Ethanol Effects on Dopaminergic Ventral Tegmental Area Neurons During Block of $I_h$: Involvement of Barium-Sensitive Potassium Currents

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McDaid J, McElvain MA, Brodie MS. Ethanol effects on dopaminergic ventral tegmental area neurons during block of $I_h$: involvement of barium-sensitive potassium currents. J Neurophysiol 100: 1202–1210, 2008. First published July 9, 2008; doi:10.1152/jn.00994.2007. The dopaminergic neurons of the ventral tegmental area (DA VTA neurons) are important for the rewarding and reinforcing properties of drugs of abuse, including ethanol. Ethanol increases the firing frequency of DA VTA neurons from rats and mice. Because of a recent report on block of ethanol excitation in mouse DA VTA neurons with ZD7288, a selective blocker of the hyperpolarization-activated cationic current ($I_h$), we examined the effect of ZD7288 on ethanol excitation in DA VTA neurons from C57Bl/6J and DBA/2J mice and Fisher 344 rats. Ethanol (80 mM) caused only increases in firing rate in mouse DA VTA neurons in the absence of ZD7288, but in the presence of ZD7288 (30 μM), ethanol produced a more transient excitation followed by a decrease of firing. This same biphasic phenomenon was observed in DA VTA neurons from rats in the presence of ZD7288 only at very high ethanol concentrations (160–240 mM) but not at lower pharmacologically relevant concentrations. The longer latency ethanol-induced inhibition was not observed in DA VTA neurons from mice or rats in the presence of barium (100 μM), which blocks G protein–linked potassium channels (GIRKs) and other inwardly rectifying potassium channels. Ethanol may have a direct effect to increase an inhibitory potassium conductance, but this effect of ethanol can only decrease the firing rate if $I_h$ is blocked.

Introduction

Dopaminergic pathways are important in mediating the rewarding and reinforcing properties of numerous drugs of abuse (Koob et al. 1998; Wise 1996). Ethanol increases dopamine in target brain regions of the ventral tegmental area (VTA) in rats and mice (Di Chiara and Imperato 1988; Zapata et al. 2006). Ethanol directly stimulates dopaminergic (DA) VTA neurons, increasing the spontaneous firing frequency in dissociated neurons from rats (Brodie et al. 1999b) with an excitatory potency that is similar to that observed in brain slices of rats (Brodie et al. 1990) and mice (Brodie and Appel 2000). Excitation of DA VTA neurons may underlie the rewarding/reinforcing effects of ethanol, so it is important to understand how alcohol produces this effect. The mechanism of this excitation is under study, but it is likely that an ethanol-induced reduction of potassium current is involved because the potassium channel blocker quinidine can block ethanol excitation (Appel et al. 2003). In addition, ethanol decreases M-current in DA VTA neurons (Koyama et al. 2007), and this action may be responsible in part for ethanol-induced excitation of DA VTA neurons. A hyperpolarization activated cationic current ($I_h$) is a characteristic of mesencephalic dopamine neurons (Grace 1987; Neuhoff et al. 2002), and we have reported that ethanol increases the amplitude of $I_h$ (Brodie and Appel 1998).

A recent report indicated that ethanol excitation in DA VTA neurons of C57Bl/6J mice could be reduced by the compound ZD7288, which is a selective blocker of $I_h$ (Okamoto et al. 2006). This observation is different from that reported in rats, in which no significant change in ethanol excitation was observed in the presence of ZD7288 (Appel et al. 2003). In light of this controversy, we felt it necessary to study this phenomenon further. This study presents the results of studies in two mouse strains and in F344 rats in an effort to determine the role $I_h$ may play in ethanol-induced excitation of DA VTA neurons.

Methods

Animals

Both rats and mice were used in these studies. Fischer 344 (F344; 90–150 g) were obtained from Harlan Sprague-Dawley (Indianapolis, IN); C57Bl/6J (C57; 3–5 wk old) and DBA/2J (DBA; 3–5 wk old) mice were obtained from Jackson Laboratories (Bar Harbor, ME). Animals used in this study were treated in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all experimental methods were approved by the Animal Care Committee of the University of Illinois at Chicago.

Preparation of brain slices

Brain slices containing the VTA were prepared from the subject animals as previously described (Brodie 2002; Brodie et al. 1999a). Briefly, after rapid removal of the brain, the tissue was blocked coronally to contain the VTA and substantia nigra; the cerebral cortices and a portion of the dorsal mesencephalon were removed. The tissue block was mounted in the vibratome and submerged in chilled artificial cerebrospinal fluid (ACSF). Coronal sections (400 μm thick) were cut, and the slice was placed onto a mesh platform in the recording chamber. The slice was totally submerged in ACSF maintained at a flow rate of 2 ml/min; the temperature in the recording chamber was kept at 35°C. The composition of the ACSF in these experiments was (in mM) 126 NaCl, 2.5 KCl, 1.24 NaH2PO4, 2.4 CaCl2, 1.3 MgSO4, 26 NaHCO3, and 11 glucose. The ACSF was saturated with 95% O2-5% CO2 (pH = 7.4). Equilibration time of at least 1 h was allowed after placement of tissue in the recording chamber before electrodes were placed in the tissue.

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Cell identification

The VTA was clearly visible in the fresh tissue as a gray area medial to the darker substantia nigra and separated from the nigra by white matter. Recording electrodes were placed in the VTA under visual control. DA neurons have been shown to have electrophysiological characteristics very different from non-DA neurons in the mesencephalon (Grace and Bunney 1984; Lacey et al. 1989). Only those neurons that were anatomically located within the VTA and that conformed to the criteria for DA neurons established in the literature and in this laboratory (Lacey et al. 1989; Mueller and Brodie 1989) were studied. These criteria include broad action potentials, slow spontaneous firing rate (0.5–5 Hz), and a regular interspike interval. Cells were not tested with opiate agonists as has been done by other groups to further characterize and categorize VTA neurons (Margolis et al. 2006a). It should be noted that some neurons with the characteristics we used to identify DA VTA neurons may not, in fact, be dopamine containing (Margolis et al. 2006b).

Drug administration

Drugs were added to the ACSF by means of a calibrated infusion pump from stock solutions 100–1,000 times the desired final concentrations. The addition of drug solutions to the ACSF was performed in such a way as to permit the drug solution to mix completely with ACSF before this mixture reached the recording chamber. Final concentrations were calculated from ACSF flow rate, pump infusion rate, and concentration of drug stock solution. The small volume chamber (~300 µl) used in these studies permitted the rapid application and washout of drug solutions. Typically, drugs reach equilibrium in the tissue after 2–3 min of application.

A stock solution of 95% ethanol (vol/vol USP) was used in the pump, and infusion of ethanol never exceeded 1% of the flow rate of the ACSF. Ethanol was administered for 6 min to ensure that measurements were made after the full ethanol concentration was reached in the tissue, and the peak drug effect was attained. The behaviorally active range for blood ethanol concentrations in the rat extends from 40 (sedation) to 90 mM (loss of righting reflex) (Majchrowicz and Hunt 1976); the lethal blood ethanol concentration in rats is ~200 mM (LD₅₀ = 202 mM) (Haggard et al. 1940). Rats will self-administer 44–55 mM ethanol directly into the VTA, indicating that this concentration is reinforcing in the whole animal (Rodd-Henricks et al. 2000), whereas mice with continuous access to ethanol can achieve plasma ethanol concentrations as high as 120 mM (Jelic et al. 1998). This study primarily examined an ethanol concentration of 80 mM, a pharmacologically relevant, sublethal concentration in both the rat and mouse. Note that lower ethanol concentrations will self-administer 44–55 mM ethanol directly into the VTA, indicating that this concentration is reinforcing in the whole animal (Rodd-Henricks et al. 2000), whereas mice with continuous access to ethanol can achieve plasma ethanol concentrations as high as 120 mM (Jelic et al. 1998). This study primarily examined an ethanol concentration of 80 mM, a pharmacologically relevant, sublethal concentration in both the rat and mouse. Note that lower ethanol concentrations have been shown to have effects on behavioral performance. We used 80 mM ethanol and higher concentrations in these studies because these concentrations of ethanol produce reliable and robust excitation. Lower concentrations of ethanol (20–40 mM) have been shown to produce qualitatively similar excitation.

ZD7288 was purchased from Tocris (Ellisville, MO). Most of the chemicals used to prepare the extracellular media were purchased from Sigma (St. Louis, MO). Sulpiride and bicuculline were also purchased from Sigma. Barium chloride was purchased from Fisher Scientific (Fair Lawn, NJ).

Extracellular recording

Extracellular recording electrodes were made from 1.5-mm-diam glass tubing with filament and were filled with 0.9% NaCl. Tip resistance of the microelectrodes ranged from 4 to 8 MΩ. The Fintronics amplifier used in these recordings includes a window discriminator, the output of which was fed to both a rectilinear pen recorder, and a computer-based data acquisition system that was used for on-line and off-line analysis of the data. The multiplexed output of the Fintronics amplifier was displayed on an analog storage oscilloscope for accurate adjustment of the window levels used to monitor single units. An IBM-PC-based data acquisition system was used to calculate, display, and store the frequency of firing over 5-s and 1-min intervals. Firing rate was determined before and during drug application. Firing rate was calculated over a 1-min interval immediately before drug administration and a 1-min interval during the peak drug effect; drug-induced changes in firing rate were expressed as the percentage change from the control firing rate according to the formula \[ \frac{[FR_D - FR_C]}{FR_C} \times 100 \] , where FRD is the firing rate during the peak drug effect and FRc is the control firing rate. The change in firing rate thus is expressed as a percentage of the initial firing rate, which controls for small changes in firing rate that may occur over time. This formula was used to calculate both excitatory and inhibitory drug effects. Excitation was defined as the peak increase in firing rate produced by the drug (e.g., ethanol) greater than the predrug baseline. Inhibition was defined as the lowest firing rate below the predrug baseline; in cases in which no inhibition of firing rate was observed, the value of 0% inhibition was used.

For comparison of the time course of effects on firing rate, the data were normalized and averaged. Firing rates over 1-min intervals were calculated and normalized to the 1-min interval immediately before ethanol administration. These normalized data were averaged by synchronizing the data to the ethanol administration period, and graphs of the averaged data were made.

Statistical analysis

Averaged numerical values were expressed as the mean ± SE. The significance of firing rate changes before and after a single drug concentration was assessed with a paired t-test. For effects of multiple drug concentrations or more than one drug, an appropriate one- or two-way ANOVA was used, followed by Student-Newman-Keuls post hoc comparisons when needed (Kenakin 1987). Statistical analyses were performed with SigmaStat software (Systat, San Jose, CA).

RESULTS

Neuronal characteristics

Spontaneously active neurons that conformed to the criteria set by this study and the literature for DA VTA neurons (see Methods) were recorded in a series of extracellular recording experiments. Data from one slice per animal and one neuron per slice are used in these results. Baseline firing rates of DA VTA neurons from F344 rats and C57 and DBA mice were similar to those previously reported (Brodie and Appel 2000; Brodie et al. 1995). Baseline firing rates for F344 rats were in the range of 0.83–2.21 Hz, with a mean of 1.39 ± 0.15 Hz, n = 12. For C57 mice, baseline firing rates ranged from 0.66 to 4.49 Hz, with a mean firing rate of 1.89 ± 0.16 Hz, n = 31, whereas for DBA mice, the baseline firing rate was from 0.66 to 3.06 Hz, with a mean of 1.76 ± 0.13 Hz, n = 21. Ethanol was added to the superfusate at concentrations of 80–240 mM. Most experiments conducted in this study involved the use of ethanol at a concentration of 80 mM. In F344 rats, 80 mM ethanol reversibly increased the spontaneous firing rate of DA VTA neurons by 19.76 ± 1.68%. For C57 mice, 80 mM ethanol increased firing by 16.55 ± 5.63%, and for DBA mice, the increase was 24.68 ± 2.89%; the excitatory effect of ethanol was significantly greater in DBA mice than C57 mice (1-way ANOVA; F(2,59) = 5.24, P < 0.01). The larger effect of ethanol on DA VTA neurons from DBA mice compared with those from C57 mice is consistent with data from a previous study from this laboratory (Brodie and Appel 2000).
Effect of ZD7288 on ethanol excitation of C57 and DBA mouse DA VTA neurons

ZD7288 (30 μM) has been shown to completely block the hyperpolarization activated cationic current (I_K) in DA VTA neurons of both mice and rats (Neuhoff et al. 2002; Okamoto et al. 2006); based on this evidence from the literature, we used ZD7288 at 30 μM to assess the effect of block of I_K on ethanol excitation in our experiments. Superfusion of ZD7288 (30 μM) completely blocks I_K within 10 min of bath application (Okamoto et al. 2006); because ZD7288 significantly decreased the firing rate in both C57 and DBA DA VTA neurons, we tested ethanol 15–20 min after application of ZD7288 to allow the firing rate to stabilize at a new, lower baseline. In C57 mice, ZD7288 decreased spontaneous firing by 33.28 ± 5.33% (paired t-test, t = 5.01, df = 7, P < 0.005, n = 8); in DBA mice, there was a decrease of 28.94 ± 5.44% (paired t-test, t = 3.99, df = 14, P < 0.005, n = 15). Ethanol was not tested until the lower baseline firing rate induced by ZD7288 was established and stable.

As shown in Figs. 1 and 2A, I_K blockade with ZD7288 did not significantly reduce the peak excitatory effect of 80 mM ethanol in DA VTA neurons of C57 mice, but a significant inhibitory effect of ethanol was observed [2-way repeated-measures ANOVA; F(7,31) = 6.33, P < 0.05, n = 8; post hoc Student-Newman-Keuls, P > 0.05 for ethanol excitation before and after ZD7288, P < 0.05 for inhibition before and after ZD7288]. This observation that, in ZD7288, the excitatory effect of ethanol was followed by an inhibitory effect is important because this phenomenon was not observed before I_K block. The inhibitory effect of ethanol in ZD7288 was quantified using the same method used to calculate the excitatory effect of ethanol. As seen in Figs. 1A and 2A, the excitatory effect of 80 mM ethanol in the C57 DA VTA neuron consists typically of a sustained plateau of excitation during the period of drug application; however, in the presence of ZD7288, similar peak excitatory effect is reached, but this is not sustained and gradually becomes an inhibitory effect before the end of the 6-min period of application. A similar time course of ethanol effect on firing rate in ZD7288 was observed in DA VTA neurons of DBA mice (Fig. 2B); for DA VTA neurons from DBA mice, the peak excitatory effect of ethanol was reduced significantly [2-way repeated-measures ANOVA, F(14,59) = 26.76, P < 0.001, n = 15; post hoc Student-Newman-Keuls, P < 0.05]. Of the eight C57 DA VTA neurons tested, only one neuron did not show ethanol-induced inhibition in ZD7288; mean data presented include the results in all eight cells tested, whether they were inhibited or not. Of 15 DBA neurons similarly tested, 2 neurons did not show ethanol-induced inhibition, and 2 neurons stopped firing completely in the presence of ethanol and ZD7288; mean data presented include the results in all 15 cells tested.

To compare the results of these studies more directly to the earlier study of ZD7288 and ethanol in DA VTA neurons from C57 mice (Okamoto et al. 2006), we averaged the normalized responses over 1-min intervals to facilitate comparison of responses in the absence and presence of ZD7288. These normalized data were averaged and are displayed in the graphs in Fig. 2. For DA VTA neurons from C57 mice under control conditions, the typical pure excitation induced by 80 mM ethanol is observed; in the presence of 30 μM ZD7288, a transient excitation is followed by inhibition, which reversed at 7–9 min after washout (Fig. 2A). In DA VTA neurons from DBA mice (Fig. 2B), clear excitation was observed under

FIG. 1. Effects of the selective I_K blocker ZD7288 (30 μM) on the firing rate response to 80 mM ethanol in dopaminergic neurons of the ventral tegmental area (DA VTA neurons) from C57 or DBA mice. A: a typical ratemeter graph of an extracellular single unit recording of firing rate of a single C57 DA VTA neuron. Firing rate is plotted as a function of time (vertical bars), and the horizontal bars indicate the duration of application of ethanol. Before application of ZD7288, 80 mM ethanol produced an increase in the firing rate with no subsequent inhibition. Note that 30 μM ZD7288 (ZD7288) decreased the baseline firing rate by 42.72%. In the presence of ZD7288, there was a biphasic effect of ethanol; brief ethanol-induced excitation was followed by inhibition of firing below baseline levels. This inhibition recovered on washout of ethanol. B and C: pooled data from experiments such as that shown in Fig. 1A. For each ethanol response, both excitatory (e) and inhibitory (i) responses were measured. Bars labeled “e” represent mean peak excitatory responses, and bars labeled “i” represent mean peak inhibitory responses to ethanol. B: ethanol (80 mM) effect on firing frequency of DA VTA neurons from C57 mice in the absence (Control) and presence (ZD7288) of 30 μM ZD7288. C: ethanol (80 mM) effect on firing frequency of DA VTA neurons from DBA mice in the absence (Control) and presence (ZD7288) of 30 μM ZD7288.
control conditions, but in the presence of 30 μM ZD7288, a brief excitation was also followed by a profound and prolonged inhibition. Note that, in DA VTA neurons from both strains of mice, the normalized firing rate was <1.0 in the presence of ethanol, and this inhibition of firing persisted after the ethanol was removed from the perfusate but did show reversal with washout. It is unclear why the inhibition outlasts the duration of ethanol application in DA VTA neurons from both strains of mice.

Effect of barium on ethanol excitation/inhibition of C57 DA VTA neurons in the absence and presence of ZD7288

Figure 3 shows the effect of the prior application of barium (100 μM) on the response to 80 mM ethanol in the absence and presence of ZD7288 (30 μM) in DA VTA neurons of C57 mice. Barium has been shown to block dopamine and baclofen-mediated GIRK currents in Xenopus oocytes (Werner et al. 1996) and in DA neurons from substantia nigra and VTA (Cruz et al. 2004; Lacey et al. 1988) while having no effect on \( I_h \) (van et al. 2005). Barium also blocks other inwardly rectifying

FIG. 2. Pooled time-course of the effects of 80 mM ethanol on firing frequency for C57 and DBA VTA DA neurons before (●) and during (○) the application of ZD7288 (30 μM). Firing rate over 1-min intervals for each cell was calculated and normalized to the firing rate immediately before the administration of ethanol (time = 0). The data for the firing rates over these 1-min intervals was averaged across the DA VTA neurons tested in the absence and presence of ZD7288. A: in C57 VTA DA neurons, the initial peak excitatory effect of 80 mM ethanol was not altered significantly by ZD7288 (30 μM), but during ethanol application, there was a decrease in the sustained excitatory effect followed by a decrease of the firing rate below baseline; this effect gradually recovered during the washout of ethanol. B: in DA VTA neurons from DBA mice, there was a decrease in the peak and sustained excitatory effect of 80 mM ethanol in the presence of ZD7288 (30 μM), and, as in C57 mice, there was a statistically significant decrease of the firing rate below baseline, which did not fully recover.

FIG. 3. Effects of barium (100 μM) on the response to ethanol of DA VTA neurons from C57 mice in the absence and presence of 30 μM ZD7288. A: a typical ratemeter graph of an extracellular single unit recording of firing rate of a single C57 DA VTA neuron. Firing rate is plotted as a function of time (vertical bars), and the horizontal bars indicate the duration of application of the agents as labeled. Barium (100 μM) was present during the entire time of the recording shown. Ethanol (80 mM) increased the firing rate before the addition of ZD7288 and also in the presence of ZD7288 without subsequent ethanol-induced inhibition. Note that the baseline firing rate was reduced by 31.01% in the presence of 30 μM ZD7288 (ZD7288), but the inhibitory effect of 80 mM ethanol was not observed. B: pooled time-course data from experiments such as that shown in A, illustrating the effects of 80 mM ethanol on firing frequency for C57 VTA DA neurons in the presence of barium (100 μM) before (●) and during (○) the application of ZD7288 (30 μM). Firing rates over 1-min intervals for each cell was calculated and normalized to the firing rate immediately before the administration of ethanol (time = 0). The data for the firing rates over these 1-min intervals was averaged across the DA VTA neurons tested in the absence and presence of ZD7288. C: pooled data comparing peak excitatory effects of 80 mM ethanol in control conditions (Control), in the presence of 100 μM barium (Barium), and in the presence of 100 μM barium and 30 μM ZD7288 (Barium + ZD7288). There was no difference statistical difference observed in the ethanol-induced excitation observed under these 3 conditions (1-way repeated-measures ANOVA, \( P > 0.05 \)).
potassium channels (Morishige et al. 1993, 1994). Barium (100 
\mu M) increased the baseline firing rate of C57 DA VTA neurons by 45.93 ± 7.43\% (paired t-test, \( t = -3.89, df = 4, P < 0.05, n = 5 \)) but did not significantly increase the excitatory response to 80 mM ethanol [1-way repeated-measures ANOVA, \( F(2,14) = 2.72, P > 0.05, n = 5 \)]. In the presence of barium, there was no inhibitory effect of ethanol in ZD7288 in any of the cells tested (5/5), and ethanol produced a pattern of sustained excitation as is usually seen in the absence of ZD7288.

**Effect of ZD7288 on ethanol excitation of F344 rat DA VTA neurons**

In DA VTA neurons from F344 rats, the peak excitatory effect of 80 mM ethanol was not altered by \( I_h \) blockade with 30 \mu M ZD7288 (paired t-test, \( P > 0.05 \)), nor was there any inhibitory effect of 80 mM ethanol on the firing of F344 rats neurons (Fig. 4), in contrast to the ethanol-induced inhibition seen in the presence of ZD7288 in DA VTA neurons of C57 and DBA mice (cf., Fig. 1). These results are consistent with data from a previous study from this laboratory (Appel et al. 2006). A small but significant increase in firing rate was produced by ZD7288 alone (1.19 ± 0.14 Hz before ZD7288; 1.38 ± 0.16 Hz in 30 \mu M ZD7288; paired t-test, \( P < 0.05, n = 6 \)). Some effects of ethanol in rat brain slices were only observed at ethanol concentrations far in excess of 200 nM, a concentration associated with respiratory failure in rats (Haggard et al. 1940). As we have recently shown depolarization blockade of firing produced by high ethanol concentrations (200–400 mM) and concentrations of longer chain alcohols (Appel et al. 2006), we examined the effect of ZD7288 on excitation produced by high ethanol concentrations (160–240 mM) (Fig. 5). As shown in Fig. 5A, the excitatory effect of a high concentration of ethanol (200 mM) on a F344 DA VTA neuron in the absence of ZD7288 typically consisted of a sustained plateau of excitation during the period of drug application; however, in the presence of 30 \mu M ZD7288, a similar peak excitatory effect was reached, but this was not sustained, and gradually the firing rate was reduced below baseline before the end of the 6-min period of ethanol application. This effect is similar to that observed with 80 mM ethanol in DA VTA neurons from C57 and DBA mice. For the population of cells tested, an increase of firing of 45.56 ± 4.08 (n = 3) was produced by 160 mM ethanol; 200 (n = 3) and 240 mM (n = 3) elicited similar large and reversible increases in the firing frequency (Fig. 5B). In the presence of ZD7288, the excitatory effect of ethanol at 200 and 240 mM ethanol was reduced, and each ethanol excitation was followed by significant inhibition [2-way repeated-measures ANOVA, \( F(1,35) = 19.97, P < 0.05 \); post hoc Student-Newman-Keuls, \( P < 0.05 \)].

![FIG. 4. Effects of the selective \( I_h \) blocker ZD7288 (30 \mu M) on the firing rate response to 80 mM ethanol in DA VTA neurons from F344 rats. A: a typical ratemeter graph of an extracellular single unit recording of firing rate of a single F344 rat DA VTA neuron. Firing rate is plotted as a function of time (vertical bars), and the horizontal bars indicate the duration of application of ethanol. Note that 80 mM ethanol (EtOH 80) produced excitation without subsequent inhibition in the absence and presence of 30 \mu M ZD7288 (ZD7288), in contrast to the biphasic response to ethanol seen in DA VTA neurons from C57 and DBA mice in the presence of ZD7288 (cf., Fig. 1). B: the pooled responses of DA VTA neurons from F344 rats to ethanol (80 mM) alone (Control) or in the presence of 30 \mu M ZD7288 (ZD7288).](http://jn.physiology.org/)

**DISCUSSION**

Ethanol excitation of DA VTA neurons is likely to be important in the rewarding and reinforcing properties of ethanol (Koob et al. 1998; Wise 1996). We have previously shown that the firing frequency of DA VTA neurons is increased by ethanol in vitro, both in brain slices (Brodie et al. 1990) and in dissociated neurons (Brodie et al. 1999b); ethanol increases the firing rate of DA VTA neurons in vivo as well (Gessa et al. 1985). This study indicated that blockade of \( I_h \) using the selective \( I_h \) blocker ZD7288 shows an inhibitory action of ethanol that follows the excitatory action of ethanol. This inhibition was produced only in the presence of ZD7288, and,
Although it was observed in mouse DA VTA neurons at close to pharmacological concentrations, it was only observed in rat DA VTA neurons at high concentrations that would be lethal in vivo. This inhibition of firing produced by ethanol could be blocked by barium in both mouse and rat DA VTA neurons, suggesting an involvement of inwardly rectifying potassium (IRK) channels in this phenomenon, possibly GIRK channels. A recent report indicated that ethanol excitation in DA VTA neurons from C57 mice could be antagonized by ZD7288 (Okamoto et al. 2006). Our study demonstrated no significant effect of ZD7288 on the peak excitatory response of DA VTA neurons in C57 mice. However, we did observe inhibition in firing after ethanol excitation in both C57 and DBA mice. Because ethanol produces both excitation and inhibition in C57 mice in the presence of ZD7288, these effects may cancel out overall, resulting in an apparent blockade of ethanol-induced excitation of firing rate. This may be the case with the results of Okamoto et al. 2006. Other technical reasons for the discrepancy between that previous study and this study may include the plane of the brain slice section (coronal vs. horizontal) and recording method used (extracellular vs. whole cell patch), but it is clear from this study and our earlier work (Appel et al. 2003) that ZD7288 does not block ethanol excitation but may reveal an inhibitory phenomenon that can counteract the ongoing excitation.

Effects of ZD7288 on DA VTA firing rate

In both C57 and DBA DA VTA neurons, ZD7288 decreased the baseline firing rate by similar amounts; this effect may have been caused by the blockade of I_h because the firing rate effect of ZD7288 showed the same dose dependency as the reduction in I_h (Okamoto et al. 2006). A link between spontaneous firing rate and I_h is reinforced by the time required for the firing to reach a new baseline in ZD7288, an effect that closely correlates with the time taken for ZD7288 to fully block I_h (Harris et al. 2003). Ethanol produced a greater increase in firing rate, but no subsequent inhibition. In the presence of ZD7288, 200 mM ethanol (EtOH 200) produced an increase in the baseline firing rate of 27.7% immediately before 200 mM ethanol was reapplied. In the presence of 30 μM ZD7288, ethanol excitation was followed by an ethanol-induced inhibition. B: the pooled responses of DA VTA neurons from F344 rats to 200 mM ethanol (160, 200, and 240 μM) alone (Control) or to ethanol in the presence of 30 μM ZD7288 (ZD7288). Bars labeled “e” represent mean peak excitatory responses, and bars labeled “i” represent mean peak inhibitory responses to ethanol. ZD7288 decreased the peak excitatory (e) response to 200 and 240 mM ethanol (2-way repeated-measures ANOVA, P < 0.05) in DA VTA neurons from F344 rats. There was no inhibitory effect (i) of ethanol at any of these concentrations before the application of ZD7288.

FIG. 5. Effects of the selective I_h blocker ZD7288 (30 μM) on the firing rate response to high concentrations of ethanol (160, 200, and 240 mM) in DA VTA neurons from F344 rats. A: a typical ratemeter graph of an extracellular single-unit recording of the firing rate of a single F344 rat DA VTA neuron.

Firing rate is plotted as a function of time (vertical bars), and the horizontal bars indicate the duration of application of the agents as labeled. Before application of ZD7288, 200 mM ethanol produced a firing rate increase without subsequent inhibition. The addition of 30 μM ZD7288 produced an increase in the baseline firing rate of 27.7% immediately before 200 mM ethanol was reapplied. In the presence of 30 μM ZD7288, ethanol excitation was followed by an ethanol-induced inhibition. B: the pooled responses of DA VTA neurons from F344 rats to 200 mM ethanol (160, 200, and 240 μM) alone (Control) or to ethanol in the presence of 30 μM ZD7288 (ZD7288). Bars labeled “e” represent mean peak excitatory responses, and bars labeled “i” represent mean peak inhibitory responses to ethanol. ZD7288 decreased the peak excitatory (e) response to 200 and 240 mM ethanol (2-way repeated-measures ANOVA, P < 0.05) in DA VTA neurons from F344 rats. There was no inhibitory effect (i) of ethanol at any of these concentrations before the application of ZD7288.

FIG. 6. Effects of barium (100 μM) on the response to 200 mM ethanol of DA VTA neurons from F344 rats in the absence or presence of 30 μM ZD7288. A: a typical ratemeter graph of an extracellular single-unit recording of firing rate of a single F344 rat DA VTA neuron. Firing rate is plotted as a function of time (vertical bars), and the horizontal bars indicate the duration of application of the agents as labeled. Before ZD7288 administration, 200 mM ethanol (EtOH 200) produced an increase in firing rate. In the presence of ZD7288, 200 mM ethanol produced a greater increase in firing rate, but no subsequent inhibition. B: the pooled responses of DA VTA neurons from F344 rats to 200 mM ethanol (EtOH 200) produced an increase in firing rate. In the presence of ZD7288, 200 mM ethanol produced a greater increase in firing rate, but no subsequent inhibition.

Effects of barium (100 μM) on the response to 200 mM ethanol of DA VTA neurons from F344 rats in the absence or presence of 30 μM ZD7288. A: a typical ratemeter graph of an extracellular single-unit recording of firing rate of a single F344 rat DA VTA neuron. Firing rate is plotted as a function of time (vertical bars), and the horizontal bars indicate the duration of application of the agents as labeled. Before ZD7288 administration, 200 mM ethanol (EtOH 200) produced an increase in firing rate. In the presence of ZD7288, 200 mM ethanol produced a greater increase in firing rate, but no subsequent inhibition. B: the pooled responses of DA VTA neurons from F344 rats to 200 mM ethanol (EtOH 200) produced an increase in firing rate. In the presence of ZD7288, 200 mM ethanol produced a greater increase in firing rate, but no subsequent inhibition.
and Constanti 1995; Okamoto et al. 2006). Note that excessively high (≥100 μM) concentrations of ZD7288 have been shown to shut off spontaneous firing of DA neurons completely (Neuhoff et al. 2002; Seutin et al. 2001), an effect that may be related to the ability of ZD7288 to decrease synaptic transmission (Chen 2004; Chevaleyre and Castillo 2002; Inaba et al. 2006) and/or exocytosis (Gonzalez-Iglesias et al. 2006). Careful studies of the kinetics of ZD7288 and the effects on firing rate may be needed to determine the relationship between blockade of \( I_h \) and firing rate changes. In contrast to the effects in DA VTA neurons from mice, in DA VTA neurons from Fischer344 rats, ZD7288 produced a small but significant increase in the baseline firing rate; ZD7288 produced decreases in firing in other similar studies (Appel et al. 2003; Seutin et al. 2001).

The difference in the magnitude of the inhibitory responses of rat and mouse VTA neurons may be caused by a greater role of \( I_h \) in mouse DA VTA neurons. Relative age differences between the rats and mice are unlikely to be the reason for these differences. Although careful comparison of age-related and strain/species-related differences is beyond the scope of this study, qualitatively similar results were observed in DA VTA neurons from C57 mice 8–12 wk old (data not shown). Clearly, the large decrease in firing rate produced by ZD7288 alone in this study suggests that firing frequency is significantly regulated by \( I_h \) in mice. In contrast, in the rat DA VTA neurons, ZD7288 had a small excitatory (this study) to a small inhibitory (Appel et al. 2003) effect on spontaneous firing rate. The greater role of \( I_h \) in mouse DA VTA neurons may also account for the observation of the apparent ethanol activation of barium-sensitive potassium channels at lower ethanol concentrations in mouse neurons than in rat neurons.

**Role of \( I_h \) in DA VTA neurons**

In many neurons, \( I_h \) is a pacemaker current (Pape 1996) and is necessary for regular firing of some central neurons. This does not seem to be true in DA VTA neurons, because 30 μM ZD7288 reduces but does not completely inhibit the spontaneous activity of these neurons. This inhibition produced by ZD7288 is noticeably larger in DA VTA neurons from mice but more modest and variable in DA VTA neurons from rats. Higher concentrations of ZD7288 have been shown to block spontaneous activity, but the conclusion of that study suggested a non-\( I_h \)-related mechanism for that cessation of firing (Seutin et al. 2001). Although the effect of opening of \( I_h \) channels is to produce a depolarizing influence, a more important role of these channels in the VTA may be their characteristic opening in response to hyperpolarizing membrane potentials and the resultant decrease in membrane resistance. In response to a hyperpolarizing potential in a portion of the membrane (near a GIRK channel, for example), \( I_h \) channels open and decrease the membrane resistance in that membrane region. An increase in membrane conductance (decreased membrane resistance) caused by activation of that \( I_h \) may decrease the efficiency of passive transmission of that hyperpolarization over the membrane. The lowest threshold for generation of an action potential is at the axon hillock, so it is the ultimate site at which firing frequency is controlled. Decreased efficiency of passive transmission of hyperpolarizing current toward the axon hillock would decrease the influence of that hyperpolarizing current on firing rate. Simply stated, opening of \( I_h \) channels would shunt out or short circuit that hyperpolarization. A similar role for \( I_h \) in the subiculum has been proposed (van et al. 2006). Blockade of \( I_h \) by ZD7288 would eliminate this shunting, and as a result of this reduction of \( I_h \), hyperpolarizing influences (like the opening of potassium channels) would have a greater effect on firing frequency.

**Role of barium-sensitive currents in excitatory and inhibitory effects of ethanol**

Many inwardly rectifying potassium channels (IRKs) are sensitive to blockade by barium, and one candidate for the barium-sensitive current in DA VTA neurons is a GIRK current. The effect of ethanol on other IRKs has not been studied as extensively as ethanol effects on GIRKs. A comparison of the direct effects of ethanol on GIRKs and IRK channels expressed in Xenopus oocytes showed an effect on the GIRK but not IRK channels (Kobayashi et al. 1999). An enhancement of GIRK function by ethanol has been shown at concentrations as low as 10 mM in Xenopus oocytes and 75 mM in rat cerebellar granule cells in culture, and this enhancement is thought to be strongest in the GIRKs expressing the GIRK2 subunit (Lewohl et al. 1999), which is the predominant GIRK subunit expressed in the VTA (Karschin et al. 1996; Liao et al. 1996). Stimulation of dopamine \( D_2 \) receptors leads to inhibition of DA VTA firing because of activation of GIRK channels (Lacey et al. 1988); this raises the possibility that ethanol-induced excitation of dopamine neurons could lead to the increased release of dopamine onto postsynaptic \( D_2 \) autoreceptors, which may inhibit firing. We applied the \( D_2 \) receptor antagonist sulpiride (10 μM) at a concentration that blocks the inhibitory effects of bath applied dopamine, but a significant inhibitory effect of ethanol persisted in the presence of 10 μM sulpiride and 30 μM ZD7288 [data not shown, 2-way repeated-measures ANOVA, \( F(2,47) = 8.81, P < 0.005 \); post hoc Student-Newman-Keuls, \( P < 0.05, n = 8 \)]. Because numerous neurotransmitters activate GIRKs in the VTA (e.g., adenosine, GABA via GABA\(_A\) receptors, etc.), it is not possible to rule out the specific action of ethanol on all possible receptors that might produce inhibition by GIRK activation. One important non–GIRK-mediated inhibitory influence on DA VTA neurons is GABA acting at GABA\(_A\) receptors. Because the sensitivity of GABA\(_A\) receptors may be altered by ethanol (Weight et al. 1992), we tested the GABA\(_A\) antagonist bicuculline against the inhibitory effect of ethanol, but this did not block the inhibitory effect of ethanol in the presence of ZD7288 [data not shown, 2-way repeated-measures ANOVA, \( F(2,23) = 2.43, P > 0.05, n = 4 \)]. Barium was the only agent we found that completely blocked the inhibitory effect of ethanol in ZD7288; this may indicate that ethanol can directly affect a barium-sensitive current, thus bypassing any second messenger system (Kobayashi et al. 1999). Previously, we showed that \( I_h \) blockade with 30 μM ZD7288 increased the inhibitory effect of dopamine on DA VTA cell firing (Liu et al. 2003); this action of ZD7288 may also be a consequence of the reduced ability of the cell membrane to shunt out the hyperpolarizing effects of \( D_2 \) receptor–mediated GIRK currents after a reduction of \( I_h \) (Liu et al. 2003).

A similar enhancement of the inhibitory effect of dopamine has been observed in the presence of a reduced \( I_h \) after repeated
ethanol exposure (Wang et al. 2006). A reduction in \( I_h \) may lead to a reduction in the ability of the DA VTA cell membrane to shunt out the inhibitory effects of ethanol, leading to the inhibitory effects of ethanol mediated by a barium-sensitive current, as observed in this study. Several recent studies have shown that repeated ethanol treatment leads to decreased \( I_h \) in DA VTA neurons of rats and mice (Hopf et al. 2007; Okamoto et al. 2006). This phenomenon may also explain the decrease in the excitatory effect of ethanol in DA VTA neurons of C57 mice subjected to repeated ethanol treatment (Okamoto et al. 2006). A decrease in \( I_h \) produced by chronic ethanol treatment may reduce the shunting of inhibitory currents, and under these conditions, inhibitory effects of ethanol (e.g., activation of barium-sensitive currents) may have a greater influence on firing rate, resulting in an apparent decrease in ethanol-induced excitation. Longer ethanol treatment periods than those used in some earlier studies (Okamoto et al. 2006) may lead to additional cellular adaptations such as a greater decrease in \( I_h \) and possibly may result in a reduction in the sensitivity of IRK (or other barium-sensitive currents) to ethanol; this later IRK adaptation may supersede the apparent partial tolerance produced by a decrease in \( I_h \) and could result in a sensitization to ethanol excitation, as has been reported (Brodie 2002). Reduction of an inhibitory component of ethanol excitation should produce an apparent increase in ethanol-induced excitation (see Fig. 3).

In conclusion, the excitatory effects of ethanol in DA VTA neurons of rats and mice may be attenuated by the effect of ethanol on barium-sensitive potassium channels. Although an ethanol-induced increase in \( I_h \) is not directly responsible for the excitatory effects of ethanol, it may play a role in facilitating excitation by reducing concurrent inhibitory influences of ethanol on barium-sensitive currents.

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