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# Differential involvement of GABA system in mediating behavioral and neurochemical effect of acupuncture in ethanol-withdrawn rats

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ABSTRACT

In our previous study we demonstrated that acupuncture at Shenmen (HT7) points suppressed a decrease of accumbal dopamine (DA) release in ethanol-withdrawn rats. Furthermore, here we found that it inhibited behavioral withdrawal signs of ethanol. In an effort to better understand the mechanisms underlying this inhibition, the potential role of GABA receptor system in acupuncture was investigated. Male Sprague–Dawley rats were treated with 3 g/kg/day of ethanol (20%, w/v) or saline by intraperitoneal injection for 21 days. Following 48 or 72 h of ethanol withdrawal, acupuncture was applied at bilateral HT7 for 1 min. The selective $GABA_A$ antagonist bicuculline and the selective $GABA_B$ antagonist SCH 50911 were injected intraperitoneally 20 min before acupuncture, respectively. Importantly, suppressive effects of acupuncture on DA deficiency were completely abolished by SCH 50911, but not by bicuculline, whereas ameliorating effects of acupuncture on ethanol withdrawal syndrome were completely blocked either by SCH 50911 or bicuculline. These results suggest that acupuncture at specific acupoint HT7 may normalize the DA release in the mesolimbic system and attenuate withdrawal syndrome through the $GABA_B$ receptor system in ethanol-withdrawn rats.



Withdrawal from chronic ethanol administration produced a reduction in dopamine (DA) outflow in the nucleus accumbens (NAc) that has long been associated with withdrawal signs including tremor and hypermotility [4] and may represent the mechanism, at least in part, underlying dysphoria and anhedonia that contribute to the intense ethanol craving experienced by addicts [17]. Chronic administration of ethanol has been shown to enhance the baseline activity of ventral tegmental area (VTA) GABA neurons underlying the decrease in accumbal DA release following withdrawal from chronic ethanol [3]. Furthermore, it has been demonstrated that activation of inhibitory $GABA_A$ or $GABA_B$ receptors located on VTA GABA neurons increases DA release by a disinhibition of the DA neurons [2,19], whereas activation of these GABA receptors reduces the activity of DA system via direct actions on VTA DA neurons [18,19].

In a previous study we showed that acupuncture at a specific acupoint Shenmen (HT7) attenuated acute ethanol-induced DA release in the NAc through the $GABA_B$ receptor [22]. This result raises the possibility that GABA receptors may be involved in acupuncture's action on DA release in the NAc. Most importantly, our previous study demonstrated that acupuncture at HT7 prevented a decrease of accumbal DA release following withdrawal from chronic ethanol treatment [24]. Here we found that acupuncture at HT7 inhibited the expression of ethanol withdrawal syndrome. Based on these observations, it was hypothesized that acupuncture at HT7 could suppress behavioral withdrawal signs of ethanol by inhibition of DA depletion through actions on $GABA_A$ or $GABA_B$ receptors located on VTA GABA neurons. To test this hypothesis, we used a pharmacological approach directed at elucidating the possible mechanism of acupuncture on the GABA pathway.

Subjects were male Sprague–Dawley rats (Daehan Animal, Seoul, Korea) weighing between 280 and 300 g at the start of the experiment. All rats were maintained on a 12-h light–dark cycle with free access to food and water throughout the course of the study. Measures were taken to minimize pain and discomfort and

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were in compliance with NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 80-23).

Behavioral responses were measured using the same paradigm of ethanol treatment as DA study described previously [24]. Rats were treated chronically with 3 g/kg/day of ethanol (20%, w/v) or saline by intraperitoneal injection for 21 days. Following 48 h of ethanol withdrawal, rats (ethanol + HT7 group) received acupuncture at bilateral HT7 points. The tail points were used as a stimulation control site in some animals (ethanol + tail group) to determine the effects of mechanical stimulation at non-acupoints. In similar fashion, bilateral Neiguan (PC6) points were used as non-specific control point (ethanol + PC6 group). In oriental medicine, Shenmen (HT7) on the heart channel is commonly used for the treatment of mental disorders and Neiguan (PC6) is the point that is clinically used to treat cardiac disorders [16]. Ethanol group or saline group was treated with ethanol or saline receiving the same light restriction without insertion of acupuncture needles. Stainless-steel needles with a diameter of 0.16 mm and a length of 20 mm were inserted vertically to a depth of 3 mm into each treatment points for 1 min with the light restraint of rats by hands. All rats were prehandled for 2 min/day for 3 consecutive days prior to acupuncture treatments to reduce stress and facilitate handling. The acupuncture stimulation was manually delivered by twisting acupuncture needles at a frequency of twice per s for a total of 2 s of stimulation while needles were inserted and withdrawn from acupoints. The anatomical locations of stimulated acupuncture points in rats were equivalent to the acupoints in animal acupuncture atlas [15]. HT7 is anatomically located on the transverse crease of the wrist of the forepaw, radial to the tendon of the m. flexor carpi ulnaris. PC6 is located between the tendons of the m. palmaris longus and flexor carpi radialis, 4 mm proximal to the transverse crease of the wrist of the forepaw. In addition, needles were placed into non-acupoints apart one-fifth of tail length from the proximal region of the tail to avoid the two tail acupoints (proximal tail and tip of the tail). These non-acupoints are distal to the proximal tail acupoints.

Rats were observed for 5 min to determine behavioral responses with locomotor activity, tremor, and grooming. The assessment protocol for overt withdrawal signs was referred to other studies with a slight modification [7,14]. Horizontal locomotor activity was expressed as incidence and converted into scores ranging from 0 to 5 (0, no moving; 1, 3–4 min no moving; 2, 2–3 min no moving; 3, 1–2 min no moving; 5, always moving). Tremor was scored ranging from 0 to 3 (0, not present; 1, slight; 2, moderate; 3, severe). Grooming was scored ranging from 0 to 3 (0, not present; 1, transient face grooming, 2, several times face grooming; 3, continuous face grooming and body grooming). Behaviors were scored by two independent trained observers (rater correlation $r = 0.90$). All experiments were carried out at the same time every day during the light period.

To pharmacologically characterize the effects of acupuncture on behavioral responses during the withdrawal period, experiments were carried out on different groups of rats using the same paradigm of ethanol treatment. Following 48 h of ethanol withdrawal, rats were intraperitoneally injected with SCH 50911 [1], the highly selective $GABA_B$ receptor antagonist, at a dose of 3 mg/kg, or bicuculline, the highly selective $GABA_A$ receptor antagonist, at a dose of 1 mg/kg, and then acupuncutre at bilateral HT7 (SCH 50911 or bicuculline + ethanol + HT7 group) for 1 min was given after 20 min. For comparative purposes, HT7 + ethanol group, ethanol alone group, SCH 50911 or bicuculline alone group, and SCH 50911 or bicuculline + ethanol group were also included. Additionally, in order to investigate acupuncture-mediated suppression of chronic ethanol-induced DA release in the NAc through the GABA system extracellular DA levels were determined by microdialysis using the same paradigm of bicuculline or SCH 50911 treatment after 48 h ethanol withdrawal period. Five days before start of chronic ethanol treatment, microdialysis probe guide cannulae were stereotaxically implanted into rats under anesthesia (sodium pentobarbital, 50 mg/kg, i.p.) using the coordinates of nucleus accumbens shell (AP 1.7, ML 0.8, DV 6.0) according to the atlas of Paxinos and Watson [12]. On the 4th day after the last daily injection, microdialysis probes (CMA/11, Cuprophane dialysis membrane, 6000 Dalton, 2 mm length) were inserted through the guide cannula into the nucleus accumbens shell of unanesthetized rats for collection of microdialysis samples in microcentrifuge tubes connected to the outlet of microdialysis probe by a 40-cm tube (i.d., 0.12 mm). Modified Ringer's solution (150 mM NaCl, 3.0 mM KCl, 1.4 mM $CaCl_2$, 0.8 mM $MgCl_2$ in 10 mM phosphate butter at pH 7.1) was perfused at a constant rate of 1.5 μl/min (CMA 100, Microinjection pump). Samples were collected at 20-min intervals and injected directly into an HPLC apparatus. The concentration of DA in the microdialysis samples was assayed using HPLC with a coulometric detector (ESA, Coulochem II, Model 5200A) (Supplementary Fig. 1). A guard cell was set at +400 mV and the screen electrode was set at −100 mV with the detection electrode at +350 mV. The mobile phase contained 75 mM sodium phosphate monobasic, 1.7 mM, sodium octane sulfonate, 25 μM EDTA, 0.714 mM triethylamine, 10% acetonitrile, with the pH adjusted to 3.0. The mobile phase was pumped at the flow rate of 1.0 ml/min using HPLC pump (Sykam 7121) connected to the analytical column (HR-80, 80 mm × 4.6 mm, 3 μm particle size; ESA). All reported results are based on levels corrected for individual probe recovery. Baseline DA levels were operationally defined as the mean DA level from three consecutive samples with less than 10% variation. Typically, this required 3–4 h after start of perfusion.

Following the establishment of baseline DA levels, animals were subjected to acupuncture. The DA concentration data were transformed to percentage of the basal values. To confirm the location of the microdialysis probe rats were sacrificed and perfused with formalin, and then brains were prepared on completion of the microdialysis experiment. Coronal brain sections (30 μm) were stained with cresyl violet and evaluated by light microscope.

Animals were excluded from the statistical analysis if the histological examination revealed that the probe placement was not localized to the NAc shell (Supplementary Fig. 2). Neurochemical data were analyzed using repeated-measures ANOVA followed by post hoc Tukey test and behavioral data were analyzed using one-way ANOVA followed by post hoc the least significant difference (LSD) test.

The first experiment was designed to study the effect of acupuncture at HT7 on the behavioral signs of ethanol withdrawal. Following 48 h of ethanol withdrawal, there was a significant increase in the locomotor activity, tremor, and grooming scores in ethanol group to 193.9, 589.3, and 238.1% of the saline group, respectively. In our study we selected 48 h after withdrawal from chronic ethanol treatment as withdrawal time point based on the previous study with the ethanol intoxication model [7]. Most importantly, our results show that acupuncture at HT7, significantly suppressed the locomotor activity, tremor, and grooming scores to 35.1, 42.4, and 46.7% of ethanol group, respectively (Fig. 1). In addition, results indicate that acupuncture at the specific acupoint HT7 produced the greatest decrease in withdrawal syndrome compared to non-specific acupoints insertion and control points insertion. Nevertheless, acupuncture at HT7 did not show a significant difference in behavioral response in the vehicle (saline)-treated rats, suggesting the interaction between acupuncture and ethanol withdrawal syndrome. In the previous study, our results demonstrated that acupuncture at HT7 prevented a decrease of extracellular DA