Author’s response to reviews

Title: Electroacupuncture activates corticotrophin-releasing hormone-containing neurons in the paraventricular nucleus of the hypothalammus to alleviate edema in a rat model of inflammation

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Author’s response to reviews: see over
Dear Dr. Norton:

Attached please find the revised manuscript MS #3439623171784939, entitled "Electroacupuncture activates corticotrophin-releasing hormone-containing neurons in the paraventricular nucleus of the hypothalamus to alleviate edema in a rat model of inflammation" by Li et al. We would like to express our cordial thanks to the editors and reviewers for their constructive comments. We have revised the manuscript carefully to incorporate their comments and suggestions. The following responses address the reviewers’ point-by-point concerns and are reflected in the text.

The first Reviewer’s report:

Major Compulsory Revisions
1) (page 4-5) Experimental design: in experiment 2 & 3, experimental groups were divided into three groups, but groups should be divided into 4 groups including 'CFA + ACTH(11-24) + Sham EA'

Two control experiments, CFA + ACTH(11-24) + Sham EA and CFA + Astressin + Sham EA, have been performed and the data have been added to the manuscript on page 10 and figures 2-5.

2) (page 5, line 2) : you mentioned that 2 mg/kg of ACTH was intravenously (i.v.) injected. in general, 2 mg/kg injection is too much dosage in the aspect of intravenous injection. confirm about this.

We appreciate the reviewer’s comments and agree that micrograms of ACTH work efficiently. We injected ACTH(11-24), which is different from ACTH and has substantial competitive antagonist activity (Kapas et al., 1996). ACTH(11-24) is inactive for steroidogenesis and has little influence on circulating plasma corticosterone values (Brain and Evans, 1977; Hinson and Birmingham, 1987). In order to completely block the ACTH receptor, we injected 2 mg/Kg of ACTH(11-24).


3) (page 2) Abstract: 'Key Words' should be included
Key words, Acupuncture, ACTH, CRH, Inflammation, and Rats, have been included.

4) (page 4, line 20) : 'CFA only' -> 'CFA + no treatment' 'EA only' -> Naive + EA’ would be better
These two groups have been renamed according to the suggestion.

5) (page 19) Figure 6 legend : you’d better explain more detaily including treatment method of A & B & C & D group.
Panels A, B and C used naïve, EA-treated rats. Panels A and B are microphotographs from the same section, which was stained simultaneously with anti-CRH (red for A) and anti-p-NR1 (green for B). Panel C is a merger of A and B. Panel D shows naïve, untreated rats. It is a microphotograph of a section single-labeled with anti-p-NR1 and shows no P-NR1 labeling.
6) Figure 3 & 5: Asterisks should be marked in group of EA treatment
The figures have been revised according to the reviewer’s comment.

The second Reviewer’s report:
This study was performed to see whether CRH and ACTH receptors involve in EA-produced anti-edema effects in CFA-injected pain model. The end points were EA activates CRH neuron to increase plasma ACTH levels and suppress edema through CRH and ACTH receptors. Although the presentation of the data is clear, there is one major and some minor concerns.

Major points
The authors observed CRH and ACTH antagonism for inflammatory edema and hyperalgesia in CFA-induced pain model. Authors explains that EA stimulation have no stress effects in pain model. But if EA stimulation started after CFA injection, CFA and EA synergistically induce stress effects. Some possibility for stress effects of EA could not rule out based on experimental procedures (EA stimulation with 10 Hz frequency and 3 mA intensity, twice in a day) and the present results (increase of plasma ACTH, phosphorylation of NR1 receptor in PVN). It should be mentioned that some possibility of EA stress effects in the discussion section.

We have revised the discussion (2nd paragraph) according to the comments.

Minor points
1. Abstract section: Add more clear description for animal model in the conclusion section as in CFA-induce pain model or in inflamed rat model.

The phrase “in a rat model of inflammation” has been added to the conclusion.

2. Method 2.5 section: Clearly describe acupuncture needle thickness, the depth of insertion and units of rat plastic chamber.

A #32 gauge (0.25 mm) needle was inserted to a depth of the insertion of 0.5 inches. The plastic chamber is 5 x 8 x 11 inches.

3. Method 2.7 section, line 4: * as X
The error has been corrected.

4. Conclusion section: Add “in CFA-induce pain model” or “in inflamed rat model”
The phrase “in a rat model of inflammation” has been added at the end of the conclusion.

The third Reviewer’s report:
The submission of Li et al. demonstrated that electroacupuncture (EA) significantly decreased CFA-induced edema possibly through inhibition of ACTH in blood. Authors also tried to prove that this inhibition of EA was through NR1 phosphorlalation of NMDA receptors in CRH-containing neurons of the paraventricular nucleus.

The research is of interest, but the reviewer has the following concerns.

Major concerns:
1. As stated by reference 5, electrical stimulation of the PVN significantly increased the pain threshold and enhanced acupuncture analgesia. But in the present study, author claimed that no involvement of ACTH was found in EA anti-hyperalgesic effect. How to explain this discrepancy?
It is well known that beta-endorphin inhibits pain. Electrical stimulation of the PVN may induce the secretion of CRH and the subsequent secretion of beta-endorphin to inhibit pain. Previous studies also demonstrate that EA increases plasma levels of beta-endorphin to inhibit pain. Concordantly, our study showed that a CRH antagonist partially prevented EA anti-hyperalgesia, possibly by inhibiting an EA-induced release of beta-endorphin. In contrast, an ACTH antagonist did not prevent EA anti-hyperalgesia. We have revised the related discussion on page 13.

2. ACTH (11-24) and astressin were injected intravenously. Could they enter into the brain (paraventricular nucleus)?
Neither ACTH (11-24) nor astressin can pass the blood brain barrier, and they only work peripherally in the adrenal and pituitary glands, respectively, to prevent the effects of ACTH and CRH.

3. If ACTH (11-24) and astressin could penetrate brain-blood barrier, how authors exclude the possibility that ACTH takes role peripherally?
See above.

4. On page 5, “CFA-inflamed rats were divided into three groups (n = 7 per group): 1) CFA + Vehicle + Sham EA, 2) CFA + Vehicle + EA, and 3) CFA + Astressin (Sigma) + EA.” It seems that one more group “CFA+Astressin+Sham EA” was ignored?
A CFA + Astressin + Sham EA group has been performed and the data have been added to Figs 4-5.

5. Although authors strongly suggested that there was no stress reaction in the present study, but under EA stimulation at intensity as high as 3 mA, stress should not be avoid completely. Authors could give out their opinion, but should not draw this conclusion absolutely. Please refer to relative references like Wan Y, et al., Acupuncture Research 2001; 26: 234-240.
We have revised the discussion (2\textsuperscript{nd} paragraph) according to the comments.

6. Result of Fig. 6 should be added more in detail. In the text, author claimed that “some neurons were single-labeled with CRH, suggesting that they were not activated by EA.” It is encouraged that authors count and give out the exact number or percentage of the double-stained neurons.

\textbf{Seventy-three percent of phosphorylated NR1 staining was localized in the CRH-containing neurons, indicating that EA mainly activated CRH-containing neurons.}

7. In Fig. 6, a bigger amplification to doubly stained neurons is suggested, for example a bigger amplification is inserted as an inset of Fig. 6A, B or C. In the present picture, it is hard to see clearly the positive staining of a neuron.
\textbf{Three insets, indicated by squares, have been added to Figs. 6A, B and C respectively.}

8. Conclusion is simple and not supported strongly by authors’ evidences. It is better to state like following: “Electroacupuncture (EA) significantly decreased CFA-induced edema through inhibition of ACTH in blood. This inhibition of EA was possibly through activation of NMDA receptors in CRH-containing neurons of the paraventricular nucleus.”
\textbf{We appreciate the reviewer’s comments regarding the conclusion. The results suggest that EA decreased CFA-induced edema by activating ACTH in the blood. The conclusion has been revised according to the reviewer’s comments.}

\textbf{Minor concerns:}
1. “3.1 EA increased plasma levels of ACTH” should be “3.1 EA increased plasma levels of ACTH in CFA rats.”
\textbf{It has been revised.}
2. Figure 1 axis should be “-points”
   It has been revised.

3. Page 10: “However, paw thickness in EA-treated rats plus ACTH (11-24) was no different from that in sham EA rats plus vehicle” should be “However, paw thickness in EA-treated rats plus ACTH (11-24) was not different from that in sham EA rats plus vehicle.”
   It has been corrected.

Sincerely yours,

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