Effects of Acupuncture at Zu-San-Li (ST36) on the Activity of the Hypothalamic–Pituitary–Adrenal Axis during Ethanol Withdrawal in Rats

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Abstract
The current study investigated the effects of acupuncture at Zu-San-Li (ST36) on the hypothalamic–pituitary–adrenal axis during ethanol withdrawal in rats. Rats were intraperitoneally treated with 3 g/kg/day of ethanol or saline for 28 days. Following 24 hours of ethanol withdrawal, acupuncture was applied at bilateral ST36 points or non-acupoints (tail) for 1 minute. Plasma levels of corticosterone (CORT) and adrenocorticotropic hormone (ACTH) were measured by radioimmunoassay (RIA), and the corticotropin-releasing factor (CRF) protein levels in the paraventricular nucleus of the hypothalamus were also examined by RIA 20 minutes after the acupuncture treatment. RIA showed significantly increased plasma levels of CORT and ACTH in the ethanol-withdrawn rats compared with the saline-treated rats, which were inhibited significantly by the acupuncture at the acupoint ST36 but not at the non-acupoint. Additionally, ethanol withdrawal promoted CRF protein expressions in the paraventricular nucleus of the hypothalamus, which were also blocked by the acupuncture at ST36. These findings suggest that acupuncture at the
specific acupoint ST36 can inhibit ethanol withdrawal-induced hyperactivation of hypothalamic—pituitary—adrenal axis, and it may be mediated via the modulation of hypothalamic CRF.

1. Introduction

Converging evidence from preclinical and clinical studies has demonstrated that in both rodents and humans, negative emotion associated with ethanol withdrawal promotes a relapse in drinking [1,2]. Numerous studies have also shown that the damage of the central nervous system, one of the most serious consequences of alcohol abuse and dependence, is markedly exacerbated by ethanol withdrawal [3], highlighting the critical role of withdrawal in the development of ethanol dependence and pathology. The hypothalamic—pituitary—adrenal (HPA) axis is a major component of the neuroendocrine system that controls reactions to stress. Ethanol withdrawal can be a stressful event and produces hyperactivation of the HPA axis. People undergoing acute ethanol withdrawal have higher levels of cortisol, and the magnitude of the increased cortisol response appears to be proportional to the severity of the withdrawal syndrome [4]. Animal experiments also revealed that high levels of corticotropin-releasing factor (CRF) could influence the severity of withdrawal. For instance, intracerebroventricular infusion of a CRF antagonist blocked the enhanced stress sensitivity induced by ethanol withdrawal [5], and plasma corticosterone (CORT) levels increased during ethanol withdrawal in rats [6]. Excessive HPA axis activation during ethanol withdrawal underlies some of the clinical complications of alcoholism [7,8]. Considering that biochemical disorders precede pathological changes, it is conceivable that medical inhibition of the hyperactivated HPA axis during ethanol withdrawal may be a promising means of avoiding the emergence of withdrawal syndrome, further to treating alcoholism.

Acupuncture, a widely known alternative medicine therapy, has been used in Eastern countries for the treatment of several mental disorders, including drug abuse withdrawal syndrome [9]. Although the biological mechanisms of acupuncture are not yet clear, several lines of evidence suggest that acupuncture can contribute to the maintenance of biochemical balance in the central nervous system and recovery of homeostasis in the body [10,11]. Previously, we reported that acupuncture at the specific acupoint Shen Men (HT7) improved ethanol withdrawal-induced anxiety-like behavior and reduced plasma CORT levels by normalizing amygdaloid catecholamines [6]. More recently, we also demonstrated that the anxiolytic effect of acupuncture at HT7 is mediated by the modulation of amygdaloid CRF during ethanol withdrawal [12]. In traditional Chinese medicine (TCM), similar to HT7, the ST36 acupoint is used extensively to treat mental disorders, and modulation of HPA axis is one of the mechanisms [13]. Therefore, in the present study, we investigated the effect of acupuncture at ST36 on the activity of HPA axis during ethanol withdrawal, to elucidate its possible therapeutic effect on alcoholism.

2. Materials and methods

2.1. Animals and experimental design

Adult male Sprague–Dawley rats (250–270 g) were obtained from the Laboratory Animal Center at the Medical College of Yanbian University (Yanji, China). The rats were given food and water ad libitum and maintained on a 12-hour light, 12-hour dark cycle throughout the course of the study. All animal procedures were conducted in accordance with the National Institutes of Health guidelines on the care and use of laboratory animals and approved by the Animal Care and Use Committee of Yanbian University.

The rats were treated with 3 g/kg/day ethanol (20% w/v) or saline by intraperitoneal injection for 28 days. After the last dose of ethanol, the rats underwent ethanol withdrawal for 24 hours. The acupuncture groups were subjected to acupuncture at either acupoint ST36 or non-acupoints (tail; Fig. 1) for 1 minute.

For acupuncture stimulation, stainless-steel needles (0.2 mm in diameter) were inserted into two identical ST36 acupoints (or tail non-acupoints) located on the left and right sides of the animal (depth of 2–3 mm). The acupuncture stimulation process was divided into two parts: reduction and reinforcement. In the reduction session (the first session), the needles were twisted significantly (>360°/C14) twice per second for 30 seconds; in the reinforcement session (the second session), the needles were twisted (<180°) gently once per second for another 30 seconds. Anatomical locations of the stimulated acupuncture points in rats were equivalent to the acupoints in humans, as described by Stux and Pomeranz [14] and in the animal acupuncture atlas [15]. Two groups of rats treated
with saline or ethanol were held for 1 minute without insertion of acupuncture needles, to achieve immobilization equivalent to that in acupuncture-treated rats, and were designated as a saline-treated or an ethanol-treated control group, respectively. Thus, the pattern of drugs and acupuncture treatment in the present study yielded four experimental groups: the saline-treated control group (28 days of saline + 24 hours of saline withdrawal + 1 minute of immobilization), the ethanol-treated control group (28 days of ethanol + 24 hours of ethanol withdrawal + 1 minute of immobilization), the ethanol + ST36 group (28 days of ethanol + 24 hours of ethanol withdrawal + 1 minute of acupuncture at ST36), and the ethanol + tail group (28 days of ethanol + 24 hours of ethanol withdrawal + 1 minute of acupuncture at tail non-acupoints).

2.2. Plasma adrenocorticotropic hormone and CORT assays

Twenty minutes after the acupuncture/sham treatment, the rats were euthanized and decapitated, the entire brain was removed and stored at −80°C for the hypothalamic CRF assay, and 1.0 mL blood was collected in a chilled tube containing EDTA (20 mg/mL, 20 μL) and centrifuged (1000 × g) at 4°C for 10 minutes. Plasma was separated and stored at −80°C until assayed. Adrenocorticotropic hormone (ACTH) and CORT were measured in the plasma samples using the ImmuneChem Double Antibody 125I radio-immunoassay (RIA) kit from MP Biomedicals (Orangeburg, NY, USA), and values were expressed in picograms (for ACTH) or nanograms (for CORT) per milliliter [16].

2.3. Hypothalamic CRF assay

To determine hypothalamic CRF protein levels, the paraventricular nucleus of the hypothalamus (PVN) was punched out from the stored brains according to the protocol established by Wang et al [17], and the coordinates of the PVN were based on the rat brain atlas developed by Paxinos and Watson [18] (Fig. 2). The PVN sample was sonicated in a hot 0.1 M acetic acid solution, heated at 90°C for 10 minutes, and cooled to room temperature. The cooled sample was sonicated again and centrifuged at 4000 × g, at 4°C for 10 minutes, and then a 100 μL supernatant aliquot was dried in a vacuum centrifuge for the RIA. The CRF level was measured using a commercially available CRF 125I RIA kit (Phoenix Pharmaceuticals, Belmont, CA, USA) and expressed in nanograms per gram. Protein concentration of the PVN homogenate was determined using the bicinchoninic acid (BCA) protein assay.

2.4. Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM), and statistically analyzed by one-way analysis of variance (ANOVA) followed by the Newman–Keuls multiple comparison test using the commercially available GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). A p value of <0.05 was considered to indicate statistical significance.

3. Results

3.1. Acupuncture at ST36 blocks the increased plasma levels of CORT and ACTH induced by ethanol withdrawal

The effects of acupuncture at ST36 on the secretion of CORT and ACTH are illustrated in Figs. 3 and 4,
3.2. Acupuncture at ST36 decreases the elevated CRF protein levels in PVN induced by ethanol withdrawal

To further explore the effect of acupuncture at ST36 on the activity of the central CRF system, CRF protein expression in the PVN was examined during ethanol withdrawal, and the results are illustrated in Fig. 5. One-way ANOVA and post hoc tests indicated significantly enhanced CRF protein levels in the ethanol-treated control rats compared with the saline-treated control rats \([F(3, 18) = 5.43; p = 0.008;\) saline- \((n = 5)\) vs. ethanol-treated \((n = 6)\) control group (ng/g protein): 658.39 ± 92.75 vs. 1138.47 ± 128.36; \(q = 3.36; p < 0.05\), reflecting excessive activation of the hypothalamic CRF system during early-stage ethanol withdrawal. Similar to CORT and ACTH, acupuncture at the specific ST36 acupoint [ethanol-treated control group vs. ethanol + ST36 group \((701.33 ± 101.11, n = 6); q = 3.49; p < 0.05\)] but not at the non-acupoint [ethanol-treated group vs. ethanol + tail group \((1273.66 ± 189.43, n = 5); q = 1.03; p > 0.05\)] blocked the ethanol-withdrawal-induced protein expression in the PVN. These results indicate that acupuncture treatment at ST36 can modulate all three major neuroendocrine components of the HPA axis during ethanol withdrawal.

4. Discussion

We report here that acupuncture treatment at the specific ST36 acupoint blocked the increase in plasma CORT and ACTH levels during ethanol withdrawal and antagonized the overexpression of hypothalamic CRF protein.

In our experiment, markedly increased secretion of CORT was observed 24 hours after the termination of ethanol withdrawal in both saline- and ethanol-treated control rats (Fig. 4). As expected, one-way ANOVA and post hoc analysis showed significant increases in plasma CORT levels in the ethanol-treated control rats compared with the saline-treated control rats \([F(3, 18) = 5.68; p = 0.006;\) saline- \((n = 5)\) vs. ethanol-treated \((n = 6)\) control groups (ng/mL): 162.33 ± 28.53 vs. 529.36 ± 98.12; \(q = 4.46; p < 0.05\)], indicating withdrawal-induced hypersecretion of CORT during early-stage ethanol withdrawal (24 hours after ethanol withdrawal). However, acupuncture treatment at the specific acupoint ST36 [ethanol-treated control group vs. ethanol + ST36 group \((265.83 ± 60.06, n = 6); q = 3.36; p < 0.05\)] but not at the non-acupoint (tail) [ethanol-treated control group vs. ethanol + tail group \((570.12 ± 112.12, n = 5); q = 0.50; p > 0.05\)] greatly inhibited the enhanced secretion of CORT caused by ethanol withdrawal (Fig. 3).

Similar to CORT, one-way ANOVA and post hoc analysis showed that early-stage ethanol withdrawal produced a significant increase in plasma ACTH levels \([F(3, 18) = 6.23; p = 0.004;\) saline- \((n = 5)\) vs. ethanol-treated \((n = 6)\) control groups (pg/mL): 86.17 ± 14.25 vs. 192.56 ± 26.12; \(q = 5.01; p < 0.05\)]. Post hoc analysis also revealed that acupuncture at ST36 suppressed the increase in plasma ACTH secretion [ethanol-treated control group vs. ethanol + ST36 group \((108.72 ± 18.41, n = 6); q = 4.14; p < 0.05\)], whereas that at the non-acupoint had no effect [ethanol-treated group vs. ethanol + tail group \((183.18 ± 21.93, n = 5); q = 0.44; p > 0.05\)] (Fig. 4).

Therefore, acupuncture treatment at the specific acupoint but not at the non-acupoint had an inhibitory effect on the increased secretion of CORT and ACTH caused by ethanol withdrawal. In addition, a preliminary study revealed that 1-minute immobilization had no effect on the HPA axis in either saline- or ethanol-treated rats (data not shown).
ethanol administration, consistent with the observation of Borlikova et al. [19] showing that early-stage ethanol withdrawal leads to excessive secretion of CORT in mice after its prolonged use. In our experiment, ethanol withdrawal also elevated plasma ACTH levels in rats, but the magnitude of this increase was smaller than that of CORT, indicating that negative feedback occurs between CORT and ACTH within the HPA axis.

CRF is a major neuropeptide involved in the stress response of the body, and its main function is to stimulate pituitary synthesis and secretion of ACTH, triggering activation of the HPA axis. CRF is secreted mainly by the parvocellular neurons of the PVN in response to stress, and the increased level of CRF protein in the PVN is an important marker of stressful stimulation. In our experiment, we detected significantly enhanced CRF expression in the PVN 24 hours after the discontinuation of ethanol use. At present, it is not clear whether there is up- or downregulation of CRF activity in the PVN in the early stages of ethanol withdrawal, but there is a general consensus that withdrawal of abused drugs, including ethanol, causes excessive activation of the CRF system in the brain [20,21]. Some researchers argue that hyperactivated CRF in the brain is mainly derived from increased activity of extra-hypothalamic CRF systems, such as the central nucleus of the amygdala and the bed nucleus of the stria terminalis [22–25]. However, it cannot be excluded that over-activation of CRF in the PVN contributes to hyperactivation of the HPA axis during early-stage ethanol withdrawal, because in our preliminary experiment, we did not observe significantly increased CRF protein levels in the central nucleus of the amygdala 24 hours following the administration of the last dose of ethanol (data not shown), which was closely related to anxiety-like behavior during ethanol withdrawal. Taken together, our results support the notion that ethanol withdrawal can be an internal stressor that initiates an increased HPA axis response.

Ethanol withdrawal increases the release of CRF from the PVN, inducing ACTH release from the anterior pituitary, which in turn stimulates the secretion of CORT from the adrenal cortex. CORT is bound to glucocorticoid receptors in peripheral tissues and in the brain, and is associated with both motivational and physical symptoms of ethanol withdrawal, leading to a relapse in ethanol use after periods of abstinence. CRF not only acts as a major secretagogue of ACTH, but also participates in the pathophysiological process of ethanol withdrawal syndrome [20]. Dysregulation of the CRF system can lead to a variety of psychiatric disorders, such as depression, anxiety, obsessive-compulsive disorder, post-traumatic stress disorder, and substance abuse disorders. Moreover, excessive activation of the HPA axis can cause brain damage. For example, chronic glucocorticoid treatment leads to dendritic atrophy in the hippocampus and medial prefrontal cortex, whereas acute CORT treatment induces dendritic hypertrophy in the basolateral amygdaloid nucleus [26–28]. Therefore, blocking the hyperactivation of the HPA axis is a promising method to prevent relapse and facilitate treatment of alcoholism.

Acupuncture has been used in TCM for over 3000 years. Acupuncture treatment has recently gained worldwide acceptance and is used for a variety of disorders. The general principle of acupuncture treatment was established under the guidance of the holistic concept of the body and empirically tested knowledge on the differentiation of syndromes in TCM. Fundamentally speaking, a loss of balance between Yin and Yang is considered the cause of disease in TCM, which occurs due to stagnation of Qi in certain meridians. Acupuncture corrects the imbalances between Yin and Yang to maintain homeostasis of the body by facilitating the flow of stagnant Qi, caused by adverse forces, via stimulation of certain acupoints along the meridians [29,30]. Early-stage ethanol withdrawal causes excessive activation of the HPA axis, which is regarded as an imbalance between Yin and Yang in TCM; therefore, acupuncture stimulation at an appropriate acupoint can improve the Qi flow to rectify the dysfunction of the HPA axis.

ST36 is the He-Sea point in the “stomach” meridian (ST) that tonifies “Qi” and “blood,” harmonizes and strengthens the “spleen” and “stomach,” and ultimately strengthens the body. In TCM, the stomach paired with the spleen is associated with the element of earth related to emotions such as anxiety and stress; therefore, ST is widely used for treating psychological/mental disorders. Although the exact mechanisms of acupuncture at ST36 are not yet clear, numerous studies indicate that modulation of the neuroendocrine system is one important mechanism. Eshkevari et al [31] reported that acupuncture at ST36 blocks cold stress-induced elevation of HPA axis activity in rats. Park et al [32] demonstrated that acupuncture at ST36 ameliorates restraint stress-induced anxiety through modulation of plasma CORT and tyrosine hydroxylase expression in rats. In our experiment, acupuncture at ST36 significantly antagonized the increased plasma levels of CORT and ACTH, and prevented the elevated expression of hypothalamic CRF during ethanol withdrawal in rats. Ethanol intake affects almost all organic systems in the body, and ethanol withdrawal produces a variety of biochemical and clinical phenotypes, the most significant being hyperactivated HPA axis. In TCM, the cause and treatment of illness are based on a holistic understanding of the human body, and the HPA axis is a major component of the neuroendocrine system that is critical for maintaining internal homeostasis; thus, it is reasonable to expect that acupuncture at ST36 modulates the function of the HPA axis during ethanol withdrawal and so may play a therapeutic role in alcoholism.

In conclusion, in the present study we showed the preventative effect of acupuncture treatment at ST36 on the oversecretion of CORT and ACTH during ethanol withdrawal in rats. Additionally, acupuncture at ST36 inhibited ethanol withdrawal-enhanced protein expression in the PVN. These results suggest that acupuncture at ST36 blocks hyperactivation of the HPA axis during ethanol withdrawal and is therefore a possible medical treatment for alcoholism.

Disclosure statement

The author affirms there are no conflicts of interest and the author has no financial interest related to the material of this manuscript.
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