Molecular Tolerance of Voltage-Gated Calcium Channels is Evident After Short Exposures to Alcohol in Vasopressin-Releasing Nerve Terminals

Andrzej Z. Pietrzykowski*, Sonia Ortiz-Miranda*, Thomas K. Knott, Edward Custer, Sylvie Puig, José R. Lemos, and Steven N. Treistman

Background: Voltage-gated calcium channels (VGCCs) in rat neurohypophysial terminals exhibit molecular tolerance to alcohol, including desensitization to the drug and increased current density, after 3 weeks of alcohol drinking. Moreover, after this time, terminals from drinking rats exhibit diminished alcohol inhibition of vasopressin (AVP) release.

Methods: We took advantage of organotypic cultures (explants) of the hypothalamo-neurohypophysial system (HNS) to extend our analysis of molecular tolerance to 2 classes of the VGCC. The isolated HNS explant allows much finer temporal resolution of molecular tolerance than do voluntary drinking paradigms. After exposure of the HNS explant to alcohol, terminals are isolated by mechanical treatment and plated in a dish. Patch clamp recording techniques are used to obtain VGCC currents, and immunohistochemistry is used to determine VGCC distribution. A release assay is used to provide functional readout of AVP release.

Results: We show that even a brief, 1-hour exposure to a clinically relevant concentration of alcohol is sufficient to evoke similar changes to those observed after several weeks of exposure. Acute ethanol (EtOH) exposure inhibits high K^+-induced AVP release from naïve terminals. However, terminals pre-exposed to 20 mM EtOH for 1 hour become tolerant to EtOH, and subsequent exposure has significantly less effect on high K^+-induced AVP release. Electrophysiological recordings indicate that among different types of VGCCs present in the neuronal terminal, the L-type is the most affected by alcohol. The current density of L-type current is significantly increased (approximately 50%), while its responsiveness to alcohol is significantly diminished (approximately 50%), after brief alcohol exposure. Fluorescent imaging results were consistent with the electrophysiology and suggest that the increased current density of VGCCs after brief exposure is attributable to combined synthesis of 1.2 and 1.3 subtypes of the L-type VGCC and redistribution of channel protein into terminal plasma membrane.

Conclusions: These data indicate that a brief alcohol exposure affects subsequent alcohol sensitivity of VGCCs and neuropeptide release from presynaptic terminals.

Key Words: Alcohol, Molecular Tolerance, Calcium Channels, Neurohypophysial Terminals, Vasopressin.

ALCOHOL (ETHANOL [ETOH]) has a profound effect on synaptic function, affecting neurons in many brain regions, leading to intoxication and in many cases to alcohol abuse and addiction. Voltage-gated calcium channels (VGCCs) are pivotal determinants of neuronal activity, playing an especially important role in the release of neurotransmitters from presynaptic neuronal terminals. At resting membrane potential, they are normally closed, but become activated at depolarized membrane potentials, allowing calcium ions to enter into cells (Katz and Miledi, 1969). Previous research has indicated that the action of alcohol on neuronal channels is primarily via effects on channel gating, rather than on other functional attributes such as conductance or ion selectivity (Dopico et al., 1999; Wang et al., 1993). The hypothalamo-neurohypophysial system (HNS) preparation is unusual in allowing direct study of the nerve terminal, typically inaccessible except by indirect means in most neuronal preparations. Moreover, the ability to examine channel activity, morphology, and peptide release in tandem provides a particularly powerful approach. Previously, we assayed the release of the neurohormones, vasopressin (AVP), and oxytocin from posterior pituitary terminals of the HNS and showed that 3-week-long alcohol drinking in rats diminishes the effects of subsequent challenge with alcohol on release of these hormones (Knott et al., 2002). In parallel, we presented evidence that chronic in vivo alcohol

From the Department of Animal Sciences (AZP), Rutgers University, New Brunswick, New Jersey; Department of MaPS & Program in Neuroscience (SO-M, TKK, EC, SP, JRL, SNT), University of Massachusetts Medical School, Worcester, Massachusetts; and Institute of Neurobiology (SNT), University of Puerto Rico Medical School, San Juan, Puerto Rico.

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Reprint requests: Steven N. Treistman, PhD, University of Puerto Rico Medical School, Institute of Neurobiology, 201 Blvd. del Valle, San Juan, PR 00901; Tel.: 787-721-4149; Fax: 787-721-4584; E-mail: steven.treistman@upr.edu

**Both authors contributed equally to this work.*

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exposure increases density but diminishes alcohol sensitivity of VGCCs in these terminals. The large-conductance calcium-activated potassium (BK) channel in HNS terminals (part of a functional and morphological dyad with VGCCs) showed qualitatively similar changes to those observed in the VGCCs, but opposite in effect (i.e., immediate response to the drug was potentiation of channel activity, while the compensatory changes included a decreased current density (Knott et al., 2002). Subsequent research focused on BK channels has uncovered a number of molecular mechanisms that contribute to rapid changes in BK contributing to molecular tolerance (Treistman and Martin, 2009). Here, we use organotypic cultures of the HNS, which allow much greater temporal resolution than alcohol ingestion, to determine whether short alcohol exposure can induce molecular tolerance in VGCCs as well, and the time course of this tolerance. We show that even a brief, 1-hour exposure to a clinically relevant concentration of alcohol evokes changes similar to those observed after several weeks of alcohol drinking.

The finding that short alcohol exposure in vitro produces effects in the VGCC similar to those observed in BK raises the possibility that similar mechanisms may underlie the molecular tolerance observed in both channel proteins. For example, it has been suggested that miRNA, which is affected by EtOH in neurons after short exposures, might provide a "molecular master switch" (Pietrzykowski, 2010; Pietrzykowski et al., 2008, p. 284) integrating the response of a number of downstream targets, including the BK channel and VGCCs.

MATERIALS AND METHODS

Explant Preparation

Male Sprague–Dawley rats (Taconic Farms, Germantown, NY) weighing 200 to 250 g were housed and sacrificed in accordance with the Guide for the Care and Use of Laboratory Animals, created by the Institute of Animal Resources, National Research Council, DHHS, and NIH, and with the study protocol approved by the University of Massachusetts Animal Care and Use Committee as described previously (Pietrzykowski et al., 2004). Briefly, immediately after decapitation, the whole brain including the pituitary with intact pituitary stalk were removed very rapidly from the skull and chilled in oxygenated dissection buffer (in mM): 120 NaCl, 5 KCl, 1.0 $MgCl_2$, 1.9 $CaCl_2$, 2.0 EGTA, 10 HEPES, 5.0 $NaHCO_3$, 15.0 glucose, 0.5 glutamine (Glutamax; Invitrogen, Gaithersburg, MD), and 100 U/ml to 0.1 mg/ml penicillin–streptomycin (Invitrogen), pH 7.2, 310 mOsm. To increase cell survival, dissection buffer also contained: (i) low levels of free calcium (3 μM), (ii) B27 supplement with antioxidants (1:50; Invitrogen), and (iii) 1 mM nitroarginine and kynurenic acid. After removal of the anterior pituitary under a dissection microscope, a hexagonal block of tissue was carved out (by cutting rostral to the optic chiasm, parallel to the optic tract, lateral to the median eminence and caudal to the stalk, and undercutting at a depth of 1 to 2 mm). This allowed trimming the tissue to the final thickness of 550 to 650 μm necessary for adequate penetration of nutrients and oxygen despite the lack of vascular perfusion (Fujii et al., 1982; Nicholson and Hounsgaard, 1983). Explants were cultured individually in an actively humidified incubator, at 34°C, in 5% CO_2, on Millicell CM inserts (Millipore, Bedford, MA), placed in Petri dishes (60 mm; Corning, Corning, NY). Explants were cultured in Neurobasal A medium (310 mOsm) with B27, glutamine, penicillin–streptomycin (as in dissection buffer), and 10% fetal calf serum (Sigma, St. Louis, MO) in the air–medium interface (Brewer, 1997; House et al., 1998; Stoppini et al., 1991; Xiang et al., 2000).

EtOH Exposure

Medium was changed a few hours after dissection to EtOH-free medium (naïve control) or medium containing 20 mM EtOH (EtOH group) and explants were cultured for 15, 30, or 60 minutes. Next, the posterior pituitary was removed from the dish, washed with several changes of EtOH-free, low-calcium (3 μM) Locke's solution (see Solutions) to ensure complete removal of EtOH, and neurohypophysial (NH) terminals were prepared for either high K^+-induced AVP release experiments or electrophysiological recordings.

AVP Release Assay

For AVP release experiments, the posterior pituitary from each explant incubated with or without EtOH for 1 hour was removed, washed in low-calcium (3 μM) Locke's solution (see Solutions) and homogenized in sucrose buffer (Cazalis et al., 1985) to obtain a purified population of NH terminals. The NH terminals were loaded equally onto filters (Acrodisc, LC13, 0.45 μm; Gelman Scientific, Ann Arbor, MI) and perfused at 100 ul/min with normal Locke's buffer, containing in (mM): NaCl 140; KCl 5; $MgCl_2$ 1.2; $CaCl_2$ 2.2; glucose 10; HEPES–Tris 10; pH 7.2; at 37°C, at 295 to 300 mOsml/l osmolarity, for 1 hour, to stabilize AVP release. Samples were then collected every 3 minutes for 18 minutes to determine basal AVP release. The NH terminals were then perfused with 50 mM KCl to stimulate active release of AVP in the presence or absence of acute EtOH (50 mM) exposure. EtOH was present 6 minutes prior to and during the KCl depolarization. The solution containing elevated potassium was prepared by replacing an equimolar amount of NaCl with 50 mM KCl. The KCl solution contained in (mM): NaCl 90; KCl 55; $MgCl_2$ 1.2; $CaCl_2$ 2.2; glucose 10; HEPES–Tris 10; pH 7.2; at 37°C, and the osmolarity was 295 to 300 mOsml/l. Basal AVP release was determined by averaging the 6 samples prior to KCl induced stimulation. High K^+-induced AVP release was determined by subtracting the mean basal release from each sample during the high K^+ stimulation. Samples were frozen and stored at −20°C, until AVP was determined by a specific AVP ELISA (Assay Designs, Inc., Ann Arbor, MI). The sensitivity of the ELISA assay was 0.25 pg AVP (Custer et al., 2007).

Electrophysiology

Terminal Preparation. The posterior pituitary was excised either from the HNS explant cultured for a specified amount of time: 15, 30, or 60 minutes in 20 mM EtOH or from the control explant cultured in parallel without EtOH. Excised posterior pituitaries were washed briefly in EtOH-free low-calcium (3 μM) Locke's solution (see Solutions), and NH terminals were isolated as described previously (Knott et al., 2002; Lemos and Nordmann, 1986). The dissociated terminals were first placed within a plastic ring centered in a sterile polystyrene dish. The surrounding dish was then filled with low (3 μM) calcium Locke's solution. The ring was removed from the dish after 1 minute, and the terminals were allowed to descend to the bottom of the dish for 3 minutes before they were perfused slowly (1 ml/min) with 2.2 mM calcium Locke's for 3 to 5 minutes, followed by a fast perfusion (8 to 10 ml/min) for a minimum of 10 minutes. This change in flow rate allows the terminals to adhere to the bottom of the dish but remain attached loosely enough to be lifted from the bottom after formation of a 1 GΩ seal. The dissociated terminals were 6 to 12 μm in diameter and easily identified

using phase and interference (Hoffmann) optics. During these procedures (approximately 45 minutes) prior to the acute EtOH challenge in the experiment, the terminals were not exposed to EtOH.

Perforated-Patch Whole-Cell Recordings. Ba^{2+} current recordings were obtained using the perforated-patch technique (Ortiz-Miranda et al., 2005; Wang et al., 1997). After isolating the terminals as mentioned above and rinsing with normal Locke's solution, the terminals were further rinsed with 5 mM barium (in place of calcium) Locke's for 5 minutes and all subsequent current acquisition performed in the Ba^{2+} Locke's. Perforation of the terminals was obtained by the addition of amphotericin B to the pipette solution (see Solutions). Electrodes (David Kopf Instruments, Tujunga, CA) were pulled from 100-μl glass pipettes (Drummond Scientific Co., Broomall, PA) and the tips fire-polished on a microforge (Narashige, Tokyo, Japan) to give a resistance of 4 to 8 MΩ when filled with pipette solution (see Solutions). All experiments were performed at room temperature and currents recorded using a BVC-700A amplifier (Dagan Corp., Minneapolis, MN), filtered at 1 kHz and stored in computer files for later analysis with pClamp5 (Axon Instruments, Foster City, CA). We used capacitance measurements to determine the membrane area of the terminals and then calculated the current density in naïve versus terminals with EtOH exposure. Capacitance was correlated with the diameter of the terminal, and this relationship was unaltered by drug treatment, suggesting that infolding was not induced by drug treatment (Knott et al., 2002).

Onset and Reversibility of Alcohol Effects. For the currents examined, the short-term effects of EtOH were evident within 5 seconds of exposure, and the effects were reversed within 30 seconds of washout of the drug, for both EtOH preexposed and nonpreexposed explants.

Solutions. In whole-cell recordings, the terminals were bathed in Locke's solution consisting of 130 mM NaCl, 15 mM glucose, 10 mM HEPES, 5 mM KCl, 2.2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.3, and 305 mOsM. In Barium Locke's, the calcium was replaced with 5 mM $BaCl_2$. In low-calcium (3 μM) Locke's, the 2.2 mM $CaCl_2$ was reduced to 1.96 and 2 mM EGTA was added.

The pipette solution consisted of 130 mM CsGlut, 15 mM $CsCl_2$, 5 mM glucose, 5 mM tetraethylammonium chloride, 1 mM $MgCl_2$, 300 μM amphotericin B, pH 7.3, and 315 mOsM. In all perforated-patch recordings, the terminals were bathed in Locke's solution followed by a 5 mM barium Locke's solution, which consisted of 130 mM NaCl, 10 mM glucose, 10 mM HEPES, 5 mM KCl, 5 mM $BaCl_2$, 2 mM $MgCl_2$, pH 7.3, and 305 mOsM. An osmotic difference of 10 mOsM was maintained between the pipette and bath solutions to enhance seal formation. Sustained L-type calcium currents were enhanced by 1 μM Bay K.

Immunostaining of α1.2 and α1.3 Subunits

Control and EtOH-treated posterior pituitaries were dissociated in parallel but independently in a buffer containing (in mM): sucrose, 270; EGTA, 0.01; HEPES–Tris (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid–Tris(hydroxymethyl)aminomethane), 10, pH 7.3; and 295 to 300 mOsm/L at 37°C. The homogenates containing the dissociated terminals were aliquoted into 35 mm polypropylene glass-bottom microwell dishes (MatTek Corporation, Ashland, MA). The tissue was fixed overnight in 4% paraformaldehyde-phosphate buffer (PB) solution (pH 7) and washed repetitively in PB afterward. Six dishes were obtained per experiment, 3 EtOH-treated and 3 controls. Two dishes, containing EtOH-treated terminals were exposed to a 1% Triton X-100 PB solution containing 10% donkey serum, an affinity purified goat anti-Neurophysin-II (AVP) at 2 μg/ml (Santa Cruz Biotechnology, Santa Cruz, CA), and either an affinity purified rabbit anti-$Ca_v1.2$ (α_{1C}) at 4 μg/μl (Alomone Labs Ltd, Jerusalem, Israel) or an affinity purified rabbit anti-$Ca_v1.3$ (α_{1D}) at 5 μg/μl (Alomone Labs Ltd). Two dishes from the control group were treated with an identical antibody mix. Primary antibodies were omitted in the third dish from each group, to assess the fluorescence level contributed by nonspecific binding. After overnight incubation and wash with PB, all dishes were exposed to a mixture of fluorescently tagged secondary antibodies (Molecular Probes, Carlsbad, CA) for 3 to 5 hours at room temperature: Alexa Fluor 350 donkey anti-goat IgG, and Alexa Fluor donkey anti-rabbit IgG, diluted 1/200 in PB solution containing 10% donkey serum and 1% Triton X-100. After a final series of washes, the dishes were mounted with antifade media (Prolong; Molecular Probes) and sealed with a glass coverslip. Images were obtained with an Axiovert 200 M Inverted microscope (Carl Zeiss Inc., Thornwood, NY) interfaced to a Zeiss AxioCam MRm camera fitted to a 63-X Plan/Apochromat oil immersion lens. Axiovert 40 (V.4.6.3.0) software was used for acquisition and image analysis. Images of terminals were obtained in automated Z-stacks with 0.27 μm interval. Images were deconvoluted using a high stringency fast iterative algorithm and corrected for fluorescence crosstalk between stacks.

L-type channel distribution was estimated from the $Ca_v1.2$ and $Ca_v1.3$ immunofluorescent signals. The amount of fluorescence within 0.45 μm of the outer terminal's boundary (established from the differential interference contrast images) was obtained by calculating the difference in fluorescent signal between 2 tightly fitted circles: one at the terminal outer perimeter and another circle 0.45 μm inside of the first one. This distance was the highest attainable resolution limit and was corroborated in independent experiments using nonpermeabilized terminals exposed to antibodies against extracellular epitopes of other membrane-associated proteins (i.e., μ-opioid receptors and *N*-type channels; data not shown). This approach has also been used by others (Chiu et al., 2002) and by us (Lemos et al., 2011; Ortiz-Miranda et al., 2010; Pietrzykowski et al., 2004) to estimate amount of antigen labeling associated with a cell's plasma membrane. Percent values were calculated for individual terminals and means ± errors obtained for each group/treatment. The fluorescence signal in 2 control dishes was at or below nonspecific levels for all 3 wavelengths: $\leq 284 \pm 28$ grey fluorescent units (gfu; $n = 32$ terminals).

Statistics

Unless otherwise indicated all data are given as means ± SEM. Statistical significance using 2-tailed Student's *t*-test for all analyses was established as $p < 0.05$.

RESULTS

EtOH Modulation of AVP Release from NH Terminals

HNS explants were cultured for 1 hour in control media or media containing 20 mM EtOH. After this incubation period, the posterior pituitary (also known as the neurohypophysis) from each explant was carefully removed, homogenized and the isolated NH terminals were loaded onto filters (see Materials and Methods). Subsequent EtOH (50 mM) challenges had no effect on *basal* AVP release from NH terminals obtained from either control or EtOH-treated HNS explants (data not shown). However, EtOH reduced (to 66.06 ± 3.17% of control, $n = 3$) high K^+-induced AVP release from NH terminals maintained in control media (Fig. 1). In contrast, a subsequent EtOH challenge had a sig-

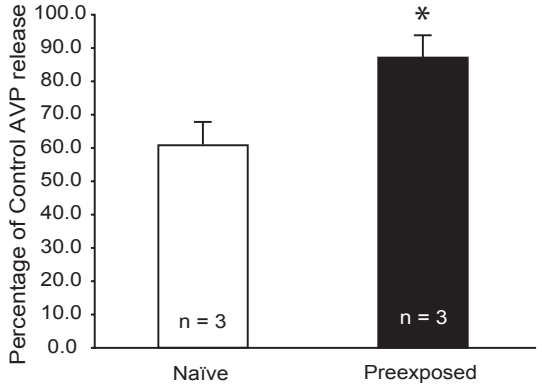

Fig. 1. Effect of ethanol (EtOH) exposure on vasopressin (AVP) release from presynaptic terminals. Rat neurohypophysial terminals obtained from hypothalamo-neurohypophysial system (HNS) explants incubated for 1 hour in 20 mM EtOH (Preexposed) and exposed to a 50 mM EtOH challenge released significantly more AVP in response to high K^+-induced depolarization than terminals obtained from HNS explants incubated for 1 hour without 20 mM EtOH (Naïve). *$p = 0.041$.

nificantly smaller inhibitory effect (to 92.64 ± 4.72% of control, $n = 3$, $p < 0.05$) on high K^+-induced AVP release from terminals from explants preexposed to EtOH (Fig. 1). This indicates that a 1 hour exposure of the HNS explant to 20 mM EtOH was sufficient to induce tolerance to a subsequent acute challenge of 50 mM EtOH on high K^+-induced AVP release.

Alcohol Effects on Calcium Currents in Presynaptic Terminals

The amount of neuropeptide released from terminals is directly coupled to the level of calcium inflow through VGCCs in the plasma membrane. Therefore, we next examined whether molecular tolerance in VGCCs contributes to the tolerance observed in neuropeptide release. We divided VGCC-mediated current into 2 major types: (i) the transient current composed of N-, P/Q-, and R-subtypes (for a fuller description, see Lemos et al., 2012) and (ii) sustained L-type currents. Figure 2 (left) shows representative traces of calcium current recordings from individual presynaptic terminals. We measured 2 main features of ion currents: current amplitude and current density (calculated using membrane capacitance), during acute EtOH challenge of terminals preexposed to EtOH for 0, 15, 30, and 60 minutes. The density of the transient current was unaffected within any of the time periods examined (Fig. 2*B* upper). However, a decrease in sensitivity to EtOH challenge was observed after 30 minutes of exposure, but was not evident after 15 minutes (Fig. 2*B* lower).

In contrast to its actions on the transient current, EtOH affected both current density and channel activity of the L-type current within the time frames examined. Preexposure to 20 mM EtOH for 30 minutes resulted in an approximately 50% increase in the density of L-type calcium channels (Fig. 2*C* upper). This increase was maintained during a subsequent 30 minutes of exposure. Acute EtOH exposure inhibited L-type calcium current, and as seen with the transient current, EtOH pretreatment decreased the sensitivity of the L-type calcium channel to a subsequent acute EtOH challenge (Fig. 2*C* lower). The decrease in sensitivity to EtOH challenge by preexposure to EtOH occurred more quickly for L-type calcium currents (evident after 15 minutes) than for the transient calcium current (evident only after 30 minutes).

Regulation of L-Type Calcium Channel Subtypes by Alcohol

We used immunohistochemistry techniques to further examine whether the changes in L-type VGCC density and activity are mediating molecular tolerance to EtOH in NH terminals. This approach provided an independent method to complement the electrophysiological measurements of current density previously described. Two subtypes of L-type calcium channel (α1.2 [L_C] and α1.3 [L_D]) have been described in the HNS (Chin et al., 1992; Fisher and Bourque, 1996; Rusin et al., 1997; Soldo and Moises, 1998). We used affinity purified antibodies recognizing a unique subunit of a particular subtype to determine expression of each subtype in the HNS terminal. Immunolabeling showed significant signal intensity (SI), roughly a thousand times higher (10^5 gfu/μm^2) than nonspecific background levels (10^2 gfu/μm^2, omission of primary antibodies, preincubation with blocking peptides) for both subunits.

Terminals exposed to EtOH for 1 hour (EtOH) or kept in EtOH-free Neurobasal A medium (Control) were both labeled using a combination of antibodies against AVP and 1 of the calcium channel subtypes $Ca_v1.2$ or $Ca_v1.3$ (Fig. 3*A*). Interestingly, even this short EtOH exposure significantly increased total expression levels in the entire terminal for both subunits (Fig. 3*B*). For $Ca_v1.2$ ($n = 10$) mean SI was $1.9 \times 10^5 \pm 4.8 \times 10^4$ gfu/μm^2 in EtOH-free, control conditions, while $4.9 \times 10^5 \pm 1.1 \times 10^4$ gfu/μm^2 ($n = 10$) in terminals exposed to EtOH for 1 hour (Fig. 3*D*). Similarly, for $Ca_v1.3$ channels mean SI was $4.3 \times 10^5 \pm 9.4 \times 10^4$ gfu/μm^2 ($n = 18$) in control terminals, while $9.0 \times 10^5 \pm 1.8 \times 10^4$ gfu/μm^2 ($n = 10$) in EtOH-exposed terminals. Involvement of the L-type VGCC in the release of neurotransmitters is mainly associated with channels located in the plasma membrane of the terminal. Therefore, we have defined, as described previously by others (Chiu et al., 2002) and us (Lemos et al., 2011; Ortiz-Miranda et al., 2010; Pietrzykowski et al., 2004) the outer region of the terminal (0.45 μm thickness) containing channels located in the plasma membrane and probably also some channels in close vicinity to the membrane (Fig. 3*C*). In this region, we observed an increase in the plasmalemmal expression of both subtypes upon EtOH exposure, without, however, reaching statistical significance (data not shown). As electrophysiological recordings of functional channels make no distinction between channel subtypes and simultaneously measure activity of both, $Ca_v1.2$ and $Ca_v1.3$ channels, we combined the plasmalemmal, fluorescent signal of both subtypes. This approach indicated a

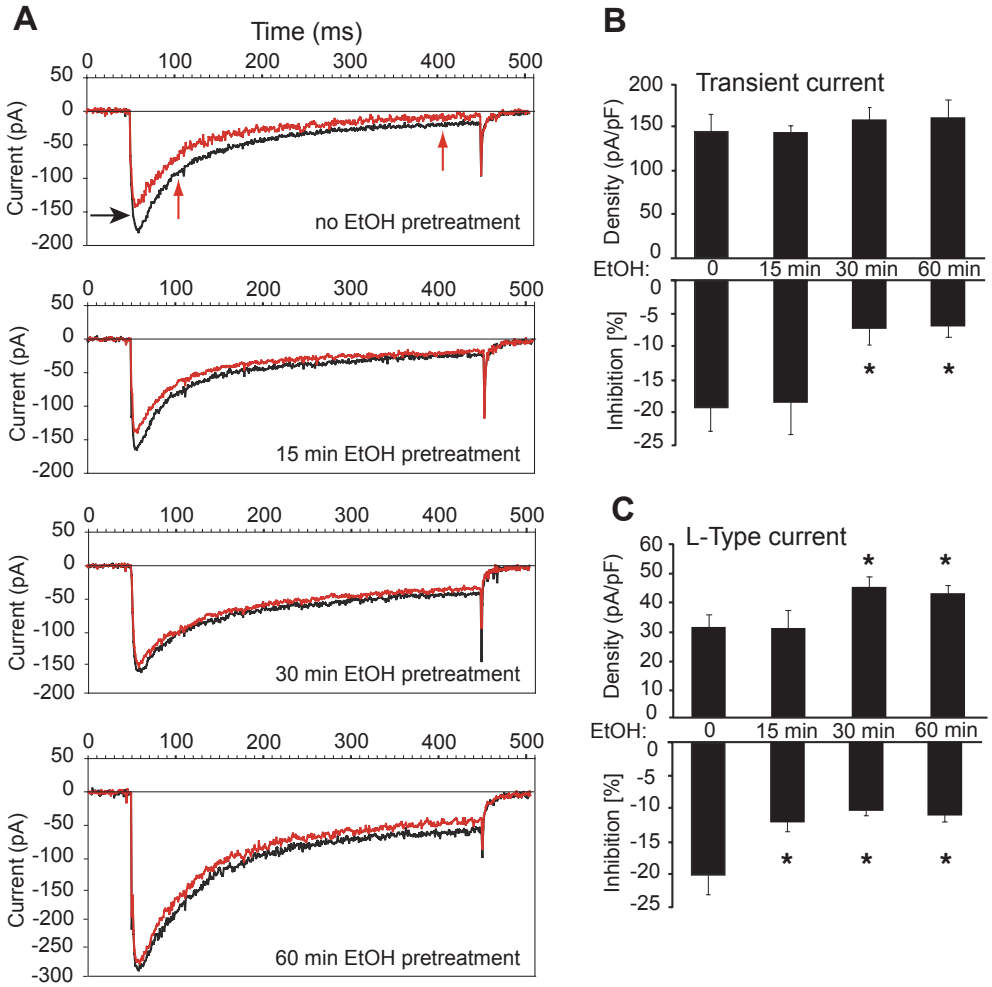

Fig. 2. Effect of ethanol (EtOH) on calcium channel currents in isolated terminals from cultured hypothalamo-neurohypophysial system (HNS) explants. (**A**) Barium current traces from isolated terminals under control conditions (black) or exposed to 50 mM EtOH (red) following preincubation of HNS explants with 20 mM EtOH for various periods of time (0 to 60 minutes). Arrows in top left-hand panel indicate where current measurements were taken for the histogram comparisons. Horizontal arrow indicates where the value for the transient currents was obtained (5 ms on either side of the peak). Two vertical arrows indicate where the value for the L-type current was obtained (mean of the current between the 2 arrows). (**B**) Summary of current density and acute EtOH effects on transient current. Histograms of current density (upward bars) and the effect of a 50 mM EtOH challenge (downward bars) in HNS explants preexposed to EtOH for increasing periods of time. (**C**) Current density and summary of EtOH challenge on L-type current. $n = 3$ to 4, $^*p < 0.5$.

significant up-regulation of the L-type VGCC in the plasma membrane of the AVP terminal by 1 hour EtOH exposure (Fig. 3*D*).

DISCUSSION

VGCCs including the L-type can be localized in presynaptic terminals (43, 44) including terminals located in the neurohypophysis (Lemos et al., 2012). They are part of the neuronal terminal machinery responsible for release of neurotransmitters (Lemos et al., 2012). We show here that molecular tolerance of alcohol regulation of AVP release from the NH terminals previously observed in VGCCs after weeks of in vivo EtOH drinking in the rat is evident in excised brain tissue after only minutes of alcohol exposure. EtOH exposure immediately inhibits AVP release from naïve terminals. However, terminals preexposed to 20 mM EtOH for 1 hour became tolerant to EtOH and a short exposure to this drug had little effect on release. Electrophysiological recordings indicated that both types of VGCCs (i.e., L-type and transient) tested in the neuronal terminal exhibited molecular tolerance to alcohol. The current density of L-type current significantly increased (approximately 50%), while its responsiveness to alcohol was significantly diminished (approximately 50%). Moreover, use of molecular imaging techniques indicates that the increase in the L-type VGCC in terminal membrane after the EtOH exposure is a result of the simultaneous increase of the 2 main subtypes of the L-type VGCC: 1.2 and 1.3. Increase in either of these 2 subtypes was insufficient to fully explain the increase of the L-type VGCC current in the terminal, while the combined expression of 1.2 and 1.3 subtypes corresponded to the elec-

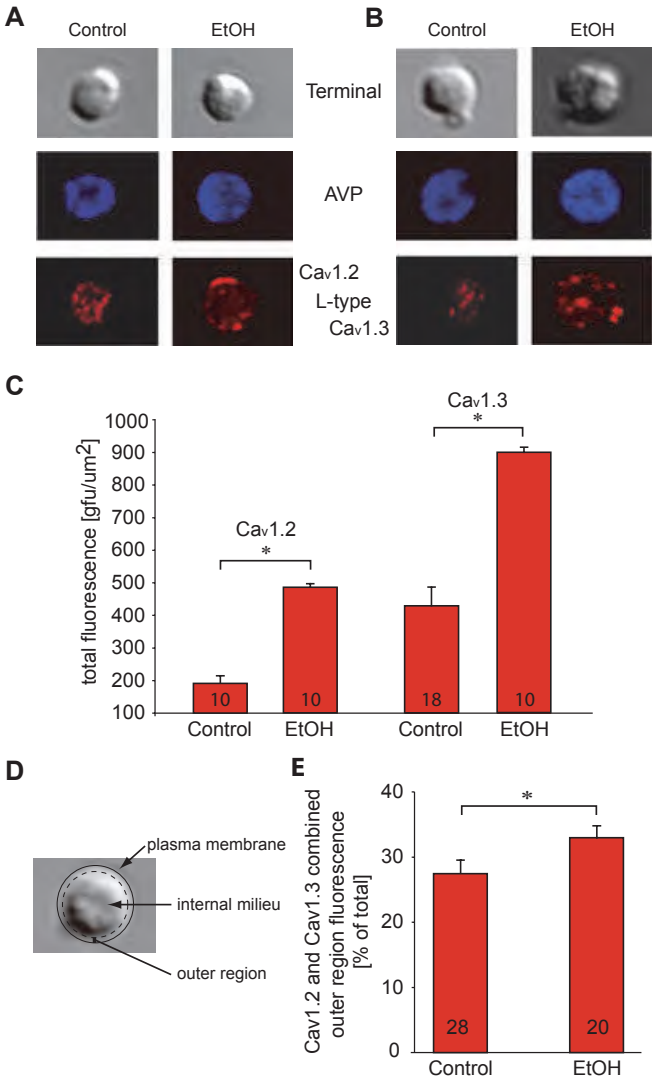

Fig. 3. Immunolabeling of L-type $Ca_V1.2$ and $Ca_V1.3$ α-subunits. (**A** and **B**) Representative images of individual neurohypophysial terminals isolated from posterior pituitary cultured in Neurobasal A medium in control, ethanol (EtOH)-free conditions (left) or exposed for 1 hour to 20 mM EtOH (right). Top row: differential interference contrast images of the terminals. Middle row: Staining showing neurotransmitter content of these terminals (AVP = vasopressin). Bottom row: Staining showing expression of the $Ca_V1.2$ (**A**) and $Ca_V1.3$ (**B**) subtypes of the L-type channel in AVP terminals. (**C**) Bar graph summary of immunofluorescent signal associated with the whole terminal of the individual subtypes of L-type calcium channel in AVP terminals. (**D**) A schematic showing the method to assess surface expression (the outer region of a terminal) of channels (Lemos et al., 2011; Ortiz-Miranda et al., 2010; Pietrzykowski et al., 2004). (**E**) Bar graph summary of L-type voltage-gated calcium channel expression ($Ca_V1.2$ + $Ca_V1.3$) in the outer region of the AVP terminal (*$p < 0.05$).

trophysiologically observed increase in density of the L current.

Interestingly, we not only observed an increase in the expression of L-type channels on the terminal surface but also within the milieu of the terminals. This would suggest that EtOH causes synthesis of new channels in addition to redistribution of preexisting channels within a terminal; that is, trafficking of channels from the terminal intracellular milieu to the terminal plasma membrane. Thus, 1-hour EtOH exposure may be sufficient to trigger de novo synthesis of these channels in the somatic compartment of the magnocellular neurons located in the supraoptic nucleus, which are then transported along axonal processes of these neurons to their terminals located in the neurohypophysis. Alternatively, local synthesis of new channels in the presynaptic terminal cannot be excluded. Regardless, whether new channels were produced remotely in the soma, or locally in the terminal, they were eventually inserted into the terminal plasma membrane. Interestingly, this mechanism seems to be very similar for both subtypes of the L-type VGCC, 1.2 and 1.3, despite their association with different scaffolding proteins (Zuccotti et al., 2011).

Both subtypes of the L-type VGCC, 1.2 and 1.3, can form a functional dyad with BK channels, in which calcium entry through the L-type VGCC activates the BK located in the immediate vicinity of the L-type VGCC: around 10 nm radius (Brandt et al., 2003; Lu et al., 2007; Marrion and Tavalin, 1998; Zuccotti et al., 2011). For the L-type VGCC, current inhibition produced by EtOH challenge is significantly reduced within 15 minutes of EtOH exposure, similar to the time frame previously reported for reduction of EtOH potentiation of the BK channel (Pietrzykowski et al., 2004). Thus, both members of the BK-VGCC functional dyad exhibit a compensatory response to EtOH within minutes of exposure (Fig. 4). For the transient I_{Ca}, the reduction of EtOH's effects is also evident within minutes, although in this case, it is not significantly reduced until after 30 minutes of EtOH preexposure. Thus, although we cannot state from our present data whether the temporal fine structure of acute tolerance for desensitization is the same for the L-type VGCC and the BK channel, we can certainly state that they both occur within minutes of exposure.

We (Pietrzykowski et al., 2004) have previously defined 2 independent components of molecular tolerance using the BK channel as a model: decreased EtOH potentiation of channel activity (component 1) and decreased channel density (component 2). For component 1 of molecular tolerance, the time course of desensitization to alcohol is very similar for BK and L-type VGCC, consistent with an upstream controller (Fig. 4). Our imaging results show that the second component of molecular tolerance of the L-type VGCC is an increase in this channel's density in the terminal plasma membrane. For this channel, the second component of molecular tolerance is evident within 30 minutes of EtOH exposure, which is significantly faster than that observed for the parallel reduction of current density in the BK channel (Pietrzykowski et al., 2004). Both channels are functionally and geographically tightly linked to each other. Therefore, a change in the amount of both, L-type VGCC (an increase) and BK (a decrease) in the plasma membrane of the terminal could serve as a compensatory mechanism to maintain a constant distance between these 2 channels, very important for the release of neurotransmitters from the terminal.

The transient I_{Ca} does not exhibit an altered current density within 1 hour of EtOH preexposure, although that may

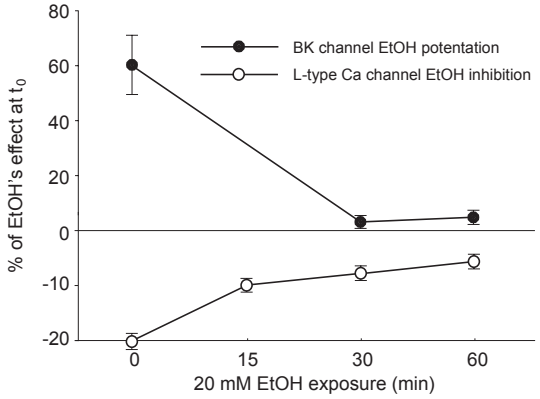

Fig. 4. Time course of the development of ethanol (EtOH) tolerance in large-conductance calcium-activated potassium (BK) versus voltage-gated calcium channels (VGCCs). Hypothalamo-neurohypophysial (NH) system explants were cultured on Millipore inserts and exposed to 20 mM EtOH for 0, 15, 30, or 60 minutes (n = 4 to 10). All 50 mM EtOH effects were normalized to control values at 0 minutes (t_0) of 20 mM EtOH preexposure, to facilitate comparison. EtOH tolerance of BK channels in the cultured NH terminals is manifested as decreased potentiation by EtOH, evident within 30 minutes of drug exposure (black circles). EtOH tolerance of L-type VGCCs in the cultured NH terminals is manifested as decreased inhibition by acute EtOH, evident within 15 minutes of drug exposure (white circles).

develop later. Although we do not have evidence for a role for miRNA in L-type VGCC molecular tolerance, we have previously suggested that miR-9, which is up-regulated in HNS neurons after short EtOH exposures, might provide a "molecular master switch" integrating the response of a number of downstream targets (Pietrzykowski et al., 2008, p. 284). Interestingly, miR-9 targets include not only the BK channel but also VGCC beta subunit (Pietrzykowski et al., 2008), which associates with both alpha subtypes of the VGCC studied here: 1.2 and 1.3.

We conclude that both the BK channel and the L-type VGCC require only a period of minutes of alcohol exposure to develop both components of molecular tolerance and that it remains a reasonable possibility that an integrated response mediated by an upstream controller may integrate alcohol molecular tolerance among different targets. In summary, synergistic EtOH effects on these 2 subtypes of the L-type VGCC lead to an important physiological consequence, that is a decrease in release of AVP.

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