Hyperpolarization-Activated Cation Current ($I_h$) Is an Ethanol Target in Midbrain Dopamine Neurons of Mice

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Ethanol stimulates the firing activity of midbrain dopamine (DA) neurons, leading to enhanced dopaminergic transmission in the mesolimbic system. This effect is thought to underlie the behavioral reinforcement of alcohol intake. Ethanol has been shown to directly enhance the intrinsic pacemaker activity of DA neurons, yet the cellular mechanism mediating this excitation remains poorly understood. The hyperpolarization-activated cation current, $I_h$, is known to contribute to the pacemaker firing of DA neurons. To determine the role of $I_h$ in ethanol excitation of DA neurons, we performed patch-clamp recordings in acutely prepared mouse midbrain slices. Superfusion of ethanol increased the spontaneous firing frequency of DA neurons in a reversible fashion. Treatment with ZD7288, a blocker of $I_h$, irreversibly depressed basal firing frequency and significantly attenuated the stimulatory effect of ethanol on firing. Furthermore, ethanol reversibly augmented $I_h$ amplitude and accelerated its activation kinetics. This effect of ethanol was accompanied by a shift in the voltage dependence of $I_h$ activation to more depolarized potentials and an increase in the maximum $I_h$ conductance. Cyclic AMP mediated the depolarizing shift in $I_h$ activation but not the increase in the maximum conductance. Finally, repeated ethanol treatment in vivo induced downregulation of $I_h$ density in DA neurons and an accompanying reduction in the magnitude of ethanol stimulation of firing. These results suggest an important role of $I_h$ in the reinforcing actions of ethanol and in the neuroadaptations underlying escalation of alcohol consumption associated with alcoholism.

I N T R O D U C T I O N

The dopaminergic projection from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and other limbic structures, termed the mesolimbic dopamine (DA) system, is critically involved in reward processing, behavioral reinforcement, and addictive behaviors. Several lines of evidence show that this system plays an important role in the reinforcing/rewarding actions of ethanol. Administration of DA antagonists, either systemically or directly into the NAc, suppresses ethanol-reinforced behavioral responses, such as ethanol drinking, self-administration, and preference (George et al. 1995; Kaczmarek and Kiefer 2000; Myers and Robinson 1999; Samson et al. 1993; Weiss et al. 1990), although neurotoxin-induced DA neuron lesion studies have produced some equivocal results (Ikemoto et al. 1997; Rassnick et al. 1993). The brain DA system has also been implicated in ethanol-induced euphoria and behavioral stimulation in humans (Ahlenius et al. 1973). Consistent with the role played by the mesolimbic DA system in the behavioral actions of ethanol, active and passive administration of ethanol elevates extracellular DA levels in the NAc (Di Chiara and Imperato 1988; Weiss et al. 1993), most likely through its effect at the VTA DA cell body region rather than the NAc terminal region (Budgyn et al. 2001; Yi and Gonzales 2000). Indeed, rats self-administer ethanol directly into the VTA (Gatto et al. 1994; Rodd et al. 2004) and ethanol facilitates action potential firing of DA neurons both in vivo (Gessa et al. 1985; More et al. 1984) and in vitro (Brodie and Appel 1998; Brodie et al. 1990). It has been shown that this stimulatory effect of ethanol results, at least partly, from its direct action on the intrinsic excitability of DA neurons (Brodie et al. 1999).

DA neurons display a slowly activating, inward current, termed $I_h$, in response to membrane hyperpolarization (Harris and Constanti 1995; Neuhoff et al. 2002). $I_h$, originally described in cardiac pacemaker cells (Brown and Difrancesco 1980), has been shown to contribute to the autonomous pacemaker activity of a variety of neurons (Chan et al. 2004; Luthi and McCormick 1998; Maccarelli and McBain 1996). DA neurons are also capable of spontaneous, low-frequency pacemaker discharge (1–5 Hz) independent of synaptic input drive (Grace 1991; Kitai et al. 1999). Recent studies have shown that $I_h$ is actively engaged in controlling the pacemaker frequency in a subset of DA neurons (Neuhoff et al. 2002; Seutin et al. 2001). A previous intracellular recording study has also demonstrated that ethanol can enhance $I_h$ in DA neurons (Brodie and Appel 1998). However, the contribution of $I_h$ to ethanol-induced modulation of DA neuron excitability is not known.

In the present investigation, we show that pharmacological blockade of $I_h$ attenuates ethanol enhancement of DA neuron pacemaker firing. Using whole cell voltage-clamp recordings, we found that ethanol facilitates the voltage gating of $I_h$ in a cAMP-dependent fashion and also increases the maximum $I_h$ conductance independent of cAMP. Furthermore, we present data demonstrating downregulation of $I_h$ density in DA neurons after repeated ethanol exposure in vivo, suggesting that $I_h$ plasticity may play an important role in the neuroadaptations underlying alcoholism.

M E T H O D S

Slices and solutions

Horizontal midbrain slices (200–220 μm) containing both the VTA and the substantia nigra pars compacta (SNc) were prepared from...
C57BL/6J mice [postnatal day 21 (P21) to P35]. Animals were anesthetized with halothane or isoflurane and then killed by cervical dislocation or decapitation in accordance with a protocol approved by the University of Texas Institutional Animal Care and Use Committee. Slices were cut using a vibratome (VT1000S; Leica, Nussloch, Germany) in ice-cold (4°C) physiological saline containing (in mM) 126 NaCl, 2.5 KCl, 1.2 NaH2PO4, 1.2 MgCl2, 2.4 CaCl2, 11 glucose, and 21.4 NaHCO3, saturated with 95% O2-5% CO2 (pH 7.4, 300 mOsm/kg) and then incubated in the same solution warmed to 35°C for ≥1 h before being used for electrophysiological recordings. MK-801 (50–100 μM) was added to the cutting and incubation solutions to block N-methyl-d-aspartate (NMDA)-mediated excitotoxicity. For recording, slices were placed in a recording chamber superfused with physiological saline (35°C) at 2.5–3 ml/min. Unless noted otherwise, pipette solutions used for whole cell and cell-attached recordings contained (in mM) 115 K-methylsulfate, 20 KCl, 1 MgCl2, 10 HEPES, 5 EGTA, 2 Mg-ATP, 0.2 Na-GTP, and 10 Na-phosphocreatine (pH 7.3, 280 mOsm/kg).

Electrophysiological recordings

Cells were visualized using a ×40 water-immersion objective on an upright microscope with IR/DIC optics (BX51WI; Olympus, Melville, NY). All recordings were performed in DA neurons identified by spontaneous pacemaker firing (1–5 Hz) and the presence of large Ih (>200 pA), evoked by a 1.5-s hyperpolarizing step from −55 to −105 mV. Whole cell voltage-clamp recordings were made at a holding potential of −55 mV unless stated otherwise. A MultiClamp 700A amplifier (Axon Instruments, Foster City, CA) was used to record the data, which were filtered at 1 kHz, digitized at 2 kHz, and collected on a personal computer using Axograph 4.9 (Axon Instruments).

Action potential firing was monitored using either perforated-patch or cell-attached recording configurations because the spontaneous firing of DA neurons is significantly distorted with a standard whole cell recording configuration (Morikawa et al. 2003). For perforated-patch recordings, the pipette was filled with a solution containing (in mM) 135 KCl and 10 HEPES (pH 7.3, 295 mOsm/kg) and then back-filled with the same solution added with gramicidin (50–250 μg/ml). The typical series resistance obtained 15–30 min after the formation of the gigaseal was 20–40 MΩ. Perforated-patch and cell-attached recordings gave equivalent results; hence, the data were pooled from both recording conditions.

Some of the firing recordings were done in the presence of 6,7-dinitroquinoxaline-2,3-dione (DNQX) (10 μM), picrotoxin (100 μM), and strychnine (1 μM) to block AMPA, GABAβ, and glycine-mediated synaptic inputs. The NMDA-mediated input was routinely blocked by pretreatment with MK-801 (50–100 μM), as described in the preceding text.

Drugs

Drugs were applied by superfusion to the slice, except for cAMP analogues, Rp-cAMPs and Sp-cAMPS, which were infused into the cytosol through the whole cell pipette. Superfusion of forskolin (10 μM), an activator of adenylyl cyclase, produced variable effects on Ih and hence was not used in the present study. This was most likely due to the well-documented facilitation of neurotransmitter release by forskolin (Chavis et al. 1998) because a variety of neurotransmitters can modulate Ih via activation of G-protein-coupled receptors in DA neurons (Cathala and Paupardin-Tritsch 1997, 1999; Jiang et al. 1993; Liu et al. 2003). DNQX, MK-801, and ZD7288 were purchased from Tocris Cookson (Ellisville, MO). All other chemicals were obtained from Sigma/RBI (St. Louis, MO).

Repeated ethanol treatment

Mice received once daily intraperitoneal injections of either normal saline or 2 g/kg ethanol (20% wt/vol in saline) for 5 consecutive days. Midbrain slices were prepared 24 h after the final injection and were used for electrophysiological recordings. No more than two data were obtained from a single animal in this series of experiments. Repeated ethanol treatment had no significant effect on the cell capacitance in both the VTA and the SNc (data not shown), suggesting that there was no significant change in the size of DA neurons.

Data analysis

Ih was evoked once per minute by a 1.5-s hyperpolarizing step to −105 mV from −55 mV. Ih amplitude was defined as the difference in the current values measured at the onset, after the capacitative transient has subsided, and the end of the voltage step. The effect of ethanol on Ih was determined with respect to the average of control and washout values. The activation time constant of Ih was determined by fitting the activating phase of the current trace with a single exponential function over the initial 500 ms of the hyperpolarizing step. This gave much more consistent values in each recorded cell than fitting the entire activating phase during the 1.5-s hyperpolarizing step with a double-exponential function.

For the construction of Ih activation curves, 1.5-s hyperpolarizing steps to various potentials (−55 to −125 mV) were applied from a holding potential of −45 mV and tail currents were measured at −105 mV. These experiments were done in TEA-Cl (10 mM), with equimolar reduction of NaCl, to block noninactivating voltage-dependent K+ conductances. Tail current amplitudes at −105 mV, after subtraction of the current following no hyperpolarizing step, were plotted as a function of test potentials. The obtained curve was fitted with a Boltzmann function: \( I = I_{\text{max}}/[1 + \exp(V - V_{1/2})/s] \), where \( I_{\text{max}} \) is the maximal tail current amplitude, \( V \) is the test potential, \( V_{1/2} \) is the half-activation potential, and \( s \) is the slope factor. \( I_{\text{max}} \) was normalized to the cell capacitance to estimate \( I_h \) density in each cell. When comparing saline- and ethanol-treated mice for \( I_h \) density and \( V_{1/2} \), these values were routinely obtained 10 min after establishing the whole cell configuration.

Data are expressed as means ± SE. Statistical significance was determined by paired or unpaired Student’s t-test. The difference was considered significant at \( P < 0.05 \).

RESULTS

Ethanol stimulates DA neuron firing via \( I_h \)

The spontaneous firing of DA neurons was monitored with perforated-patch or cell-attached current-clamp recordings in midbrain slices from C57BL/6J mice. DA neurons displayed spontaneous pacemaker-like firing at 1–5 Hz, as reported previously (Grace 1991). Superfusion of ethanol (100 mM) reversibly increased the firing frequency by 18.4 ± 1.7% (from 2.27 ± 0.10 to 2.66 ± 0.11 Hz, \( n = 33, P < 0.0001 \); Fig. 1, A and B). Eleven of these 33 recordings were done in the presence of antagonists of ionotropic neurotransmitter receptors (see METHODS). The effect of ethanol was not significantly different in the absence and presence of these antagonists (19.4 ± 2.4%, \( n = 22 \) vs. 16.3 ± 2.2%, \( n = 11, P = 0.41 \); Fig. 1C). Ethanol produced nearly identical increases in the firing frequency in the VTA and the SNc (18.5 ± 2.6%, \( n = 16 \) vs. 18.2 ± 2.3%, \( n = 17, P = 0.93 \); Fig. 1D). Ethanol (50 mM) increased the firing frequency by 10.2 ± 2.0% (from 2.38 ± 0.21 to 2.62 ± 0.23 Hz, \( n = 8, P < 0.01 \); Fig. 1B), whereas 25 mM ethanol produced a small increase (approximately 5%) in one of five cells tested.
Furthermore, ZD7288 treatment dramatically reduced the firing frequency by 30–40% (33.3 \pm 2.3% (n = 7), P < 0.01; Fig. 3B). Ethanol (50 mM) also enhanced \( I_h \) by 11.7 \pm 1.7% (from 43.6 \pm 6.8 to 48.4 \pm 7.2 pA, n = 5, P < 0.01; Fig. 3B), although 20–25 mM ethanol did not have a measurable effect. Ethanol enhancement of \( I_h \) was accompanied by an acceleration of the activation kinetics (Fig. 3AI). Thus ethanol (100 mM) reduced the activation time constant of \( I_h \) from 384 \pm 53 to 267 \pm 25 ms (n = 11, P < 0.01; Fig. 3C). It has been reported that neurotransmitters can produce an apparent inhibition of \( I_h \) in DA neurons secondary to a reduction in the membrane input resistance, which would worsen space clamp of the recorded cell in voltage-clamp recordings (Watts et al. 1996). Hence, it is possible that ethanol-induced augmentation of \( I_h \) was due to an increase in the membrane input resistance and improved space clamp. However, ethanol (100 mM) actually reduced the membrane input resistance from 344 \pm 50 to 254 \pm 44 \text{M} \Omega (n = 11, P < 0.0001). Ethanol also caused variable changes in the holding current, ranging from inward to outward, which were not further investigated in the present study.

To gain a mechanistic insight into the ethanol action, we further investigated the effect of ethanol on the voltage depen-

\text{Antg (–)} and presence [Antg (+)] of major neurotransmitter receptors. D: comparison of the effects of ethanol in the VTA and the SNc.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure1}
\caption{Ethanol facilitates dopamine (DA) neuron firing. A: time graph illustrating the effect of ethanol (100 mM) on DA neuron firing. This experiment was done with a perforated-patch configuration. Representative traces of action potential firing in control (C) and in ethanol (E) are shown in the inset. B: summary bar graph showing the effects of 50 and 100 mM ethanol. C: comparison of the effects of ethanol in the absence [Antg (–)] and presence [Antg (+)] of major neurotransmitter receptors. D: comparison of the effects of ethanol in the VTA and the SNc.}
\end{figure}

To examine the role played by \( I_h \) in ethanol stimulation of firing, we used ZD7288, an \( I_h \) blocker (Harris and Constanti 1995). Using whole cell voltage-clamp recordings, we confirmed that ZD7288 (30 \mu M) completely and irreversibly abolished \( I_h \) in \(~10\) min (n = 5; Fig. 2A). ZD7288 produced no significant changes in the holding current and the membrane input resistance at a holding potential of \(-55\) mV (data not shown). Because prolonged ZD7288 treatment (>20 min) can produce effects independent of \( I_h \) blockade (Chevaleyre and Castillo 2002), we tested the effect of 10-min superfusion of ZD7288 (30 \mu M) on firing. ZD7288 irreversibly decreased the firing frequency with a time course similar to its effect on \( I_h \) (Fig. 2B). The reduction in the firing frequency produced by ZD7288 was of similar magnitude in the VTA and the SNc (28.2 \pm 4.1%, n = 7 vs. 33.2 \pm 7.9%, n = 7, \( P = 0.58 \); Fig. 2C).

Furthermore, ZD7288 treatment dramatically reduced the stimulatory effect of ethanol on firing from 17.6 \pm 2.9 to 0.5 \pm 3.9% (n = 7, \( P < 0.01 \); Fig. 2, B and D). Ethanol produced a small inhibition of firing after ZD7288 treatment in two of seven cells, suggesting the presence of an inhibitory action of ethanol when \( I_h \) is blocked. On the other hand, injection of a hyperpolarizing current (\(-20\) to \(-60\) pA), which decreased the firing frequency by 30–40% (33.3 \pm 3.8%, n = 3), did not significantly affect the magnitude of ethanol response (from 22.6 \pm 5.8 to 28.8 \pm 9.1%, \( P = 0.20 \); Fig. 2D, right), showing that a mere reduction in the firing frequency does not attenuate the effect of ethanol. In 11 cells where both ethanol and ZD7288 were tested, the magnitude of ethanol-induced enhancement of firing was positively correlated with that of ZD7288-induced suppression (\( r = 0.74 \); Fig. 2E). Altogether, these data suggest that \( I_h \) contributes to the stimulation of pacemaker activity by ethanol.

Ethanol enhances \( I_h \) via two mechanisms

We next directly examined the effect of ethanol on \( I_h \) using whole cell voltage-clamp recordings. \( I_h \) was evoked once a minute by a 1.5-s hyperpolarizing voltage step to \(-105\) from \(-55\) mV. Superfusion of ethanol (100 mM) reversibly increased \( I_h \) amplitude by 26.2 \pm 4.2% (from 501 \pm 61 to 621 \pm 69 pA, n = 11, \( P < 0.001 \); Fig. 3, A and B). Ethanol (50 mM) also enhanced \( I_h \) by 11.7 \pm 1.7% (from 43.6 \pm 6.8 to 48.4 \pm 7.2 pA, n = 5, \( P < 0.01 \); Fig. 3B), although 20–25 mM ethanol did not have a measurable effect. Ethanol enhancement of \( I_h \) was accompanied by an acceleration of the activation kinetics (Fig. 3A). Thus ethanol (100 mM) reduced the activation time constant of \( I_h \) from 384 \pm 53 to 267 \pm 25 ms (n = 11, P < 0.01; Fig. 3C). It has been reported that neurotransmitters can produce an apparent inhibition of \( I_h \) in DA neurons secondary to a reduction in the membrane input resistance, which would worsen space clamp of the recorded cell in voltage-clamp recordings (Watts et al. 1996). Hence, it is possible that ethanol-induced augmentation of \( I_h \) was due to an increase in the membrane input resistance and improved space clamp. However, ethanol (100 mM) actually reduced the membrane input resistance from 344 \pm 50 to 254 \pm 44 \text{M} \Omega (n = 11, P < 0.0001). Ethanol also caused variable changes in the holding current, ranging from inward to outward, which were not further investigated in the present study.

To gain a mechanistic insight into the ethanol action, we further investigated the effect of ethanol on the voltage depen-

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2}
\caption{\( I_h \) is involved in ethanol stimulation of firing. A: time graph showing the effect of ZD7288 (30 \mu M) on \( I_h \). Representative traces of \( I_h \) in control (C) and in ZD7288 (ZD) are shown in the inset. B: ZD7288 (30 \mu M) irreversibly decreased the firing frequency and blocked the effect of ethanol (100 mM). This experiment was performed with a cell-attached configuration. C: comparison of the effects of ZD7288 on the firing frequency (FF) before and after ZD7288 treatment (left) and hyperpolarizing current injection (right) is plotted in each cell. E: magnitude of ethanol-induced increase in FF is plotted vs. the magnitude of ZD7288-induced suppression. - - -, linear fit to the data. ** \( P < 0.01 \).}
\end{figure}
Accordingly, the magnitude of the depolarizing shift in maximal \( I_h \) was significantly smaller in Rp-cAMPS than in control (6.0 ± 2.3% in Rp-cAMPS vs. 8.3 ± 2.2% in control, \( P = 0.49 \); Fig. 5G). The basal \( V_{1/2} \) value and the maximal \( I_h \) density in Rp-cAMPS were not significantly different from those values in control (\( P = 0.90 \) for \( V_{1/2} \) and \( P = 0.39 \) for maximal \( I_h \) density; Fig. 5, D and F); hence \( I_h \) is not modulated by tonic levels of cAMP. On the other hand, the basal \( V_{1/2} \) value was more depolarized in seven cells dialyzed with Sp-cAMPS (\( P < 0.05 \) vs. control; Fig. 5D), showing that \( I_h \) gating can be facilitated by cAMP in DA neurons. Sp-cAMPS prevented the depolarizing shift in \( I_h \) activation produced by ethanol (Fig. 5C). On the contrary, ethanol induced a small but significant hyperpolarizing shift in \( V_{1/2} \) in the presence of Sp-cAMPS (from \(-88.8 \pm 1.9 \) to \(-91.1 \pm 1.6 \) mV, \( n = 7 \), \( P < 0.05 \); Fig. 5E). The reason for this hyperpolarizing shift in \( V_{1/2} \) caused by ethanol in Sp-cAMPS is not known. Sp-cAMPS did not affect the maximal \( I_h \) density (\( P = 0.50 \) vs. control; Fig. 5F) or the magnitude of ethanol effect on maximal \( I_h \) (\( P = 0.99 \) vs. control; Fig. 5G). Altogether these results demonstrate that ethanol enhances \( I_h \) via two mechanisms: a cAMP-dependent mechanism causing a depolarizing shift in the voltage dependence of activation and a cAMP-independent mechanism resulting in augmentation of the maximal \( I_h \) conductance.

**I\(_h\) downregulation after repeated ethanol exposure**

Repeated exposure to ethanol produces various compensatory changes in the mesolimbic system to counter the stimulatory action of ethanol (Weiss and Porrino 2002). Recent studies indicate that \( I_h \) undergoes activity-dependent plasticity under various neuropathological conditions (Chaplan et al. 2003; Chen et al. 2001a; Shah et al. 2004). Therefore we asked if repeated ethanol exposure in vivo could induce plastic changes in the expression and properties of \( I_h \) in DA neurons. Mice received daily injections of either saline or ethanol (2 g/kg ip), to produce blood ethanol concentrations of 40–50 mM (Grisel et al. 2002), for 5 days, and recordings were made 1 day after the final injection. Although the neuroadaptive consequences in the brain can be different after active versus passive administration of drugs of abuse (Jacobs et al. 2003), the passive ethanol treatment protocol used in this study has been shown to cause facilitation of ethanol drinking in C57BL/6J mice (Lessov et al. 2001).

We first obtained the maximum \( I_h \) density and \( V_{1/2} \) of \( I_h \) activation in each cell using the tail current analysis illustrated...
in Fig. 4. $I_h$ density was significantly reduced after repeated ethanol treatment in both the VTA and the SNc (Fig. 6A). Overall, $I_h$ density was 11.4 ± 0.7 pA/pF ($n = 20$) and 8.8 ± 0.4 pA/pF ($n = 19$) in saline- and ethanol-treated mice, respectively ($P < 0.01$). A previous study using neonatal mice (P12–P15) has reported that DA neurons in the SNc generally have higher density of $I_h$ than in the VTA (Neuhoff et al. 2002); however, we found no significant difference between the VTA and the SNc in this study using older mice (P21–P35). The $V_{1/2}$ value (Fig. 6B) and the activation kinetics of $I_h$ (Fig. 6C) were not significantly different between saline- and ethanol-treated mice in both the VTA and the SNc, implying that the gating properties of $I_h$ were not affected by repeated ethanol exposure. These results suggest that repeated ethanol treatment induces downregulation of functional $I_h$ expression in DA neurons. The membrane input resistance at a holding potential of −55 mV was similar in saline- and ethanol-treated mice (Fig. 6D), consistent with the $I_h$ activation curve exhibiting no activation at −55 mV (Fig. 5A).

To determine the functional consequence of $I_h$ downregulation after repeated ethanol exposure, we next compared the firing activity in saline- and ethanol-treated mice. The basal firing frequency was not significantly different in these two types of mice (2.18 ± 0.10 Hz, $n = 27$ in saline-treated mice vs. 2.31 ± 0.09 Hz, $n = 29$ in ethanol-treated mice, $P = 0.35$; Fig. 7A). However, the magnitude of decrease in the firing frequency produced by ZD7288 (30 μM) was significantly reduced after repeated ethanol treatment in both the VTA and the SNc (Fig. 7B). Overall, the magnitude of ZD7288-induced suppression of firing was 32.6 ± 2.7% ($n = 10$) and 17.8 ± 2.5% ($n = 12$) in saline- and ethanol-treated mice ($P < 0.001$). Thus the contribution of $I_h$ to the pacemaker activity was diminished after repeated ethanol exposure. Furthermore, the magnitude of increase in the firing frequency caused by acute ethanol challenge (100 mM) was also significantly smaller in ethanol-treated mice (11.3 ± 2.0%, $n = 13$) than in saline-treated mice (19.0 ± 2.7%, $n = 10$, $P < 0.05$; Fig. 7C), in agreement with the involvement of $I_h$ in the stimulatory effect of ethanol. Ethanol-induced enhancement of $I_h$ was similar in
both types of animals (Fig. 7D), demonstrating that the ethanol sensitivity of $I_h$ itself was not altered. Taken together, repeated ethanol exposure in vivo resulted in tolerance to ethanol stimulation of DA neuron activity, most likely due to down-regulation of $I_h$.

**DISCUSSION**

Ethanol modulates the excitability of neurons by acting on a multitude of ion channels (Harris 1999). In this study, we have identified $I_h$, a canonical pacemaker current, as an ethanol target in mediating the stimulation of DA neuron firing. Furthermore, repeated ethanol exposure produced down-regulation of functional $I_h$ in DA neurons. The resulting reduction in the stimulatory effect of ethanol may contribute to the progressive increase in alcohol consumption in alcoholic individuals (Schuckit 1994).

$I_h$ and pacemaker activity

ZD7288 (30 μM) suppressed the firing of DA neurons in both the VTA and the SNc, consistent with the involvement of $I_h$ in the regulation of pacemaker activity. This observation is in contrast with a recent study by Neuhoff et al. (2002) demonstrating that $I_h$ is engaged in the pacemaker frequency control of a subpopulation of DA neurons only in the SNc. In that study, ZD7288 selectively inhibited pacemaker activity of SNc DA neurons that do not express the calcium-binding protein calbindin, whereas ZD7288 had no effect in calbindin-positive SNc DA neurons or in any of VTA DA neurons. In line with this, calbindin-negative SNc DA neurons had a higher density of $I_h$ compared with other subpopulations of DA neurons, whereas we detected similar $I_h$ densities in the VTA and the SNc. One difference between the two studies is the age of animals used, i.e., P21–P35 in this study versus P12–P15 in the study of Neuhoff et al., although both used the same strain of mice (C57BL/6J). In this regard, developmental increase in functional $I_h$ expression between P1 and P20 has been reported in hippocampal pyramidal neurons (Vasilyev and Barish 2002). Therefore it is conceivable that maturation of $I_h$ expression during development could affect their impact on DA neuron pacemaker activity. In support of the prominent role of $I_h$ in mature animals, it has been shown that ZD7288 can suppress the spontaneous firing of a majority of VTA DA neurons in adult rats (Seutin et al. 2001). It should also be noted that calbindin-positive VTA DA neurons, which have minimal expression of $I_h$, displayed a highly irregular firing pattern in the study of Neuhoff et al. It is likely that these VTA neurons were not included in our study because we recorded only from those neurons with regular pacemaker activity.

**Acute ethanol effect**

An initial study using brain slices demonstrated that ethanol increased the firing frequency of DA neurons in a concentration-dependent fashion at 20–320 mM with an EC$_{50}$ of ~100 mM (Brodie et al. 1990), the concentration mainly used in the present investigation. Ethanol facilitation of DA neuron firing was not significantly affected by blockade of major synaptic inputs, including GABAergic inputs, consistent with the direct stimulatory action of ethanol (Brodie et al. 1999). An in vivo study has shown that ethanol suppresses the firing of GABAergic neurons in the VTA (Stobbs et al. 2004), an effect that may lead to disinhibition of DA neurons. GABAergic tone on DA neurons may not be large enough in a brain slice preparation to detect this component of ethanol action.

Inhibition of $I_h$ by ZD7288 treatment caused marked attenuation of the stimulatory effect of ethanol. Ethanol was also able to facilitate $I_h$, further supporting the role of $I_h$ as an ethanol target. In slow pacemaking neurons like DA neurons with an interspike interval of 0.2–1 s, $I_h$ is thought to be activated by the large afterhyperpolarization (AHP) after each action potential, thus curtailing the AHP. Consistent with this scenario, augmentation of $I_h$ by ethanol reduced the size and duration of the AHP (Fig. 1A, inset), whereas blocking $I_h$ with ZD7288 had an opposite effect (data not shown).

Four genes encoding $I_h$, termed HCN1-4, have been identified, each having distinct biophysical properties and expression profiles (Kaupp and Seifert 2001). The major type expressed in the VTA appears to be HCN2 (Notomi and Shigemoto 2004), which has an activation time constant of 200–500 ms and is highly sensitive to cAMP-induced facilitation of the voltage gating. In line with this, the activation time constant of $I_h$ was ~400 ms in this study. Furthermore, ethanol facilitation of $I_h$ had a cAMP-dependent component, most likely via stimulation of adenylyl cyclase and subsequent increase in cytoplasmic cAMP levels (Yoshimura and Tabakoff 1995), mediating a depolarizing shift in the voltage dependence of activation. Ethanol also augmented the maximal $I_h$ amplitude in a cAMP-independent fashion. It is possible that ethanol can directly bind to the $I_h$ channel protein itself, as has been shown for other proteins (Harris 1999), and increase the single-channel conductance and/or maximal open probability of individual $I_h$ channels.

Ethanol stimulation of firing was not entirely eliminated after ZD7288 treatment in three of seven cells (Fig. 2D), suggesting that $I_h$ may not be the only target mediating the effect of ethanol. Our preliminary data show that ethanol can inhibit rapidly inactivating A-type K$^+$ currents in DA neurons (unpublished observation). A-type K$^+$ channels are abundantly expressed in DA neurons and play an opposing role to $I_h$ in controlling the pacemaker frequency (Liss et al. 2001), raising the possibility that these channels may be another target of ethanol. Appel et al. (2003) reported that ethanol excitation of DA neurons was blocked by quinidine, a blocker of multiple types of K$^+$ channels, but not by ZD7288, in rats. Therefore ethanol excitation of DA neurons may involve different mechanisms in different species of animals.

**Repeated ethanol exposure**

$I_h$ density was reduced by ~25% in DA neurons after repeated ethanol treatment in vivo (2 g/kg, once daily for 5 days). The reduction in functional $I_h$ resulted in a smaller contribution of $I_h$ to pacemaker activity, as shown by a significantly smaller inhibition of firing by ZD7288. The magnitude of ethanol stimulation of firing was also reduced after repeated ethanol treatment, although ethanol facilitated $I_h$ to a similar degree in saline- and ethanol-treated mice. Thus reduced expression of functional $I_h$ is likely responsible for this tolerance to the excitatory effect of ethanol. The ethanol tolerance that we observed in this study is in contrast to the development of sensitization reported previously using more vigorous and...
prolonged ethanol treatment protocol (3.5 kg/kg, twice daily for \( \approx 21 \) days) (Brodie 2002). It would be interesting to examine if differential plasticity of \( I_h \) underlies the difference in these two studies.

We confirmed that 100 mM ZD7288, which inhibits \( I_h \) amplitude by \( \approx 25\% \) (Harris and Constanti 1995), was able to depress the firing frequency by \( \approx 10\% \) in naïve mice \((n = 5)\), indicating that a small reduction in functional \( I_h \) can affect DA neuron firing. However, the basal firing frequency was not decreased after repeated ethanol exposure. This may be a consequence of plastic changes in other ion channels that counterbalance the reduction in \( I_h \). In this regard, it has been reported that A-type \( K^+ \) currents described in the preceding text are co-regulated with \( I_h \) in pylocytic neurons, resulting in homeostatic regulation of their pacemaker activity (MacLean et al. 2003).

**Functional significance**

Mereu et al. (1984) reported that intravenous injections of ethanol (0.5–2 g/kg) dose-dependently enhanced the firing of SNc DA neurons by 40–80% in awake rats. Although the actual ethanol level was not measured in the study of Mereu et al., blood and brain ethanol concentrations have been shown to reach 60–70 mM after an intravenous injection of 1 g/kg ethanol (Robinson et al. 2002). In the present investigation using a brain slice preparation, ethanol at 50–100 mM produced smaller increases (10–20%) in the DA neuron firing frequency. The firing of DA neurons in vivo is diverted from a simple pacemaker pattern due to the influence of active synaptic inputs. Thus it is likely that dual actions of ethanol on the intrinsic pacemaker activity and on synaptic inputs work in concert to attain its full stimulatory effect on DA neuron firing in vivo. It is well documented that \( I_h \) can dampen the impact of synaptic inputs by reducing the membrane input resistance (Magee 1998). Although \( I_h \) does not contribute to the input resistance of DA neurons held at \( \approx 55 \) mV, it is certainly involved in the dynamic regulation of the input resistance when DA neurons are tonically firing. The predominant input to DA neurons is GABAergic, which exerts powerful inhibitory control of firing (Tepper et al. 1998). These GABAergic inputs are thought to be suppressed by acute ethanol exposure in vivo (Mereu and Gessa 1985; Stobbs et al. 2004). Ethanol-induced enhancement of \( I_h \) may further reduce the inhibitory influence of GABAergic inputs and facilitate the disinhibition of DA neurons. On the other hand, an increase in presynaptic GABA release has been reported after withdrawal from ethanol treatment (Melis et al. 2002). The reduction in \( I_h \) density observed in this study may augment the postsynaptic impact of these facilitated GABAergic inputs, thus contributing to the marked reduction in DA neuron activity during ethanol withdrawal (Diana et al. 1993). Therefore \( I_h \) may play a role not only in the acute reinforcing action of ethanol but also in the hypodopaminergic state underlying the emotional/motivational component of ethanol withdrawal symptoms.

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