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ETHANOL DUALLY MODULATES GABAergic SYNAPTIC TRANSMISSION ONTO DOPAMINERGIC NEURONS IN VENTRAL TEGMENTAL AREA: ROLE OF μ-OPIOID RECEPTORS

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Abstract—The mesolimbic dopaminergic system, originating from the ventral tegmental area (VTA) is implicated in the rewarding properties of ethanol. VTA dopaminergic neurons are under the tonic control of GABAergic innervations. Application of GABAergic agents changes ethanol consumption. However, it is unclear how acute ethanol modulates GABAergic inputs to dopaminergic neurons in the VTA. This report describes ethanol at clinically relevant concentrations (10–40 mM) dually modulates inhibitory postsynaptic currents (IPSCs). IPSCs were mediated by $GABA_A$ receptors and were recorded from VTA dopaminergic neurons in acute midbrain slices of rats. Acute application of ethanol reduced the amplitude and increased the paired pulse ratio of evoked IPSCs. Ethanol lowered the frequency but not the amplitude of spontaneous IPSCs. Nevertheless, ethanol had no effect on miniature IPSCs recorded in the presence of tetrodotoxin. These data indicate that ethanol inhibits GABAergic synaptic transmission to dopaminergic neurons by presynaptic mechanisms, and that ethanol inhibition depends on the firing of GABAergic neurons. Application of CGP 52432, a $GABA_B$ receptor antagonist, did not change ethanol inhibition of IPSCs. Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol enkephalin (DAMGO), a μ-opioid receptor agonist, conversely, silenced VTA GABAergic neurons and inhibited IPSCs. Of note, in the presence of a saturating concentration of DAMGO (3 μM), ethanol potentiated the remaining IPSCs. Thus, ethanol dually modulates GABAergic transmission to dopaminergic neurons in the VTA. Ethanol modulation depends on the activity of VTA GABAergic neurons, which were inhibited by the activation of μ-opioid receptors. This dual modulation of GABAergic transmission by ethanol may be an important mechanism underlying alcohol addiction.

Key words: mesolimbic system, drug addiction, postsynaptic currents.

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Abbreviations: APV, DL-2-amino-5-phosphono-valeric acid; BIC, bicuculline; CGP 52432, 3-[[(3,4-dichlorophenol) methyl] amino] propyl] diethoxymethyl) phosphinic acid; DA, dopaminergic; DAMGO, Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol enkephalin; DNQX, 6,7-dinitroquinoxaline-2,3-dione; eIPSC, evoked inhibitory postsynaptic current; GACSF, glycerol-based artificial cerebrospinal fluid; I_h, inward current; IPSC, inhibitory postsynaptic current; K-S, Kolmogorov-Smirnov; mIPSC, miniature inhibitory postsynaptic current; MOR, μ-opioid receptor; NAcc, nucleus accumbens; P, postnatal; PPR, paired pulse ratio; QP, quinpirole; sIPSC, spontaneous inhibitory postsynaptic current; TTX, tetrodotoxin; VTA, ventral tegmental area.

The mesolimbic dopaminergic (DA) system, originating from the ventral tegmental area (VTA) is implicated in the rewarding effects of ethanol (Imperato and Di Chiara, 1986; Ikemoto et al., 1997a; Nowak et al., 1998). Ethanol facilitates the firing of VTA DA neurons both *in vivo* (Imperato and Di Chiara, 1986) and *in vitro* (Brodie et al., 1990, 1999; Di Chiara and North, 1992; Ye et al., 2006; Xiao et al., 2007).

VTA DA neurons are under tonic control of GABAergic inputs, which are from medium spiny neurons of the nucleus accumbens (NAcc) (Waddington and Cross, 1978; Kalivas et al., 1993) and the ventral pallidum (VP) (Kalivas et al., 1993), but the primarily inhibitory regulation is from collaterals of VTA GABAergic neurons (Johnson and North, 1992a). Blockade of GABAergic innervation enhances the activity of VTA DA neurons (Johnson and North, 1992a,b; Ye et al., 2004; Xiao et al., 2007), and causes burst firing in VTA DA neurons (Kitai et al., 1999). Previous *in vivo* studies on rodents demonstrated that VTA GABAergic neurons control ethanol consumption (Nowak et al., 1998; Gallegos et al., 1999; Koob, 2004; Stobbs et al., 2004; Besheer et al., 2006). In a recent study in midbrain slices, we found that ethanol-induced excitation of VTA DA neuron is significantly attenuated by an antagonist of $GABA_A$ receptors (Xiao et al., 2007). This result implicates that ethanol can excite VTA DA neurons indirectly, through inhibition of VTA GABAergic neurons, in addition to its direct excitatory action (Brodie et al., 1990, 1999).

The μ-opioid receptors (MORs) in VTA are mostly expressed in VTA GABAergic neurons (Mansour et al., 1995; Steffensen et al., 1998; Garzon and Pickel, 2001). Activation of MORs hyperpolarizes and inhibits VTA GABAergic neurons (Di Chiara and North, 1992; Johnson and North, 1992a; Margolis et al., 2003). Several lines of evidence indicate that MORs are involved in ethanol-induced excitation of VTA DA neurons: (1) ethanol enhances the release of β-endorphin in several brain regions, which activates MORs (Stein, 1993; Herz, 1997; Marinelli et al., 2004); (2) naloxone, an opioid receptor antagonist, greatly attenuates ethanol-induced inhibition of VTA GABAergic neurons (Xiao et al., 2007); (3) both naloxone and MOR agonist attenuated the effect of ethanol on VTA DA neurons (Xiao et al., 2007).

Numerous evidence supports that potentiation of GABAergic synaptic transmission is of great importance for the behavioral and cognitive effects of ethanol (Mihic, 1999; Siggins et al., 2005; Weiner and Valenzuela, 2006). However, previous *in vitro* studies of ethanol on inhibitory postsynaptic currents (IPSCs) in several brain regions

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generated controversial results (Siggins et al., 2005; Weiner and Valenzuela, 2006). Moreover, the effects of ethanol on GABAergic synaptic transmission to VTA DA neurons are unknown. In the present study, we asked whether and how ethanol affects GABAergic IPSCs in DA neurons in VTA. We found that ethanol decreases IPSCs when it was applied alone. However, ethanol enhanced the remaining IPSCs when VTA GABAergic neurons were fully depressed by a MOR agonist.

EXPERIMENTAL PROCEDURES

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. All efforts were made to minimize animal suffering and to reduce the number of animal used. The experiments were performed on Sprague–Dawley rats aged 14–28 postnatal (P) days.

Slice preparation

The midbrain slices were prepared as described previously (Ye et al., 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, USA). While the brain was kept in ice-cold glycerol-based artificial cerebrospinal fluid (GACSF)—containing (in mM) 252 glycerol, 1.6 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 18 $NaHCO_3$, and 11 glucose, and oxygenated with 95% O_2/5% CO_2—250–300 μm thick slices were cut in the coronal plane (Ye et al., 2006). The slices (two per animal) were allowed to recover for at least 1 h in a holding chamber in regular artificial cerebrospinal fluid (ACSF), which has the same composition as GACSF, except that glycerol was replaced with 126 mM NaCl.

Electrophysiological recording in midbrain slices

Cells in midbrain slices were visualized with an upright microscope (E600FN, Nikon, Tokyo, Japan) and near-infrared illumination. Electrical signals were obtained in whole-cell patch clamp technique with MultiClamp 700A amplifiers (Molecular Devices Co., Union City, CA, USA), a Digidata 1320A A/D converter (Molecular Devices Co.) and pCLAMP 9.2 software (Molecular Devices Co.). Data were filtered at 2 kHz and sampled at 5 kHz.

When filled with internal solutions, the patch electrodes had a resistance of 3–5 MΩ. Whole-cell currents were recorded with a CsF-based pipette solution containing (in mM): 135 CsF, 5 CsCl, 5 EGTA, 0.5 $CaCl_2$, 10 Hepes, 2 Mg-ATP, and 0.1 GTP. Whole-cell voltages were recorded with a K-gluconate-based pipette solution. This solution has the same composition as CsF-based solution except that CsF was replaced with 135 mM K-gluconate, and CsCl with 5 mM KCl. The pH of pipette solutions was adjusted to 7.2 with tris-base, and the osmolarity to 280–300 mOsm with sucrose. A single slice was transferred into the 0.4 ml recording chamber where it was stabilized by a platinum ring. Throughout the experiments, the bath was continually perfused with ACSF (1.5–2.0 ml/min).

All IPSCs were recorded at a holding potential of 0 mV. To evoke monosynaptic inhibitory postsynaptic currents (eIPSCs), a glass stimulating electrode, filled with ACSF, was placed 50–100 μm away from the recorded VTA neuron. Electrical stimuli (100–200 μs in duration) were applied once every 20 s. An input/output curve was obtained near the start of recording, and the stimulation was set at 20–30% of maximum, an intensity that evoked stable responses with no failures. Paired eIPSCs were elicited with identical double stimuli at an interval of 50 ms. Most recordings were performed at 32 °C.

Unless indicated, all recordings were obtained from putative DA neurons identified by their pharmacological and physiological properties. Specifically, spontaneous firing of VTA neurons was first recorded with the loose-patch cell attached configuration. The depression of spontaneous firing by 0.2 μM quinpirole (QP), and facilitation by 1 μM Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol enkephalin (DAMGO) (probably owing to DAMGO-induced disinhibition) are characteristic features of VTA DA neurons (Johnson and North, 1992a,b; Margolis et al., 2003). QP is a dopamine D_2/D_3 receptor agonist. DAMGO is a MOR agonist. Further suction changed the recording to the whole-cell configuration. A prominent inward current (I_h) activated by hyperpolarizing voltage steps (between −60 and −160 mV) or a corresponding voltage-sag in response to a hyperpolarizing current pulse (−100 pA) (Lacey et al., 1989) confirmed the identity of putative DA neurons. The identification of VTA DA neuron by the responses to QP and the expression of I_h has been challenged (Margolis et al., 2006). However, we believe that in the population of neurons reported in this study (except the GABA neuron shown in Fig. 4A), (1) there were no GABAergic neurons because VTA GABAergic neurons have no *prominent* I_h (Jones and Kauer, 1999; Ibanez-Sandoval et al., 2006; Lee and Tepper, 2007) and they are inhibited by MOR agonists (Margolis et al., 2003), and (2) all the recorded VTA neurons were probably DA because in VTA, DA neurons are uniquely excited by DAMGO, via a mechanism of disinhibition (Margolis et al., 2003; Xiao et al., 2007).

Chemicals and applications

Most of the chemicals, including bicuculline (BIC), DAMGO, tetrodotoxin (TTX), DL-2-amino-5-phosphono-valeric acid (APV), 6,7-dinitroquinoxaline-2,3-dione (DNQX), and QP were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). 3-[[(3,4-Dichlorophenol) methyl] amino] propyl] diethoxymethyl) phosphinic acid (CGP 52432) was from Tocris Bioscience (Ellisville, MO, USA). Ethanol (95% v/v, prepared from grain) was from Pharmco (Brookfield, CT, USA) and stored in glass bottles. Chemicals were added in known concentrations to the superfusate.

Data analysis

Spontaneous discharges and inhibitory postsynaptic currents (sIPSCs) were counted and analyzed with Clampfit 9.2 (Molecular Devices Co). For each experimental condition, we averaged the amplitudes of evoked IPSCs or paired pulse ratio (PPR=$IPSC_2$/$IPSC_1$) in 10–20 traces. $IPSC_1$ and $IPSC_2$ are the IPSCs in response to the first and second stimulus of the paired pulses, respectively. In the figures, we showed averaged single or paired eIPSCs. sIPSCs or miniature inhibitory postsynaptic currents (mIPSCs) were screened automatically (5 pA amplitude threshold), checked visually, and accepted or rejected according to their rise and decay times. Cumulative probability plots of the incidence of various inter-event intervals and amplitudes (for 100–1500 sIPSCs), recorded under different conditions from the same neuron, were compared with the Kolmogorov-Smirnov (K-S) test. For other plots, data obtained over a 1–2 min period at the peak of a drug response were normalized to the average values of the frequency and amplitude of sIPSCs or mIPSCs during the initial control period (4–5 min). Data were expressed as means (±S.E.M.). The statistical significance of drug effects was assessed by a paired two-tailed *t*-test on normalized data. Values of $P<0.05$ were considered significant.