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Ethanol-induced enhancement of inhibitory synaptic transmission in the rat spinal substantia gelatinosa

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Abstract

Recent studies have shown that ethanol produces a widespread modulation of neuronal activity in the central nervous system. It is not fully understood, however, how ethanol changes nociceptive transmission. We investigated acute effects of ethanol on synaptic transmission in the substantia gelatinosa (lamina II of the spinal dorsal horn) and mechanical responses in the spinal dorsal horn. In substantia gelatinosa neurons, bath application of ethanol at low concentration (10 mM) did not change the frequency and amplitude of spontaneous inhibitory postsynaptic currents. At medium to high concentrations (20–100 mM), however, ethanol elicited a barrage of large amplitude spontaneous inhibitory postsynaptic currents. In the presence of tetrodotoxin, such enhancement of spontaneous inhibitory postsynaptic currents was not detected. In addition, ethanol (20–100 mM) increased the frequency of spontaneous discharge of vesicular GABA transporter-Venus-labeled neurons and suppressed the mechanical nociceptive response in wide-dynamic range neurons in the spinal dorsal horn. The present results suggest that ethanol may reduce nociceptive information transfer in the spinal dorsal horn by enhancement of inhibitory GABAergic and glycinergic synaptic transmission.

Keywords

Alcohol, acute ethanol, spinal cord, antinociceptive action, vesicular GABA transporter, after discharge, wide-dynamic range neuron

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Introduction

Ethanol is commonly consumed, and elevated blood concentrations of ethanol produces changes in mood, cognition, locomotion and causes sedation and analgesia. Recent studies have shown that acute ethanol administration exerts actions throughout the central nervous system (CNS) including prefrontal cortex, amygdala, hippocampus, ventral tegmental area, and spinal ventral horn.[1,2] As ethanol has hypofunctional and sedative actions, one of the main actions of ethanol is considered to be the inhibition of neuronal activities in part through enhancement of inhibitory synaptic transmission. In the prefrontal cortex, which is thought to be important for mood and cognition, ethanol enhanced the GABA-mediated Cl^- current and reduced the neuronal activities.[3–5] It also enhanced GABAergic currents evoked in CA1 pyramidal neurons in the hippocampus,[6] which is implicated in learning and memory, and in the amygdala,[7,8] which is important for fear and stress. In the cerebellum and the spinal ventral horn, which are related to motor function, ethanol potentiated inhibitory synaptic transmission.[9,10] An inhibitory action on excitatory synaptic responses was also reported in the prefrontal cortex[11] and hippocampus.[12] In addition to synaptic ethanol actions, ethanol effects on neuronal intrinsic excitability are also observed, but only in

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restricted groups of neurons in the CNS. Dopaminergic neurons in the ventral tegmental area that innervate the nucleus accumbens and are a critical component of reward system are directly excited by ethanol to increase their firing frequency.[13,14] Ethanol increased spontaneous firing of Cerebellar Golgi cells (GABAergic neurons).[10] The excitability of hippocampal inhibitory interneurons was increased by ethanol.[15] Thus, ethanol has modulatory actions on synaptic transmission to suppress neuronal activities widely in the CNS, but its excitatory actions on intrinsic neuronal excitability are cell-type specific. Ethanol has been used as an analgesic[16] and therefore there has been a long-standing interest in the analgesic properties of ethanol. Although ethanol actions on neuronal activities in the amygdala, which play a role in emotional aspects of pain, are studied,[7,8] it is not still understood how ethanol induces its analgesic action.

The substantia gelatinosa (SG), in the spinal superficial dorsal horn (lamina II), plays an important role in the transmission and modulation of nociceptive information.[17,18] SG neurons are second-order neurons receiving input from nociceptive primary afferents and also inhibitory interneurons which are mostly located in lamina I-III.[19–22] In this study, we used three protocols to clarify the acute effects of ethanol on nociceptive transmission in the spinal dorsal horn. First, we investigated actions of ethanol on inhibitory and excitatory synaptic transmission in the SG using the whole-cell patch-clamp recording technique[23] and found that ethanol preferentially enhanced inhibitory synaptic transmission. Then, we used a transgenic rat expressing the fluorescent protein Venus under the control of vesicular GABA Transporter (VGAT)[24–26] and examined how ethanol acts on the VGAT-Venus-labeled neurons in the spinal dorsal horn. Finally, we assessed whether ethanol-modulated spinal sensory responses evoked by cutaneous mechanical stimulation in anesthetized rats.

Material and methods

Animals

Male Sprague-Dawley (SD) rats (SLC, Hamamatsu, Japan) and VGAT-Venus Wister rats were used in this study. Animals were housed in cages with food and water available ad libitum. The room was maintained with 12-h light/dark cycle and kept at 20°C. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of Hyogo College of Medicine and National Institutes of Natural Sciences in Japan and were performed in accordance with the institutional guidelines for animal experiments and were consistent with the ethical guidelines of the International Association for the Study of Pain. Every effort was made to reduce the number of animals. At the end of the study, the animals were killed with supplemental injection of urethane (2–4 g/kg, i.p.) or by exsanguination under the urethane anesthesia (1.2–1.5 g/kg, i.p.)

Spinal cord slice preparations

The method for obtaining spinal cord slices has been described previously.[23,27] Briefly, two- to four-week-old SD and VGAT-Venus rats were deeply anesthetized with urethane (1.2–1.5 g/kg, i.p.), and then thoracolumbar laminectomy was performed. The lumbosacral spinal cord was removed and placed in a pre-oxygenated cold Krebs solution containing (in mM): 117 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, and 11 glucose at 1°C to 3°C. The pia-arachnoid membrane was removed after cutting all of the ventral and dorsal roots. The spinal cord was mounted on a vibratome, and a 500-µm (for blind whole-cell recordings) or 300-µm (for recordings from VGAT-Venus-labeled cells)-thick transverse slice was cut. The slice was placed in the recording chamber and then perfused with Krebs solution saturated with 95% O_2 and 5% CO_2 at 36°C at a flow rate of 10 ml/min.

Whole-cell patch-clamp recordings

For blind whole-cell recordings, the SG was easily discernible with transmitted illumination as a relatively translucent band across the dorsal horn in the transverse slice preparation. Blind whole-cell patch-clamp recordings were made from SG neurons.[23] The patch pipettes were filled with a solution containing (mM): potassium solution (K-gluconate 135, $CaCl_2$ 0.5, $MgCl_2$ 2, KCl 5, EGTA 5, 5 Mg-ATP, and HEPES 5; pH: 7.2) for recordings of excitatory postsynaptic currents (EPSCs) and membrane potentials, or cesium solution (Cs_2SO_4 110, TEA-Cl 5, $CaCl_2$ 0.5, $MgCl_2$ 2, EGTA 5, ATP-Mg 5, and HEPES-CsOH 5; pH: 7.2) for recordings of inhibitory postsynaptic currents (IPSCs). EPSCs and IPSCs were recorded under voltage-clamp conditions at a holding potential of –70 mV and 0 mV, respectively. Membrane potentials and action potentials (APs) were recorded under current-clamp conditions. The passive membrane and active properties were examined by passing hyperpolarizing and depolarizing current pulses through the recording electrode from a membrane potential of –60 mV. The firing frequency was calculated from the firings in response to 1 s depolarizing current pulse with an amplitude of 1.5 to 2 times higher than the threshold. Input membrane resistance was calculated from the hyperpolarized membrane potentials ranging from –60 to –80 mV. VGAT-Venus-labeled neurons in the slice preparation were visualized using an upright microscope (BX51WI; Olympus Optical Tokyo, Japan)

equipped with infrared differential interference contrast Nomarski with a fluorescence filter (U-MWIGA3; Olympus). Signals were amplified with a patch-clamp amplifier (Axopatch 200B; Molecular Devices, Sunnyvale, CA, USA), and data were low-pass filtered at 5 kHz, digitized with a analog-to-digital converter (Digidata 1322; Molecular Devices), and stored on a personal computer at 10 to 20 kHz using a data acquisition program (pCLAMP version 12.3; Molecular Devices). Ethanol and tetrodotoxin (TTX) were dissolved in Krebs solution. Synaptic events were analyzed using a program (Minianalysis version 6.0.7; Synaptosoft, Fort Lee, NJ, USA). We a priori defined neurons as being sensitive to ethanol when the frequency of synaptic responses was altered by more than ±50% of control.

Extracellular recording from spinal dorsal horn neurons in vivo

The methods for making *in vivo* preparation were similar to those in our previous studies.[20,28,29] Briefly, six-week-old SD rats were anesthetized with urethane (1.2–1.5 g/kg, i.p.) and placed on a warm plate. A thoracolumbar laminectomy at table13-1744806918817969-L2 levels was performed to expose the dorsal surface of the lumber enlargement of spinal cord at L3-L5 levels. The rat was then placed in a stereotaxic apparatus (ST-7; Narishige, Tokyo, Japan). The dura matter was removed, and the pia-arachnoid membrane was cut, making a small window to insert a tungsten electrode with an impedance of 10 MΩ (FHC, Bowdoin, ME, USA) using a micromanipulator (MHW-4-1; Narishige) at a fixed angle. The electrode was placed into the spinal dorsal horn, and multiunit neuronal firing was amplified with a differential extracellular amplifier (EX1; Dagan, Minneapolis, MN, USA).[30,31] The signal was bandpass-filtered at 300–3 kHz and sampled at 25 kHz. Recorded signals were spike-sorted with a software (Spike2 version 6; Cambridge Electronic Design, Cambridge, UK). As shown previously,[32,33] neurons were classified as a wide dynamic range (WDR) neuron if they elicited firing in response to light touch (brushing or tapping the ipsilateral hind paw) and displayed increased firing to pinching with toothed forceps (11022-14; Fine Science Tools, Heidelberg, Germany). Mechanical noxious stimulation was applied with using a von Frey filament (60 g) which induced a withdrawal reflex in awake rats. The surface of the exposed spinal cord was irrigated with Krebs solution, and ethanol dissolved in Krebs solution was applied by the superfusion.[29,34]

Statistical analysis

All numerical data are shown as mean ± *SEM*. Statistical significance was determined as $p < 0.05$ using student's paired and unpaired *t* test. The Kolmogorov–Smirnov test was used to compare the cumulative distributions of synaptic responses. In all cases, *n* refers to the number of neurons studied.

Results

Ethanol increased spontaneous but not miniature IPSCs in SG neurons

First, we examined effects of acute ethanol on inhibitory synaptic transmission and used three rages of ethanol concentrations at 10, 20 to 50, and 100 mM defined as low, medium, and high concentrations, respectively.[2] SG neurons tested exhibited sIPSCs with a frequency and amplitude of 5.4 ± 1.2 Hz and 39.3 ± 6.3 pA ($n = 24$), respectively. During stable recording of sIPSCs, ethanol was applied by bath application. Low-concentration ethanol did not change the frequency and amplitude of sIPSCs (frequency: 105.9 ± 9.8% of control; amplitude: 101.3 ± 9.8% of control; $n = 8$; $p > 0.05$). As shown in Figure 1(a), however, medium-concentration ethanol elicited a barrage of sIPSCs. The amplitude distribution of sIPSCs shows that ethanol increases the proportion of events having the same amplitudes detected in control, and further that of large events (>25 pA) (Figure 1(b)). The actions of medium- to high-concentration ethanol on the frequency and amplitude of sIPSCs in all SG neurons tested are shown in Figure 1(d) and (e). Enhancement of sIPSCs by medium-concentration ethanol (frequency increase to more than 150%) was detected in 6 out of 21 (28.6%) SG neurons (20 mM, 2 out of 7; 30 mM, 3 out of 10; 50 mM, one out of four) (Figure 1(c)), and in the neurons sensitive to ethanol, the frequency and amplitude of sIPSCs were 227.5 ± 38.8% and 129.6 ± 17.9% of control ($n = 6$), respectively. In remaining neurons ($n = 15$), medium concentration of ethanol did not change the frequency and amplitude (107.1 ± 6.2% and 101.9 ± 5.6% of control). High-concentration ethanol also increased sIPSCs in 56.5% (13 out of 23) of SG neurons tested (Figure 1(c)), and the frequency and amplitude of sIPSCs were 254.0 ± 40.1% and 116.6 ± 12.0% of control, respectively ($n = 13$). In remaining neurons ($n = 10$), high concentration of ethanol did not change the frequency and amplitude (106.4 ± 12.0% and 107.5 ± 11.6% of control). SG neurons elicit GABAergic and glycinergic IPSCs which are sensitive to either strychnine or bicuculline. We first examined action of high-concentration ethanol on sIPSCs. In SG neurons sensitive to the ethanol ($n = 10$), ethanol was then applied in the presence of either 3 μm strychnine or 10 μm bicuculline. As shown in Figure 2, in the presence of strychnine, the frequency of strychnine-insensitive (GABAergic)

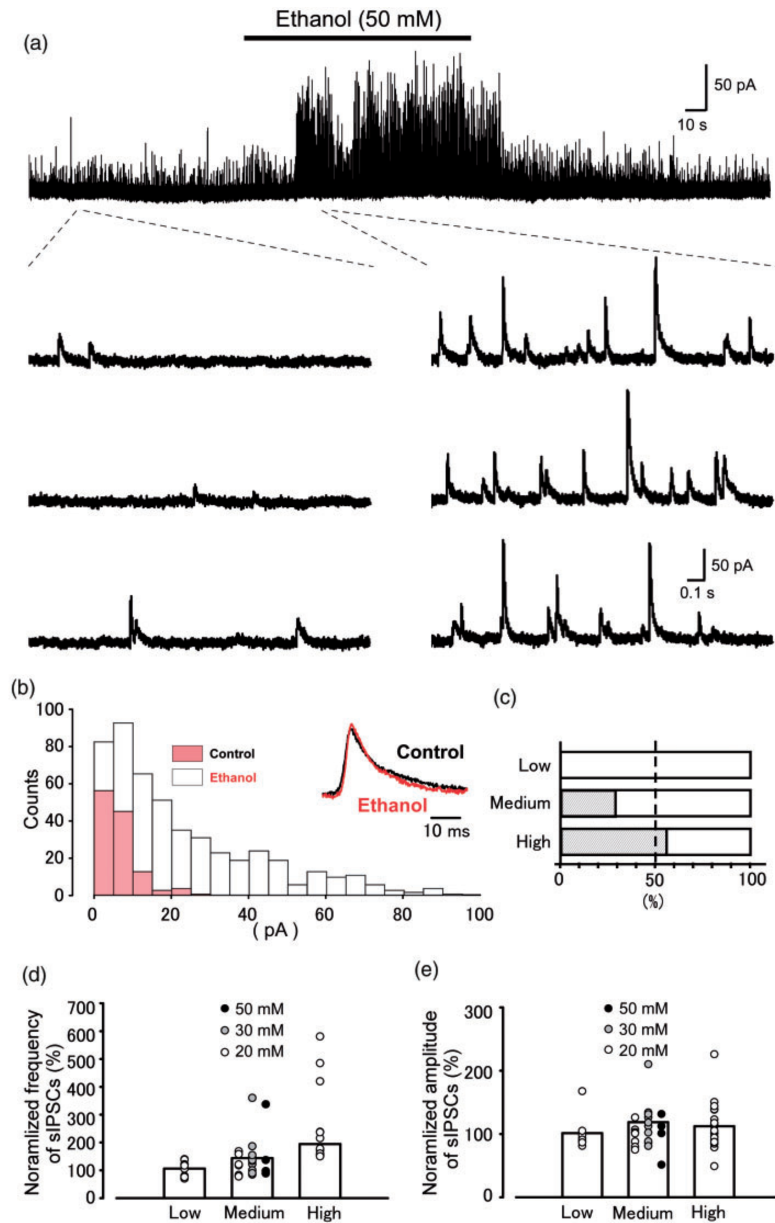

Figure 1. Effects of ethanol on spontaneous IPSCs in the SG of the spinal dorsal horn. (a) An example trace showing that a medium concentration of ethanol (50 mM) elicited a barrage of sIPSCs in SG neurons under voltage-clamp at a holding potential of 0 mV. Lower three traces in control and in the presence of ethanol are shown on an expanded timescale. (b) Histograms of the amplitude distribution of sIPSCs in control and with ethanol (50 mM). Insets showed an averaged sIPSC from control and under the action of ethanol (average of 30 events, normalized for amplitude). (c) The percentage of cells sensitive to ethanol at low (10 mM), medium (20, 30, and 50 mM), and high (100 mM) concentrations. (d) and (e) Summary showing effect of low to high concentrations (low: 10 mM; medium: 20, 30, and 50 mM; high: 100 mM) of ethanol on the frequency and amplitude of sIPSCs.
IPSCs: inhibitory postsynaptic currents.

sIPSCs was increased by high-concentration ethanol (control: 1.1 ± 0.4 Hz; ethanol: 2.3 ± 0.5 Hz; $n = 5$). In the presence of bicuculline, ethanol also increased the frequency of bicuculline-insensitive (glycinergic) sIPSCs (control: 1.8 ± 0.4 Hz; ethanol: 3.3 ± 0.7 Hz; $n = 5$). These results suggest that ethanol enhances inhibitory synaptic transmission in a subset of SG neurons by reversibly evoking a barrage of GABAergic and glycinergic sIPSCs with large amplitudes.

We next examined ethanol action on miniature inhibitory postsynaptic events. The sodium channel blocker, TTX (1 μm) was used to eliminate AP-dependent inhibitory synaptic responses. SG neurons exhibited

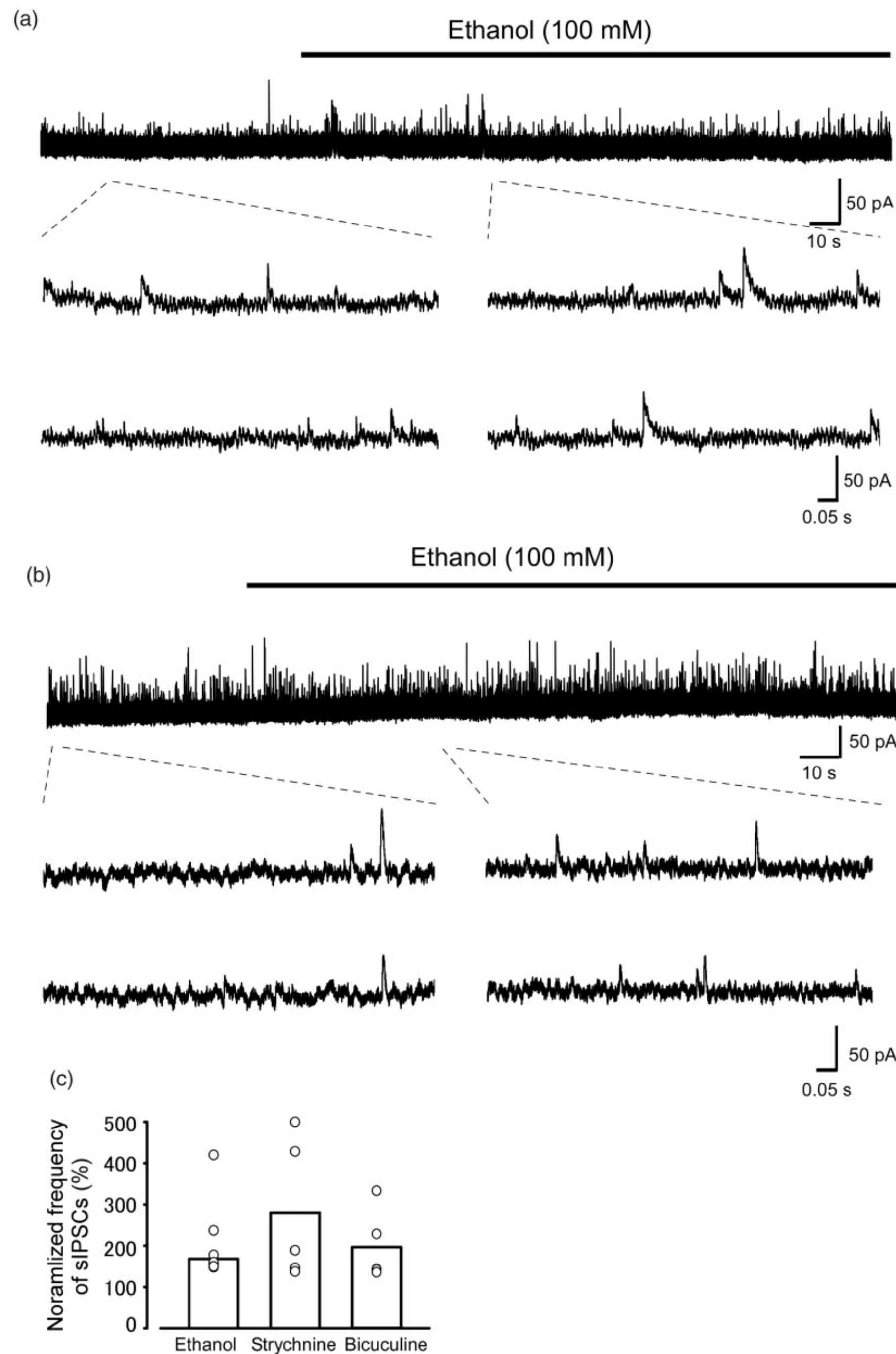

Figure 2. Effect of ethanol on spontaneous strychnine- and bicuculline-insensitive (GABAergic and glycinergic) IPSCs. (a) An example trace showing that high concentration of ethanol (100 mM) increased sIPSCs in the presence of strychnine (3 μm) under voltage-clamp conditions at a holding potential of 0 mV. Lower three traces in control and under the action of ethanol are shown on an expanded timescale. (b) An example trace showing that high concentration of ethanol (100 mM) enhanced sIPSCs in the presence of bicuculline (10 μm). Lower two traces in control and under the action of ethanol are shown on an expanded timescale. (c) Summary showing the relative change of high concentrations (100 mM) of ethanol actions on the frequency of sIPSCs. IPSCs: inhibitory postsynaptic currents.

miniature IPSCs (mIPSCs) with a frequency and amplitude of 1.9 ± 0.5 Hz and 23.5 ± 2.6 pA, respectively ($n = 16$). No SG neurons showed a change in mIPSCs in response to ethanol. Even high-concentration ethanol did not change mIPSCs (Figure 3). The frequency and amplitude in the presence of high-concentration ethanol were $111.1 \pm 9.3\%$ of control and $98.5 \pm 3.9\%$ of control ($n = 6$).

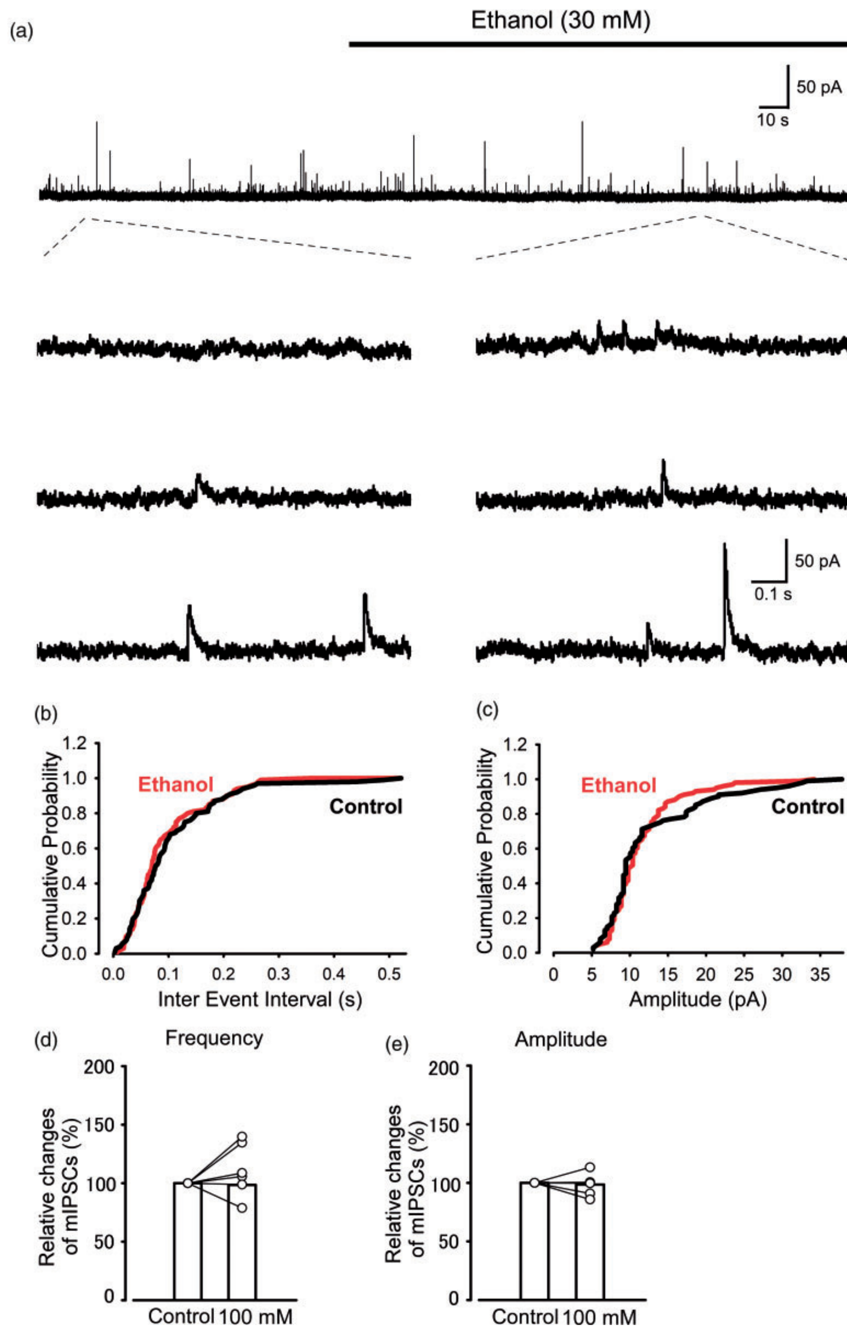

Figure 3. Lack of effect of ethanol on miniature IPSCs. (a) Example of mIPSCs recorded in SG neurons in the presence of TTX (1 μm) under voltage-clamp conditions at a holding potential of 0 mV. Ethanol (30 mM) had no visible effect. Lower three traces in control and under the action of ethanol are shown on an expanded timescale. (b,c) Cumulative histograms of the inter-event interval and amplitude of mIPSCs in control and ethanol obtained from the trace shown in (a). Ethanol did not shift the curves ($p = 0.56$ for inter-event interval; $p = 0.35$ for amplitude). (d,e) Summary showing the relative change of high concentration (100 mM) of ethanol actions on the frequency and amplitude of mIPSCs.
IPSCs: inhibitory postsynaptic currents.

Ethanol did not affect the spontaneous EPSCs

SG neurons tested exhibited spontaneous EPSCs (sEPSCs) with a frequency and amplitude of 9.2 ± 2.4 Hz and 23.7 ± 5.9 pA, respectively ($n = 14$). Three neurons out of seven neurons were sensitive to high-concentration ethanol, but high-concentration ethanol did not exert any typical effects on sEPSCs (an increase in the sEPSC frequency in two of them; a decrease in that in one of them) (Figure 4). These data indicate that ethanol does not induce any consistent influence on sEPSCs.

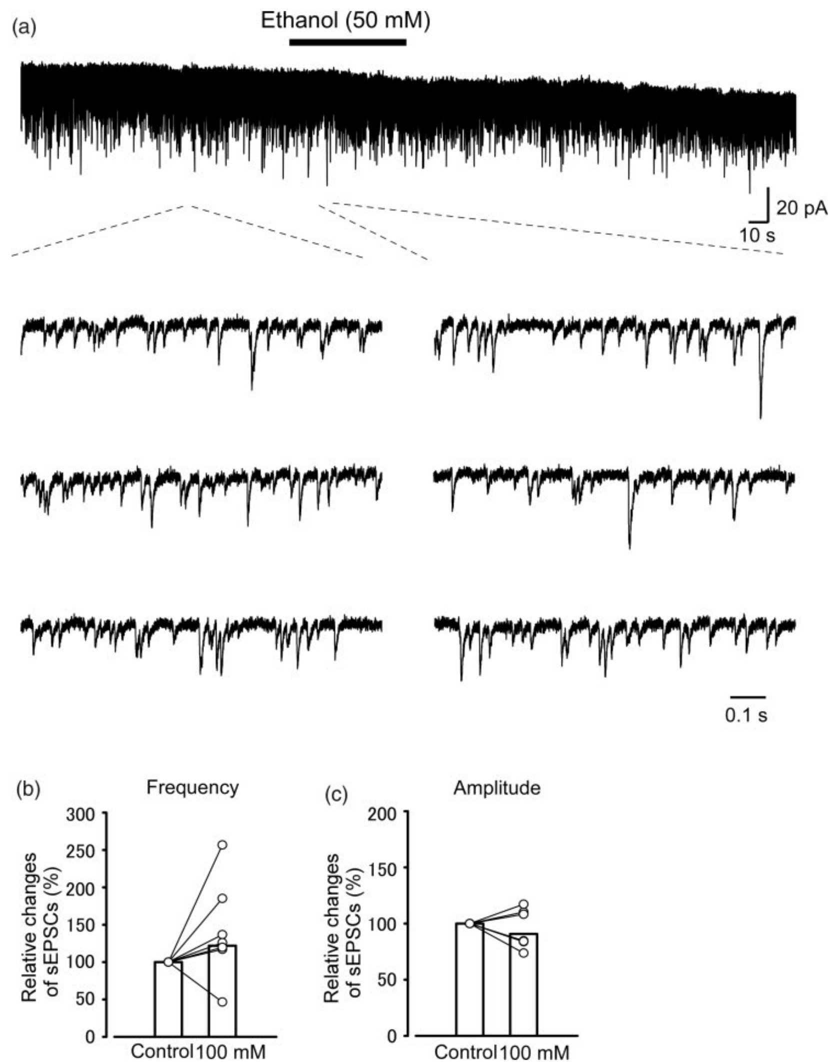

Figure 4. Effects of ethanol on spontaneous EPSCs. (a) An example of effects of ethanol (30 mM) on sEPSCs recorded in SG neuron under voltage-clamp conditions at a holding potential of –70 mV. Lower three traces in control and ethanol are shown on an expanded timescale. (b,c) Summary showing the relative change by high concentration (100 mM) of ethanol on the frequency and amplitude of spontaneous EPSCs.
IPSCs: inhibitory postsynaptic currents.

Ethanol-evoked APs in VGAT-Venus-labeled neurons

As noted above, ethanol facilitated inhibitory synaptic transmission by increasing the incidence of large amplitude IPSCs in slice preparations, suggesting that ethanol might generate APs in spinal inhibitory interneurons. We therefore addressed ethanol action on inhibitory interneurons by using a transgenic VGAT-Venus rat, which expresses the fluorescent protein Venus under the promotor for VGAT.[24–26] Using an infrared differential interference contrast fluorescence microscope, we identified VGAT-Venus expressing neurons in spinal cord slices and performed whole-cell patch-clamp recordings from the Venus-labeled neurons (Figure 5(a) and (b)). In current clamp mode, the VGAT-Venus-labeled neurons fired spontaneous APs with a frequency of 0.7 ± 0.4 Hz ($n = 7$). As shown in Figure 5(c) and (d), medium concentrations (30 mM) of ethanol elicited a number of APs. Under the action of ethanol, the AP frequency was increased to $308.8 \pm 132.8\%$ of control ($p < 0.05$, $n = 7$). However, the frequency of APs elicited by current injection through the recording electrode was not changed by ethanol (control: 21.3 ± 4.8 Hz; ethanol: 21.6 ± 5.0 Hz; $p > 0.05$, $n = 7$) (Figure 5(e)). The input membrane resistance was not also altered by ethanol (control: 0.8 ± 0.2 MΩ; ethanol: 0.7 ± 0.2 MΩ; $p > 0.05$, $n = 7$). These results suggest that ethanol increased spontaneous firing of inhibitory interneurons in the spinal dorsal horn.

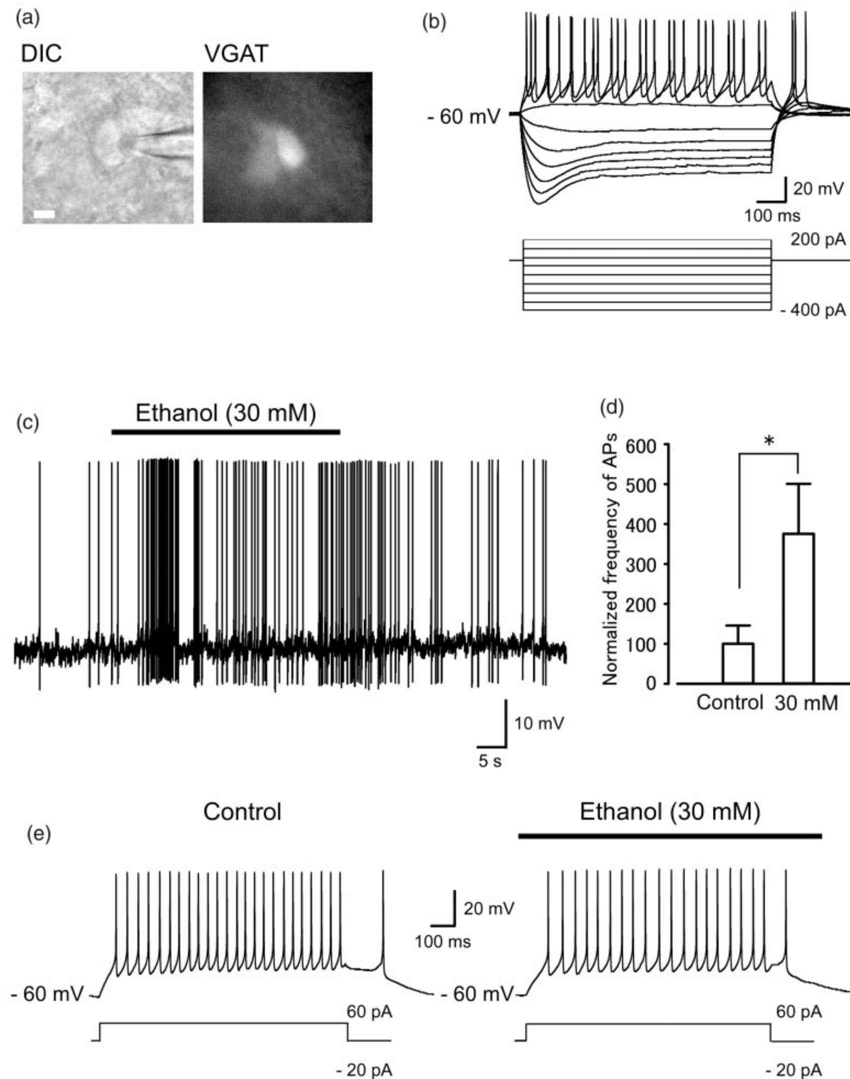

Figure 5. Ethanol-evoked action potentials in VGAT-Venus-labeled neurons. (a) An example of recording from the same neuron observed with a differential interference contrast optics (left) and labeled with VGAT-Venus fluorescent protein (right). (b) An example of firing properties of Venus-labeled neurons. In response to current injections from the recording pipette, Venus-labeled neuron showed a tonic firing. (c) VGAT-Venus neurons exhibited spontaneous APs and a medium concentration of ethanol (30 mM) increased the frequency of discharge. (d) Summary data showing the effect of ethanol on the frequency of APs. Ethanol (30 mM) increased the spontaneous firing frequency ($n = 7$, $*p < 0.05$). (e) An example of active membrane property of VGAT-Venus neurons in control and in the presence of ethanol (30 mM) showing that it had no action on the firings.
DIC: differential interference contrast; VGAT: vesicular GABA transporter.

Ethanol suppressed mechanical sensory responses of spinal dorsal horn neurons in vivo

So far, we found that ethanol evoked large amplitude of sIPSCs in the SG and increased the firing frequency of spinal inhibitory interneurons. Finally, we examined whether ethanol can suppress spinal nociceptive transmission. In our previous studies using *in vivo* preparations, drugs applied to the surface of the spinal cord had significant action within a depth of 250 μm in which inhibitory interneurons are located.[20,29] Therefore, we applied high concentration of ethanol to the surface of the spinal cord. In WDR neurons in the spinal dorsal horn, mechanical noxious responses were elicited by a von Frey filament (60 g) applied to the skin. High-concentration ethanol applied to the surface of the spinal cord did not have any inhibitory actions on the responses during the mechanical noxious stimulation. However, we found that ethanol suppressed the after discharge that followed mechanical stimulation. As shown in Figure 6(a) and (b), application of a series of different concentrations of ethanol (10, 30, and 100 mM)

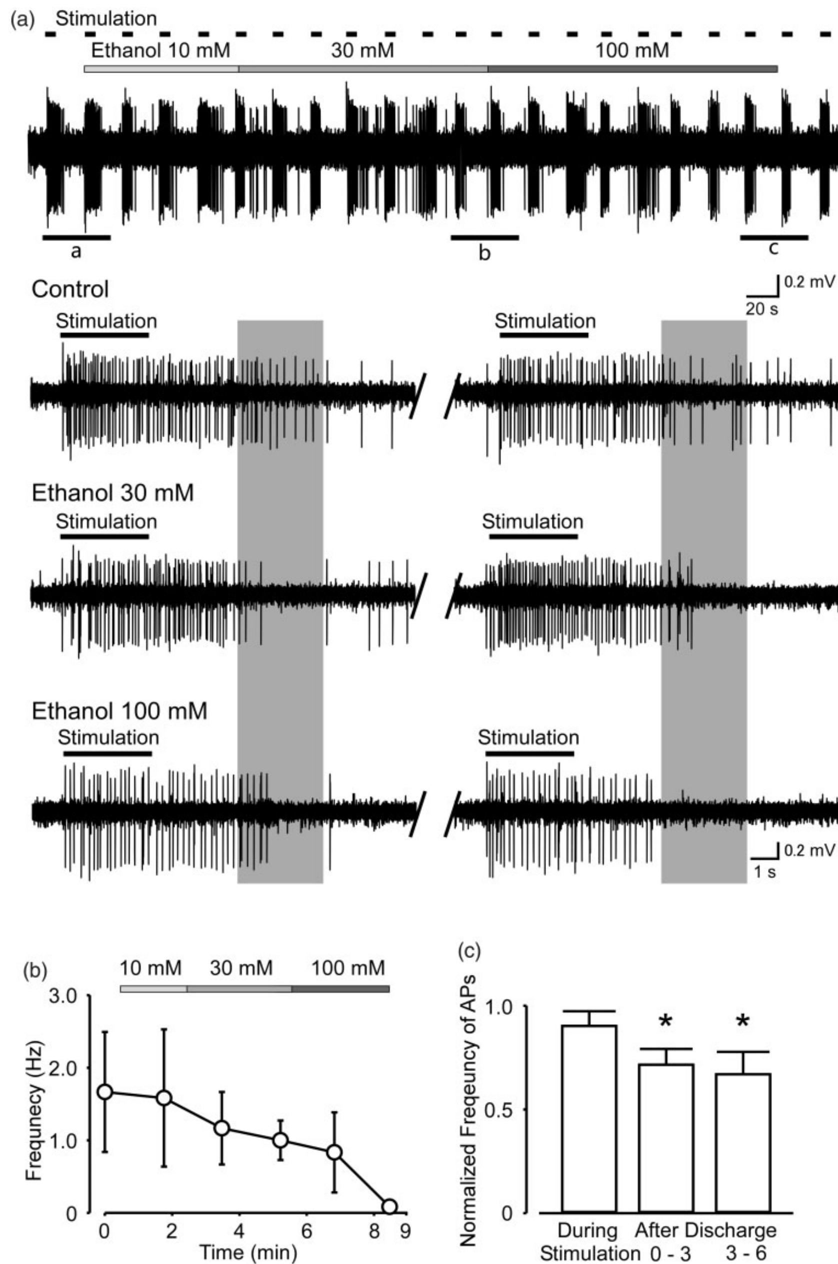

Figure 6. Ethanol suppressed spinal nociceptive responses evoked by cutaneous mechanical stimuli *in vivo*. (a) A continuous recording showing actions of increasing concentrations of ethanol on APs elicited by mechanical stimulation in a wide-dynamic range neuron (top trace). A von Frey filament (60 g) stimulation was repeatedly applied to the ipsilateral hind paw at an interval of 20 s. Lower three traces show the mechanical nociceptive responses on an expanded timescale in control and in the presence of 30 mM and 100 mM ethanol indicated by *a*, *b*, and *c*, respectively. Note that typical after-discharges were elicited following each mechanical stimulation and suppressed by 30 and 100 mM ethanol. Gray shows after discharge responses at 3 to 6 s after the stimulation. (b) The time-course of averaged after-discharge frequency at 3 to 6 s after the mechanical stimulation in control and ethanol (10, 30, and 100 mM) obtained from the same neuron shown in (a). The frequency was gradually decreased by ethanol. (c) Normalized AP frequency of nociceptive responses during the stimulation, 0 to 3 and 3 to 6 s after the stimulation under the action of high concentration of ethanol (100 mM, $n = 14$).
APs: action potentials.

incrementally suppressed the after discharge. High-concentration ethanol significantly reduced the after discharge firing frequency at 0 to 3 and 3 to 6 s after cessation of the mechanical stimulation (0–3 s, $76.0 \pm 4.2\%$ of control, $n = 13$; 3–6 s, $67.0 \pm 10.8\%$, $n = 12$; $p < 0.05$) (Figure 6(c)). High-concentration ethanol also shortened the duration of after discharged APs (control: 25.6 ± 4.6 s; ethanol: 14.6 ± 2.6 s, $n = 5$; $p < 0.05$).

Discussion

Recent studies have shown that acute ethanol modulates neuronal activities widely in the CNS. In this study, we revealed for the first time a direct spinal action of acute ethanol on synaptic activity in the SG of spinal cord slices and on single neuronal mechanical nociceptive responses elicited in the spinal dorsal horn of rats *in vivo*. Our major findings are as follows: (1) ethanol at medium to high but not low concentrations enhanced spontaneous inhibitory (GABAergic and glycinergic) synaptic transmission in the SG, eliciting large amplitude synaptic currents; (2) VGAT-Venus-labeled neurons showed an ethanol-induced increase in their spontaneous firing; and (3) after discharges following to cutaneous mechanical stimuli in spinal WDR neurons were suppressed by ethanol. The present results suggest that acute ethanol exerts an analgesic action in the spinal dorsal horn by a preferential excitation of inhibitory interneurons.

Ethanol concentrations and their actions on behavior and inhibitory synaptic transmission in the SG

The degree of acute intoxication and behavioral changes induced by ethanol is dependent on blood ethanol concentrations.[1,35,36] In general, ethanol concentrations of ∼10 mM produces anxiolytic and euphoric effects. Higher ethanol levels (more than 15 mM, defined here as medium concentration) induce a degree of sedation and motor incoordination. At concentrations more than 50 mM defined as high concentration, ethanol induces locomotor disruption and marked cognitive impairments associated with increasing sedation. A large number of behavioral studies using animals have shown equivalent ethanol-induced behavioral changes including an analgesic action.[37–40] Tail-flick latency provoked by noxious heat stimulation is increased in rats after intraperitoneal administration of ethanol at doses of more than 2 g/kg.[37] This dose would be predicted to increase blood ethanol concentration for more than 2 h to approximately 20 to 30 mM based on a previous study of blood ethanol concentration profiles in rodents after intraperitoneal administration.[41]

In this study, we showed a direct action of ethanol at different concentrations on synaptic activity in the SG of superficial dorsal horn in slice preparations. Low concentration of ethanol (10 mM) did not have any detectable actions on spontaneous inhibitory or excitatory synaptic transmission. However, concentrations of ethanol above 20 mM produced enhancement of spontaneous inhibitory but not excitatory synaptic transmission; consistent with the blood ethanol concentration showing an analgesic action on tail-flick latencies in the previous study.[37] However, it is hard to assess analgesic action of high dose (blood concentration) of ethanol in behaving animals, there is a confound of ethanol-induced sedation as these concentrations of ethanol can induce sleep.[42] Our results obtained from spinal cord slice preparations clearly demonstrated that facilitatory action of ethanol on spontaneous inhibitory synaptic transmission in the SG was still detected at high concentrations of ethanol. Indeed, the enhancement of sIPSCs was concentration dependent (percentage of SG neurons sensitive to ethanol, 28.6% for medium ethanol vs. 41.6% for high-concentration ethanol; averaged increase in sIPSC frequency, 227% of control for medium ethanol vs. 338% of control for high ethanol).

Ethanol does not have pre- or postsynaptic actions on inhibitory synaptic transmission in the SG but excites spinal inhibitory interneurons

Ethanol is reported to potentiate $GABA_A$ receptor-mediated currents.[43] Enhancement of the postsynaptic currents of inhibitory synaptic transmission was also observed in the amygdala, hippocampus, and spinal ventral horn.[8,44,45] In the present study, ethanol did not have any actions on the frequency and amplitude of mIPSCs elicited in SG neurons in the presence of TTX, suggesting that ethanol has no pre- or postsynaptic actions on inhibitory synaptic transmission. In the hippocampus, ethanol prolonged the decay time constant of IPSCs.[44] Such ethanol-induced changes in the decay of IPSCs were not observed in this study (see an example of sIPSC kinetics of control and in the presence of ethanol in the *inset* of Figure 1(b)). The ethanol-induced barrage of large amplitude sIPSCs (see Figure 1(a) and (b)) was not detected in the presence of TTX. We therefore propose that ethanol may induce IPSCs directly by an increase in the intrinsic excitability of spinal inhibitory interneurons. As predicted, recordings from VGAT, VGAT-Venus neurons demonstrated that ethanol increased their spontaneous firing frequency. A similar action of ethanol on intrinsic excitability was seen in dopamine neurons in the ventral tegmental area[13,14] and GABAergic Cerebellar Golgi cells.[10] This raises the question of how ethanol can modulate intrinsic excitability? Previous studies have suggested that a putative molecular target of ethanol is the large-conductance calcium-activated potassium channel (BK channel). The BK channel is known to inhibit and excite neurons and is thought to be potentiated by ethanol.[46–48] In the spinal dorsal horn, the BK channel was only expressed in a small population of dorsal horn neurons.[49] G protein-coupled receptor inwardly rectifying K+ channel is also reported to be one of the possible direct molecular targets.[50,51] Although ethanol enhancements of inhibitory synaptic transmitter release were also observed in the CNS as

described above, the underlying mechanism for the ethanol enhancement are also not well-understood. However, in mice lacking protein kinase A or protein kinase C epsilon, ethanol enhancement of GABA release was prevented.[52–54] These suggest that ethanol interacts with intercellular signaling molecules. The firing properties of the VGAT-Venus neurons in response to current injections were not changed by ethanol in the present study. However, further experiments are needed to elucidate how ethanol could excite spinal inhibitory interneurons to increase the synaptic release.

Ethanol suppresses the after discharge response of WDR neurons to noxious mechanical stimuli

To test whether ethanol could inhibit nociceptive transmission, we examined the effects of ethanol on sensory responses in the spinal dorsal horn. Ethanol at medium concentration applied to the surface of the spinal cord suppressed the after discharge elicited in WDR neurons by mechanical stimulation (see Figure 6), suggesting that ethanol has a spinal analgesic action on mechanical nociceptive transmission. Given that ethanol elicited a barrage of large amplitude IPSCs, and inhibitory postsynaptic responses are known to modulate spinal nociceptive transmission by shunting excitatory currents.[55,56] It is known that firing in response to mechanical stimulation in WDR neurons is attenuated by inhibitory synaptic transmission.[57,58] Taken together with the current and previous studies, an increase in the frequency of spontaneous IPSCs by excitation of spinal inhibitory interneurons may account for the suppression of the after discharges of WDR neurons. However, the firings of WDR neurons during the mechanical stimulation were not inhibited (Figure 6 (c)), suggesting that ethanol-induced IPSCs does not have such a strong suppressive effect. One possible reason for this could be due to differences between the excitatory currents evoked in WDR neurons during the stimulation and in the period of the after discharge. During mechanical stimulation, fast excitatory mono- and polysynaptic currents with large amplitudes are mainly evoked through the activation of afferent fibers.[20,59,60] In contrast, after discharges of WDR are elicited by an intrinsic plateau potential.[61] To effectively inhibit an EPSP, there needs to be tight temporal synchronicity of the inhibitory synaptic events to produce summation to counter the excitatory drive.[62] Ethanol induced a barrage of IPSCs in an episodic manner, and summated sIPSCs were not detected (see an example of IPSC traces under the action of ethanol on an expanded timescale in Figure 1(a)). The average event-interval of sIPSCs under the ethanol action was 83.0 ± 3.4 ms which was longer than the half decay time ($\sim$ 40 ms) for inhibitory postsynaptic potentials evoked in SG neurons shown in previous study.[56] These suggest that the ethanol-induced IPSC facilitation does not produce any summating outward currents. Thus, ethanol may have induced inhibitory postsynaptic responses sufficient to shunt the plateau potentials in WDR neurons spontaneously, to decrease the number of after discharge firings. We postulate that this may be sufficient to account for the analgesic effect of acute ethanol consumption where it "takes the edge off the pain" without being able to completely suppress pain altogether.

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Author Contributions

AY, MO, and HF designed research. AY, KK, and HF performed experiments, and AY analyzed data. AY, KK, MO, and HF wrote the paper. KK and HF supervised the experiments.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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