

Original Article

Ethanol inhibits histaminergic neurons in mouse tuberomammillary nucleus slices via potentiating GABAergic transmission onto the neurons at both pre- and postsynaptic sites

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Aim: Ethanol, one of the most frequently used and abused substances in our society, has a profound impact on sedation. However, the neuronal mechanisms underlying its sedative effect remain unclear. In this study, we investigated the effects of ethanol on histaminergic neurons in the tuberomammillary nucleus (TMN), a brain region thought to be critical for wakefulness.

Methods: Coronal brain slices (250 μm thick) containing the TMN were prepared from GAD67-GFP knock-in mice. GAD67-GFP was used to identify histaminergic neurons in the TMN. The spontaneous firing and membrane potential of histaminergic neurons, and GABAergic transmission onto these neurons were recorded using whole-cell patch-clamp recordings. Drugs were applied through superfusion.

Results: Histaminergic and GAD67-expressing neurons in the TMN of GAD67-GFP mice were highly co-localized. TMN GFP-positive neurons exhibited a regular spontaneous discharge at a rate of 2–4 Hz without burst firing. Brief superfusion of ethanol (64, 190, and 560 mmol/L) dose-dependently and reversibly suppressed the spontaneous firing of the neurons in the TMN; when synaptic transmission was blocked by tetrodotoxin (1 $\mu\text{mol/L}$), ethanol caused hyperpolarization of the membrane potential. Furthermore, superfusion of ethanol markedly increased the frequency and amplitude of spontaneous and miniature inhibitory postsynaptic currents (sIPSCs and mIPSCs), which were abolished in the presence of the GABA_A receptor antagonist bicuculline (20 $\mu\text{mol/L}$). Finally, ethanol-mediated enhancement of sIPSCs and mIPSCs was significantly attenuated when the slices were pretreated with the GABA_B agonist baclofen (30 $\mu\text{mol/L}$).

Conclusion: Ethanol inhibits the excitability of histaminergic neurons in mouse TMN slices, possibly via potentiating GABAergic transmission onto the neurons at both pre- and postsynaptic sites.

Keywords: ethanol; tuberomammillary nucleus; histaminergic neurons; GABA receptor; IPSCs; bicuculline; baclofen; tetrodotoxin

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Introduction

Ethanol affects sleep, daytime alertness, physiological function during sleep and enhances sleep disorders. Acute ethanol intake of social drinkers who are not alcohol-dependent reduces the time to fall asleep (sleep onset latency) and

increases slow-wave sleep in the first half of the night^[1], followed by sleep disruption during the second half of the night^[2]. Ethanol-associated sleep problems have a significant socio-economic impact on individuals and society. In the United States, it is estimated that the social cost of ethanol-related sleep disorders exceeds 18 billion dollars every year^[2]. Although the effects of ethanol on human sleep were first described more than 70 years ago^[1], little is known about how and where ethanol acts in the brain to alter sleep and wakefulness.

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Over the past 30 years, much attention has focused on the acute potentiating effects of ethanol on fast inhibitory synaptic transmission mediated by GABA in various brain regions such as the hippocampus^[3, 4] and basolateral amygdala^[5]. GABA receptors are divided into GABA_A and GABA_B receptors. GABA_A receptors are localized at the postsynaptic site and serve as the primary target for a variety of sedative and hypnotic drugs, such as barbiturates and benzodiazepines, which allosterically enhance GABA_A receptor function^[6]. GABA_B receptors are expressed at the presynaptic site and act as autoreceptors to modulate GABAergic synaptic transmission. Studies have examined the effects of ethanol on GABAergic synaptic transmission and reported that ethanol potentiates^[7], inhibits^[8], or does not affect^[9] the transmission on GABA_A receptors. On the other hand, blockade of presynaptic GABA_B receptors can dramatically enhance the acute potentiating effect of ethanol on GABA_A receptor-mediated IPSCs in the rat hippocampus^[3] and basolateral amygdala^[5]. Therefore, whether ethanol directly modulates GABA receptor function remains controversial.

The tuberomammillary nucleus (TMN) of the posterior hypothalamus is one of the brain regions that are thought to play a critical role in sleep-wake regulation^[10–12]. The histaminergic neurons mainly reside in the TMN and send widespread projections to various brain areas. Strong and consistent evidence has implicated histamine as a crucial player in promoting wakefulness through histamine H₁ and/or H₃ receptors^[12, 13]. In recent years, more attention has been paid to the role of brain histamine on the sedative effects of ethanol. Lintunen *et al* reported that rats genetically selected for their high tolerance to the ataxic effects of ethanol show higher levels of brain histamine along with a higher density of histamine-immunoreactive nerve fibers^[14]. In addition, pretreatment with the histamine precursor *L*-histidine significantly reduced ethanol-induced sedation^[15]. However, whether ethanol affects the activity of histaminergic neurons in the TMN remained unknown. Given that GABA_A and GABA_B receptors are present in GABAergic synapses on histaminergic TMN neurons^[16, 17], we hypothesized that ethanol may alter GABAergic transmission in the TMN.

In the present study, we used whole-cell patch-clamp recordings to examine the effects of ethanol on neurons in slices prepared from the TMN in glutamic acid decarboxylase 67-green fluorescent protein (GAD67-GFP) knock-in mice. GAD67-GFP was used to identify histaminergic neurons because GAD67 is expressed in the histaminergic neurons in the TMN^[18]. We found that ethanol suppressed the activity of histaminergic neurons in the TMN by directly hyperpolarizing the membrane potential and by potentiating GABAergic transmission mediated by GABA_A receptors and under the tight regulation of GABA_B autoreceptors.

Materials and methods

Animals

Male GAD67-GFP knock-in mice (weighing 16–20 g, 4–8 weeks old) in which GFP is expressed in GABAergic neurons under

the control of the endogenous GAD67 promoter^[18, 19] were used for electrophysiological and immunohistochemical studies. Mice were maintained in the Laboratory Animal Center of Fudan University. All of the animals were housed at a constant temperature (24±0.5°C) with *ad libitum* food and water and were exposed to a 100-lux light/dark cycle of 12:12 h (lights on from 7:00 AM to 7:00 PM). Experimental protocols were approved by the Medical Experimental Animal Administrative Committee of Shanghai Medical College of Fudan University. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to use only the number of animals required for the production of reliable scientific data.

Slice preparation

Coronal brain slices (250 μm thick) containing the TMN were prepared as described previously^[18]. Briefly, the GAD67-GFP mice were anesthetized using isoflurane and killed by decapitation. The brain was quickly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) saturated with carbogen (95% O₂/5% CO₂), in which NaCl had been replaced with 207 mmol/L sucrose. TMN was identified according to the stereotaxic coordinates^[20]. Slices were cut using a vibratome (Leica VT 1200S) in ice-cold ACSF containing (in mmol/L): Sucrose 207, KCl 5, CaCl₂ 2.4, MgSO₄ 1.3, NaH₂PO₄ 1.24, NaHCO₃ 20 and glucose 10. Slices were then quickly transferred to the recording bath, where they were continuously perfused with oxygenated ACSF containing (in mmol/L): NaCl 130, KCl 5, CaCl₂ 2.4, MgSO₄ 1.3, NaH₂PO₄ 1.24, NaHCO₃ 20 and glucose 10, allowed to recover for 1 h at 32°C, and then kept at room temperature before recording^[21].

Patch-clamp recordings

Patch electrodes were pulled from borosilicate glass capillaries (1.5 mm od, 0.8 mm id, Harvard Apparatus, USA) on a Flaming/Brown Micropipette Puller (Model P-97, Sutter Instrument, Novato, CA, USA). The patch electrodes had a resistance of 4–6 MΩ when filled with pipette solutions containing (in mmol/L): 130 K-gluconate, 10 KCl, 2 MgCl₂, 10 HEPES, 2 Mg-ATP, and 0.3 Na₂-GTP for the examination of neuronal firing properties in current-clamp mode or 140 KCl, 4 NaCl, 0.5 CaCl₂, 10 HEPES, 5 EGTA, and 2 Mg-ATP for the examination of IPSCs in voltage-clamp mode (held at -80 mV), and the pH was adjusted to 7.3 with KOH^[22]. The micropipettes were attached to an electric microdrive (MP 285, USA) and placed in contact with the soma of the selected cell under visual control.

TMN neurons were identified under visual guidance using infrared-differential interference contrast (IR-DIC) video microscopy with a 40× water-immersion objective lens (BX51WI, Olympus). The images were detected with an IR-sensitive CCD camera (IR1000, DAGE MTT) and displayed on a monitor. Throughout this study, we only selected brightly fluorescent neurons as GAD67-positive neurons. Recordings were conducted in whole-cell configurations at 30–32°C using a Multiclamp 700B amplifier (Molecular Devices Co, USA), a

Digidata CED1401 converter and Spike2 software (Cambridge Co, UK). Signals were filtered at 1 kHz and sampled at 10 kHz. Data were acquired and analyzed with Spike2 software (Cambridge Co, UK). When needed, *D*(-)-2-amino-5-phosphonovaleric acid (D-AP5, 30 $\mu\text{mol/L}$), 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX, 10 $\mu\text{mol/L}$), strychnine (1 $\mu\text{mol/L}$), (-)-bicuculline methiodide (20 $\mu\text{mol/L}$) and baclofen (30 $\mu\text{mol/L}$) were added in the ACSF to block NMDA, AMPA/kainate, glycine, and GABA_A receptors and to activate GABA_B receptors, respectively.

Immunohistochemistry and confocal microscopy

The brains of GAD67-GFP mice were removed, post-fixed for 6 h in 4% PFA, and then immersed in 20% sucrose overnight. Thereafter, frozen sections were cut at 30 μm in coronal planes using a freezing microtome (Leica Microsystems, Wetzlar, Germany). Immunohistochemistry was performed in accordance with the free-floating method described previously^[18, 23]. In general, the sections were incubated with rabbit anti-histidine decarboxylase (HDC) antibody (1:800; Progen, Germany) for 24 h on a rotary shaker at 4°C. Then, sections were washed in 0.01 mol/L PBS and incubated in a secondary Texas Red-conjugate Affinipure Goat anti-Rabbit IgG (H+L) (1:500; Proteintech Group, USA) prepared with 0.3% Triton X-100 in 0.01 mol/L PBS for 2 h on a rotary shaker at room temperature. Slices were mounted on slides, cover-slipped and sealed with nail polish.

All images were taken using a confocal laser-scanning microscope (Leica TCS-NT, Heidelberg, Germany) with excitation/emission wavelengths set to 488/520 nm for GFP and 561/620 nm for Texas Red in the sequential mode. The images were acquired at 0.5 mm steps and analyzed with Leica TCS NT/SP SCANWARE (version 1.6.587) software^[24].

Chemicals

Absolute ethanol (99.99%), (-)-bicuculline methiodide, TTX, CNQX, D-AP5, strychnine and baclofen were purchased from Sigma-Aldrich (Sigma-Aldrich, St Louis, MO, USA). Immediately before use, ethanol was diluted with fresh ACSF to following concentrations at 64 (1:270, *v:v*), 190 (1:90) or 560 (1:30) mmol/L, based on our preliminary results and previous *in vitro* studies^[25, 26]. All drugs were diluted in fresh ACSF to the final concentration immediately before the experiment. Drugs used in patch-clamp recording were delivered to the slice at a flow rate of 2 mL/min.

Statistical analysis

All data are expressed as the mean \pm SEM. Data analysis was performed using GraphPad Prism version 4.03 for Windows. The changes in the firing rate of histaminergic neurons and the frequency and amplitude of IPSCs induced by ethanol were analyzed by one-way repeated-measures analysis of variance (ANOVA) followed by Dunnett's *post hoc* test or Student's *t*-test. The significance level was set at $P < 0.05$ for all statistical tests.

Results

Identification of histaminergic TMN neurons in GAD67-GFP mouse brain slices

To determine whether the GAD67-expressing neurons in the TMN are histaminergic neurons, we prepared brain slices containing the TMN from GAD67-GFP knock-in mice according to the atlas of Paxinos and Franklin (Figure 1A and 1B)^[20]. We found that histaminergic and GAD67-expressing neurons in the TMN of GAD67-GFP mice are highly co-localized. HDC is the key enzyme for histamine synthesis. Approximately 80% of the GFP-positive cells were also positive for HDC (112/140, two mice), and 82.4% of the HDC-positive cells were also positive for GFP (112/136, two mice; Figure 1C). Figure 1D shows representative TMN GFP-positive neurons, which are multipolar with three to four long dendrites and diameters of approximately 20–30 μm . In the current-clamp configuration of whole-cell recordings, TMN GFP-positive neurons exhibited a regular spontaneous discharge at a typical rate of 2–4 Hz and no burst firing (Figure 1E). Additionally, the inwardly rectifying current activated by hyperpolarization (I_h) and the transient outward current (I_A) are present in TMN GFP-positive neurons (Figure 1F), while in GFP-negative neurons, they are rarely encountered. In addition, these cells displayed rather broad action potentials with a Ca²⁺ shoulder on the downstroke (Figure 1G). Furthermore, the application of the histamine H₃ receptor agonist *R*- α -methylhistamine significantly reduced the firing rate of GFP-positive neurons (Figure 1H). This evidence is consistent with previously reported immunohistochemical, morphological, electrophysiological and pharmacological properties^[27], showing that GFP-positive neurons in the TMN of GAD67-GFP mice are histaminergic neurons.

Ethanol depressed the firing rate and membrane potential of histaminergic TMN neurons

To examine whether ethanol affects the activity of histaminergic TMN neurons, whole-cell patch-clamp recordings were carried out in acute brain slices of GAD67-GFP mice. As illustrated in Figure 2A–2E, ethanol at concentrations of 64, 190 and 560 mmol/L suppressed the firing rate of histaminergic neurons, whereas the firing rate recovered after the washout. We found that ethanol significantly inhibited the firing of histaminergic neurons in a concentration-dependent manner. Ethanol superfused at 64, 190, and 560 mmol/L reduced the firing rate by 45.2 \pm 3.7% (from 4.7 \pm 0.5 to 2.5 \pm 0.3 Hz, $n=5$, $P < 0.01$, paired *t*-test), 73.0 \pm 7.6% (from 4.0 \pm 0.4 to 1.0 \pm 0.3 Hz, $n=5$, $P < 0.01$), and 93.1 \pm 1.6% (from 4.1 \pm 0.5 to 0.3 \pm 0.1 Hz, $n=5$, $P < 0.01$) of their baseline controls, respectively (Figure 2F). Additionally, we noted that the inhibition of firing rate induced by ethanol at 560 mmol/L was stronger than that by ethanol at 64 and 190 mmol/L. Moreover, the effect of ethanol at 190 mmol/L was higher than that at 64 mmol/L. These findings indicate that the suppression of the firing rate of histaminergic neurons by ethanol is both concentration dependent and reversible.

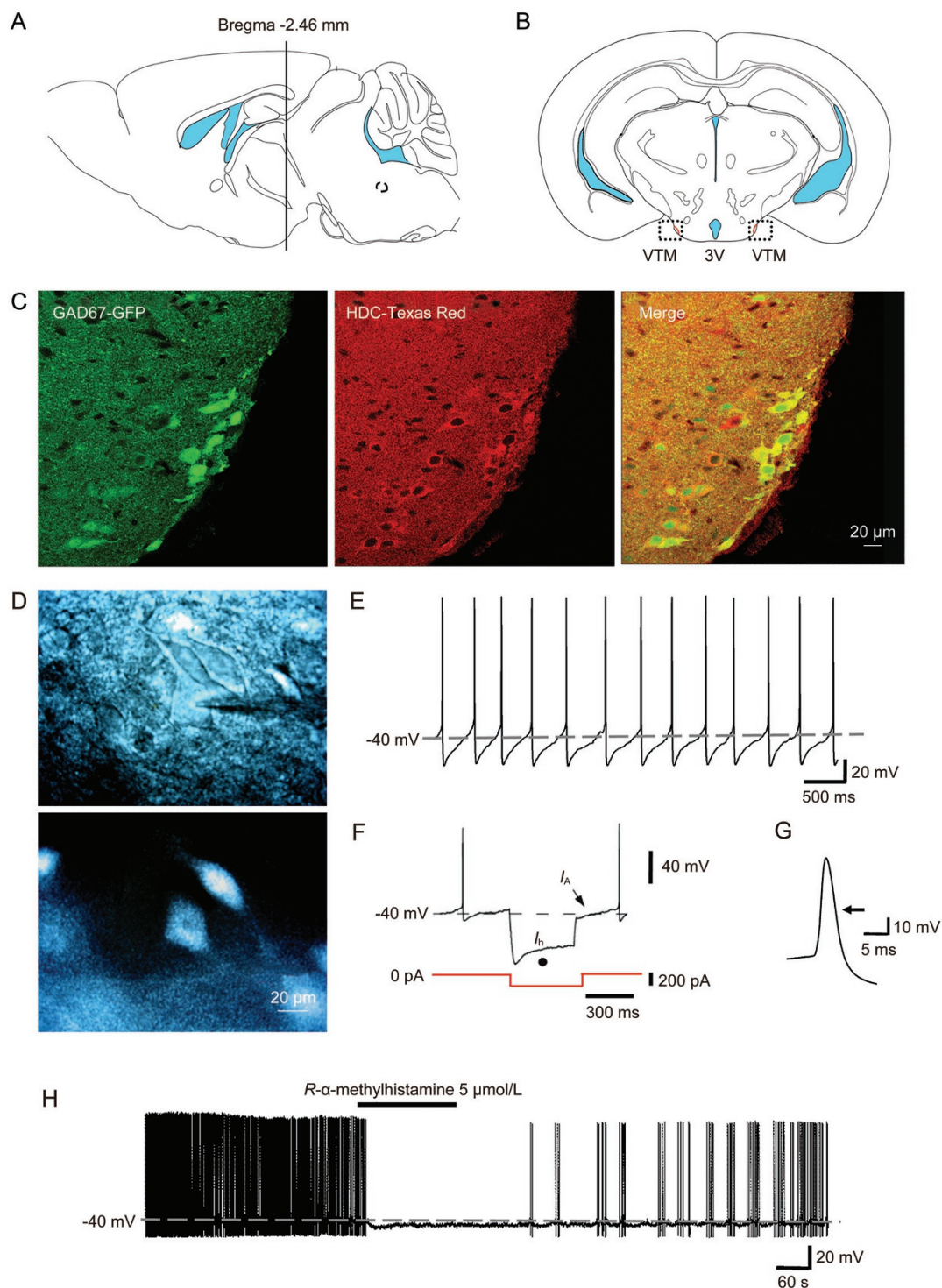


Figure 1. Characteristics of histaminergic neurons in a TMN slice of GAD67-GFP knock-in mice. (A, B) Schematic drawings of sagittal (A) and coronal (B) sections of the mouse brain showing the location of the TMN according to the atlas of Paxinos and Franklin^[20]. 3V, third ventricle; VTM, ventral tuberomammillary. (C) Confocal image of TMN brain slice containing cells expressing GAD67-GFP (left panel) and cells immunoreactive for HDC-Texas Red (center panel). A high degree of colocalization can be seen in the merged image (right panel). (D) Morphological properties of histaminergic neurons: large soma (20–30 μm in diameter), multipolar with three to four long dendrites. The differential interference contrast image (top panel) and fluorescence image (bottom panel) show a patched neuron and the visible patch pipette. (E–G) Electrophysiological identifications of histaminergic TMN neurons: (E) Regular firing (2–4 Hz); (F) the inwardly rectifying current activated by hyperpolarization (I_h , dot) and the transient outward current (due to A-type K^+ currents; I_A , arrow); (G) Broad action potentials (4 ms, arrowhead). (H) Pharmacological application of histamine H_3R agonist (5 μmol/L *R*-α-methylhistamine) confirmed that recorded GAD67-positive cells are histaminergic neurons.

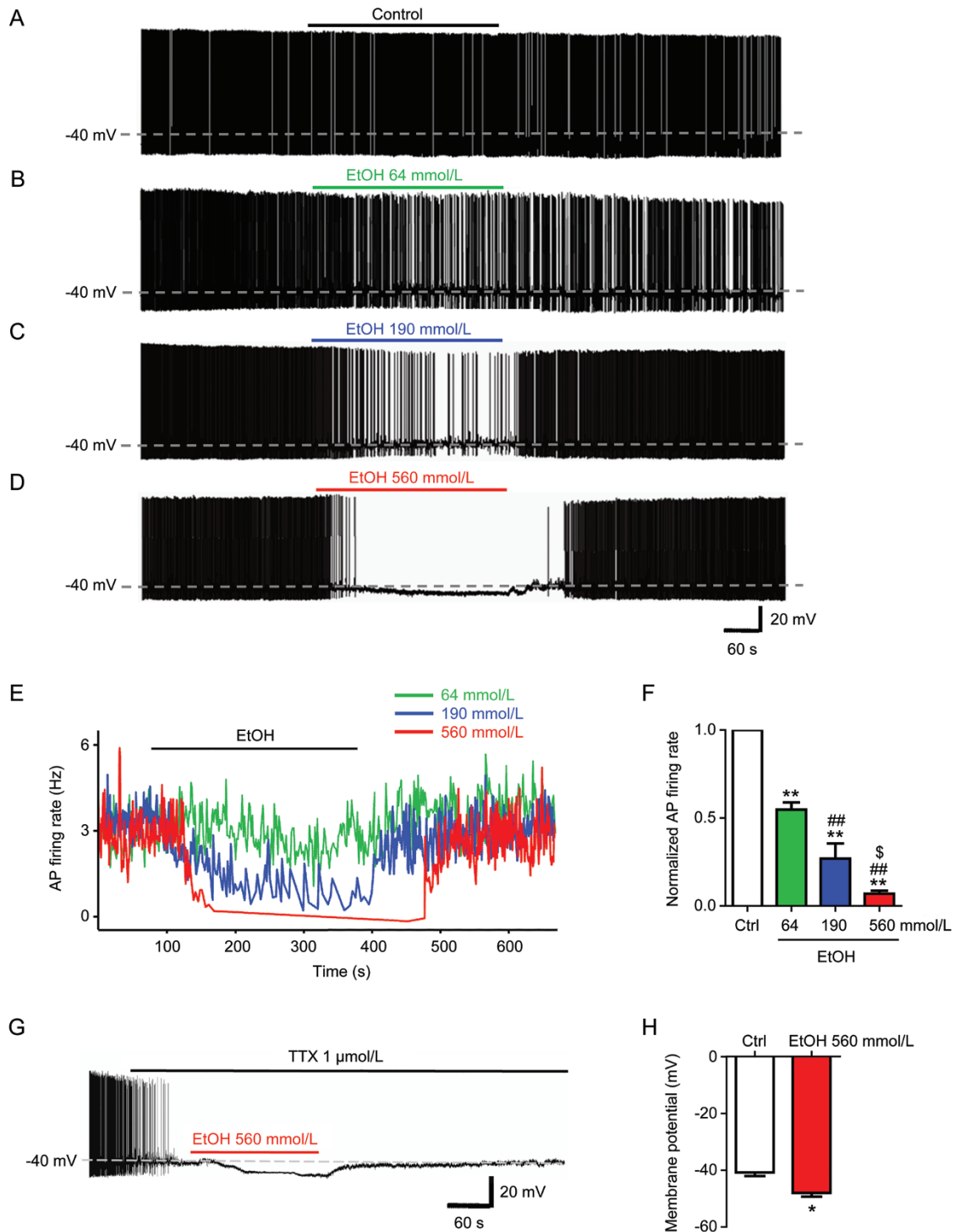


Figure 2. Ethanol inhibited the firing rate and membrane potential in histaminergic TMN neurons. (A–D) Typical examples of histaminergic TMN neurons' firing properties recorded before, during and after bath application of ethanol at 64, 190 and 560 mmol/L for approximately 6 min. (E) Time course of firing rates showing that bath application of ethanol at 64 mmol/L (green), 190 mmol/L (blue) and 560 mmol/L (red) inhibited the firing rates of histaminergic TMN neurons in a concentration-dependent manner and that the firing rates recovered after washout. Data are from three different neurons shown in B–D. (F) Normalized firing rate change of histaminergic TMN neurons after ethanol application at 64, 190 and 560 mmol/L. Mean±SEM. $n=5$. ** $P<0.01$ vs control by paired t -test. ## $P<0.01$ vs ethanol at 64 mmol/L by one-way ANOVA. \$ $P<0.05$ vs ethanol at 190 mmol/L by one-way ANOVA. (G) Bath application of 560 mmol/L ethanol significantly hyperpolarized the histaminergic TMN neuron in the presence of tetrodotoxin (TTX) to block action potentials. (H) Membrane potential changes before (control) and during application of ethanol at 560 mmol/L in histaminergic TMN neurons in the presence of 1 μ mol/L TTX. Mean±SEM. $n=4$. * $P<0.05$ vs control, assessed by paired t -test.

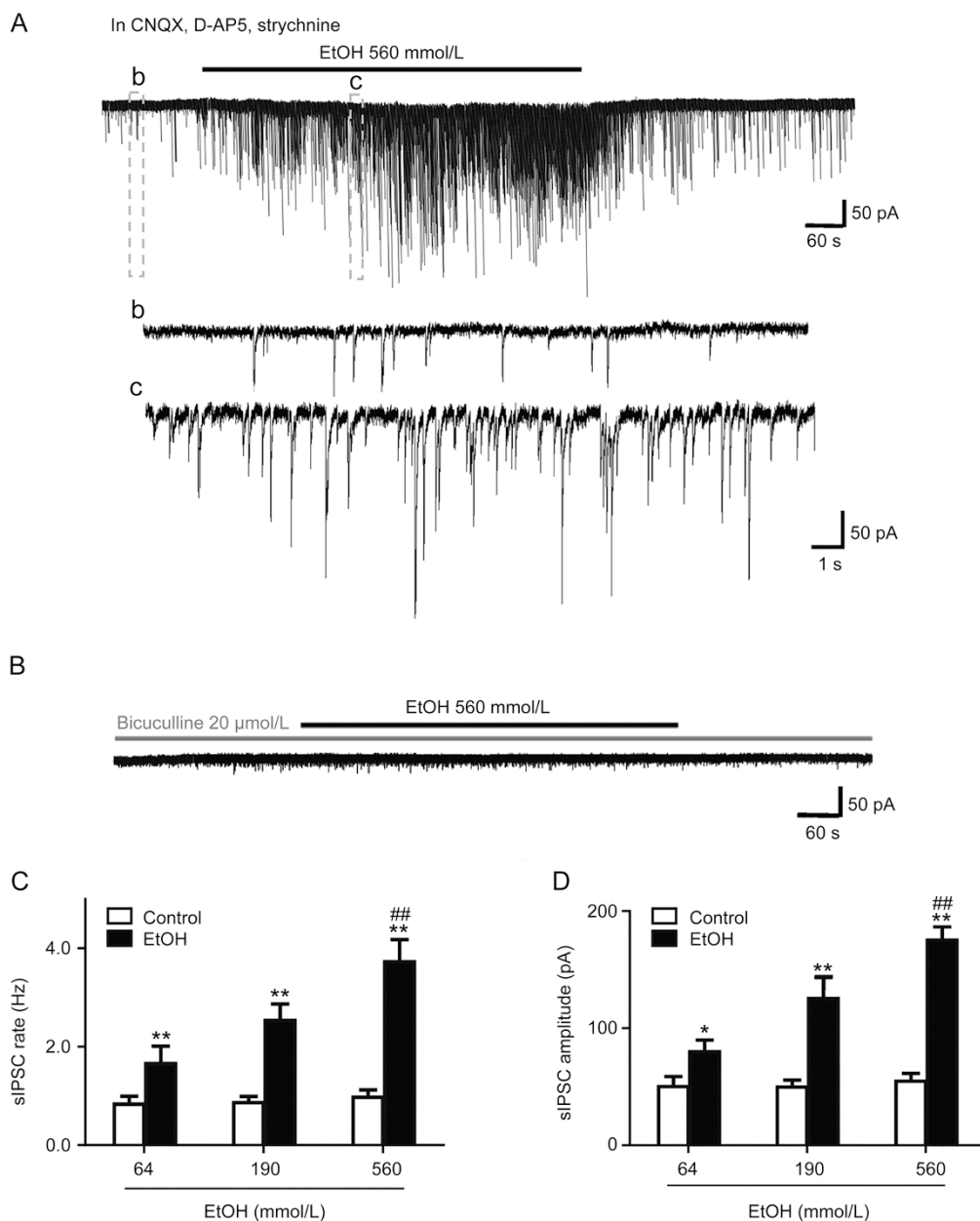


Figure 3. Ethanol increased the amplitude and frequency of GABA_A receptor-mediated spontaneous IPSCs (sIPSCs) recorded from histaminergic TMN neurons in a concentration-dependent manner. (A) A representative sIPSC trace recorded from a histaminergic TMN neuron before, during and after bath application of 560 mmol/L ethanol in the presence of D-AP5 (30 μmol/L), CNQX (10 μmol/L) and strychnine (1 μmol/L). Enlarged sIPSC trace from (A) shows the baseline sIPSCs (b) and the sIPSCs during bath application of 560 mmol/L ethanol (c). (B) sIPSCs and the effect of ethanol were abolished by bicuculline at 20 μmol/L. Ethanol increased the frequency (C) and amplitude (D) of sIPSCs in histaminergic TMN neurons in a concentration-dependent manner. Data are expressed as the mean±SEM. $n=7-9$. * $P<0.05$, ** $P<0.01$ vs baseline control, paired t -test. ## $P<0.01$ vs ethanol at 64 mmol/L, one-way ANOVA.

To further investigate the effect of ethanol on the membrane potential of histaminergic neurons, we added TTX (1 μmol/L) to block action potential-dependent transmitter release and recorded the membrane potential under whole-cell current-clamp mode. We found that ethanol at 560 mmol/L significantly hyperpolarized histaminergic TMN neurons (from -40.8 ± 1.1 in the control to -48.1 ± 1.2 mV during ethanol treatment, $n=4$, $P<0.05$, paired t -test) (Figure 2G and 2H), suggest-

ing a direct effect of ethanol on the intrinsic properties of histaminergic TMN neurons.

Ethanol potentiated GABA_A receptor-mediated spontaneous IPSCs in histaminergic TMN neurons

To determine whether ethanol changes GABAergic inputs to histaminergic TMN neurons, we recorded GABA_A receptor-mediated spontaneous inhibitory postsynaptic currents

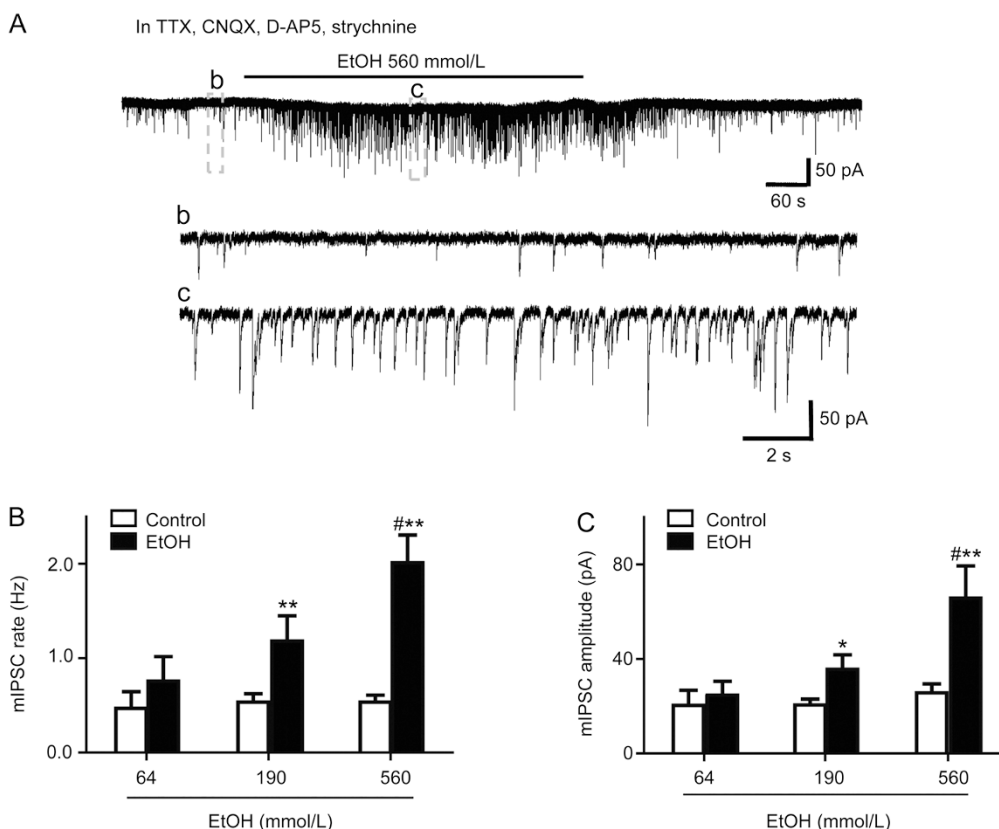


Figure 4. Ethanol augmented the amplitude and frequency of GABA_A receptor-mediated miniature IPSCs (mIPSCs) recorded from histaminergic TMN neurons in a concentration-dependent manner. (A) A representative mIPSC trace recorded before, during and after bath application of 560 mmol/L ethanol in the presence of 1 μ mol/L TTX, 30 μ mol/L D-AP5, 10 μ mol/L CNQX and 1 μ mol/L strychnine. Enlarged mIPSC trace from (A) showing the baseline mIPSCs (b) and the mIPSCs during bath application of 560 mmol/L ethanol (c). Ethanol increased the frequency (B) and amplitude (C) of mIPSCs in histaminergic TMN neurons in a concentration-dependent manner. Data are expressed as the mean \pm SEM. $n=4-5$. * $P<0.05$, ** $P<0.01$ vs baseline control, paired t -test. # $P<0.05$ vs ethanol at 64 mmol/L, one-way ANOVA.

(sIPSCs) in GFP-positive neurons from GAD67-GFP mice in the presence of D-AP5 (30 μ mol/L), CNQX (10 μ mol/L) and strychnine (1 μ mol/L) to block the NMDA and AMPA glutamate receptors and the glycine receptors, respectively. Under whole-cell voltage-clamp mode, bath application of ethanol at 560 mmol/L for 8 min significantly increased the frequency and amplitude of sIPSCs recorded in histaminergic neurons, and after the washout, the effect of ethanol was eliminated (Figure 3A). Figure 3B shows that bath application of bicuculline (20 μ mol/L), a GABA_A receptor antagonist, completely blocked sIPSCs, suggesting that the recorded sIPSCs are mediated by GABA_A receptors.

As shown in Figure 3C, ethanol at concentrations of 64, 190, and 560 mmol/L increased the frequency of sIPSCs by 92.1% \pm 16.4% (from 0.8 \pm 0.1 to 1.6 \pm 0.3 Hz, $n=7$, $P<0.01$, paired t -test), 213.8% \pm 29.4% (from 0.9 \pm 0.1 to 2.5 \pm 0.3 Hz, $n=9$, $P<0.01$), and 305.6% \pm 25.9% (from 1.0 \pm 0.1 to 3.7 \pm 0.4 Hz, $n=9$, $P<0.01$) compared with the corresponding baseline control. Additionally, ethanol at concentrations of 64, 190 and 560 mmol/L potentiated the amplitude of sIPSCs by 84.3% \pm 31.9% (from 50.1 \pm 7.9 to 79.9 \pm 9.3 pA, $n=7$, $P<0.05$, paired t -test), 150.1% \pm 21.6% (from 49.7 \pm 5.6 to 125.4 \pm 16.9 pA, $n=9$, $P<0.01$),

and 260.8% \pm 50.9% (from 54.7 \pm 6.3 to 175.0 \pm 10.8 pA, $n=9$, $P<0.01$) compared with the corresponding baseline control (Figure 3D). Moreover, ethanol at 560 mmol/L produced stronger increases in the frequency and amplitude of sIPSCs than did ethanol at 64 mmol/L ($P<0.01$, one-way ANOVA), whereas there was no statistic significant difference between ethanol at 560 mmol/L and at 190 mmol/L ($P>0.05$, Figure 3C, 3D). These results demonstrated that ethanol enhanced GABAergic inputs to histaminergic TMN neurons, indicating that ethanol inhibits the activities of histaminergic TMN neurons by potentiating synaptic inhibition via GABA_A receptors.

In addition, we tested the effect of ethanol on the firing rate of histaminergic TMN neurons in the presence of bicuculline at 20 μ mol/L. Ethanol at 560 mmol/L still decreased the firing rate of histaminergic neurons by 69.4% (from 3.7 \pm 0.6 to 1.4 \pm 0.1 Hz, $n=7$, $P<0.01$, paired t -test). However, the normalized AP firing rate in the presence of ethanol at 560 mmol/L and bicuculline was significantly increased compared to that in the presence of 560 mmol/L ethanol in normal ACSF (from 6.9% \pm 1.6% to 40.2% \pm 4.3%, $n=5-7$, $P<0.01$, one-way ANOVA), suggesting that ethanol inhibits the activity of histaminergic TMN neurons partly by potentiating GABAergic transmission.

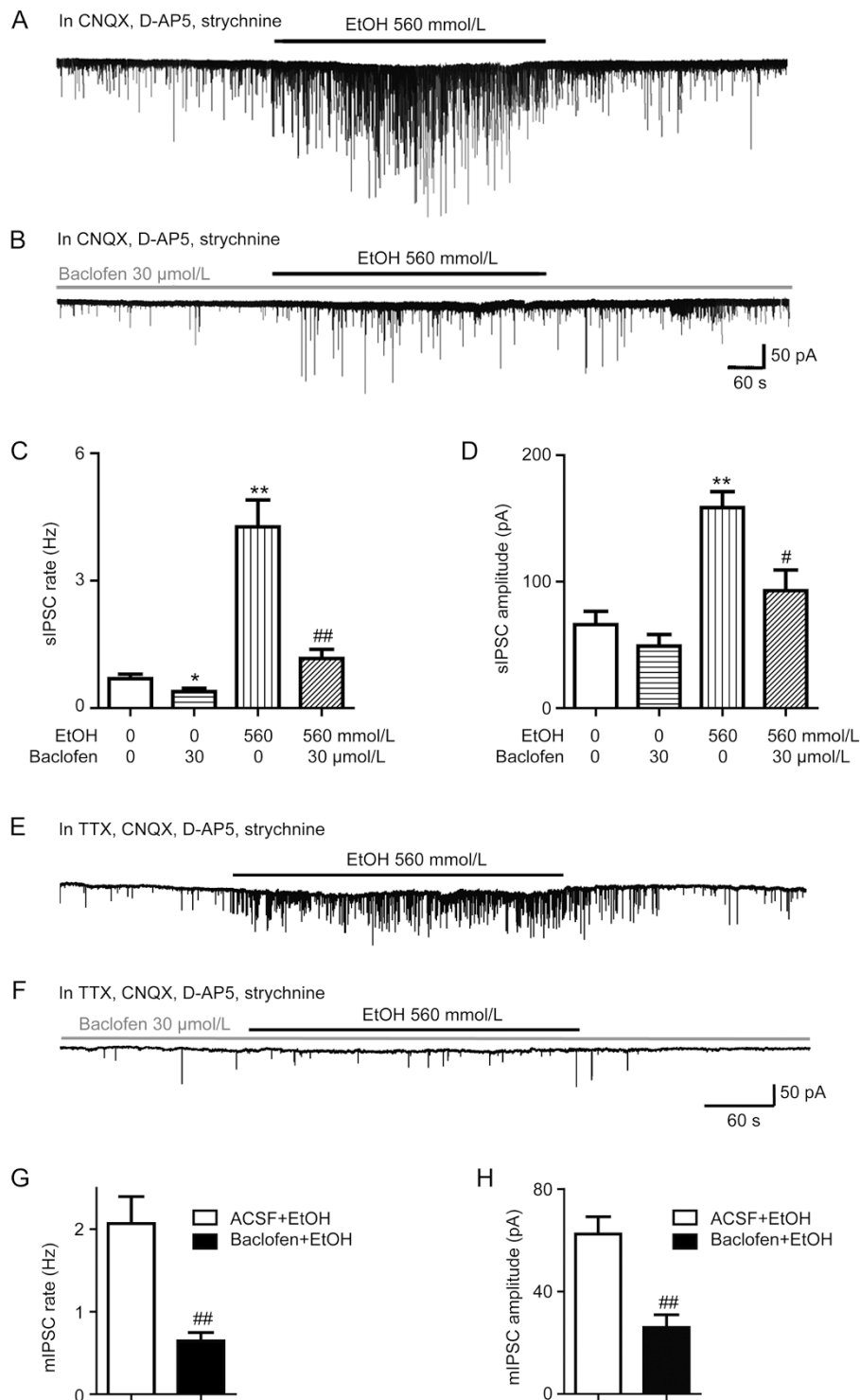


Figure 5. Ethanol increased the frequency and amplitude of sIPSCs and mIPSCs in histaminergic TMN neurons partially via presynaptic GABA_B receptors. (A) Bath application of 560 mmol/L ethanol increased the frequency and amplitude of sIPSCs. (B) In the presence of 30 μ mol/L baclofen, the effect of ethanol on sIPSCs was significantly blocked. (C) The frequency and (D) amplitude of sIPSCs, which were increased by 560 mmol/L ethanol, decreased significantly in the presence of 30 μ mol/L baclofen. (E) Bath application of 560 mmol/L ethanol increased the frequency and amplitude of mIPSCs in histaminergic TMN cells, but (F) with the addition of 30 μ mol/L baclofen, the effect of ethanol on mIPSCs was abolished. The frequency (G) and amplitude (H) of mIPSCs increased by ethanol at 560 mmol/L decreased significantly in the presence of 30 μ mol/L baclofen. Data are expressed as the mean \pm SEM. $n=5$. * $P<0.05$, ** $P<0.01$ vs baseline control, paired t -test. # $P<0.05$, ## $P<0.01$ vs 560 mmol/L ethanol treatment, unpaired t -test.

Ethanol enhanced GABA_A receptor-mediated miniature IPSCs in histaminergic TMN neurons

It is accepted that a change in the frequency of miniature IPSCs (mIPSCs) implies an altered probability of transmitter release at the presynaptic site and that a change in the amplitude of mIPSCs reflects alterations in the sensitivity of postsynaptic receptors—in this case, GABA_A receptors^[7, 28]. To separate the pre- and postsynaptic components of the effects of ethanol on sIPSCs recorded in histaminergic TMN neurons, we added TTX (1 μmol/L) in the bath solution to block action potential-dependent transmitter release and recorded mIPSCs in histaminergic neurons. Figure 4A shows that application of ethanol at 560 mmol/L for 8 min increased the frequency and amplitude of mIPSCs recorded in a histaminergic neuron, whereas the effect of ethanol was removed after the washout. We found that ethanol at 64, 190, and 560 mmol/L increased the frequency of mIPSCs by 79.1%±19.4% (from 0.5±0.1 to 0.76±0.22 Hz, *n*=4, *P*>0.05), 115.3%±17.5% (from 0.5±0.1 to 1.2±0.2 Hz, *n*=5, *P*<0.01), and 276.2%±10.7% (from 0.5±0.1 to 2.0±0.3 Hz, *n*=5, *P*<0.01, paired *t*-test) compared with the corresponding baseline controls (Figure 4B), suggesting a presynaptic site for the action of ethanol. Additionally, bath application of 64, 190, and 560 mmol/L ethanol enhanced the amplitude of mIPSCs by 34.3±18.7% (from 20.4±5.6 to 24.7±5.1 pA, *n*=4, *P*>0.05, paired *t*-test), 78.1%±21.8% (from 20.5±2.2 to 35.7±5.5 pA, *n*=5, *P*<0.05), and 151.4%±17.2% (from 25.7±3.3 to 65.7±12.2 pA, *n*=5, *P*<0.01) compared with the corresponding baseline control (Figure 4C), suggesting a postsynaptic site of the action of ethanol. Both the frequency and the amplitude of mIPSCs showed significant increases with the two higher concentrations of ethanol. These mIPSCs were totally blocked by bicuculline, suggesting that they are mediated by GABA_A receptors (data not shown). Moreover, the increases in the frequency and amplitude of mIPSCs induced by ethanol at 560 mmol/L were stronger than those induced by ethanol at 64 mmol/L (*P*<0.05, one-way ANOVA), whereas there were no statistic significant differences between ethanol at 560 and at 190 mmol/L (Figure 4B and 4C). These findings demonstrated that ethanol increased the frequency and amplitude of mIPSCs, indicating that ethanol acts on both pre- and postsynaptic sites of GABAergic synapses on histaminergic neurons.

Activation of GABA_B receptors by baclofen reduced the ethanol-induced enhancement of GABAergic transmission in histaminergic neurons

The presynaptic GABA_B autoreceptor has been reported to control GABAergic inhibition in rat histaminergic neurons *in vitro*^[29]. Because we found that ethanol increased the frequency of mIPSCs, resulting from a presynaptic modification of synaptic vesicle release probability, we hypothesized that this presynaptic effect would be sensitive to the modulation by presynaptic GABA_B autoreceptors. To address this hypothesis, brain slices were pretreated with the GABA_B receptor agonist baclofen (30 μmol/L). First, we demonstrated that baclofen at 30 μmol/L significantly reduced the frequency of sIPSCs, by 44.8%, from a baseline of 0.7±0.1 Hz in ACSF to

a baseline of 0.4±0.1 Hz in ACSF with baclofen (*n*=5, *P*<0.05, paired *t*-test; Figure 5A, 5B, and 5C), but baclofen did not affect the amplitude of sIPSCs (66.2±9.3 vs 49±8.2 pA, *n*=5, *P*>0.05, paired *t*-test; Figure 5A, 5B, and 5D). Next, we found that ethanol at 560 mmol/L in the presence of baclofen significantly decreased the frequency of sIPSCs, by 70.2%±5.3%, when compared to treatment with ACSF+560 mmol/L ethanol (from 4.3±0.6 to 1.2±0.2 Hz, *n*=5, *P*<0.01, unpaired *t*-test; Figure 5A, 5B, and 5C), and baclofen reduced the potentiation effect on the amplitude of sIPSCs by 40.7%±10.7% (from 158.6±11.4 in ACSF+560 mmol/L ethanol to 93.0±14.5 pA in ACSF+baclofen+560 mmol/L ethanol, *n*=5, *P*<0.05; Figure 5A, 5B, and 5D).

Then, we tested the effect of ethanol on mIPSCs in histaminergic neurons. In the presence of baclofen, the facilitatory effect of ethanol at 560 mmol/L on the mIPSC frequency was significantly decreased, by 68.0%±2.3% compared to the treatment with ACSF+560 mmol/L ethanol (from 2.1±0.3 to 0.6±0.1 Hz, *n*=5, *P*<0.01, unpaired *t*-test), and the potentiation effect on the mIPSC amplitude was also significantly attenuated, by 59.0%±5.0% (from 62.4±6.0 in ACSF+560 mmol/L ethanol to 25.9±4.5 pA in baclofen+560 mmol/L ethanol, *n*=5, *P*<0.01). Taken together, our results show that ethanol may act at presynaptic GABA_B autoreceptors to enhance GABAergic transmission in histaminergic neurons.

Discussion

In this study, we report for the first time that ethanol directly suppresses the firing rate of histaminergic TMN neurons in a concentration-dependent and reversible manner. These findings suggest that histaminergic TMN neurons may serve as one of the main targets in the brain for ethanol-induced sedation and hypnosis.

The activity of histaminergic neurons correlates with an animal's vigilance state, locomotion, and exploratory activity in a novel environment^[10, 30]. During wakefulness, histaminergic neurons in the TMN discharge tonically and specifically^[31]. The extracellular histamine level in the frontal cortex and the amount of wakefulness are positively correlated in rats^[32]. In rats, up-regulation of c-Fos, representing neuronal activity, was observed during the active phase, whereas down-regulation occurred in the resting phase^[33]. Recent studies have reported that ethanol promotes sleep by increasing adenosine levels in the orexinergic perifornical hypothalamus, thus resulting in A₁ receptor-mediated inhibition of orexin neurons^[34]. Although the orexinergic system is important for wakefulness, there is evidence showing that histaminergic TMN neurons may be among the essential downstream targets for the arousal effect of orexin^[35-37]. Therefore, ethanol may promote sleep by inhibiting the activity of histaminergic TMN neurons.

Here, we provide direct evidence that ethanol inhibited the excitability of histaminergic TMN neurons by hyperpolarizing the membrane potential. It is likely that ethanol-induced changes in the membrane potential in histaminergic TMN neurons may be mediated by adenosine via A₁ receptors, which

activate G-protein-coupled inwardly rectifying potassium channels^[38]. Thakkar *et al* and Sharma *et al* reported an ethanol-induced increase in adenosine in several wake-promoting brain regions, such as the basal forebrain and orexinergic perifornical hypothalamus^[26,34]. Oishi *et al* previously reported the expression of adenosine A₁ receptors in histaminergic TMN neurons^[39]. In addition to the adenosine A₁ receptor system, the remarkable membrane hyperpolarization in histaminergic TMN neurons may also be mediated by increased GABAergic inhibition, possibly reinforced by ethanol-induced sensitization of extrasynaptic GABA_A receptors, which mediate tonic inhibitory currents.

We report for the first time that ethanol significantly increased the frequency of sIPSCs and mIPSCs in histaminergic TMN neurons. This finding is consistent with other studies demonstrating ethanol-mediated presynaptic enhancement of action potential-dependent sIPSCs^[40] and action potential-independent mIPSCs^[41] in the hippocampus, an important brain structure for learning and memory, and the central amygdala, which is involved in anxiety and stress responses^[7,9], as well as in the ventral tegmental area, which mediates motivation and drug addiction^[42]. Moreover, in the cerebellum, a brain region that plays an important role in motor control, acute ethanol application increased the frequency of sIPSCs recorded from granule cells^[43]. Therefore, ethanol modulation of GABAergic transmission exists in many CNS structures, and the enhancement of GABAergic transmission by ethanol in the TMN may play a crucial role in the sedative effect of ethanol.

We show that pretreatment with baclofen, a GABA_B receptor agonist, significantly reduced the effect of ethanol on the frequency and amplitude of sIPSCs and mIPSCs, suggesting that ethanol-induced presynaptic enhancement of action potential-dependent and -independent GABA release is primarily mediated by GABA_B autoreceptors in the TMN. Activation of presynaptic GABA_B receptors at many inhibitory synapses in the mammalian CNS produces a well-characterized inhibition of GABA release along with a decrease in the size of action potential-dependent IPSCs^[44,45]. Combined with our data, we speculate that ethanol enhances GABA release possibly by antagonizing the activity or the intracellular signaling pathways of presynaptic GABA_B autoreceptors at GABA synapses onto histaminergic TMN neurons.

In addition, ethanol significantly increased the amplitude of mIPSCs in our study, possibly reflecting postsynaptic actions of ethanol on GABA_A receptors. Moreover, both sIPSCs and mIPSCs were antagonized by bicuculline, confirming that these currents were mediated by GABA_A receptors. Therefore, we hypothesized that ethanol may act allosterically at GABA_A receptors to increase the affinity of GABA for GABA_A receptors, resulting in more Cl⁻ influx through GABA_A receptors, thus potentiating the amplitude of GABA_A receptor-mediated currents on histaminergic TMN cells. The wake-active TMN receives inhibitory GABAergic inputs from the sleep-active ventrolateral preoptic area. The GABAergic synapses formed on histaminergic TMN neurons express at least nine different GABA_A receptor subunits: α 1, α 2, α 5, β 1, β 2, β 3, γ 1, γ 2, and

ϵ ^[46]. Although the γ 1-subunit of GABA_A receptors likely contributes to the action of common sedatives in TMN neurons^[17], Wafford *et al* reported that the α 1 β 1 γ 2 GABA_A receptor subunit combination, which forms a type-1 benzodiazepine receptor, allowed ethanol enhancement of GABA responses, whereas other GABA_A receptor subunit combinations did not, even at high ethanol concentrations^[47]. Hence, the specific GABA_A receptor subtype responsible for the effect of ethanol in the TMN remains to be examined in further studies.

Based on our present findings that ethanol directly inhibited the firing rate of histaminergic TMN neurons, hyperpolarized the membrane potential, increased the frequency and amplitude of sIPSCs and mIPSCs and that the enhancement of these two IPSCs could be abolished by a GABA_B autoreceptor agonist, we hypothesized that ethanol reduces the activity of histaminergic neurons, facilitates the release of GABA to histaminergic cells via GABA_B autoreceptors and enhances the sensitivity of GABA_A receptors. Taken together, we concluded that ethanol acts at both presynaptic (GABA_B autoreceptors) and postsynaptic sites (GABA_A receptors) to enhance GABAergic transmission and to decrease the activities of histaminergic TMN neurons, providing a plausible mechanism for the sedative effect of ethanol.

Abbreviation

GAD67-GFP, glutamic acid decarboxylase 67-green fluorescent protein; HDC, histidine decarboxylase; IPSC, inhibitory postsynaptic current; mIPSC, miniature IPSC; sIPSC, spontaneous IPSC; tuberomammillary nucleus (TMN); TTX, tetrodotoxin.

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Author contribution

Yu SUN and Lu WANG designed the study and conducted the experiments; Shi-yu JIANG, Jian NI, Yan-jia LUO, and Chang-rui CHEN coordinated the experiments; Zong-yuan HONG and Wei-min QU were involved in data analysis and discussion of the experiments; Yuchio YANAGAWA provided GAD67-GFP knock-in mice; Yu SUN, Lu WANG, and Zhi-li HUANG were involved in discussion of the experiments and writing the manuscript. All authors read and approved the final manuscript.

References

- 1 Roehrs T, Roth T. Sleep, sleepiness, sleep disorders and alcohol use and abuse. *Sleep Med Rev* 2001; 5: 287–97.
- 2 Thakkar MM, Sharma R, Sahota P. Alcohol disrupts sleep homeo-

- stasis. *Alcohol* 2015; 49: 299–310.
- 3 Wan FJ, Berton F, Madamba SG, Francesconi W, Siggins GR. Low ethanol concentrations enhance GABAergic inhibitory postsynaptic potentials in hippocampal pyramidal neurons only after block of GABA_B receptors. *Proc Natl Acad Sci U S A* 1996; 93: 5049–54.
 - 4 Kang MH, Spigelman I, Olsen RW. Alteration in the sensitivity of GABA(A) receptors to allosteric modulatory drugs in rat hippocampus after chronic intermittent ethanol treatment. *Alcohol Clin Exp Res* 1998; 22: 2165–73.
 - 5 Silberman Y, Ariwodola OJ, Weiner JL. Differential effects of GABA_B autoreceptor activation on ethanol potentiation of local and lateral paracapsular GABAergic synapses in the rat basolateral amygdala. *Neuropharmacology* 2009; 56: 886–95.
 - 6 Thompson SM. Modulation of inhibitory synaptic transmission in the hippocampus. *Prog Neurobiol* 1994; 42: 575–609.
 - 7 Roberto M, Madamba SG, Moore SD, Tallent MK, Siggins GR. Ethanol increases GABAergic transmission at both pre- and postsynaptic sites in rat central amygdala neurons. *Proc Natl Acad Sci U S A* 2003; 100: 2053–8.
 - 8 Siggins GR, Pittman QJ, French ED. Effects of ethanol on CA1 and CA3 pyramidal cells in the hippocampal slice preparation: an intracellular study. *Brain Res* 1987; 414: 22–34.
 - 9 Nie Z, Schweitzer P, Roberts AJ, Madamba SG, Moore SD, Siggins GR. Ethanol augments GABAergic transmission in the central amygdala via CRF1 receptors. *Science* 2004; 303: 1512–4.
 - 10 Huang ZL, Urade Y, Hayaishi O. Prostaglandins and adenosine in the regulation of sleep and wakefulness. *Curr Opin Pharmacol* 2007; 7: 33–8.
 - 11 Huang ZL, Urade Y, Hayaishi O. The role of adenosine in the regulation of sleep. *Curr Top Med Chem* 2011; 11: 1047–57.
 - 12 Huang ZL, Zhang Z, Qu WM. Roles of adenosine and its receptors in sleep-wake regulation. *Int Rev Neurobiol* 2014; 119: 349–71.
 - 13 Huang ZL, Mochizuki T, Qu WM, Hong ZY, Watanabe T, Urade Y, *et al*. Altered sleep-wake characteristics and lack of arousal response to H3 receptor antagonist in histamine H1 receptor knockout mice. *Proc Natl Acad Sci U S A* 2006; 103: 4687–92.
 - 14 Lintunen M, Raatesalmi K, Sallmen T, Aничтchik O, Karlstedt K, Kaslin J, *et al*. Low brain histamine content affects ethanol-induced motor impairment. *Neurobiol Dis* 2002; 9: 94–105.
 - 15 Didone V, Quoilin C, Nyssen L, Closon C, Tirelli E, Quertemont E. Effects of L-histidine and histamine H3 receptor modulators on ethanol-induced sedation in mice. *Behav Brain Res* 2013; 238: 113–8.
 - 16 Misgeld U, Bijak M, Jarolimek W. A physiological role for GABA_B receptors and the effects of baclofen in the mammalian central nervous system. *Prog Neurobiol* 1995; 46: 423–62.
 - 17 May AC, Fleischer W, Kletke O, Haas HL, Sergeeva OA. Benzodiazepine-site pharmacology on GABA_A receptors in histaminergic neurons. *Br J Pharmacol* 2013; 170: 222–32.
 - 18 Chen CR, Sun Y, Luo YJ, Zhao X, Chen JF, Yanagawa Y, *et al*. Paeoniflorin promotes non-rapid eye movement sleep via adenosine A1 receptors. *J Pharmacol Exp Ther* 2016; 356: 64–73.
 - 19 Tamamaki N, Yanagawa Y, Tomioka R, Miyazaki J, Obata K, Kaneko T. Green fluorescent protein expression and colocalization with calretinin, parvalbumin, and somatostatin in the GAD67-GFP knock-in mouse. *J Comp Neurol* 2003; 467: 60–79.
 - 20 Paxinos G, Franklin KBJ. *The mouse brain in stereotaxic coordinates*. San Diego: Academic Press; 2001.
 - 21 Wang Q, Yue XF, Qu WM, Tan R, Zheng P, Urade Y, *et al*. Morphine inhibits sleep-promoting neurons in the ventrolateral preoptic area via mu receptors and induces wakefulness in rats. *Neuropsychopharmacology* 2013; 38: 791–801.
 - 22 Kolaj M, Coderre E, Renaud LP. Orexin peptides enhance median preoptic nucleus neuronal excitability via postsynaptic membrane depolarization and enhancement of glutamatergic afferents. *Neuroscience* 2008; 155: 1212–20.
 - 23 Qu WM, Yue XF, Sun Y, Fan K, Chen CR, Hou YP, *et al*. Honokiol promotes non-rapid eye movement sleep via the benzodiazepine site of the GABA(A) receptor in mice. *Br J Pharmacol* 2012; 167: 587–98.
 - 24 Kukko-Lukjanov TK, Panula P. Subcellular distribution of histamine, GABA and galanin in tuberomammillary neurons *in vitro*. *J Chem Neuroanat* 2003; 25: 279–92.
 - 25 Ericson M, Molander A, Lof E, Engel JA, Soderpalm B. Ethanol elevates accumbal dopamine levels via indirect activation of ventral tegmental nicotinic acetylcholine receptors. *Eur J Pharmacol* 2003; 467: 85–93.
 - 26 Sharma R, Sahota P, Thakkar MM. Role of adenosine and the orexinergic perifornical hypothalamus in sleep-promoting effects of ethanol. *Sleep* 2014; 37: 525–33.
 - 27 Sergeeva OA, Klyuch BP, Fleischer W, Eriksson KS, Korotkova TM, Siebler M, *et al*. P2Y receptor-mediated excitation in the posterior hypothalamus. *Eur J Neurosci* 2006; 24: 1413–26.
 - 28 Otis TS, De Koninck Y, Mody I. Lasting potentiation of inhibition is associated with an increased number of gamma-aminobutyric acid type A receptors activated during miniature inhibitory postsynaptic currents. *Proc Natl Acad Sci U S A* 1994; 91: 7698–702.
 - 29 Stevens DR, Kuramasu A, Haas HL. GABA_B-receptor-mediated control of GABAergic inhibition in rat histaminergic neurons *in vitro*. *Eur J Neurosci* 1999; 11: 1148–54.
 - 30 Burgess CR. Histamine and orexin in the control of arousal, locomotion, and motivation. *J Neurosci* 2010; 30: 2810–1.
 - 31 Vanni-Mercier G, Gigout S, Debilly G, Lin JS. Waking selective neurons in the posterior hypothalamus and their response to histamine H3-receptor ligands: an electrophysiological study in freely moving cats. *Behav Brain Res* 2003; 144: 227–41.
 - 32 Chu M, Huang ZL, Qu WM, Eguchi N, Yao MH, Urade Y. Extracellular histamine level in the frontal cortex is positively correlated with the amount of wakefulness in rats. *Neurosci Res* 2004; 49: 417–20.
 - 33 Scammell T, Gerashchenko D, Urade Y, Onoe H, Saper C, Hayaishi O. Activation of ventrolateral preoptic neurons by the somnogen prostaglandin D2. *Proc Natl Acad Sci U S A* 1998; 95: 7754–9.
 - 34 Sharma R, Engemann SC, Sahota P, Thakkar MM. Effects of ethanol on extracellular levels of adenosine in the basal forebrain: an *in vivo* microdialysis study in freely behaving rats. *Alcohol Clin Exp Res* 2010; 34: 813–8.
 - 35 Hong ZY, Huang ZL, Qu WM, Eguchi N. Orexin A promotes histamine, but not norepinephrine or serotonin, release in frontal cortex of mice. *Acta Pharmacol Sin* 2005; 26: 155–9.
 - 36 Huang ZL, Qu WM, Li WD, Mochizuki T, Eguchi N, Watanabe T, *et al*. Arousal effect of orexin A depends on activation of the histaminergic system. *Proc Natl Acad Sci U S A* 2001; 98: 9965–70.
 - 37 Mochizuki T, Arrigoni E, Marcus JN, Clark EL, Yamamoto M, Honer M, *et al*. Orexin receptor 2 expression in the posterior hypothalamus rescues sleepiness in narcoleptic mice. *Proc Natl Acad Sci U S A* 2011; 108: 4471–6.
 - 38 Liu ZW, Gao XB. Adenosine inhibits activity of hypocretin/orexin neurons by the A1 receptor in the lateral hypothalamus: a possible sleep-promoting effect. *J Neurophysiol* 2007; 97: 837–48.
 - 39 Oishi Y, Huang ZL, Fredholm BB, Urade Y, Hayaishi O. Adenosine in the tuberomammillary nucleus inhibits the histaminergic system via A1 receptors and promotes non-rapid eye movement sleep. *Proc Natl Acad Sci U S A* 2008; 105: 19992–7.

- 40 Carta M, Partridge LD, Savage DD, Valenzuela CF. Neurosteroid modulation of glutamate release in hippocampal neurons: lack of an effect of a chronic prenatal ethanol exposure paradigm. *Alcohol Clin Exp Res* 2003; 27: 1194–8.
- 41 Sanna E, Talani G, Busonero F, Pisu MG, Purdy RH, Serra M, *et al.* Brain steroidogenesis mediates ethanol modulation of GABA_A receptor activity in rat hippocampus. *J Neurosci* 2004; 24: 6521–30.
- 42 Theile JW, Morikawa H, Gonzales RA, Morrisett RA. Ethanol enhances GABAergic transmission onto dopamine neurons in the ventral tegmental area of the rat. *Alcohol Clin Exp Res* 2008; 32: 1040–8.
- 43 Carta M, Mameli M, Valenzuela CF. Alcohol enhances GABAergic transmission to cerebellar granule cells via an increase in Golgi cell excitability. *J Neurosci* 2004; 24: 3746–51.
- 44 Bowery NG, Enna SJ. Gamma-aminobutyric acid(B) receptors: first of the functional metabotropic heterodimers. *J Pharmacol Exp Ther* 2000; 292: 2–7.
- 45 Couve A, Moss SJ, Pangalos MN. GABA_B receptors: a new paradigm in G protein signaling. *Mol Cell Neurosci* 2000; 16: 296–312.
- 46 Sergeeva OA, Andreeva N, Garret M, Scherer A, Haas HL. Pharmacological properties of GABA_A receptors in rat hypothalamic neurons expressing the epsilon-subunit. *J Neurosci* 2005; 25: 88–95.
- 47 Wafford KA, Burnett DM, Leidenheimer NJ, Burt DR, Wang JB, Kofuji P, *et al.* Ethanol sensitivity of the GABA_A receptor expressed in *Xenopus* oocytes requires 8 amino acids contained in the gamma 2L subunit. *Neuron* 1991; 7: 27–33.