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Electroacupuncture Attenuates Hyperalgesia in Rats Withdrawn From Chronic Alcohol Drinking via Habenular Mu Opioid Receptors

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Abstract

Background—Hyperalgesia or increased sensitivity to pain is often found in alcoholics during alcohol withdrawal and may contribute to relapse drinking. Alternative therapies such as acupuncture and electroacupuncture (EA), through mechanisms involving opioid receptors, may reduce pain and substance dependence and withdrawal syndromes. The lateral habenula (LHb), an epithalamic structure rich in mu opioid receptors (MORs), is a critical target for both drugs of abuse and pain. We previously observed hyperalgesia in rats withdrawn from chronic ethanol drinking, and found that EA at the acupoint Zusanli (ST36) reduced ethanol intake. This raised question of whether EA can alleviate hyperalgesia during alcohol withdrawal and, if so, whether the mechanism involves MORs in the LHb.

Methods—We trained male rats to drink ethanol using intermittent access to 20% ethanol in a two-bottle free choice paradigm for eight weeks, after which the alcohol supply was discontinued. We measured pain sensitivity using radiant heat (a light beam directed at the hind paw of rats) and compared the paw withdrawal latencies (PWL) with and without EA at ST36.

Results—The PWLs were significantly shorter in rats at 24, 48, 72 hours and 7 days after the discontinuation of ethanol when compared to ethanol-naïve rats. After a single administration of 2 Hz EA for 20 minutes at ST36, the PWLs at 24 hours after the withdrawal of ethanol were significantly greater than those of the sham group (2 Hz EA at the tail). Furthermore, the effect of EA on PWLs was significantly attenuated by bilateral intra-habenula infusion of the MOR antagonist naltrexone.

Conclusion—These results suggest that EA can alleviate hyperalgesia during ethanol withdrawal through a mechanism involving MORs in the habenula. Based on this, EA could be of potential value as a therapy for hyperalgesia in alcohol dependence.

Abstract

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Electroacupuncture may lessen pain during alcohol withdrawal. During withdrawal from chronic alcohol drinking (b), when a radiant light beam aimed at the paw, rats removed their paw faster, indicating increased pain sensitivity. This was alleviated by electroacupuncture (EA) (c). EA's effect may involve μ opioid receptors (MORs), since it was attenuated by the MOR antagonist naltrexone in a brain region called habenula (Hb) (d). These results suggest that electroacupuncture could be a potential therapy for pain in alcoholic humans.

Keywords

Electroacupuncture; pain; ethanol withdrawal; mu opioid receptor; lateral habenula

INTRODUCTION

About 5% of the world's adult population suffers from alcohol use disorder (AUD) (Gowing et al., 2015). However, currently available therapeutic options for AUD are far from satisfactory. Therefore, finding novel effective therapies is of paramount importance. Increasing evidence suggests that chronic pain states could significantly affect alcohol use patterns, which in turn could influence pain sensitivity (Egli et al., 2012). Hyperalgesia, which refers to an increased sensitivity to pain, may be one of the major withdrawal symptoms contributing to relapse drinking (Gatch, 2009, Gatch and Lal, 1999, Jochum et al., 2010, Gatch, 2006).

Acupuncture, a well-known form of alternative therapy, has been used effectively for treating various disorders including pain, certain drug-addiction and withdrawal syndromes (Stux and Pomeranz, 1987). Electroacupuncture (EA) is a modified method that adds electrical stimulation to improve the therapeutic efficacy of acupuncture. Clinical and preclinical studies have demonstrated that EA produces an analgesic effect on different types of acute and chronic pain (Baek et al., 2005, Chang et al., 2004, Kim et al., 2000, Li et al., 2007, Ulett et al., 1998), mediated mainly via μ and δ , but not κ opioid receptors (Zhang et al., 2004, Kim et al., 2004, Kim et al., 2009). Previous rat studies have demonstrated that EA reduces voluntary alcohol intake through the activation of μ opioid receptors (MORs)

(Overstreet et al., 2008). Recent rat studies in our laboratory have observed hyperalgesia during withdrawal from chronic alcohol consumption (Fu et al., 2015), and a reduction in ethanol intake after giving EA at the acupoint Zusanli (ST36) to rats chronically consuming high amounts of ethanol (Li et al., 2011d).

The lateral habenula (LHb), a small epithalamic structure, plays a critical role in processing nociceptive signals and generating aversive states, evidenced by the fact that aversive stimuli such as tail pinches (Zhang et al., 2013), sciatic nerve stimulation (Benabid and Jeaugey, 1989), and air puffs to the face (Matsumoto and Hikosaka, 2009) excite LHb neurons, while lesions to the habenula can block the analgesic effect of electrical stimulation in the hypothalamus (Fuchs and Cox, 1993). Further supporting the LHb's involvement in pain is the high expression of MOR mRNA in the habenula (Zastawny et al., 1994, Bunzow et al., 1995, Aizawa et al., 2012). It has been observed that the MOR selective agonist DAMGO inhibits a subset of LHb neurons by inhibiting glutamate release into these cells (Margolis and Fields, 2016). Intra-habenula infusion of the MOR agonist morphine produces analgesia (Ma et al., 1992), suggesting that MORs act locally within the habenula. To access whether EA can alleviate hyperalgesia during ethanol withdrawal and if it achieves this effect via MORs located in the habenula, we examined the effect of EA on pain sensitivity in rats withdrawn from chronic ethanol drinking, and the effect of blocking habenular MOR function on the efficacy of EA.

MATERIALS AND METHODS

All experiments were conducted in accordance with the guidelines of the National Institutes of Health for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Rutgers University, New Jersey Medical School.

Animals

Experiments were performed on male Long-Evans rats (Harlan lab, NY). They were housed individually in ventilated cages on a standard 12/12 hour light/dark cycle (with lights on at 11:00 p.m. and lights off at 11:00 a.m.) in a climate-controlled room (20–22). Food and water were available ad libitum unless otherwise stated.

Intermittent Access 20% Ethanol in a Two-Bottle Free Choice Drinking Paradigm (IA2BC)

After 7-day acclimation to the animal facility of the New Jersey Medical School, rats (200–250 g) were trained to drink 20% ethanol for 8 weeks, using the IA2BC paradigm (Li et al., 2011a, Li et al., 2016, Simms et al., 2008). Briefly, animals were given 24-hour concurrent access to one bottle of 20% (v/v) ethanol in water and one bottle of water, starting at 11:00 a.m. on Monday. After 24 hours, the ethanol bottle was replaced with a second water bottle that was available for the next 24 hours. This pattern was repeated on Mondays, Wednesdays, and Fridays. The other days of the week, the rats had unlimited access to two bottles of water. On the days when ethanol was accessible to the rats, the placement of the ethanol bottle was alternated to avoid any side preferences. Animal body weight was measured weekly on Mondays. Ethanol consumption was determined by calculating the grams of alcohol consumed per kilogram of body weight. The preference ratio of ethanol

intake was calculated by the following formula: Preference ratio (%) = ethanol solution intake (ml/ 24 hours)/total fluid intake (ml/ 24 hour ethanol solution + ml/ 24 hour water). All ethanol solutions were prepared in tap water from 95% (v/v) ethanol (Pharmco, Brookfield, CT, USA). A bottle containing water in a cage without rats was used to evaluate the spillage due to the experimental manipulations during the test sessions. The spillage was consistently below 1.0 ml (< 2.5% of the total fluid intake). These animals were referred to as IA2BC rats. Ethanol-naïve rats (similar body weight) were individually housed in ventilated Plexiglas cages and allowed to access to two bottles of water for eight-weeks.

Pain Threshold Test

Paw withdrawal latencies (PWLs) were measured with an Analgesia Meter (Model 336, IITC Life Science Instruments, Woodland Hills, CA, USA) as described previously (Fu et al., 2015). Briefly, each rat was placed in a Plexiglas chamber on a glass plate located above a light box. After 15 minute acclimation to the environment, these rats were subjected to radiant heat, which was applied by aiming a light beam to the middle of the plantar surface of the hind paw. When the rat lifted its paw in response to the heat, the light beam was turned off. The PWL, measured in the unit of seconds, was defined as the length of time between the start of the light beam and the foot-lift. Each measure was repeated five times allowing an interval of 5 minutes between successive tests to avoid sensitization to the stimulus. A cutoff time of 20 seconds was used to prevent paw tissue damage. The mean PWL of each rat was calculated by averaging the value of five tests. PWL was measured at 24, 48, 72 hours and 7 days after ethanol withdrawal, as well as in ethanol-naïve rats.

Electroacupuncture Procedure (EA)

After eight weeks of drinking in the IA2BC paradigm, ethanol supply was discontinued, then the animals were divided randomly into three groups: EA-ST36 (EA), EA-tail (sham) and control (no EA). Prior to the treatment, rats were handled for 5 minutes/day for 3 consecutive days, during which the animals became familiar with the experimenters and the handling. At 24h after ethanol withdrawal, rats were lightly restrained on a rack with a towel covering their eyes after light anesthetization with isoflurane (Ohio medical Products), as described previously (Li et al., 2011d). Under these conditions, rats were calm and their limbs and tails could be moved freely, allowing for the application of EA (ST36 or tail) safely and effectively in the conscious rats.

EA-ST36 group—EA was administered at ST36, located near the knee joint of the hind limb, 2 mm lateral to the anterior tubercle of the tibia. Specifically, two stainless-steel needles with a diameter of 0.35 mm and a length of 13 mm were inserted vertically to a depth of about 3 mm into the ST36 of both legs. After the animals woke up from anesthesia they received 20 minutes of EA. A constant current with square-wave produced by a programmed pulse generator (Han Actens WQ 1002F, Aeron Optoelectronic Technology Corp., Beijing, China) was given via the two needles. Current intensity was adjusted to provoke light trembling of muscles (about 0.2–0.3 mA).

Sham (EA tail) group—The non-acupoints in the tail are often used as the sham points for EA studies (Zhao et al., 2006, Yang et al., 2002). This group of rats received the same

handling as those in EA-ST36 group except that EA was applied to the no-acupoints in the tail (1/5 length from the proximal region of the tail), instead of ST36.

No-EA control group—this group received the same handling as the above two groups, except that they did not receive any needle insertion or electrical stimulation. PWL was measured in these three groups 30 minutes after the end of the treatment.

Stereotaxic Surgery and Microinjection

A cannula was implanted under a stereotaxic apparatus (Li et al., 2012, Li et al., 2016) to the LHb in a subgroup of rats ($n = 12$) that had been in the IA2BC paradigm for eight weeks. Briefly, rats under isoflurane anesthesia received bilateral guide cannula (C235G-3.8/Spc, 23 gauge; Plastics One, Roanoke, VA) implantation 1 mm dorsal to the habenula (3.8 mm posterior to bregma, ± 0.7 mm mediolateral, 4.2 mm ventral to the skull surface), according to Paxinos and Watson (Paxinos and Watson, 2007). Seven days after recovery from the surgery, these rats resumed alcohol drinking in the IA2BC paradigm. When their ethanol intake was again stable, microinjections and EA were began.

To decrease stress and habituate subjects to the microinjection procedures, before habenula-microinjection and EA, animals were taken from the colony, brought to the experimental room, and handled for 5 minutes/day for 5 days, during which they became accustomed to the experimenter, the experimental room, and the manipulation procedure.

On the test days, obstructers were removed and injectors were inserted bilaterally to a depth of 1 mm beyond the end of the guide cannula. Naltrexone (20 μ g) or vehicle (artificial cerebral spinal fluid (aCSF)) in a volume of 400 nl was infused into each side of the habenula of a gently restrained rat over 60 seconds. The injector was left in place for an additional 60 seconds to allow for diffusion. After removal of the injector, a new sterile obstructer was inserted. Ten minutes after the microinjection, rats received 20 minutes of EA treatment. Thirty minutes after EA, rats were subjected to the PWL test. PWL also be measured in naïve rats 30 min after intra-habenular infusion of naltrexone (20 μ g/ 400 μ l/ side) or aCSF (400 μ l/side).

Histological Verification of Cannulae Placements

After completion of the behavioral sessions, each rat was given an overdose of a ketamine-xylazine mixture and were then transcardially perfused with ice-cold 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and then transferred to a solution containing 20% sucrose in 4% paraformaldehyde for 3 days. Brains were then frozen and cut into 40- μ m sections and stained with cresyl violet to verify probe and cannulae placement. Data from rats with injection sites located outside the habenula were excluded from data analysis.

Immunohistochemistry

We examined MOR protein distribution in the habenula in 3 naïve adult rats, which were euthanized under deep anesthesia with sodium pentobarbital (50 mg/kg, i.p.), then perfused transcardially with ice-cold saline, followed by 4% paraformaldehyde in 0.1 M phosphate

buffer (pH 7.4). The brains were then removed and transferred to a solution containing 30% sucrose in 4% paraformaldehyde overnight at 4°C for cryoprotection. Coronal brain sections (30 µm) containing the habenula were cut on a freezing microtome (Microm HM550, Walldorf, Germany) and then processed for immunohistochemistry as described in our recent study (Li et al., 2016). MORs were detected using a MOR primary antibody (AB5511, Millipore, Billerica, MA, USA) at a 1:1,000 dilution as described in a previous study (Sargeant et al., 2007). MOR staining was analyzed with a Nikon Eclipse 80i bright field microscope (Micron Optics, Cedar Knolls, New Jersey).

Statistical Analysis

All data are expressed as mean $\pm$ SEM. A one-way repeated measures analysis of variance (RM ANOVA) with *Holm-Sidak post hoc* comparisons were used to analyze the data of ethanol drinking and PWL at different ethanol withdrawal time points. Data of EA, sham treatment, and control were subjected to two-way RM ANOVA with *Tukey post hoc* comparisons. Data from the naltrexone treatment were subjected to paired *t* test. Statistical significance was declared at $p < 0.05$.

RESULTS

Increased Pain Sensitivity in Rats Withdrawn from Chronic Ethanol Drinking

Ethanol consumption of rats in the IA2BC paradigm for eight-weeks was significantly escalated (24 drinking sessions, $F_{135, 23} = 2.24$, $p = 0.002$, Fig. 1A), consistent with previous reports (Li et al., 2011a, Simms et al., 2008). *Post-hoc* analysis revealed that ethanol intake was significantly increased from the 13th to 24th drinking sessions when compared to the first session (2.6 ± 0.2 g/kg/24 hours). The drinking levels reached a plateau (5.2 ± 0.13 g/kg/24 hours) at the 13th session and there were no significant differences in the subsequent drinking sessions. In parallel with the escalation of ethanol intake, ethanol preference also increased over time ($F_{135, 23} = 3.53$, $p < 0.001$, Fig. 1B), reaching a stable level ($48.6 \pm 1.4\%$) after 8-weeks of drinking. There was not difference of total fluid intake between IA2BC and ethanol-naïve rats (data not shown).

To determine whether pain sensitivity of rats withdrawn from chronic drinking has been changed, we measured PWL at 24, 48, 72 hours and 7 days after ethanol supply was discontinued, and found that pain sensitivity was significantly increased during withdrawal (Figure 1), consistent with our recent report (Fu et al., 2015). One-way ANOVA revealed a main effect of withdrawal on pain sensitivity ($F_{44, 4} = 6.53$, $p < 0.001$, Fig. 2). The PWL of IA2BC rats was significantly shorter than that of ethanol naïve rats at 24, 48, 72 hours and 7 days after ethanol withdrawal, suggesting hyperalgesia occurred during ethanol withdrawal (Figure 2, all $p < 0.05$ vs. naïve rats).

EA (2 Hz) Alleviates Hyperalgesia in Rats Withdrawn from Chronic Ethanol Drinking

It has been well documented that acupuncture can relieve a variety of acute and chronic pain conditions. To determine whether EA could relieve hyperalgesia induced by ethanol withdrawal, we administered low frequency (2 Hz) EA at ST36 or at non-acupoints in the tail or no EA treatment, to IA2BC rats at 24 hours after ethanol withdrawal. Two-way RM

ANOVA showed a main effect of treatment [$F_{27, 2} = 4.92, p = 0.015$] with no effect of treatment time and/or treatment $\times$ time interaction. *Post hoc* analysis revealed no difference in the baseline PWL among EA at ST36, EA at non-acupoints in the tail, and no-EA groups before treatment. EA at ST36 significantly prolonged PWL in ethanol-withdrawn rats compared with EA at non-acupoints in the tail ($p = 0.005$) and the no-EA group ($p = 0.04$) (Fig. 3), suggesting an analgesic effect produced by EA at ST36. In addition, PWL in ethanol-withdrawn rats was significantly prolonged after EA at ST36 compared to their response before EA treatment (baseline, $p = 0.006$) (Fig. 3). Conversely, no significant difference in PWL was found before and after EA at tail and no-EA groups.

Intra-Habenular Infusion of Naltrexone Reduces Effect of EA at ST36 on Hyperalgesia

Previous evidence indicates that the MOR gene is richly expressed in the habenula (Zastawny et al., 1994, Bunzow et al., 1995). Consistent with these findings, using immunostaining, we found that MOR protein is richly expressed in the habenula of rats, with a high density (dark brown) in the medial part of the LHb and lateral part of the medial habenula (Fig. 4 A). To test whether the analgesic effect of EA in ethanol-withdrawn rats involved habenular MORs, we injected naltrexone (20 μ g/400 nl/side) to the habenula 10 minutes before EA treatment. Histological verification showed that injection sites of 9 out of 12 IA2BC rats and 8 out of 10 naïve rats were within the habenula (Fig. 4B). The PWL was significantly lower in withdrawal rats that received bilateral intra-habenula infusion of naltrexone compared to that received aCSF infusion ($p = 0.006$, Student's *t* test Fig. 4C). Additionally, the PWL of naïve rats was not affected by bilateral habenular injection of naltrexone ($t = 1.67, p = 0.12$, Fig. 4D).

DISCUSSION

In the current study, we report hyperalgesia in Long-Evans rats during withdrawal from chronic intermittent voluntary ethanol drinking, which is consistent with our previous finding in Sprague Dawley (SD) rats (Fu et al., 2015). Hyperalgesia was alleviated by 2 Hz EA at ST36, which was reduced by intra-habenula infusion of naltrexone, suggesting the involvement of habenular MORs.

Accumulating evidence suggests that excessive chronic ethanol use followed by withdrawal increases pain sensitivity (Gatch, 1999, Dina et al., 2007, Edwards et al., 2012). As a type of subjective, negative experience, pain may affect alcohol reinforcement, and may facilitate the transition to alcohol abuse/addiction (Egli et al., 2012). Hyperalgesia during ethanol withdrawal has been observed in various strains of rats and mice with different methods of ethanol exposure (Gatch, 2009). For example, Long-Evans rats on an ethanol-containing liquid diet (6.5%) for 10 days showed hyperalgesia 3–12 hours after ethanol withdrawal (Gatch, 1999). Similarly, Wistar rats on an ethanol-containing liquid diet for 10 days showed an increased response to the formalin test 12 hours after ethanol withdrawal (Gameiro et al., 2003). Using a bingeing model, SD rats on an ethanol-containing liquid diet (6.5%) in cycles of 4 days on ethanol and 3 days off showed hyperalgesia 48 h after withdrawal (Dina et al., 2006). We previously reported hyperalgesia in SD rats withdrawn from chronic drinking in the IA2BC paradigm (Fu et al., 2015). The current study shows that Long-Evans rats in the

IA2BC paradigm for eight weeks significantly elevated ethanol intake and preference, which is consistent with previous reports (Li et al., 2011a, Simms et al., 2008). It is unclear why rodents in the IA2BC paradigm, when compared to the continuous drinking paradigm shows a higher ethanol intake and preference. Although it has been speculated that the repeated withdrawals in the IA2BC paradigm may exacerbate the negative aversive effects of ethanol (Lopez et al., 2012). Importantly, the PWLs of these rats were significantly decreased at 24, 48, 72 hours and 7 days after withdrawal from ethanol. These data suggest that the IA2BC drinking procedure is a useful paradigm to facilitate the development of hyperalgesia during ethanol withdrawal in adult rats.

It has been well demonstrated that acupuncture or EA is effective in alleviating certain pain symptoms. The analgesic effect of EA depends on specific, low-frequency (2 Hz) stimulation, which facilitates the release of enkephalin, whereas high-frequency (100 Hz) induces the release of dynorphin (Han, 2003). The acupoint ST36 is one of the most effective analgesic points (Stux and Pomeranz, 1987). In keeping with these findings, we found that 2Hz EA at ST36, but not at the tail, significantly reduced hyperalgesia in rats withdrawn from chronic alcohol consumption. Furthermore, rats that received the same handling as EA groups without needle insertion or electrical stimulation showed similar PWLs as that of EA-tail group. These data further support the hypothesis that EA applied at ST36 is specific for relieving hyperalgesia during ethanol withdrawal. However, a weakness of this study is that we did not test whether EA changes the normal PWL in ethanol naïve rats. Given that hyperalgesia during alcohol withdrawal may significantly contribute to relapse (Gatch, 2009, Gatch and Lal, 1999, Jochum et al., 2010, Gatch, 2006), it is not surprising to see a significant reduction in ethanol intake after EA at ST36 in rats 24 hours into withdrawal (Li et al., 2011d). EA-induced reduction in ethanol intake may result from EA-induced reduction of hyperalgesia occurring during ethanol withdrawal.

The habenula is a small structure in the posterior–medial aspect of the dorsal thalamus. It receives afferents from forebrain structures and sends projections to brainstem regions, including well-known pain modulatory regions such as the periaqueductal gray, the raphe nuclei and the ventral tegmental area. MORs are widely distributed in circuits that transmit and modulate nociceptive signals, and drugs acting on MORs have been considered the most powerful and broadly effective analgesics. Consistent with a previous report showing that MORs are richly expressed in the habenula (Darcq et al., 2012), our immunostaining data revealed a dense MOR protein expression in the habenula, primarily distributed in the medial part of LHb and the lateral part of medial habenula. It has been well demonstrated that low-frequency EA produces analgesia by stimulating the release of endorphins in the CNS (Han, 2003, Han et al., 1999, Cheng and Pomeranz, 1979), and that EA at ST36 significantly increases MOR expression/activity (Gao et al., 1997, Yun et al., 2002). Since MOR activation in the LHb produces analgesia (Ma et al., 1992), we hypothesized that the analgesic effects of EA in ethanol withdrawn rats may be mediated by the MORs in the habenula. This hypothesis is supported by our finding that intra-habenular administration of the MOR antagonist naltrexone reduced the analgesic effect of EA in ethanol-withdrawn rats. Our data suggest that activation of MORs in the habenula, contributes at least in part, to EA-induced reduction of hyperalgesia in ethanol-withdrawn rats. A recent study showed that MOR activation can inhibit glutamate release into LHb neurons (Margolis and Fields, 2016).

We therefore speculate that EA-induced MOR activation may inhibit glutamate release in the LHb, which is increased during ethanol withdrawal (Li et al., 2016), which could then block descending nociceptive signals from the LHb to the periaqueductal gray (Narita et al., 2008, Li et al., 1993) and in turn reduce pain. Further experiments are needed to test this possibility.

Taken together, the current study indicates that EA at ST36 is an effective therapy to lessen pain during ethanol withdrawal, which may be mediated at least in part by MORs in the habenula. The results of this study improve our understanding of mechanisms underlying EA therapy in alcohol use disorders.

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Figure 1.

Ethanol intake and preference are increased in Long-Evans rats in the intermittent access 20% ethanol two-bottle choice drinking (IA2BC) paradigm. One-way RM ANOVA revealed that rats significantly escalated (A) ethanol intake ($F_{135, 23} = 2.24$, $p = 0.002$) and (B) preference for ethanol ($F_{135, 23} = 3.53$, $p < 0.001$) after two-month drinking in the IA2BC paradigm. The values are expressed as mean $\pm$ SEM. $n = 10$. * $P < 0.05$ compared with the first session.

Figure 2.

Hyperalgesia in rats withdrawn from chronic consumption of high amounts of ethanol. The paw withdrawal latency in rats that had been drinking ethanol in the IA2BC paradigm for 2 months decreased at 24, 48, 72 hours and 7 days after ethanol supply was discontinued, compared with that in naïve controls. The values were expressed as mean $\pm$ SEM. ** $p < 0.01$; *** $p < 0.001$ compared with naïve rats. Naïve: $n = 15$, Ethanol withdrawal: $n = 8$.

Figure 3.

EA alleviates hyperalgesia in rats at 24 hours withdrawal from chronic ethanol drinking. One time administration of EA for 20 minutes at ST36 significantly increased PWL 24 hours after ethanol withdrawal compared with sham (EA-tail) or no-EA control. # $p < 0.01$ compared with sham (EA-tail) or no-EA control. EA treatment also significantly prolonged PWL in ethanol-withdrawn rats compared to the response before EA treatment (baseline). ** $p < 0.05$ compared with baseline. EA-ST36, $n = 15$; Sham, $n = 7$; Control, $n = 8$. The values were expressed as mean $\pm$ SEM.

Figure 4.

Intra-habenula infusion of naltrexone reduces EA-induced analgesia on rats at 24 hours withdrawal from chronic ethanol drinking. A, Representative photomicrographs indicates that MOR protein (dark brown) is mainly distributed in the medial part of lateral habenula (LHb) and a small amount in the lateral part of the medial habenula (MHb). Magnification: 40×. B, Placement of cannula aimed at the habenula. Cresyl violet stained section demonstrating the trajectory and placement of the guide cannula above the habenula; Scale bars = 1 mm. C, 24h after ethanol withdrawal, naltrexone (20 µg/400 nl/side) or aCSF was administered into the habenula 10 min before EA. ** $p < 0.01$, Student's t test. $n = 9$. D,

Naltrexone or aCSF was administered into the habenula in naïve rats. PWL was tested 30 min after microinjection. n = 8.

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