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Arginine Vasopressin and Arginine Vasopressin Receptor 1b Involved in Electroacupuncture-Attenuated Hypothalamic-Pituitary-Adrenal Axis Hyperactivity in Hepatectomy Rats

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Objective: The study aims to know the effect of electroacupuncture (EA) in maintenance of the homeostasis of the neuroendocrine system in hepatectomy rats and the involvement of arginine vasopressin (AVP) signaling in hypothalamus after EA was observed.

Materials and Methods: Rats were randomly assigned to four groups, including the intact group, model group, sham-EA group, and EA group. EA was given during the perioperative period at the Zusanli (ST36) and Sanyinjiao (SP6) points after hepatectomy. The serum adrenocorticotropic hormone (ACTH) and corticosterone (CORT) levels were detected via radioimmunoassay. The expression of AVP, arginine vasopressin receptor 1a (AVPR1a), arginine vasopressin receptor 1b (AVPR1b), and glucocorticoid receptor (GR) was detected by Western blot after surgery.

Results: Compared with the intact group, the ACTH and CORT levels in the serum of model group were increased, whereas the ACTH and CORT levels were decreased in the EA group compared with the model group. Moreover, AVP and AVPR1b protein levels in the pituitary gland were increased in the model group and decreased in the EA group. Further, a distinct increase in the AVP and AVPR1a protein levels was observed in the model group, whereas they were significantly decreased in the EA group. Blockade of AVPR1b by nelivaptan reduced the increase of ACTH and CORT. D [Leu^4, Lys^8] vasopressin can inhibit the effect of EA in rectification of the hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis.

Conclusions: EA application at ST36 and SP6 can ameliorate the hyperactivity of the HPA axis via AVP signaling during the perioperative period.

Keywords: Arginine vasopressin, AVPR1b, electroacupuncture, hepatectomy, HPA axis

Conflict of Interest: The authors reported no conflict of interest.

INTRODUCTION

The hypothalamic-pituitary-adrenal (HPA) axis is a neuroendocrine system that regulates the circulating levels of adrenocorticotropic hormone (ACTH) and glucocorticoid hormone (human: cortisol; rodent: corticosterone, CORT), which are vital for normal homeostasis. Surgery, particularly laparotomy, can render a disorder of the HPA axis, which can result in fatigue, mental disorder and other symptoms. Therefore, the improved management of the HPA axis to modulate homeostasis is important in surgery-induced disorder (1).

Critical amounts of evidence have suggested that electroacupuncture (EA) at the appropriate acupoints with low-frequency stimulation can attenuate the hyperactivity of the HPA axis (2,3). EA treatment may provide a novel nonpharmacological treatment for surgery-induced HPA axis disorder. However, clear *in vivo* evidence for the role of EA in normalizing the HPA axis is still lacking.

The paraventricular nucleus (PVN) is the key regulator of HPA activity, which releases corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) into the pituitary portal circulation. CRH and AVP in the PVN exert synergistic effects on ACTH secretion from the adenohypophysis (AP) by binding to CRHR1 and AVPR1b, respectively. Recent research has shown that CRH was significantly

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decreased in the hypothalamus after EA treatment in stressed rats (4), thus providing a potential mechanism of EA on regulating stress. Given that AVP is known to potentiate CRH effects on stress, if the hypothalamic AVP level contributes to the maladaptive homeostatic condition in surgery, the mechanism of EA in alleviating the hyperactivity of the HPA axis in a surgery model must be clarified.

In this study we observed the AVP level during the perioperative period and investigated the ability of EA to alleviate the hyperactivity of the HPA axis in hepatectomy rats.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (120 ± 20 g) were purchased from the Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China) and allowed to acclimate for one week. All animals were housed in a quiet room with a 12:12 light/dark cycle and ad libitum access to food and water. Room temperature was maintained at 25 ± 2°C. All experimental procedures involving the use of animals were conducted in accordance with NIH Guidelines (NIH Publications No. 8023, revised 1978) and approved by the Animal Use and Care Committee of Fudan University.

Rats were randomly assigned to four periods (two hours, four hours, one day, and three days after hepatectomy), and each period had four groups, including the intact, model, sham-EA + model, and EA + model.

Surgery and Drug Application

Rats were divided into the following four groups: intact, model, sham-EA + model, and EA + model. Rats in the model, sham-EA + model and EA + model groups were given partial hepatectomies according to Cipriano et al. (5), under anesthesia by pentobarbital sodium (30 mg/kg). The surgical incision was approximately 7 cm long from the cartilago ensiformis to the symphysis pubis along the linea alba, and 10% of the liver was cut out from the right lobe. Then the incision was closed after exhaustive hemostasis. All rats were kept warm during and after the hepatectomy. All surgeries were performed between 8:00 and 10:00 AM.

To elucidate the molecular mechanism of EA application, rats were given intraperitoneal (i.p.) injection of the vehicle (1 mL/kg, 5% DMSO, 95% saline) or nelivaptan (6) (5 mL/kg, Axon 1114, Axon Medchem BV Amsterdam, Groningen, the Netherlands) or d [Leu^4, Lys^8] vasopressin (7) (0.3 μg/kg, 42061-33-6, Sigma, St. Louis, MO, USA) dissolved in the vehicle 24 hours before the surgery and immediately after surgery.

EA Treatment

EA application was consistent with Feng et al. (8). Rats from the EA + model group received EA stimulation via two sterile stainless steel needles (0.3 mm in diameter and 25 mm long) that were connected to the output terminals of an EA apparatus, the HANS Acupoint Nerve Stimulator (LH202H, Beijing, China). The needles were vertically inserted into the right Zusanli acupoint (ST36, located in the posterolateral knee joint, approximately 0.5 cm below the capitulum fibulae) at a depth of 5 mm and the Sanyinjiao acupoint (SP6, at the superior border of the medial malleolus, between the posterior border of the tibia and anterior border of the Achilles tendon) at a depth of 3 mm. The stimulation lasted for 30 min (started and ended time in 8:00–10:00 AM) at an intensity of 2 mA and alternating strings of dense-sparse frequencies (2 Hz for 1.05 sec and 15 Hz for 2.85 sec, alternating).

Needles were inserted into the right ST36 and SP6 points, with no stimulation for rats in the sham-EA + model group. EA and sham-EA application was given 24 hours before the surgery and immediately after surgery.

Tissue Collection

Rats in each group ($N = 6$) at one day and three days after surgery were sacrificed by decapitation, their brains were immediately removed, and the pituitary glands were dug up, thus the hypothalamus was separated from the brain. The pituitary gland and hypothalamus were snap-frozen in liquid nitrogen and then stored at −80°C until further processing. The rats in each group at one day after surgery ($N = 4$) were exsanguinated with normal saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer saline (PBS).

Radioimmunoassay (RIA)

Blood samples were collected by decapitation at the time of sacrifice, and the serum was separated by centrifugation. The concentrations of ACTH and CORT were determined by double-antibody RIA kits purchased from the Beijing Sinouk Institute of Biological Technology (Beijing, China). All of the serum samples were assayed together, and each sample was analyzed in duplicate. The sensitivity of the kit for ACTH was less than 5 pg/mL, and the intra- and interassay coefficients were less than 4.1% and 8.4%, respectively. The sensitivity of the kit for CORT was 1 pg/mL, and the intra- and interassay coefficients of variation were 7.5% and 9.5%, respectively.

Real-Time Polymerase Chain Reaction (RT-PCR)

RT-PCR was conducted following previous method in our laboratory (9). For mRNA analysis, total hypothalamus RNA was extracted by TRIzol Reagent (Invitrogen Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The purity and integrity of the RNA were checked spectroscopically before obtaining cDNA using the GoScript Reverse Transcription System (Promega, Madison, WI, USA).

To obtain cDNA, 2.0 μg of the total RNA was reverse-transcribed by the GoScript Reverse Transcription System (Promega) according to the manufacturer's instructions. Real-Time PCR was carried out by IQ5 Real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The reactions were set up with 10 μl SYBR Green Real Master Mix (Promega), 1.6 μl primer mixture (200 nM), and 1.6 μl cDNA template. The thermal cycling conditions were as follows: 95°C, 3 min for denaturation, followed by 38 cycles of 95°C, 10 sec, and 60°C, 30 sec, and 72°C for 30 sec. After the cycles, a melting curve analysis was performed to ensure the purity of PCR products.

The primers used for AVP mRNA analysis were designed and synthesized by Invitrogen and purified by HPLC (AVP, Forward: TGCCTGCTACTTCCAGAACTGC, Reverse: AGGGGAGACACTGTCTC AGCTC; GAPDH, Forward: GTA TGA CTC TAC CCA CGG CAA GT, Reverse: TTC CCG TTG ATG ACC AGC TT). All experiments were run in triplicate and relative mRNA levels were analyzed by means of the formula $2^{-\Delta\Delta Ct}$ method and normalized to the GAPDH.

Western Blot

Western blot was assayed based on previous method in our laboratory (10). Briefly, rats pituitary and hypothalamus were isolated after decapitation and homogenized via RIPA buffer (9806, CST,

Danvers, MA, USA) within Phenylmethanesulfonyl Fluoride (8553, CST). After calibration by the BCA protein assay kit (23225, Pierce Pharmaceuticals, Melbourne, VIC, Australia), the supernatant was denatured for 10 min at 100°C in a solution of 4× Laemmli sample buffer (161–0747, Bio-Rad). The proteins were separated using Bio-Rad equipment and transferred to polyvinylidene fluoride (PVDF) membranes (ISEQ00010, Merck Millipore, Darmstadt, Germany). The PVDF membranes were then incubated in 5% defatted milk for one hour at room temperature and incubated at 4°C in primary antibodies overnight (AVP: 1:1000, AB1565, Merck Millipore; AVPR1a: 1:200, sc-18096, Santa Cruz, Santa Cruz, CA, USA; AVPR1b: 1:10000, PAB11503, Abnova, Taipei, Taiwan; glucocorticoid receptor (GR): 1:200, AB2768, Abcam, Cambridge, UK).

After washing in buffer (TBS-0.1% Tween 20), the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, diluted at 1:10000, for two hours at 4°C. Target protein signals were detected using an ECL detection kit (Immobilon western chemiluminescent HRP substrate p90720, Merck Millipore) and exposed in an Image Quant LAS 4000 mini (GE Healthcare, Buckinghamshire, UK). The signals were quantified using the Quantity One software (Bio-Rad, Hercules, CA, USA). The results for signal intensity were expressed in arbitrary densitometric units, after normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH, ab181602, Abcam) as an internal standard.

Immunofluorescence

Immunofluorescence was observed based on previous method from our laboratory (10). After perfusion with normal saline followed by 4% paraformaldehyde (PFA) in 0.1 M PB, rat brains were dissected out and postfixed in 4% PFA overnight followed by 20% sucrose in a 4°C refrigerator. Cross-sections were sliced at a thickness of 30 μm via a cryostat microtome (Leica, Wetzlar, GER).

Slices were washed in PBS for 30 min at RT and blocked with 10% normal goat serum before being incubated in a rabbit polyclonal antibody against AVP (1:1000, AB1565, Merck Millipore) diluted in 0.01 M PBS containing 1% bovine serum albumin, 0.02% sodium azide, and 0.03% Triton X-100 at 4°C overnight. After rinsing, the sections were incubated in secondary antibody solutions (1:200, Alexa Flour 488 donkey anti-rabbit IgG [H + L] antibody, Life Technologies, Rockville, MD, USA) for one hour at RT. Then the sections were examined by a fluorescence microscope (Leica, Germany). The numbers of AVP-positive cells in the PVN were quantified via computer-assisted image analysis (Image J, NIH, Bethesda, MD, USA). The specificity of AVP staining was determined by preincubation of antiserums at various concentrations for 48 hours at 4°C in the absence of primary antibody to identify nonspecific staining.

Statistical Analysis

Data were presented as the mean ± standard deviation (SD) and analyzed by SPSS 17.0 (IBM Corporation, Armonk, NY, USA). For statistical comparisons, the values were subjected to one-way analysis of variance (ANOVA) between groups at each time point followed by Bonferroni multiple comparisons after analyzing time as repeated measure using two-way ANOVAs. A p value of <0.05 was considered statistically significant.

RESULTS

Effects of EA on Serum ACTH and CORT Concentrations in Hepatectomy Rats

The effects of EA on the blood ACTH and CORT concentrations were detected by RIA. There were significant main effects of the serum ACTH levels at two hours ($F_{(3,\ 24)} = 3.956$, $p = 0.02$), one day ($F_{(3,\ 24)} = 38.471$, $p = 0.000$), and three days ($F_{(3,\ 24)} = 7.14$, $p = 0.015$) after surgery. The blood ACTH concentration was increased significantly in the model group compared with the intact group at two hours ($p = 0.013$), one day ($p = 0.000$), and three days ($p = 0.012$) after hepatectomy. There was no significant difference among the groups at four hours after hepatectomy ($F_{(3,\ 24)} = 0.907$, $p = 0.452$) (Fig. 1a). Compared with the intact group, the ACTH in the Sham-EA + model group increased at three days ($p = 0.000$) after surgery, whereas it decreased in the EA + model group compared with the model group at one day after surgery ($p = 0.004$).

The serum CORT concentration revealed significant main effects of the serum CORT levels at two hours ($F_{(3,\ 24)} = 48.186$, $p = 0.000$), four hours ($F_{(3,\ 24)} = 9.488$, $p = 0.000$), and one day ($F_{(3,\ 24)} = 41.458$, $p = 0.000$) after surgery. CORT was increased significantly in the model group at two hours ($p = 0.000$), four hours ($p = 0.034$), one day ($p = 0.000$), and three days ($p = 0.006$) after surgery compared with the intact group; compared with the model group, CORT was decreased in the EA + model group at two hours ($p = 0.000$) and one day ($p = 0.034$) after hepatectomy. There was also a significance between the model group and the sham-EA + model group ($p = 0.025$) at two hours after surgery (Fig. 1b). There was a decreasing trend of CORT expression in sham-EA + model group compared with the model group at four hours ($p = 0.673$), one day ($p = 0.164$), and three days ($p = 0.187$), but with no significance. Compared with the sham-EA + model group, CORT level showed no statistical difference but only descending tendency at two hours ($p = 0.251$), four hours ($p = 0.162$), and one day ($p = 0.535$) in the EA + model group.

Effect of EA on the Expression of AVPR1b and AVP in the Pituitary of Hepatectomy Rats

To investigate whether AVP and AVPR1b are involved in the hyperactivity of the HPA axis and in the ability of EA to rectify the hyperactivity of the HPA axis, Western blotting was used to observe the expression levels of AVP and AVPR1b in the pituitary. Statically significant differences were observed among the groups for the AVP protein at one day ($F_{(3,\ 20)} = 24.854$, $p = 0.000$) and three days ($F_{(3,\ 20)} = 8.924$, $p = 0.003$) postsurgery. An obvious increase in the AVP protein level was observed at one day ($p = 0.000$) and three days ($p = 0.005$) after surgery in the model group compared with the intact group. Compared with the model group, the expression of AVP in the EA + model group showed a significant decrease at one day ($p = 0.001$) and at three days ($p = 0.000$) after surgery; the decrease also showed in the sham-EA + model group compared with the model group at one day following surgery ($p = 0.000$). There was no difference between the sham-EA + model group and the EA + model group in the AVP protein expression at one day ($p = 0.315$) or three days ($p = 0.072$) after hepatectomy (Fig. 2a,b). In addition, the AVPR1b protein showed statistically significant difference among the groups at one day ($F_{(3,\ 20)} = 40.14$, $p = 0.000$) and three days ($F_{(3,\ 20)} = 32.928$, $p = 0.000$) postsurgery. An obvious increase of AVPR1b was also observed at one day ($p = 0.016$) and three days ($p = 0.006$) after surgery. Compared with the model group, the expression of AVPR1b in the EA + model group showed a statically significant decrease at one day ($p = 0.025$) and three days ($p = 0.036$) after surgery (Fig. 2a,c). An obvious increase in AVPR1b protein level was observed at one day ($p = 0.026$). There was no significance between the sham-EA + model group and the EA + model group at one day or three days after surgery in the pituitary (Fig. 2a,c).

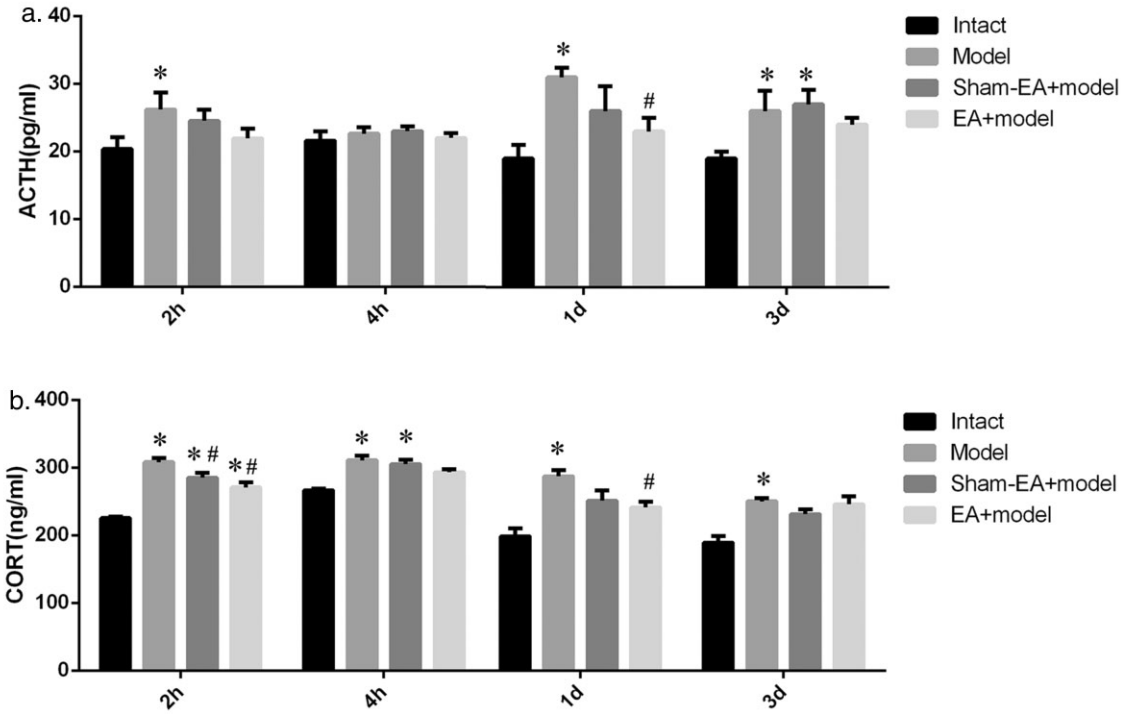

Figure 1. Stress-related hormones determined by RIA in the peripheral blood. Blood ACTH (a) and CORT (b) levels in intact, model, sham-EA + model, and EA + model groups at two hours, four hours, one day, and three days after hepatectomy. Data are presented as the mean ± SD ($N = 7$). *, vs. intact ($p < 0.05$); #, vs. model ($p < 0.05$). ACTH, adrenocorticotropic hormone; CORT, corticosterone; EA, electroacupuncture; RIA, radioimmunoassay.

Effects of EA on AVP Expression in the Hypothalamus of Hepatectomy Rats

The effect of EA on the expression of hypothalamic AVP after hepatectomy was investigated using RT-PCR, Western blot, and immunofluorescence.

AVP-positive neurons were mainly concentrated in the magnocellular cells of the PVN; most of the cells were round or prismatic or had an irregular size of approximately 20~30 μm. In this study, a number of AVP immunoreactive cell bodies and their varicose axon fibers were found in the magnocellular part of the PVN. The number of AVP-positive neurons was significantly different among the groups at one day ($F_{(3, 12)} = 21.020$, $p = 0.000$) after surgery (Fig. 3a). The number of AVP-positive neurons increased in the model group at one day ($p = 0.000$) after the surgery compared with the intact group. Compared with the model group, the AVP-positive neurons in the EA + model group were significantly decreased at one day ($p = 0.003$) after hepatectomy (Fig. 3a). The AVP-positive neurons in the sham-EA + model group showed a decreasing trend compared with the model group at one day after surgery in the PVN ($p = 0.085$), and there was no difference between the sham-EA + model and EA + model groups ($p = 0.372$) (Fig. 3a).

AVP mRNA was significantly increased at both one day ($F_{(3, 20)} = 9.012$, $p = 0.001$) and three days ($F_{(3, 20)} = 6.171$, $p = 0.004$) after surgery. The expression of AVP mRNA was upregulated in the model group compared with the intact group at one day ($p = 0.019$) and three days ($p = 0.018$) after surgery. The expression of AVP mRNA in the EA + model group was significantly lower than that observed in the model group at one day ($p = 0.002$) and three days ($p = 0.006$) postsurgery. The AVP level in the sham-EA + model group was of statistical decrease compared with that in the model group at one day ($p = 0.014$) and three days ($p = 0.000$) postsurgery, and no significance compared with EA + model group at one day ($p = 0.739$) and three days ($p = 0.572$) after surgery (Fig. 3b).

The expression of the AVP protein was significantly different among the groups at one day ($F_{(3, 20)} = 5.664$, $p = 0.006$) and three days ($F_{(3, 20)} = 48.998$, $p = 0.000$) postsurgery (Fig. 3b). The protein was significantly increased in the model group compared with the intact group at both one day ($p = 0.004$) and three days ($p = 0.000$) after surgery in the hypothalamus. AVP protein expression was significantly down-regulated in the EA + model group compared with the model group at one day ($p = 0.000$) and three days ($p = 0.006$) following surgery. A significant decrease was also found in the sham-EA + model group compared with the model group at one day ($p = 0.000$) and three days ($p = 0.019$) postsurgery. There was no difference of hypothalamus AVP protein level between sham-EA + model group and EA + model group at one day ($p = 0.814$) or three days ($p = 0.838$) after surgery (Fig. 3b,c).

The protein expression of hypothalamus AVPR1a, AVPR1b and GR at one day and three days after hepatectomy was determined via Western blot. There were significant changes in the AVPR1a protein at one day ($F_{(3, 20)} = 25.215$, $p = 0.000$) and three days ($F_{(3, 20)} = 4.644$, $p = 0.013$) postsurgery. The AVPR1a protein levels increased in the model group at one day ($p = 0.000$) and three days ($p = 0.016$) postsurgery compared with the intact group. The AVPR1a protein level in the EA + model group decreased compared with the model group at one day ($p = 0.000$) and three days ($p = 0.041$) following the surgery. A significant decrease was shown in the EA + model group compared with the sham-EA + model group at three days ($p = 0.020$) postsurgery in the hypothalamus (Fig. 3c,d).

The hypothalamic AVPR1b protein level decreased in the model group compared with the intact group at one day ($p = 0.001$) and three days ($p = 0.000$) after hepatectomy. Compared with the intact group, AVPR1b level decreased in the model group ($p = 0.000$), sham-EA + model group ($p = 0.013$), and EA + model group ($p = 0.000$) at one day postsurgery. The AVPR1b protein level in the EA + model group was decreased compared with the model group at one

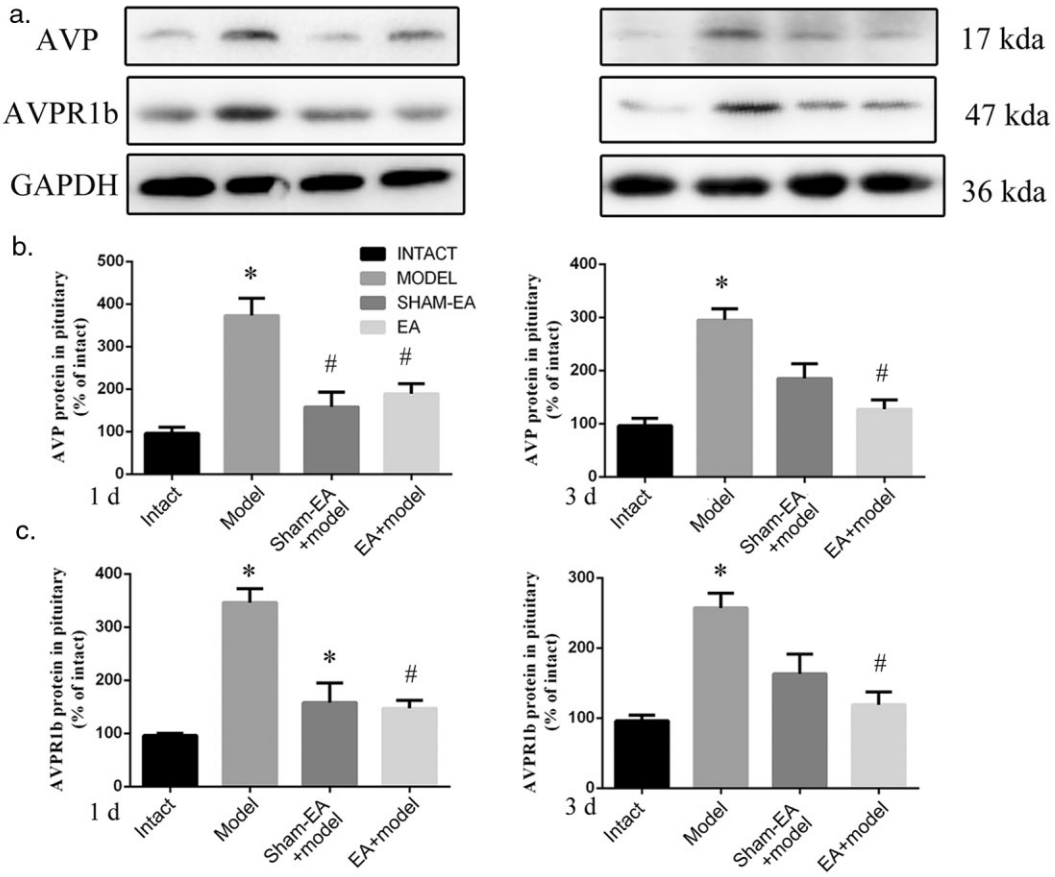

Figure 2. AVP and AVPR1b protein expression in the pituitary among intact, model, sham-EA + model, and EA + model groups. Representative bands (a) and quantification of AVP (b) and AVPR1b (c) protein expression in the pituitary at one day and three days after surgery. Data are presented as the mean ± SD ($N = 6$). *, vs. intact ($p < 0.05$); #, vs. model ($p < 0.05$). AVP, arginine vasopressin; AVPR1b, arginine vasopressin receptor 1b; EA, electroacupuncture.

day ($p = 0.018$) and three days ($p = 0.001$) after hepatectomy. Compared with the sham-EA + model group, AVPR1b level decreased in the EA + model group at one day ($p = 0.036$) and three days ($p = 0.019$) postsurgery (Fig. 3c,e).

In the hypothalamus, there were statistically significant differences in the GR protein levels among groups one day after surgery ($F_{(3, 20)} = 8.124$, $p = 0.001$). The protein was increased in the model group one day after hepatectomy compared with the intact group ($p = 0.019$). The expression of GR was decreased in the EA + model group compared with the model group ($p = 0.002$), and there was no difference between the sham-EA + model group and the EA + model group ($p = 0.419$). There were no significant differences among the four groups three days postsurgery ($F_{(3, 20)} = 0.68$, $p = 0.575$) (Fig. 3c,f).

The Ability of EA to Rectify Surgery-Induced Hyperactivity of the HPA Axis Was Altered by AVPR1b Manipulation

To evaluate the role of AVP/AVPR1b signaling in the HPA axis hyperactivity induced by surgery, serum ACTH and CORT were chosen to evaluate HPA axis activity by RIA at one day postsurgery after drug application. There was no difference between the model + vehicle and the model + agonist groups of ACTH ($p = 0.823$) and CORT expression ($p = 0.515$). After blocking AVPR1b by nelivaptan, ACTH ($p = 0.002$) and CORT ($p = 0.000$) levels decreased compared with the model + vehicle group, and there was no difference of ACTH ($p = 0.753$) and CORT level ($p = 0.240$) in the model + EA+ agonist group compared with the model + vehicle group (Fig. 4a). Besides, compared with model + EA group, CORT level in the model + EA + agonist group increased significantly ($p = 0.008$) (Fig. 4b).

DISCUSSION

EA is a complementary and alternative medicine that has been accepted worldwide. In this study, we demonstrated that EA at ST36 and SP6 could modulate the HPA axis via decreasing AVP system in the hypothalamus in a rat model of partial hepatectomy.

In response to a stressor, AVP is released from the magnocellular neurons of the PVN and can control ACTH secretion from the adenohypophysis, which in turn stimulates both the synthesis and secretion of corticosterone from the adrenal cortex. Further, corticosterone exerts a negative feedback effect to reduce the synthesis and release of ACTH, CRH, and AVP. Increasing CORT results in immunosuppression and hippocampus injury after intense stress (11,12). EA has been reported to relieve immunosuppression in patients undergoing supratentorial craniotomy (13). In the present study, partial hepatectomy was used as a stressor to excessively activate the HPA axis because studies demonstrated an evident increase of CORT in rodent after hepatectomy (5,14). Besides, the function of the powerful liver regeneration and the hepatectomy as a common clinical surgery made this research feasible and valuable.

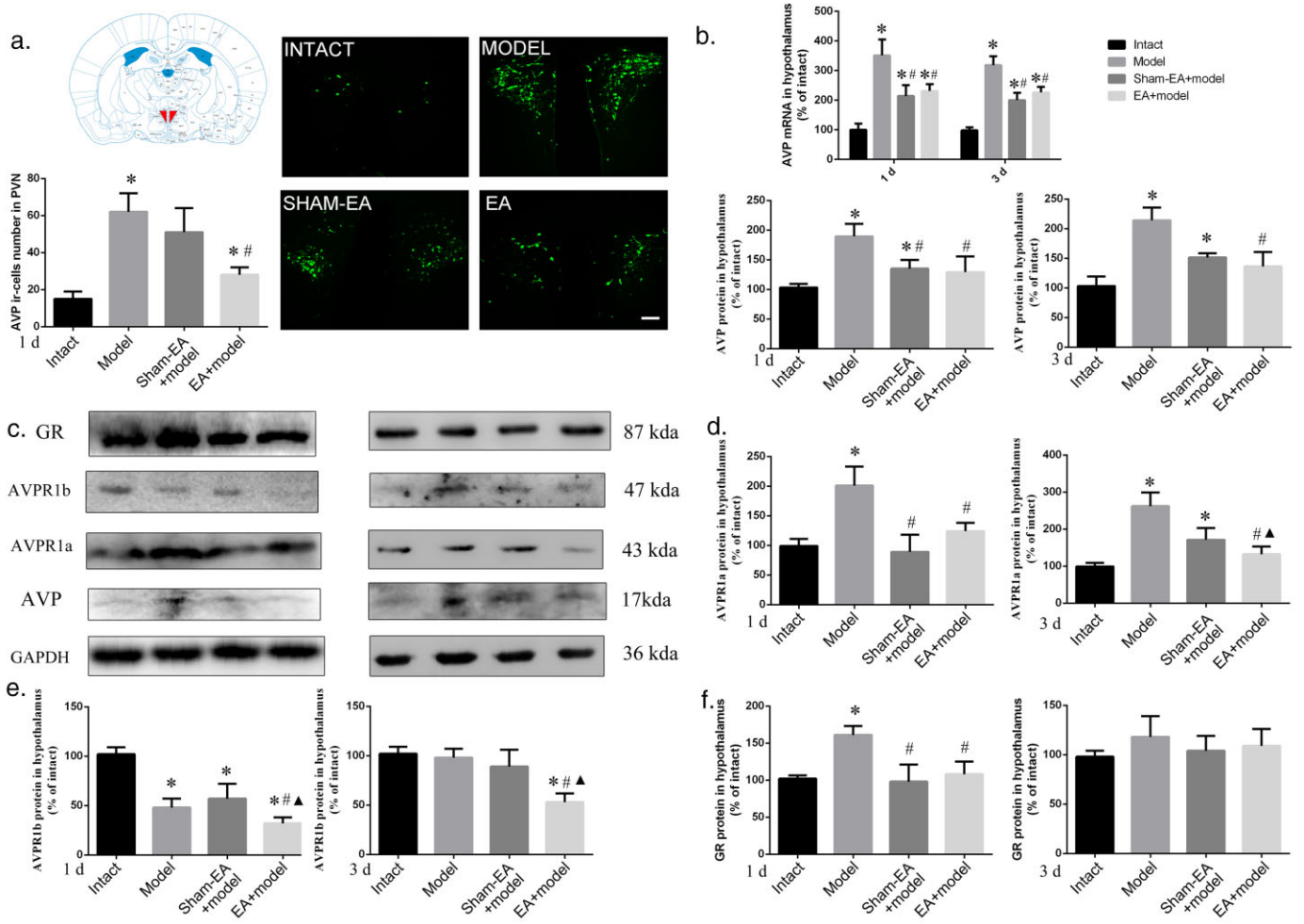

Figure 3. AVP expression levels in the hypothalamus among intact, model, sham-EA, and EA groups at one and three days after surgery. Immunofluorescent assay for AVP expression (a) in the hypothalamic PVN nuclei among groups ($N = 4$, Bar = 100 μm). Quantification of AVP mRNA (b) in the rat hypothalamus at one and three days after surgery; representative bands (c) and quantification of AVP (b), AVPR1a (d), AVPR1b (e), and GR (f) protein expression in the hypothalamus at one day and three days after surgery. Data are presented as the mean ± SD ($N = 6$). *, vs. intact ($p < 0.05$); #, vs. model ($p < 0.05$); ▲, vs. sham-EA + model ($p < 0.05$). AVP, arginine vasopressin; EA, electroacupuncture; AVPR1a, arginine vasopressin receptor 1a; AVPR1b, arginine vasopressin receptor 1b; GR, glucocorticoid receptor; PVN, paraventricular nucleus.

Result in this study showed a marked increase in serum ACTH and CORT levels in hepatectomy rats, which indicated hyperactivity of the HPA axis after hepatectomy.

AVPR1b is primarily expressed in the pituitary and plays a critical role in the mediation of the HPA axis (6). Stress induces the expression of AVP in PVN neurons, which project their axon terminals into the external layer of the median eminence and secrete AVP into the portal vein. After combination with AVPR1b in the anterior lobe of the pituitary, AVP induces secretion of ACTH from corticotropes. A previous study has shown that proximal colon distension could increase AVP expression in the rat brain (15). In this study, the AVP and AVPR1b protein levels increased in the pituitary after surgery. Moreover, both AVP mRNA and protein were increased in the hypothalamus after surgery. As the synthesis sites of AVP were PVN and supraoptic nuclei and accessory nuclei in the hypothalamus (16), AVP mRNA and protein level could not reflect adequately the PVN AVP expression, so immunofluorescence assay was conducted which showed an activation evidently of AVP positive cells in the PVN of the hypothalamus after surgery.

Accumulating evidence supports that EA can rectify the dysregulation of the HPA axis (4,17). EA has been used in many instances in TCM, for example, pain (18), obesity (13), sepsis (19), and surgery (3,20–22). EA can maintain homeostasis by promoting the ability of the organism to cope with the stressor. EA at ST36 and SP6 had been proven to regulate hypothalamus peptide (23,24). ST36 application had been proven to inhibit the elevation of the HPA axis (4). The afferent impulse of ST36 had been found to be mainly transmitted by Aβ and Aδ fibers (25). Neuroimaging showed that its modulation mechanism was related to limbic and cerebro-cerebellar system (26). EA at SP6 was proven to relieve postoperative pain with ST36 in laparotomy (8). Besides, they could affect the intestinal myoelectric activity which would be beneficial in laparotomy (27). Therefore, ST36 and SP6 were chosen in this study. It was reported that EA stimulates neuropeptide release by peripheral electrical stimulation of different frequencies (28). Gene expression demonstrated low frequency at ST36 and SP6 affects more genes than at high frequency (23). Besides, high frequency could increase AVP level through lengthening the action potential duration and increasing the Ca^{2+} entry (29) while low frequency as 2/15 Hz had been proven useful in pain relief during postoperative period (8). In addition, there was successful treated case of EA intervention before and after surgery in clinical

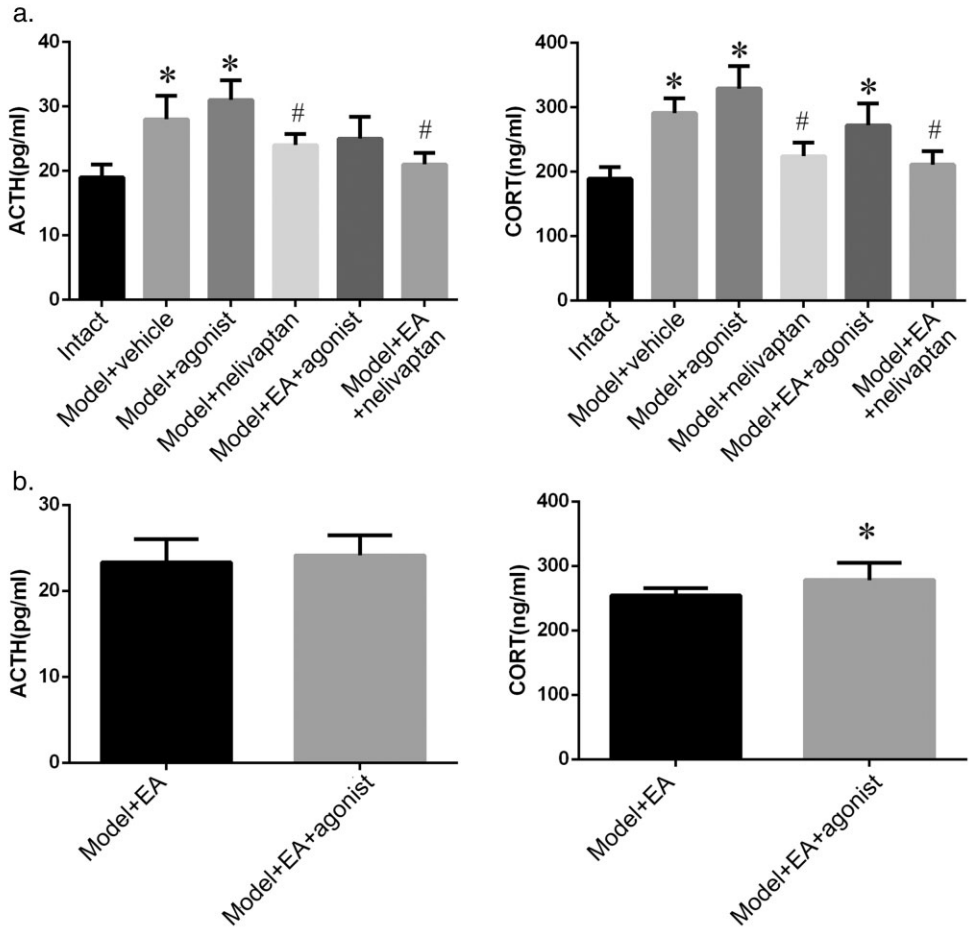

Figure 4. Stress-related hormones determined by RIA in peripheral blood after application of an AVPR1b agonist and nelivaptan. (a) Blood ACTH and CORT levels in intact, model + vehicle, model + agonist, model + nelivaptan, model + EA + agonist, and EA + nelivaptan groups at one day after hepatectomy. Data are presented as the mean ± SD ($N = 7$). *, vs. intact ($p < 0.05$); #, vs. model + vehicle ($p < 0.05$). (b) Blood ACTH and CORT levels in model + EA, model + EA + agonist groups. Data are presented as the mean ± SD ($N = 7$). *, vs. model + EA ($p < 0.05$). ACTH, adrenocorticotropic hormone; AVPR1b, arginine vasopressin receptor 1b; CORT, corticosterone; EA, electroacupuncture; RIA, radioimmunoassay.

(3). Both pretreatment and postsurgery EA could ameliorate stress effects (4).

In this study, EA decreased the elevated serum ACTH and CORT levels in hepatectomy rats, which suggests that EA may be an effective way to prevent hepatectomy-induced hyperactivity of the HPA axis. In addition, the pituitary AVPR1b and hypothalamus AVP protein levels that were significantly increased after hepatectomy were decreased by EA treatment. These results indicate that EA may alleviate surgery-induced hyperactivity of the HPA axis via decreasing AVP/AVPR1b expression.

Nelivaptan (SSR149415) is a selective AVPR1b antagonist (30). Blockade of AVPR1b reduced the increase of ACTH and CORT (31). D [Leu^4, Lys^8] vasopressin is a selective agonist of AVPR1b, which can reinforce the HPA axis. In this study, nelivaptan and d [Leu^4, Lys^8] vasopressin were given with EA application simultaneously. Result showed that blockade of AVPR1b by nelivaptan could decrease surgery-induced CORT and that application of d [Leu^4, Lys^8] vasopressin could inhibit the ability of EA to rectify the hyperactivity of the HPA axis induced by surgery while application of EA without d [Leu^4, Lys^8] vasopressin could improve dysfunction of the HPA axis. The present study indicated the involvement of AVP/AVPR1b signaling in decreasing hyperactivity of the HPA axis by EA.

AVP is known to potentiate CRF effects on stress. Research has illustrated that the AVP regulation of HPA axis was of equal importance to the CRH system (32). At the neuronal level, AVP enhances the membrane excitability and modulates synaptic transmission via its receptors AVPR1a and AVPR1b (33,34). In this study, AVPR1a tended to increase after surgery, and EA could decrease the AVPR1a expression. This result indicates that EA may regulate the HPA axis via the AVP/AVPR1a signaling pathway in the hypothalamus and via AVPR1b in the pituitary and in turn reduce the ACTH and CORT levels.

In this study, sham-EA could decrease the serum CORT at two hours postsurgery, tended to decrease AVP and AVPR1b level in the pituitary, and there was no significance between sham-EA and EA treatment after surgery of the AVP and AVPR1b expression, which indicated that sham-EA could alleviate the hyperactivity of the HPA axis to some extent. The acupuncture has been used in various diseases, and its mechanism still remains unclear (35). According to traditional Chinese medicine (TCM), insertion and retaining of needles into the acupoint could produce biological effects without electric stimulation. Acupuncture is a treatment based on empirical medicine while EA has been established in recent decades based on it. However, EA intervention was proven more significant and stable

than sham-EA in this study. In addition, there are also other valuable peripheral nerve stimulation methods; external noninvasive peripheral nerve stimulation treatment has been used in pain relief (36), percutaneous nerve stimulation in testicular torsion (37), and transcutaneous electrical nerve stimulation in postoperative side-effects (38). They are widely used in clinical while their molecular mechanisms also need further clarify.

These findings are novel as there is no published data currently on the effects of EA on AVP and AVPR1 levels in a surgery stress model such as the hepatectomy rats. Our study may provide a new application of EA on blocking the HPA axis maladaptive at the level of AVP. However, further studies are needed to identify the potential effects of EA on the interaction of CRH and AVP.

In conclusion, surgery increases the AVP, AVPR1a, and GR levels in the hypothalamus and subsequently increases AVPR1b in the pituitary. EA can ameliorate the dysregulation of the HPA axis via AVP signaling.

Authorship Statements

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the study to take public responsibility for the content. Besides, each author certifies that this manuscript has not been and will not be published in any other publication.

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COMMENTS

The study proposes that electroacupuncture (EA) can reduce hyperactivity of hypothalamic pituitary adrenal axis (HPA-axis) disorder induced by hepatectomy through arginine vasopressin signaling. The unique neurostimulation of somatic nerves such as median, deep peroneal or tibial nerves with EA using stimulation parameters of low frequency and low voltage for 30 minutes modulates neuronal activity in the hypothalamus. Based on findings observed in this study, changes in expressions involved in the HPA-axis are reasonable. To demonstrate that this type of neurostimulation decreases stress related neuronal expressions in perioperative periods warrants further investigation.

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Comments not included in the Early View version of this paper.

