = 9$ for each group). Rats treated with LV-rHCN2-GFP vector significantly increased their voluntary EtOH intake compared to control rats treated with LV-GFP, 2-way ANOVA; $F(1, 59) = 103.8$, $p < 0.001$. (**B**) Data represent the mean EtOH consumption during the 30-day period of EtOH access. The voluntary EtOH intake by the rats treated with LV-rHCN2-GFP was ~2-fold higher compared to the control group treated with LV-GFP (t-test: $t = 6.7$, df = 58, ***$p < 0.001$). HCN2, hyperpolarization-activated cyclic nucleotide-gated 2; UChB, University of Chile Drinking; VTA, ventral tegmental area.

LV-rHCN2-GFP vector prompted us to determine whether such effect had a parallel increase in NAcc dopamine release induced by systemic EtOH administration. Figure 5 shows the extracellular levels of dopamine in NAcc (% of basal levels) elicited by the administration of EtOH (1 g/kg, i.p.) in rats treated with LV-rHCN2-GFP or LV-GFP lentivectors. The results showed that animals treated with the LV-GFP control vector did not show a significant increase in dopamine levels in the NAcc after EtOH administration compared to the basal condition. However, the acute administration of EtOH to animals treated with LV-rHCN2-GFP elicited a significant increase in the relative dopamine release
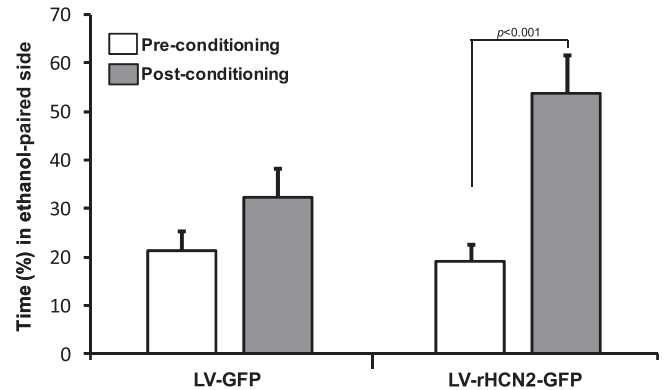

Fig. 3. HCN2 overexpression increases the development of ethanol (EtOH)-induced conditioned place preference in UChB rats. Rats treated with the LV-rHCN2-GFP ($n = 9$) or LV-GFP ($n = 9$) lentiviral vectors did not present significant differences in the time spent in the less preferred compartment before conditioning (white bars; 21.28 ± 4.08 vs. $18.98 \pm 3.56\%$ of time; t-test: $t = -2.3$, df = 16, $p = 0.34$). After conditioning with EtOH (5 daily doses on alternate days; 0.5 g/kg, i.p.), rats treated with LV-rHCN2-GFP vector developed a marked CPP to EtOH, expressed as a ~3-fold increase in the time spent in the alcohol-paired box (gray bar) compared to preconditioning value (white bar) (53.83 ± 7.84 vs. $18.98 \pm 3.56\%$ of time; t-test: $t = -4.05$, df = 16, $p < 0.001$). Control rats treated with LV-GFP vector showed a slight but nonsignificant CPP, 32.20 ± 6.06 (gray bar) versus $21.28 \pm 4.08\%$ of time (white bar); t-test: $t = -1.49$, df = 16, $p = 0.07$. CPP, conditioned place preference; HCN2, hyperpolarization-activated cyclic nucleotide-gated 2; UChB, University of Chile Drinking.

compared to that produced in control animals, 2-way ANOVA; viral vector treatment effect: $F(1, 71) = 21.09$, $p < 0.001$. A post hoc Bonferroni's test of the obtained data showed a significant difference between both experimental groups at 60 minutes (179.5 ± 36.7 vs. $84.9 \pm 6.7\%$ of basal; $p < 0.001$) and 90 minutes (190.3 ± 33.9 vs. 104.3 ± 12.9) after EtOH administration.

DISCUSSION

The present study tests the hypothesis that overexpression of the HCN ionic channels into the VTA of alcohol-preferring UChB rats results in an increase in the rewarding and stimulating properties of EtOH. Using lentiviral vectors, we overexpressed the HCN2 subunit into the VTA of UChB rats, resulting in an increase in voluntary EtOH intake and development of CPP to EtOH (rewarding effects). The alcohol-induced locomotor activity was also increased by the HCN2 overexpression (stimulant effects). In agreement with a role of dopamine on the effects of EtOH, HCN overexpression into the VTA resulted in an increase in dopamine release in NAcc following systemic administration of EtOH.

Although the 4 identified mammalian subunits of HCN channels (1 to 4) are widely expressed in the CNS, we decided to clone and overexpress the HCN2 subunit into the VTA of UChB rats. This decision was supported by previous in situ hybridization and immunohistochemical studies showing that HCN2 subunit displayed the highest level of expression in the rat VTA compared to other HCN subunits (Monteggia et al., 2000; Notomi and Shigemoto, 2004). In addition
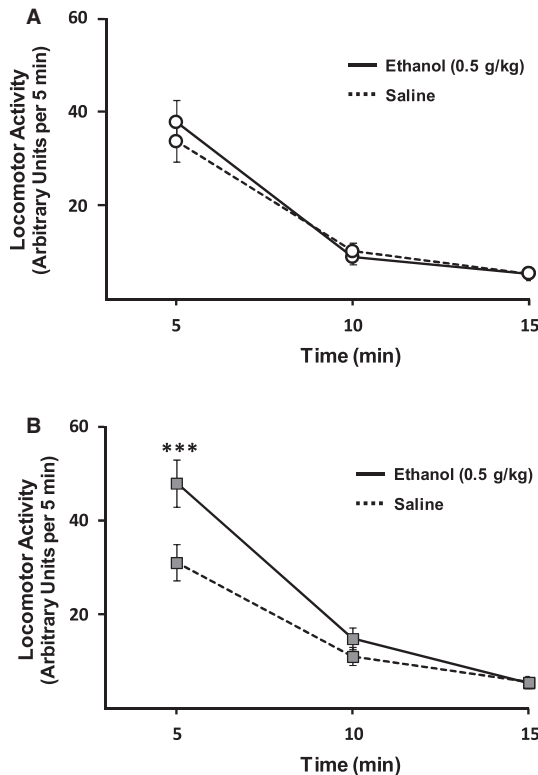
Fig. 4. HCN2 overexpression increases ethanol (EtOH)-induced loco-motor activity in UChB rats. The locomotor activity was measured for 3 successive 5-minute intervals after administration of an EtOH dose (0.5 g/kg, i.p.) or saline to rats treated with LV-rHCN2-GFP ($n = 9$) or LV-GFP ($n = 9$) lentiviral vectors. (**A**) Rats treated with LV-GFP control vector did not show any change in locomotor activity after EtOH administration (2-way ANOVA; drug treatment effect: N.S.). (**B**) Rats treated with LV-rHCN2-GFP vector and a dose of EtOH showed an overall increase in locomotor activity, 2-way ANOVA; drug treatment effect: $F(1, 53) = 8.007$, $p < 0.01$. A post hoc Bonferroni's test showed a significant difference in the first 5-minute interval (***$p < 0.001$). No significant differences in loco-motor activity were observed in the subsequent second and third 5-minute intervals. HCN2, hyperpolarization-activated cyclic nucleotide-gated 2; UChB, University of Chile Drinking.

Fig. 5. HCN2 overexpression in the VTA increases ethanol (EtOH)-induced release of dopamine in the NAcc of UChB rats. Extracellular NAcc dopamine levels were monitored under basal and EtOH (1 g/kg, i.p.) stim-ulated conditions in rats previously treated with LV-rHCN2-GFP ($n = 4$) or LV-GFP ($n = 5$) lentivectors. Data represent extracellular dopamine levels in the NAcc expressed as percentage of baseline. The arrow indicates the time of the i.p. administration of EtOH to the animals. Animals treated with the control vector did not show any significant increase in NAcc dopamine levels after EtOH administration compared to the basal condition. Acute administration of EtOH to animals treated with LV-rHCN2-GFP elicited a significant increase in the relative dopamine release compared to that produced in control animals, 2-way ANOVA; viral vector treatment effect: $F(1, 71) = 21.09$, $p < 0.001$. A post hoc Bonferroni's test showed a signifi-cant difference between both experimental groups at 60 and 90 minutes after EtOH administration (**$p < 0.01$; ***$p < 0.001$). HCN2, hyperpolari-zation-activated cyclic nucleotide-gated 2; NAcc, nucleus accumbens; UChB, University of Chile Drinking; VTA, ventral tegmental area.

conveyed by the increment of intracellular levels of cAMP. A question that arises from such proposed mechanism of action is whether the effect of overexpression of the HCN2 subunit in the rat VTA is selective for EtOH. Recent studies in mice showed that intracerebral administration of ZD7288 (a HCN antagonist) to nicotine-exposed animals precipitated somatic and affective signs of withdrawal (Görlich et al., 2013). Furthermore, it was reported that cocaine administra-tion to rats increased the expression of the HCN2 in the meso-cortico-limbic systems (Santos-Vera et al., 2013).

Recent in vitro electrophysiological studies performed in GABAergic neurons of the substantia nigra demonstrated that clinically relevant concentrations of EtOH (55 mM) enhanced HCN-mediated current (I_h) and increased neuro-nal firing rate (Tateno and Robinson, 2011). As 75% of the synaptic inputs to VTA dopamine neurons are GABAergic (Grace and Bunney, 1985), it is possible that the stimulant effects of EtOH on VTA dopamine neurons are conveyed by a combination of (i) a direct action on dopaminergic neurons and/or (ii) on GABAergic networks converging on DA neu-rons. Early studies by Brodie and colleagues (1995) showed that serotoninergic neurotransmission potentiates the EtOH-induced excitation of rat VTA dopamine neurons. Although contradictory, in vitro studies performed in dopamine VTA neurons have showed that serotonin can increase neuronal firing via the enhancement of HCN-mediated current I_h (Pessia et al., 1994). However, studies by Liu and colleagues (2003) demonstrated that the activation of 5-HT$_2$ receptors

to the activation of the HCN channels by membrane hyperpolarization, their opening is strongly modulated by intracellular cAMP. The binding of cAMP to the CNBD facilitates voltage-dependent activation by shifting the volt-age-activation threshold to more positive values (DiFrance-sco and Tortora, 1991). Interestingly, it has been reported that the HCN2 subunit exhibits the highest responsiveness to the modulatory effects of cAMP (Kusch et al., 2011). Regarding a possible mechanism for the action of EtOH on HCN channels, it is well established that EtOH increases the intracellular levels of cAMP (see Newton and Messing, 2006). In vitro studies showed that EtOH-induced cAMP generation is mediated by an indirect activation of adenosine A2a receptors (Sapru et al., 1994). Another mechanism by which EtOH might regulate intracellular cAMP levels involves the stimulation of specific isoforms of adenylyl cyclase (Yoshimura and Tabakoff, 1995). As an increase in cAMP levels modulates positively the activation HCN, it may be possible that EtOH action on HCN channels is
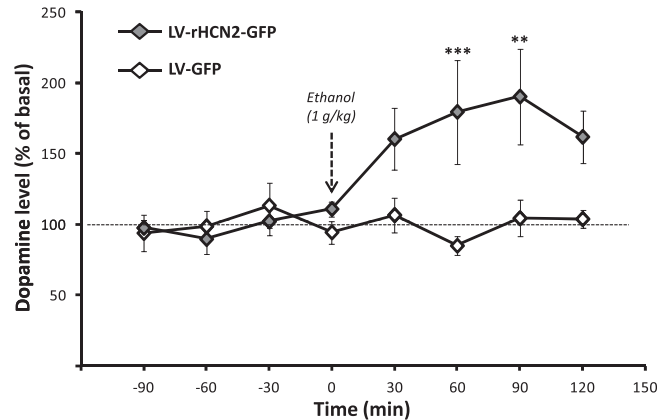
by serotonin on VTA dopamine neurons reduced in a dose-dependent fashion the amplitude of I_h; such inhibitory effects are probably mediated by PKC signaling.

As reported here, we found that intracerebral administration of a lentiviral vector coding for the HCN2 subunit results in ~2-fold increase in HCN2 protein levels compared to animals receiving the control vector. The lentiviral construction was designed to drive the expression of HCN2 subunit under the control of the ubiquitous EF1α promoter. Therefore, all the cells transduced by the lentiviral vector into the VTA, neuronal and glial cells, could potentially express the HCN2 subunit. However, in vivo studies have indicated that transduction of rat brain cells using lentiviral vectors showed a preferential neuronal tropism (Jakobsson et al., 2003). The mechanism of this effect is unknown, but some studies have attributed it to the presence on the viral capside of the vesicular stomatitis virus glycoprotein (VSV-G), which is used for pseudotype lentiviral vector constructions (Sanders, 2002).

To study the effects of increased HCN levels in the VTA on the rewarding and stimulant effects of EtOH, we used an animal model of alcoholism developed at our institution at the beginning of the 1950s, the bibulous (UChB) and abstinent (UChA) rats, selectively bred for more than 80 generations (Mardones and Segovia-Riquelme, 1983; Quintanilla et al., 2006). UChB rats are characterized by a high voluntary EtOH intake (>5 g EtOH/kg/d). In this study, we found that increasing HCN2 levels with a selective HCN2-lentiviral vector injected into the VTA led to an increase in voluntary EtOH consumption, suggesting that HCN levels are important for the rewarding effects of EtOH. A recent in vitro electrophysiological study showed that concentrations of EtOH easily obtained by social drinking (~20 μM) elicited a reversible activation of human HCN channels and that such activating effect of EtOH was markedly attenuated by the HCN blocker ivabradine (Chen et al., 2012). Further research is needed to establish whether pharmacological blockade of HCN channels reduces the rewarding effects of EtOH. One important limitation to the referred studies is the lack of selective inhibitors of HCN channels able to cross the blood–brain barrier, thus hindering their systemic administration. The study of the effects of intracerebral administration of HCN inhibitors on EtOH self-administration paradigms will certainly aid to circumvent the inability of these compounds to enter the brain.

CPP is a behavioral procedure allowing measuring the rewarding properties of a drug. However, the studies of the effects of EtOH on CPP have generated conflicting results because the expression of EtOH-mediated CPP in alcohol-naïve rats has proven to be difficult to obtain (Ciccocioppo et al., 1999). Studies by Quintanilla and Tampier (2011) demonstrated that UChB rats develop CPP to EtOH administration (1 g/kg, i.p.) only if the animals have been previously exposed to voluntary EtOH intake for at least 2 months. In the present study, the animals treated with the lentivectors were naïve to EtOH consumption and were

exposed to a paradigm of free choice between 5% EtOH and water for 30 days; and the dose of EtOH used to induce the CPP was of 0.5 g/kg (i.p.). Under these conditions, control animals (LV-GFP) developed a very weak CPP to EtOH (not reaching statically significant levels). In contrast, animals treated with the HCN2 coding vector developed a remarkable (~3-fold increase) CPP to the EtOH-paired compartment. It is important to point out that in this study, we used a biased CPP design, because animals showed a clear preference for one compartment over the other during the preconditioning session. It has been reported that interpretation of biased EtOH-induced CPP studies may be confounded by the potential anxiolytic effects of EtOH, which may result in a reduced aversion to the nonpreferred side. However, in this study, we found that EtOH did not produce any significant CPP in control animals (LV-GFP group), ruling out a major role of EtOH-derived anxiolytic effects on the strong CPP to EtOH showed by the animals treated with LV-rHCN2-GFP vector. These results provide further support to the hypothesis that HCN levels in the VTA are relevant for the positive motivational effect of EtOH.

It has been reported that low-to-moderate doses of EtOH are excitatory and potentiate locomotor activity in rats (Colombo et al., 1998; Quintanilla, 1999). In the present study, we found that animals treated with the HCN2-coding lentivector showed an increased EtOH-induced (0.5 g/kg, i.p.) locomotor activity compared to that shown by control animals, suggesting that HCN channels in the VTA are important for mediating the stimulant effects of EtOH. Unexpectedly, control animals did not show any significant increase in locomotor activity upon EtOH administration. It is possible that the initial 30-day period of voluntary EtOH consumption would affect the basal sensitivity of UChB rats to the stimulant effects of EtOH as were observed in previous studies performed in UChB EtOH-naïve rats (Quintanilla, 1999). In line with a positive correlation between HCN channels and EtOH stimulant properties, Beckstead and Phillips (2009) showed that mice selectively bred for high EtOH-induced locomotor activity had an increased density of HCN channels on dopamine neurons, compared to that of mice selected for a low locomotor response to EtOH.

In agreement with the increased rewarding and stimulant effects of EtOH in animals treated with the HCN2-coding lentiviral vector, the administration of an acute dose of systemic EtOH resulted in a marked increase in extracellular levels of dopamine monitored in the NAcc, but not in control animals treated with LV-GFP vector. The stimulant effect of EtOH on dopamine release in the animals treated with LV-rHCN2-GFP vector was observed for 120 minutes after the i.p. administration of 1 g/kg of EtOH. It is important to note that a dose of EtOH of 1 g/kg (i.p.) results approximately in 13 mM of blood EtOH concentration (Bloom et al.,1982). Similar blood EtOH levels have been reported in rats

consuming 4 to 6 g EtOH/kg/d under a free-choice paradigm (Agabio et al., 1996; Bell et al., 2006).

In conclusion, the results obtained by this study showed that overexpression of HCN2 ionic channels into the VTA of UChB rats markedly increased (i) voluntary EtOH consumption, (ii) EtOH-induced CPP, (iii) locomotor activity, and (iv) dopamine release in the NAcc. These results support the idea that HCN channel levels in the VTA are relevant for the rewarding and stimulant properties of EtOH. The development of new HCN blockers able to cross blood–brain barrier together with appropriate animal models would provide further evidence about the role of HCN channels in alcohol addiction.

ACKNOWLEDGMENTS

REFERENCES

Agabio R, Cortis G, Fadda F, Gessa GL, Lobina C, Reali R, Colombo G (1996) Circadian drinking pattern of Sardinian alcohol-preferring rats. Alcohol Alcohol 31:385–388.

Altomare C, Bucchi A, Camatini E, Baruscotti M, Viscomi C, Moroni A, DiFrancesco D (2001) Integrated allosteric model of voltage gating of HCN channels. J Gen Physiol 117:519–532.

Beckstead MJ, Phillips TJ (2009) Mice selectively bred for high- or low-alcohol-induced locomotion exhibit differences in dopamine neuron function. J Pharmacol Exp Ther 329:342–349.

Bell RL, Rodd ZA, Sable HJ, Schultz JA, Hsu CC, Lumeng L, Murphy JM, McBride WJ (2006) Daily patterns of ethanol drinking in peri-adolescent and adult alcohol-preferring (P) rats. Pharmacol Biochem Behav 83:35–46.

Biel M, Wahl-Schott C, Michalakis S, Zong X (2009) Hyperpolarization-activated cation channels: from genes to function. Physiol Rev 89:847–885.

Bloom F, Lad P, Pittman Q, Rogers J (1982) Blood alcohol levels in rats: non-uniform yields from intraperitoneal doses based on body weight. Br J Pharmacol 75:251–254.

Brodie MS, Appel SB (1998) The effects of ethanol on dopaminergic neurons of the ventral tegmental area studied by intracellular recording in brain slices. Alcohol Clin Exp Res 22:236–244.

Brodie MS, Trifunovic RD, Shefner SA (1995) Serotonin potentiates ethanol-induced excitation of ventral tegmental area neurons in brain slices from three different rat strains. J Pharmacol Exp Ther 273:1139–1146.

Brown H, Difrancesco D (1980) Voltage-clamp investigations of membrane currents underlying pace-maker activity in rabbit sino-atrial node. J Physiol 308:331–351.

Bustamante D, You ZB, Castel MN, Johansson S, Goiny M, Terenius L, Hökfelt T, Herrera-Marschitz M (2002) Effect of single and repeated methamphetamine treatment on neurotransmitter release in substancia nigra and neostriatum of the rat. J Neurochem 83:645–654.

Chen Y, Wu P, Fan X, Chen H, Yang J, Song T, Huang C (2012) Ethanol enhances human hyperpolarization-activated cyclic nucleotide-gated currents. Alcohol Clin Exp Res 36:2036–2046.

Ciccocioppo R, Panocka I, Froldi R, Quitadamo E, Massi M (1999) Ethanol induces conditioned place preference in genetically selected alcohol-preferring rats. Psychopharmacology 141:235–241.

Colombo G, Agabio R, Lobina C, Reali R, Vacca G, Gessa GL (1998) Stimulation of locomotor activity by voluntary consumed ethanol in Sardinian alcohol-preferring rats. Eur J Pharmacol 357:109–113.

DiFrancesco D, Tortora P (1991) Direct activation of cardiac pacemaker channels by intracellular cyclic AMP. Nature 351:145–147.

Görlich A, Antolin-Fontes B, Ables JL, Frahm S, Slimak MA, Dougherty JD, Ibañez-Tallon I (2013) Reexposure to nicotine during withdrawal increases the pacemaking activity of cholinergic habenular neurons. Proc Natl Acad Sci USA 110:17077–17082.

Grace AA, Bunney BS (1983) Intracellular and extracellular electrophysiology of nigral dopaminergic neurons—1. Identification and characterization. Neuroscience 10:301–315.

Grace AA, Bunney BS (1985) Opposing effects of striatonigral feedback pathways on midbrain dopamine cell activity. Brain Res 333:271–284.

Imperato A, Di Chiara G (1986) Preferential stimulation of dopamine release in the nucleus accumbens of freely moving rats by ethanol. J Pharmacol Exp Ther 239:219–228.

Jakobsson J, Ericson C, Jansson M, Björk E, Lundberg C (2003) Targeted transgene expression in rat brain using lentiviral vectors. J Neurosci Res 73:876–885.

Karahanian E, Quintanilla ME, Tampier L, Rivera-Meza M, Bustamante D, Gonzalez-Lira V, Morales P, Herrera-Marschitz M, Israel Y (2011) Ethanol as a prodrug: brain metabolism of ethanol mediates its reinforcing effects. Alcohol Clin Exp Res 35:606–612.

Kusch J, Thon S, Schulz E, Biskup C, Nache V, Zimmer T, Seifert R, Schwede F, Benndorf K (2011) How subunits cooperate in cAMP-induced activation of homotetrameric HCN2 channels. Nat Chem Biol 8:162–169.

Liu Z, Bunney EB, Appel SB, Brodie MS (2003) Serotonin reduces the hyperpolarization-activated current (I_h) in ventral tegmental dopamine neurons: involvement of 5-HT$_2$ receptors and protein kinase C. J Neurophysiol 90:3201–3212.

Ludwig A, Zong X, Jeglitsch M, Hofmann F, Biel M (1998) A family of hyperpolarization-activated mammalian cation channels. Nature 393:587–591.

Mardones J, Segovia-Riquelme N (1983) Thirty-two years of selection of rats by ethanol preference: UChA and UChB strains. Neurobehav Toxicol Teratol 5:171–178.

McDaid J, McElvain MA, Brodie MS (2008) Ethanol effects on dopaminergic ventral tegmental area neurons during block of I_h: involvement of barium-sensitive potassium currents. J Neurophysiol 100:1202–1210.

Migliore C, Cannia C, Canavier CC (2008) A modeling study suggesting a possible pharmacological target to mitigate the effects of ethanol on reward-related dopamine signaling. J Neurophysiol 99:2703–2707.

Monteggia LM, Eisch AJ, Tang MD, Kaczmarek LK, Nestler EJ (2000) Cloning and localization of the hyperpolarization-activated cyclic nucleotide-gated channel family in rat brain. Brain Res Mol Brain Res 81:129–139.

Neuhoff H, Neu A, Liss B, Roeper J (2002) I_h channels contribute to the different functional properties of identified doapminergic subpopulations in the midbrain. J Neurosci 22:1290–1302.

Newton PM, Messing RO (2006) Intracellular signaling pathways that regulate behavioral responses to ethanol. Pharmacol Ther 109:227–237.

Notomi T, Shigemoto R (2004) Immunohistochemical localization of I_h channel subunits, HCN1-4, in the rat brain. J Comp Neurol 471:241–276.

Okamoto T, Harnett MT, Morikawa H (2006) Hyperpolarization-activated cation current (I_h) is an ethanol target in midbrain dopamine neurons of mice. J Neurophysiol 95:619–626.

Overton PG, Clark D (1997) Burst firing in midbrain dopaminergic neurons. Brain Res Rev 25:312–334.

Paxinos G, Watson C (1986) The Rat Brain in Stereotaxic Coordinates. Academic Press, New York, NY.

Pessia M, Jiang ZG, North RA, Johnson SW (1994) Actions of 5-hydroxy-tryptamine on ventral tegmental area neurons of the rat in vitro. Brain Res 654:324–330.

Quintanilla ME (1999) Effect of low doses of ethanol on spontaneous locomotor activity in UChB and UChA rats. Addict Biol 4:443–448.

Quintanilla ME, Bustamante D, Tampier L, Israel Y, Herrera-Marschitz M (2007) Dopamine release in the nucleus accumbens (shell) of two lines of rats selectively bred to prefer or avoid ethanol. Eur J Pharmacol 573:84–92.

Quintanilla ME, Israel Y, Sapag A, Tampier L (2006) The UChA and UChB rat lines: metabolic and genetic differences influencing ethanol intake. Addict Biol 11:310–323.

Quintanilla ME, Tampier L (2011) Place conditioning with ethanol in rats bred for high (UChB) and low (UChA) voluntary alcohol drinking. Alcohol 45:751–762.

Rodd ZA, Bell RL, Melendez RI, Kuc KA, Lumeng L, Li TK (2004) Comparison of intracranial self-administration of ethanol within the posterior ventral tegmental area between alcohol-preferring (P) and Wistar rats. Alcohol Clin Exp Res 28:1212–1219.

Rodd ZA, Bell RL, Zhang Y, Murphy JM, Goldstein A, Zaffaroni A, Li TK, McBride WJ (2005) Regional heterogeneity for the intracranial self-administration of ethanol and acetaldehyde within the ventral tegmental area of alcohol-preferring rats: involvement of dopamine and serotonin. Neuropsychopharmacology 30:330–338.

Sanders DA (2002) No false start for novel pseudotyped vectors. Curr Opin Biotechnol 13:437–442.

Santos-Vera B, Vázquez-Torres R, Marrero HG, Acevedo JM, Arencibia-Albite F, Vélez-Hernández ME, Miranda JD, Jiménez-Rivera CA (2013) Cocaine sensitization increases I_h current cannel subunit (HCN$_2$) protein expression in structures of the mesocorticolimbic system. J Mol Neurosci 50:234–245.

Sapru MK, Diamond I, Gordon AS (1994) Adenosine receptors mediate cellular adaptation to ethanol in NG108-15 cells. J Pharmacol Exp Ther 271:542–548.

Seutin V, Massotte L, Renette MF, Dresse A (2001) Evidence for a modulatory role of I_h on the firing of a subgroup of midbrain dopamine neurons. NeuroReport 12:255–258.

Tateno T, Robinson HP (2011) The mechanism of ethanol action on midbrain doapminergic neuron firing: a dynamic-clamp study of the role of I_h and GABAergic synaptic integration. J Neurophysiol 106:1901–1922.

Ungerstedt U (1971) Stereotaxic mapping of the monoamine pathways in the rat brain. Acta Physiol Scand Suppl 367:1–48.

Welm BE, Dijkgraaf GJ, Bledau AS, Welm AL, Werb Z (2008) Lentiviral transduction of mammary stem cells for analysis of gene function during development and cancer. Cell Stem Cell 2:90–102.

Yoshimura M, Tabakoff B (1995) Selective effects of ethanol on the generation of cAMP by particular members of the adenylyl cyclase members. Alcohol Clin Exp Res 19:1435–1440.

Zhang Q, Huang A, Lin YC, Yu HG (2009) Associated changes in HCN2 and HCN4 transcripts and I_f pacemaker current in myocytes. Biochim Biophys Acta 1788:1138–1147.

Zocchi A, Girlanda E, Varnier G, Sartori I, Zanetti L, Wildish GA, Lennon M, Mugnaini M, Heidbreder CA (2003) Dopamine responsiveness to drugs of abuse: a shell-core investigation in the nucleus accumbens of the mouse. Synapse 50:293–302.