Effects of Ethanol on Midbrain Neurons: Role of Opioid Receptors

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Background: Although ethanol addiction is believed to be mediated by the mesolimbic dopamine system, originating from the ventral tegmental area (VTA), how acute ethanol increases the activity of VTA dopaminergic (DA) neurons remains unclear.

Method: Patch-clamp recordings of spontaneous firings of DA and GABAergic neurons in the VTA in acute midbrain slices from rats.

Results: Ethanol (20–80 mM) excites DA neurons, and more potently depresses firing of local GABAergic neurons. The ethanol-induced excitation of DA neurons is considerably attenuated by DAMGO (Tyr-D-Ala-Gly-*N*-Me-Phe-Gly-ol enkephalin), a μ-opioid agonist that suppresses firing of GABAergic neurons, or by naloxone, a general opioid antagonist. The ongoing opioid-induced facilitation of DA cell firing (revealed by naloxone) is enhanced by ethanol, probably by an increase in opioid release or action.

Conclusion: Ethanol excites VTA DA neurons at least partly by increasing ongoing opioid-mediated suppression of local GABAergic inhibition. This indirect mechanism may contribute significantly to the positively reinforcing properties of ethanol.

Key Words: Electrophysiology, Mesolimbic System, Synaptic Transmission, Addiction, GABA, Naloxone, Inhibitory Interneurons, Ventral Tegmental Area.

DOPAMINERGIC (DA) NEURONS SITUATED in the ventral tegmental area (VTA) are important for the rewarding effects of ethanol and other drugs of abuse (Koob and Bloom, 1988; Rodd et al., 2004). Ethanol increases ongoing firing of VTA DA neurons in vivo (Gessa et al., 1985; Foddai et al., 2004), in brain slices (Brodie et al., 1990) and enzymatically dissociated (Brodie et al., 1999), indicating some direct excitation of these DA neurons (Diana et al., 2003). However, even high doses of ethanol (~80–120 mM) typically produce only a modest increase in firing of DA neurons in vitro—up to a maximum of 61% with 320 mM (Brodie et al., 1990)—compared with the much larger changes observed in vivo in unanesthetized (Gessa et al., 1985) and urethane-anesthetized rats (Foddai et al., 2004).

The large difference between observations in vivo and in vitro led us to seek other mechanisms that might contribute to the action of ethanol. Dopaminergic neuronal activity is strongly influenced by afferent inputs, as indicated by their different firing patterns in vivo (in bursts) and in vitro (in a regular pace-maker manner) (Grace and Bunney, 1983; Grace and Onn, 1989). Dopaminergic neurons in VTA receive many inputs, including glutamatergic and GABAergic fibers. These GABA-releasing inputs come from several sources: medium spiny neurons of the nucleus accumbens, ventral pallidum, and the pedunculopontine nucleus (Johnson and North, 1992; Walaas and Fonnum, 1980), but the primary inhibitory regulation is by collaterals of local interneurons in VTA (Johnson and North, 1992).

There is evidence that these local GABAergic neurons control ethanol consumption (Melis et al., 2002; Nowak et al., 1998). In freely behaving rats, systemic ethanol depresses GABAergic neuronal firing in VTA (Stobbs et al., 2004). Hence, ethanol could excite DA neurons *indirectly* by decreasing ongoing inhibition (Gessa et al., 1985). In this respect, ethanol could act like μ-opioids, which indirectly excite DA neurons in VTA by inhibiting GABAergic inputs (Gysling and Wang, 1983; Johnson and North, 1992). We tested this hypothesis in the current study using the patch-clamp technique on VTA neurons in midbrain slices and found that ethanol indeed excites putative VTA DA neurons, but more potently inhibits VTA GABA interneurons. Especially important was the finding that activation (or block) of μ-opioid receptors,

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which are expressed mostly on the VTA GABA neurons, reduces the increase in putative DA neuronal firing elicited in VTA by ethanol.

MATERIALS AND METHODS

All experiments were performed in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey. The experiments were performed on Sprague–Dawley rats aged 14 to 28 postnatal (P) days.

Slice Preparation

The midbrain slices were prepared as described previously (Xiao et al., 2007; Ye et al., 2004, 2006). Animals were anesthetized and then killed by decapitation. The brain was removed and a midbrain block (containing the VTA) was isolated. It was glued to the cutting stage of a VF-200 slicer (Precisionary Instruments Inc., Greenville, NC). *Coronal* midbrain slices (230–300 μm thick) were cut in ice-cold glycerol-based artificial cerebrospinal fluid (ACSF)—containing 250 mM glycerol, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, 26 mM $NaHCO_3$, and 11 mM glucose, and oxygenated with 95% O_2/5% CO_2 (carbogen, Ye et al., 2006). Before recording began, the slices (2 per animal) were allowed to recover for at least 1 hour in a holding chamber containing carbogenated regular ACSF, which has the same components as glycerol-based ACSF, except that glycerol was replaced by 125 mM NaCl.

Electrophysiological Recording

In whole-cell and loose-patch cell-attached modes, signals were recorded with MultiClamp 700A amplifiers (Axon Instruments, Forster City, CA), a Digidata 1320A A/D converter (Axon Instruments), and pCLAMP 9.2 software (Axon Instruments). Data were filtered at 1 kHz and sampled at 5 kHz. The patch electrodes had a resistance of 3 to 5 MΩ when filled with the solution containing 140 mM K-gluconate, 5 mM EGTA, 0.5 mM $CaCl_2$, 10 mM HEPES, 2 mM Mg-ATP, and 0.2 mM GTP. The pH was adjusted to 7.2 with Tris-base, and the osmolarity to 280 to 300 mOsm with sucrose. A single slice was transferred to the 0.4 mL recording chamber, where it was stabilized by a platinum ring. Throughout the experiments, the bath was continually perfused with carbogenated ACSF (1.5–2.0 mL/min). The fact that 10 μM bicuculline blocked most GABAergic inhibitory postsynaptic currents within 90 seconds is an indication of the effective bath exchange time. Cells were visualized with an upright microscope (E600FN, Nikon, Tokyo, Japan) and near-infrared illumination. The VTA was identified medial to the accessory optic tract and lateral to the fasciculus retroflexus. Spontaneous firing of VTA neurons was recorded in some experiments in the loose-patch cell-attached mode (seal resistance 200–800 MΩ). This technique yields stable data with minimal dialysis of intracellular components via the patch pipette (Brodie et al., 1999; Ye et al., 2004). Series resistance (15–30 MΩ) or input resistance (300–500 MΩ) was monitored throughout the whole-cell recording and data were discarded if the resistance changed by more than 20%. Many recordings were performed at 33 ± 1 °C, maintained by an automatic temperature controller (Warner Instruments, Hamden, CT), but in some earlier experiments at 20 to 23 °C, as indicated.

Identification of GABA and DA Neurons in the VTA

The 2 main types of neurons were identified by their distinct morphological, pharmacological, and electrophysiological characteristics (Johnson and North, 1992; Xiao et al., 2007; Ye et al., 2004; but see Margolis et al., 2006). As illustrated in Fig. 1A and 1B, the

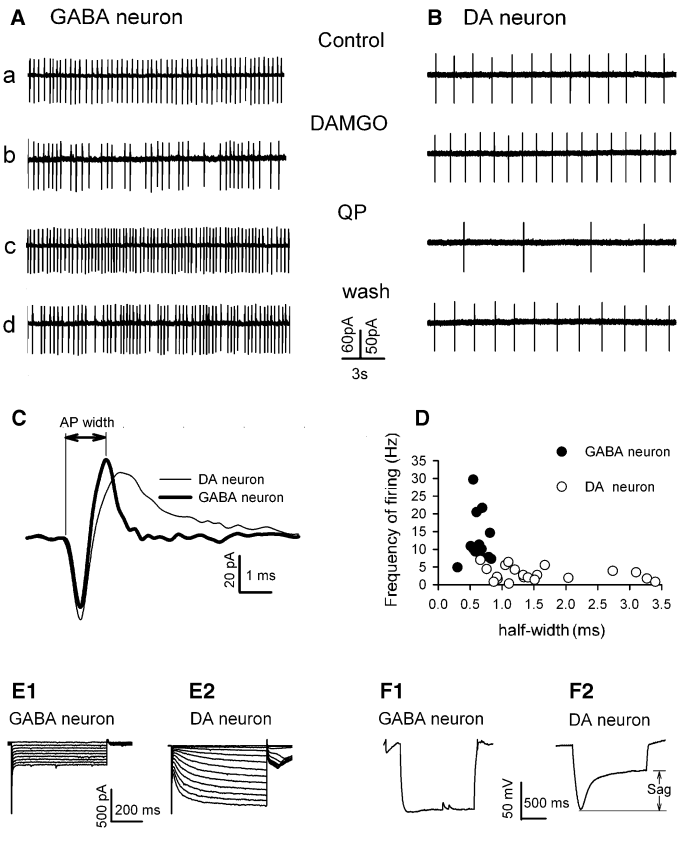

Fig. 1. Identification of ventral tegmental area GABA and dopaminergic (DA) neurons in midbrain slices. Loose-patch cell-attached recordings show selective depression of firing of (**A**) a putative GABA neuron by DAMGO (1 μM), a μ-opioid receptor agonist, and (**B**) a putative DA neuron by quinpirole (QP) (0.1 μM), a dopamine D2/D3 receptor agonist. (**C**) Comparison of single spontaneous action potentials recorded from a GABAergic neuron (thicker line, with a half-width of 0.6 ms) and a putative DA neuron (thinner line, with a half-width of 1.25 ms). Action potential widths were measured from the start of the action potential to the positive peak. (**D**) Frequency of spontaneous action potentials was plotted versus half-width. (**E**) Hyperpolarizing voltage pulses from − 60 to − 130 mV, in steps of 10 mV (not shown), elicit prominent time-dependent inward current (I_h) in DA (**E_2**) but not GABA neuron (**E_1**). (**F**) Voltage traces recorded from current-clamped DA (**F_2**) and GABAergic (**F_1**) neurons. Membrane potential of DA but not GABA neuron has a prominent time-dependent "voltage sag" during application of − 130 pA rectangular current (not shown).

μ-opioid agonist DAMGO (1 μM; Fig. 1A) selectively depresses the firing of GABAergic interneurons, whereas the dopamine D2/D3 agonist quinpirole (QP, 0.1 μM; Fig. 1B) depresses the firing of DA neurons only. Note that DAMGO accelerated the discharge of DA neurons (Figs. 1B and 4B), probably as a result of disinhibition. This observation confirms the preservation of some local circuitry in the slice. This disinhibition by DAMGO is a characteristic of DA neuron (Margolis et al., 2003).

The GABAergic neurons fired rapidly (14.4 ± 1.3Hz, $n = 22$; Fig. 1A), with narrow action potentials (half-width 0.63 ± 0.03 ms, $n = 22$; Fig. 1C and 1D). Another feature of DA neurons is a prominent hyperpolarization-activated inward current (I_h) (Fig. $1E_2$), which is lacking in GABAergic neurons (Fig. $1E_1$). Under current clamp, responses to hyperpolarizing current steps showed a prominent "voltage sag" in DA neuron (Fig. $1F_2$), but not in GABAergic neurons (Fig. $1F_1$). Using immunocytochemical methods, we recently confirmed that the DA neurons and the GABA neurons thus identified in the VTA were correspondingly positive or negative in tyrosine hydroxylase staining (Wang et al., 2005), in keeping with other studies (e.g., Brodie et al., 1999).

Chemicals and Applications

Most of the chemicals, including bicuculline methiodide, GABAzine, Tyr-D-Ala-Gly-*N*-Me-Phe-Gly-ol enkephalin (DAMGO), and (-)-Quinpirole HCl (QP), were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO). Naloxone HCl was from Du Pont Pharmaceutical (Wilmington, DE). Ethanol (95% v/v, prepared from grain) was from Pharmco (Brookfield, CT) and stored in glass bottles. Chemicals were added in known concentrations to the superfusate.

Data Analysis

Spontaneous discharges were counted and analyzed with Clampfit 9.2 (Axon Instruments). To assess the changes of spontaneous firing with drugs, data obtained over a 1- to 2-minute period at the peak of a drug response were normalized to the average values of the frequency of spontaneous discharges during the initial control period (4–5 minutes). Data were expressed as means (±SEM). The statistical significance of drug effects was assessed by a paired 2-tailed *t*-test. Values of $p<0.05$ were considered to be significant.

RESULTS

Ethanol Inhibits VTA GABAergic Interneurons in Midbrain Slices

We applied ethanol to midbrain slices, while recording the spontaneous firing of VTA neurons with the loose-patch cell-attached technique. As illustrated in Fig. $2A_{1,2}$, 40 mM ethanol sharply but reversibly depressed the ongoing firing of a GABA neuron. The concentration dependence of the ethanol-induced reduction of firing rate is shown in Fig. $2A_3$: 20, 40, and 80 mM ethanol lowered the firing rate by $41 \pm 5\%$ ($n = 5$, $p<0.001$), $63 \pm 4\%$ ($n = 6$, $p<0.001$), and $77 \pm 12\%$ ($n = 5$, $p<0.001$), respectively. A similar effect was seen in midbrain slices (from older rats, P22–P28) kept at 32 °C, in which 40 mM ethanol reduced the ongoing firing of GABAergic neurons by $65 \pm 8\%$ ($n = 5$, $p = 0.002$).

In contrast, ethanol enhanced the spontaneous activity of the putative DA neurons (Fig. $2B_{1,2}$), in keeping with the report of Brodie et al. (1990). As shown in Fig. $2B_3$, 20, 40, 80, and 120 mM ethanol increased DA neuronal firing by $14 \pm 9\%$ ($n = 6$, $p = 0.2$), $25 \pm 7\%$ ($n = 5$, $p<0.05$), $31 \pm 11\%$ ($n = 6$, $p<0.05$), and $45 \pm 7\%$ ($n = 9$, $p<0.01$), respectively. The results were comparable in slices (from older rats, P22–P28) maintained at 32 °C, in which ethanol (40 mM) enhanced DA neuronal firing rate by $37 \pm 4\%$ ($n = 9$, $p<0.001$). In the following experiments, we investigated the contribution of the action of ethanol on local GABA neurons to the excitation of DA neurons.

Ethanol-Induced Excitation of VTA DA Neurons is Considerably Attenuated by $GABA_A$ Receptor Antagonists

If ethanol excites DA neurons indirectly by reducing GABA-mediated inhibition, this excitation should be occluded by blockade of $GABA_A$ receptors. In control tests (Fig. 3A), 40 mM ethanol accelerated the spontaneous firing of DA neurons by $22 \pm 7\%$ ($n = 5$, $p<0.05$; Fig. 3Ea). When applied alone, 2 $GABA_A$ antagonists increased firing even more: 10 μM bicuculline by $45 \pm 8\%$; $n = 5$, $p = 0.005$; (Fig. 3B and 3D) and GABAzine (3 and 10 μM), a different type of antagonist, by $32 \pm 8\%$ ($n = 3$, $p<0.05$), and $57 \pm 5\%$ ($n = 3$, $p<0.01$), respectively (not illustrated). Thus, DA neurons were under strong tonic inhibition, mediated via $GABA_A$ receptors (Johnson and North, 1992). After a new stable baseline was established in the continued presence of bicuculline, adding 40 mM ethanol further enhanced firing, but only by $12 \pm 3\%$ (Fig. 3C and 3Ea, $n = 5$, $p = 0.02$). This is significantly less than the 22% increase induced by 40 mM ethanol, in the absence of bicuculline ($n = 5$, $p = 0.02$).

When $GABA_A$ receptors are blocked, the stimulating effect of ethanol on DA neurons may be underestimated owing to nonlinear summation of excitation. We therefore

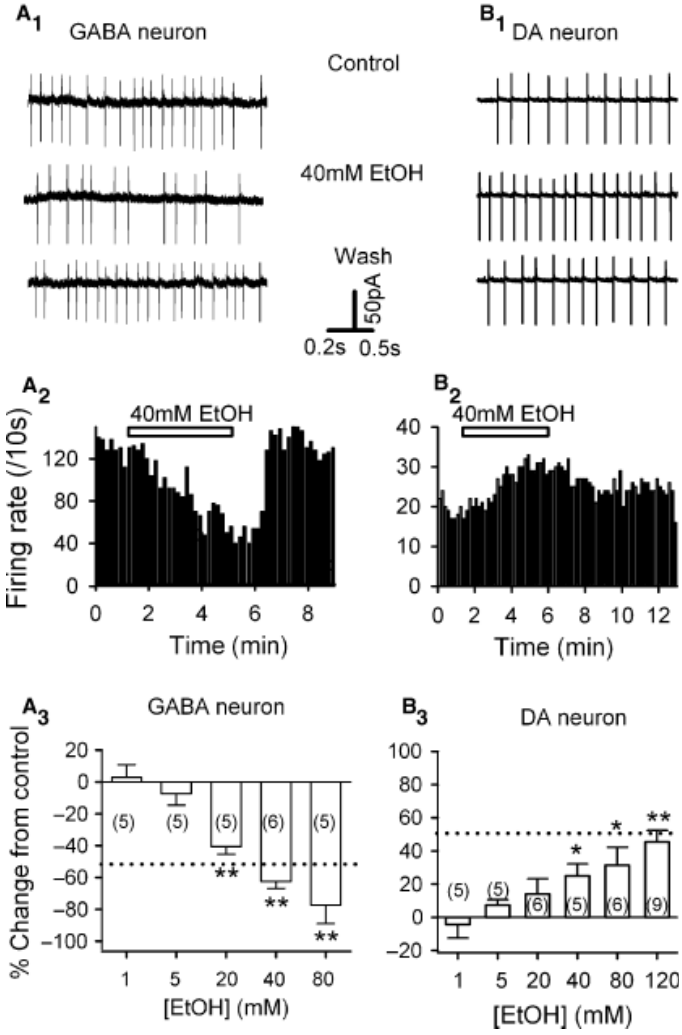

Fig. 2. Opposite effects of ethanol on spontaneous firing of GABA and dopaminergic (DA) neurons in ventral tegmental area. Ethanol depresses the firing of a GABA neuron (**A_1**) and accelerates that of a DA neuron (**B_1**). Time course of 40 mM ethanol effects on GABA (**A_2**) and DA (**B_2**) neurons. Mean changes (± SEM) in firing of 5 to 9 cells treated with different concentrations of ethanol. Note greater percentage changes in firing in GABA (**A_3**) than in DA neurons (**B_3**). Numbers of cells are indicated in brackets; $^*p<0.05$, $^{**}p<0.01$. Loose-patch cell-attached recordings in slices from P14–P15 rats.

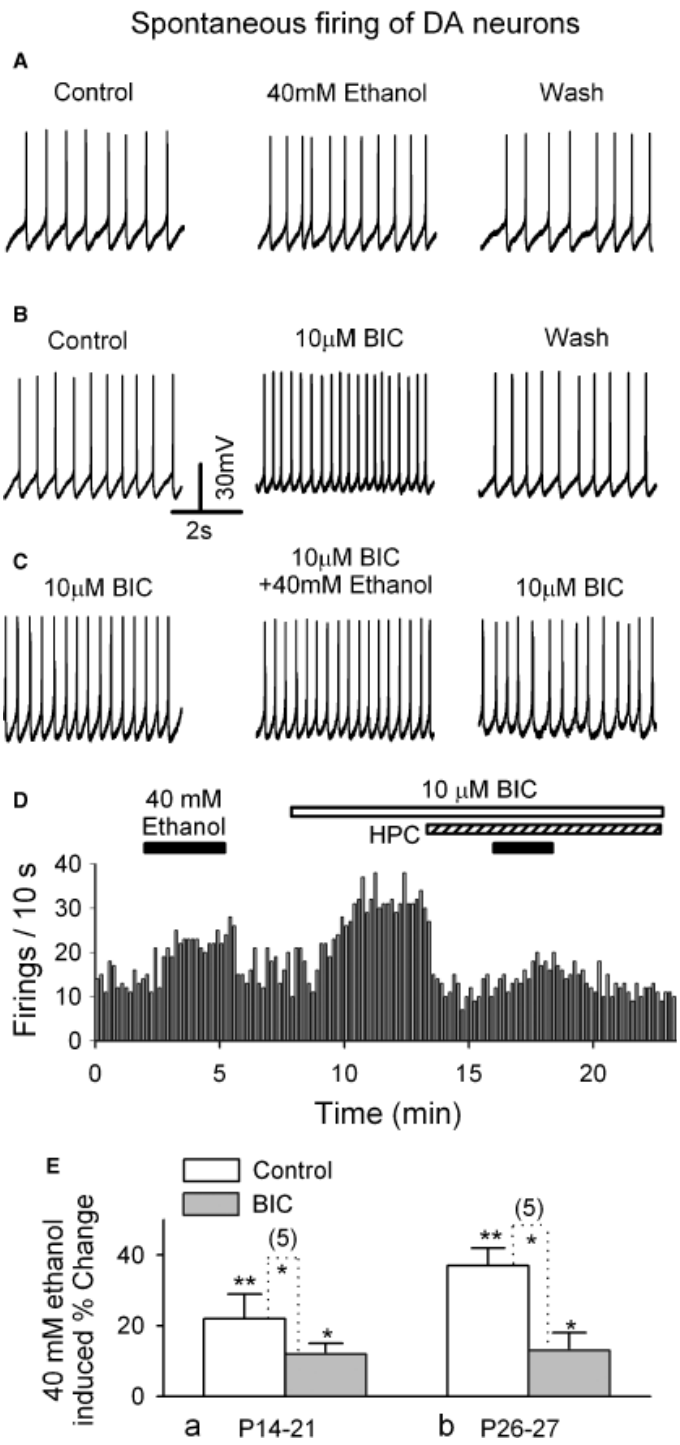

Fig. 3. Bicuculline attenuates ethanol-induced excitation of dopaminergic (DA) neurons. Ongoing firing, recorded from current clamped DA neuron in a slice from a P15 rat, was increased by ethanol (**A**), and even more by bicuculline (BIC) (**B**). In the presence of BIC, there was minimal excitation by ethanol (**C**). In another experiment, on a slice from a P26 rat (**D**), ethanol was also tested alone and in the presence of BIC, after injecting a small hyperpolarizing current (HPC, -20 pA) to restore the initial rate of firing: note again marked depression of ethanol-induced excitation. (**E**) Increases in firing (means $\pm$ SEM) produced by 40 mM ethanol alone or in the presence of BIC in P14–P21 cells (**a**) or BIC+HPC in P26–P27 cells (**b**). Recordings in slices from older (>P22) rats were at 32 °C.

repeated such tests of ethanol after restoring the firing frequency to the pre-bicuculline level by a hyperpolarizing current injection. As illustrated in Fig. 3D by the firing of a putative DA neuron in a slice from a P26 rat, even under these conditions the excitatory effect of ethanol was much smaller than in the absence of bicuculline. In experiments on 5 neurons (Fig. 3Eb), 40 mM ethanol applied alone accelerated firing by $37 \pm 5\%$ ($p = 0.004$). When reapplied in the presence of bicuculline and hyperpolarizing current (10–30 pA), ethanol increased firing by only $13 \pm 5\%$ ($p = 0.02$, Fig. 3Eb). This difference is also significant (for $n = 5$, $p < 0.05$). These results support our hypothesis that depression of GABA release contributes to ethanol-induced excitation of these DA neurons.

Ethanol-Induced Excitation of VTA DA Neurons is Attenuated by DAMGO

Acute ethanol increases the release of β-endorphin, which binds to μ-opioid receptors in the rat brain (Herz, 1997; Marinelli et al., 2004; Olive et al., 2001). In VTA, μ-opioid receptors are expressed on the GABA neurons but not the DA neurons. We therefore postulated that ethanol may increase DA neuronal firing, at least in part, by potentiating opioid-mediated inhibition of GABA neurons. To test this hypothesis, we examined the effect of ethanol on the firing of VTA DA neurons in the absence or presence of DAMGO, a selective agonist of μ-opioid receptor. Note that these experiments were conducted on putative DA neurons that were excited by DAMGO. When applied separately, ethanol (40 mM) and DAMGO (3 μM) increased the ongoing firing of DA neurons by $34 \pm 3\%$ ($n = 7$, $p < 0.001$; Fig. 4A and 4Da) and $42 \pm 11\%$ ($n = 7$, $p < 0.01$, Fig. 4B), respectively. After the response to DAMGO stabilized, adding 40 mM ethanol further increased firing by only $15 \pm 5\%$ ($n = 7$, $p = 0.02$; Fig. 4Da), less than half the increase induced by ethanol alone. DAMGO consistently increased the firing of DA neurons, in keeping with a disinhibition that has been attributed to a μ-opioid receptor mediated inhibition of spontaneously active GABAergic interneurons (Johnson and North, 1992). Thus, some critical intra-VTA circuitry is preserved in these slices, in agreement with previous reports (Johnson and North 1992; Margolis et al., 2003).

Because of possible nonlinear summation of responses (as already mentioned), ethanol was reapplied after injecting a sufficient hyperpolarizing current (-10 to -30 pA) to restore firing to its pre-DAMGO level. In Fig. 4Db, the results are presented from such experiments on slices from P26–P27 rats: applied separately, both ethanol and DAMGO (3 μM) enhanced ongoing firing of a putative DA neuron. However, in the presence of DAMGO, even when firing was restored to the pre-DAMGO level by a hyperpolarizing current, the increase in firing rate by ethanol was reduced from $32 \pm 6\%$ ($n = 5$, $p = 0.0004$) to $12 \pm 2\%$ ($n = 5$, $p < 0.005$; Fig. 4Db). Thus, DAMGO

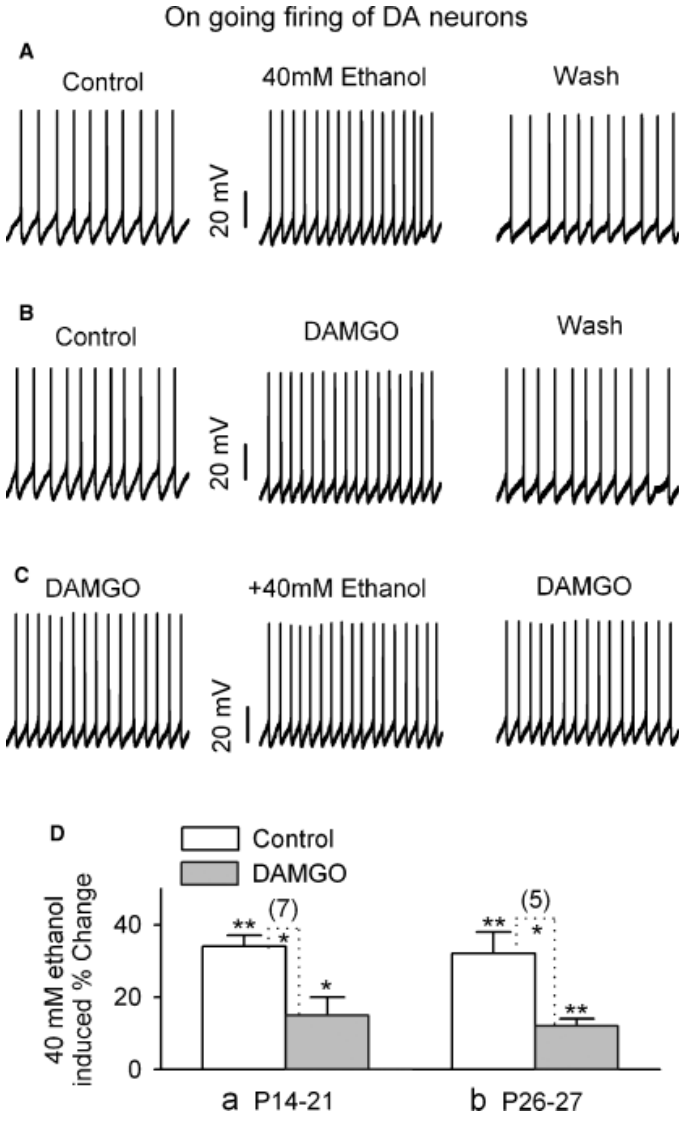

Fig. 4. DAMGO attenuates ethanol-induced excitation of dopaminergic (DA) neurons. Ongoing firing, recorded from current-clamped DA neurons in ventral tegmental area, was increased by 40 mM ethanol (**A**) and by 3 μM DAMGO (**B**). (**C**) In the presence of DAMGO, ethanol induced a smaller increase in firing. All traces were recorded over 5-s periods. (**D**) Increases in firing of DA neurons (means $\pm$ SEM) induced by 40 mM ethanol alone and in the presence of DAMGO in P14–P21 cells (**a**) and DAMGO+HPC in P26–P27 cells (**b**). All recordings in slices from older (>P22) rats were performed at 32 °C.

significantly attenuated the excitatory effect of ethanol ($n = 5$, $p = 0.02$). According to these results, agents that inactivate GABAergic inputs to DA cells significantly reduce the ethanol-induced excitation of VTA DA neurons.

Ethanol-Induced Excitation of VTA DA Neurons is Also Attenuated by Naloxone

To further probe the role of local GABAergic interneurons, we examined the effects of naloxone, a general antagonist of opioid receptors, on ethanol-induced excitation of DA neurons and inhibition of GABA neurons.

The midbrain slices studied in these experiments were from P23 to P25 rats and they were maintained at 33 ± 1 °C. Ethanol (40 mM) caused the usual reversible increase in DA neuronal firing (by $37 \pm 8\%$; for $n = 8$, $p = 0.001$; Fig. 5Ab) and a decrease in GABA neuronal firing (by $57 \pm 3\%$; for $n = 4$, $p = 0.002$; Fig. 5C). Naloxone (5 μM) markedly reduced the firing of 6 of 8 putative DA neurons (Fig. 5Ad) and increased the firing of 3 of 4 GABA neurons (not illustrated). This was direct evidence of an important opioidergic influence that tonically facilitates DA cell firing by suppressing GABAergic inhibition. After the response to naloxone stabilized, adding 40 mM ethanol increased the firing rate of DA neurons by only $16 \pm 3\%$ ($n = 4$, $p < 0.05$; Fig. 5Da). In addition, we tested the effects of ethanol in the presence of naloxone after the discharge of DA and GABA neurons was restored to the pre-naloxone level with 10 to 30 pA current injections (Fig. 5Ae). Ethanol now increased the firing rate of the DA neurons by only $15 \pm 2\%$ ($n = 8$, $p = 0.004$; Fig. 5Af), and did not change the discharge of the GABA neurons ($-7 \pm 10\%$; $n = 4$; Fig. 5C and 5Db). These results lend strong support to the idea that ethanol potentiates opioid-mediated regulation of GABAergic function in the VTA.

The observation that naloxone decreased DA neuronal firing indicates an ongoing μ-opioid receptor-mediated inhibition of spontaneously active GABAergic interneurons that facilitates tonic firing of DA neurons (Johnson and North, 1992). These data again suggest that some local circuitry is intact and functional in these slices.

DISCUSSION

Recording from VTA neurons in acute midbrain slices, we found that ethanol excites putative DA neurons, but more potently inhibits putative GABA neurons. Moreover, activation (or blocking) of μ-opioid receptors, which are mostly expressed on the GABA neurons, reduces ethanol-induced excitation of these DA neurons. Our results provide strong evidence that ethanol excites DA neurons in VTA at least in part by inhibiting local GABA neurons, evidently by a μ-opioid-mediated action.

Ethanol Excites VTA DA Neurons

Our observations are quite comparable with those reported by Brodie et al. (1990). A direct action is indicated by the excitation of enzymatically dissociated DA neurons (Brodie et al., 1999) and the augmentation of I_h of DA neurons in midbrain slices (Okamoto et al., 2006). As in those studies, the increases in firing were less pronounced than those observed in vivo (Foddai et al., 2004; Gessa et al., 1985). It suggests that ethanol may modulate the excitability of DA neurons via indirect synaptic or other mechanisms that are unavoidably damaged in brain slices.

Ethanol Inhibits VTA GABAergic Interneurons

In midbrain slices from both young and weaned rats, ethanol reduced the ongoing discharge of electrophysio-

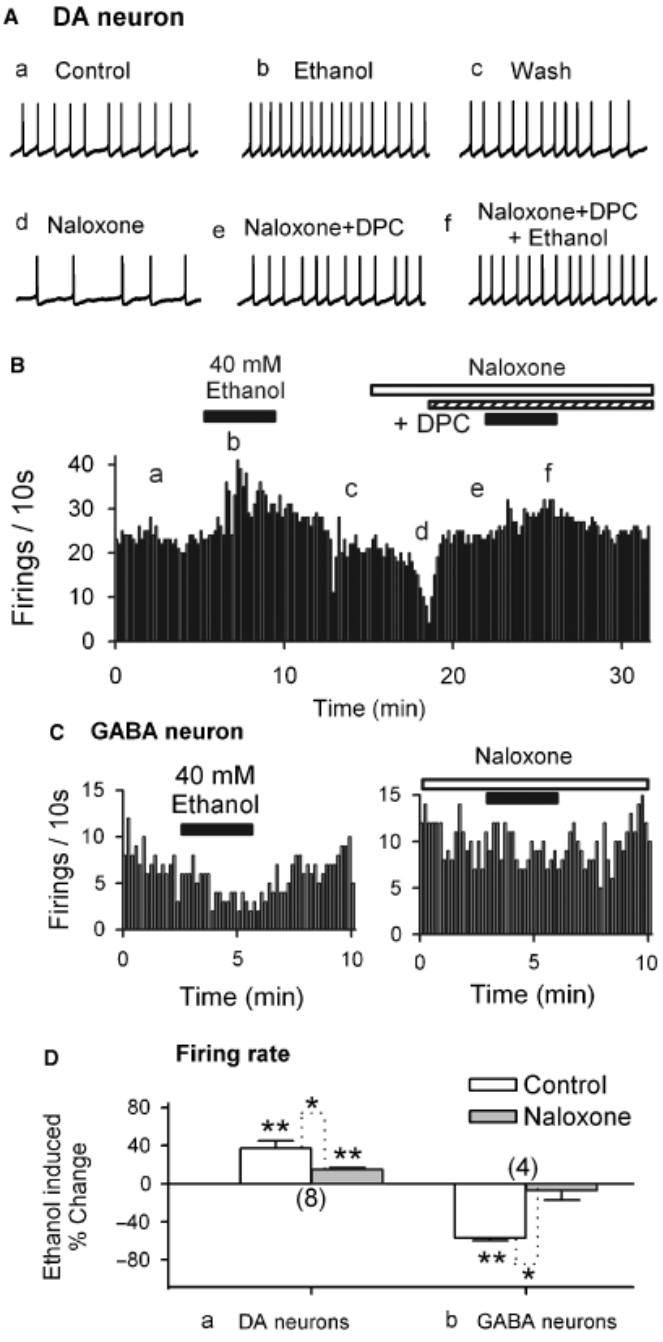

Fig. 5. Naloxone sharply reduces ongoing firing of dopaminergic (DA) neurons and attenuates ethanol-induced excitation of DA neurons. (**A**) As usual, 40 mM ethanol increased the firing of a DA neuron (in a slice from a P25 rat) (**b**); in the presence of naloxone, ongoing firing was considerably depressed (**d**) and, although the initial rate of firing was restored by depolarizing current (DPC), the effect of ethanol was nearly abolished (**f**). (**B**) For the same data, time course of 40 mM ethanol-induced changes in firing in the absence or presence of naloxone+DPC. (**C**) 40 mM ethanol robustly inhibited the firing of a GABA neuron (at left), but it had little effect in the presence of naloxone (at right). (**D**) Summary of 40 mM ethanol-induced percentage changes (means ± SEM) of the firing rate of ventral tegmental area DA (**a**) and GABA neurons (**b**) in the absence (blank) or presence (gray) of naloxone.

logically and pharmacologically identified local GABA interneurons. At concentrations as low as 20 mM, ethanol significantly depressed the firing of these GABA neurons. This potent, concentration-dependent depressant action is in agreement with reports from in vivo studies that systemic administrations of ethanol (0.2–0.5 g/kg i.p.) significantly reduce the discharge of GABA neurons in both the VTA (Stobbs et al., 2004) and the substantia nigra (Mereu and Gessa, 1985).

Disinhibition of VTA DA Neurons

Ongoing firing of DA neurons was markedly accelerated by 3 agents that suppress GABAergic inhibition, bicuculline and GABAzine (both $GABA_A$ receptor antagonists), as well as DAMGO (a specific agonist of μ-opioid receptors). In keeping with the predominant localization of μ-receptors on the local GABA neurons in VTA (Svingos et al., 2001), DAMGO is of special interest because it blocks the firing of these local GABAergic cells (Fig. 4; Gysling and Wang, 1983; Johnson and North, 1992). Clearly, the VTA DA neurons are subject to tonic GABAergic inhibition. During applications of bicuculline, GABAzine, or DAMGO, ethanol-induced excitation of VTA DA neurons was considerably attenuated, in keeping with our hypothesis that, to significant extent, ethanol acts by a similar mechanism of disinhibition.

An interesting point, however, is that the DA neurons were less sensitive than the inhibitory interneurons to the effects of ethanol—as indicated by the respective dose–response curves (Fig. 2). Possible explanations for this apparent discrepancy include: (1) potentation of tonic, extra-synaptic GABAergic inhibition (Hamann et al., 2002) by ethanol (Wallner et al., 2003) would counteract the excitatory effect of synaptic disinhibition; (2) because intra-VTA circuitry is not fully preserved in slices, the disinhibiting effect is probably reduced and therefore could be observed only in a small percentage of the DA neurons; and (3) changes in firing rate cannot be simply related to changes in inhibition (or disinhibition) as the reduction of firing depends on the degree of ongoing excitation by various inputs to the postsynaptic cell (Mathon et al., 2003)—if this is low, even a moderate increase in inhibition can suppress firing, and if high, even maximal inhibition may fail to do so.

Even saturating concentrations of DAMGO did not fully eliminate ethanol-induced excitation. Therefore, disinhibition of DA cells is probably not the only mechanism involved in the rewarding properties of ethanol. Possible indirect effects via GABAergic inputs to other monoaminergic neurons that influence DA neurons in VTA cannot be excluded without further studies. Furthermore, as already mentioned, ethanol also excites VTA DA cell directly (Brodie et al., 1990, 1999; Okamoto et al., 2006), and there is good reason to believe that indirect effects mediated via glutamatergic inputs to DA neurons also play a very significant role (Xiao et al., 2006).

Role of Opioids in Ethanol-Induced Inhibition of VTA GABA Neurons

Ethanol could inhibit GABA neurons directly, for example by activating K^+ channels (like μ-opioids, Johnson and North, 1992) or Cl^- channels (like GABA, Suzdak et al., 1986), or indirectly via NMDA receptors (Stobbs et al., 2004). Particularly relevant is the evidence that acute i.p. injections of ethanol increase the extracellular levels of β-endorphins and μ-opioid receptor binding in nucleus accumbens (Marinelli et al., 2004; Olive et al., 2001). Although comparable data are not available for the VTA, the pronounced but opposite effects of naloxone on GABA and DA neurons in our experiments are strong evidence of ongoing opioid release in the VTA. Moreover, naloxone largely eliminated the effects of ethanol on both GABA and DA neurons. The simplest explanation of our results is that ethanol potentiates either the ongoing release of an opioid or its depressant action on GABA neurons. Overall, our in vitro observations fully support the hypothesis that the addictive effects of ethanol are mediated, at least in part, by opioids (Gianoulakis, 1989; Herz, 1997; Oswald and Wand, 2004).

Relevance of These Findings for Alcohol Addiction

The changes in firing of VTA GABA and DA neurons occur at ethanol concentrations close to those observed in human drinkers. Individuals classed as ''moderately intoxicated'' have blood alcohol levels in the range 0.1 to 0.2% or 22 to 44 mM (Cherpitel et al., 2005). Especially relevant are the studies on ethanol self-administration into VTA by rats (Rodd et al., 2004), according to which the effective VTA concentrations are close to those that excited DA neurons in our slices. Some other lines of evidence link behavioral reinforcement to changes in GABA—or opioid-mediated function in the VTA. Rodents also self-administer an opioid agonist (Devine and Wise, 1994) or a $GABA_A$ antagonist infused focally into the VTA (David et al., 1997; Ikemoto et al., 1997). The crucial role of the opioid system is highlighted by the absence of ethanol self-administration in mutants lacking μ-opioid receptors (Roberts et al., 2000).

Our results in slices suggest that ethanol is more potent as an inhibitor of GABA neurons, which is consistent with previous in vivo observations (Stobbs et al., 2004). Moreover, we found that an opioid receptor agonist and an antagonist altered GABA neuronal firing, and also reduced the ethanol-induced excitation of VTA DA neurons. These results provide a link between ethanol-induced inhibition of VTA GABA neurons and ethanol-induced excitation of VTA DA neurons.

Taken together, our findings suggest that potentiation of opioid-mediated inhibition of local GABA neurons might in part account for ethanol-induced excitation of DA neurons in VTA.

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