Author's response to reviews

Title: Involvement of spinal orexin A in the electroacupuncture analgesia in a rat model of post-laparotomy pain

Authors:

Xiao-Ming Feng (fengtcm@126.com)
Wen-Li Mi (wenlimi@fudan.edu.cn)
Fang Xia (seamorning1@sina.com)
Jian-Wei Jiang (coolsunny@126.com)
Yan-Qing Wang (wangyangqing@shmu.edu.cn)
Gen-Cheng Wu (gcwu@shmu.edu.cn)
Qi-Liang Mao-Ying (maoyql@fudan.edu.cn)
Sheng Xiao (meyooo@sina.com)
Zhi-Fu Wang (wzf993002@163.com)

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Author's response to reviews: see over
Dear Dr Rowles,

Re: MS: 1037411550748040

Thank you for arranging the timely review of our manuscript titled "Involvement of spinal orexin A in electroacupuncture analgesia in a rat model of post-laparotomy pain" and for kindly approving the delay of our revisions.

We have carefully considered the critical comments and constructive suggestions, and have responded to these suggestions point-by-point. We have also performed some supplemental experiments and have revised the manuscript accordingly. In addition, we have used the Edanz language editing service to help us improve the readability of our manuscript.

Thank you very much for your consideration, and we thank the editors and reviewers for their careful review and valuable comments. We hope you will accept our revised manuscript for publication in BMC Complementary & Alternative Medicine.

Yours sincerely,

Gen-Cheng Wu, M.D.
Professor of Neurobiology
Chairman, Department of Integrative Medicine and Neurobiology
Director, Institute of Acupuncture Research
Director, WHO Collaborating Center for Traditional Medicine
Shanghai Medical College, Fudan University, P.O. Box 291
138 Yi Xue Yuan Road, Shanghai 200032, China
Tel: +86-21-5423-7526
Fax: +86-21-5423-7526
E-mail: gcwu@shmu.edu.cn
Response to reviewer’s comments:

**Editor’s comments**

**Point 1:** Please include more information on the context of your study in the background section of your manuscript.

**Response:** Thank you for the suggestion. We have rewritten the background section.

**Point 2:** Please include the email addresses of all authors on the title page of your manuscript.

**Response:** The email addresses of all authors have been added to the title page.

**Point 3:** We recommend that you ask a native English speaking colleague to help you copyedit the paper.

**Response:** Thank you for your suggestion. We have asked Edanz language editing service to help us with copyediting of our manuscript.

**Reviewer #1**

**Point 1:** Experimental groups and protocols have not been clearly described in the section of Methods.

**Response:** Thank you for bringing this to our attention. Experimental groups have been added to the Methods section (section 2.8).

**Point 2:** It is not appropriate to address the purpose of the experimental protocols in the Results.

**Response:** We have removed all descriptions of purpose of experimental protocols from the results section.

**Point 3:** It is unclear how EA was performed at 2/15Hz or 2/100Hz. Were there any differences in experimental results if EA was performed at 2, 15 or 100 Hz?

**Response:** Thank you for this question. EA was applied using a HANS Acupoint Nerve Stimulator (LH202H, Beijing, China). 2/15 or 2/100 Hz was the density wave, a type of wave that was composed of two alternative frequencies. In this regard, 2/15 Hz was an alternative frequency of 2 Hz and 15 Hz. Experimental studies have shown that different frequencies can cause the brain and spinal cord to release different types of opioid peptides, which may have different effects [1]. EA stimulation at 2 Hz accelerates the release of endorphins in the brain and large amounts of enkephalin in the spinal cord [2-4], while 15 Hz EA stimulation causes the release of endorphins, beta-endorphin, endomorphin and dynorphin, and 100 Hz EA stimulation causes the spinal cord to release dynorphin [5-9].
Thus, 2/15 Hz EA stimulation may accelerate the release of many different endogenous opioid peptides [10], each of which strengthens the effect of the others. Therefore, this frequency of EA can produce a strong analgesic effect. In the present study, we can infer from the results that 15 Hz accounts for more OXA level changes than 100 Hz.

References:
1. Han JS, Ding XZ, Fan SG: [Frequency as the cardinal determinant for electroacupuncture analgesia to be reversed by opioid antagonists]. Sheng Li Xue Bao 1986, 38(5):475-482.
5. Chen XH, Han JS: Analgesia induced by electroacupuncture of different frequencies is mediated by different types of opioid receptors: another cross-tolerance study. Behav Brain Res 1992, 47(2):143-149.

Point 4: The quality of figures needs to be improved substantially. In this regard, for instance, figure 2 is too small, most patterns of bars that indicate different groups in figure 3 and 4 are similar, and graphs demonstrating data at "05 and 1" are put together in figure 3 and 4. As such, the figures appear to be confusing, and the data presented in those figures are very difficult to be evaluated and understood.

Response: We apologize for the figure quality and composition. The figures have been adjusted according to your suggestion.

Reviewer #2
**Point 1:** This manuscript requires significant work in terms of grammar and mechanics use. I strongly encourage the authors to go through the manuscript again with a fine-comb to assess errors in grammar and mechanics use.

**Response:** Thank you for the suggestion. We have asked Edanz language editing service to help us with this issue.

**Point 2:** Introduction: The third paragraph in page 4 should be moved to the Method section.

**Response:** Thank you for this suggestion. We have adjusted the text accordingly.

**Point 3:** Accurate intrathecal injection to L6 should be confirmed with dye. The authors should mention if the intrathecally administered OXA agonist will circulate to the higher regions (i.e. brain).

**Response:** Thank you for your suggestion. We had injected Evans blue intrathecally before our experiments to confirm accurate injection. We have also performed a supplement experiment to monitor drug circulation 1 h after injection. We have added the protocol and results here. Based on previous studies where 2-3% (w/v) Evans blue was used as an intrathecal or intravenous injection dye [1-3], Evans blue (2 % (w/v); diluted in normal saline) was intrathecally injected at L6. The volume used (10 μL) was in accordance with our following experiments. The intrathecal injection method is described in the methods in the manuscript. Rats were then sacrificed at 5 min (n=2) or 1 h (n=2). Cervical laminectomy and suboccipital craniotomy were performed using blunt dissection techniques. Five minutes after injection, the dye was observed to have diffused to the lumbar enlargement (Fig 1 A, C left: bold arrows indicate the injection spot). One hour after injection, Evans blue could be seen throughout the spinal cord (Fig 1 B), superior cerebellar pool (Fig 1 D), and midbrain aqueduct (Fig 1 C right), but it was difficult to detect in the lateral ventricle and third ventricle.

References:

Point 4: The OXA concentration in the hypothalamus, PAG and spinal cord regions should be quantified by microdialysis technique and ELISA or by using mRNA expression.

Response:
Thank you for your suggestion. We performed qPCR to monitor OXA mRNA expression in the hypothalamus only.

Orexin A/B (OXA/B, hypocretin, hcrt) is a protein encoded gene located at 10q32.1. According to “Expression for HCRT gene” verified by northern blotting in http://www.genecards.org/cgi-bin/carddisp.pl?gene=HCRT&search=hcrt, hcrt mRNA is expressed only in the brain. OXA/B is produced through hydrolysis of the same precursor, Prepro-Orexin (PPO), by the proteolytic enzyme during the transport process [1, 2]. It is also reported that PPO/hcrt mRNA was produced exclusively in the lateral and perifornical areas of the posterior hypothalamus [3]. Therefore, we only tested hcrt mRNA expression in the hypothalamus at 1 h post-laparotomy or following EA application by qPCR.

Methods
Sample preparation and RNA extraction
Rats in the normal, model and EA 2/15 Hz groups were sacrificed 1 h after surgery or following EA stimulation. Samples were collected from the hypothalamus. Brain regions were dissected according to Watson and Paxinos. Each sample was homogenized in TRIZol™ reagent (Invitrogen, Carlsbad, CA, USA) and RNA was extracted according to the TRIZOL RNA Isolation Protocol (Invitrogen™, Life Technologies). RNA concentration and purity was assessed in duplicate samples using a NanoDrop™ ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA).

cDNA Synthesis and qPCR
RNA was then converted to cDNA using the M-MLV Reverse Transcriptase Protocol (Promega, Madison, WI, USA). Reactions were incubated at 70°C for 5 min, followed by 37°C for 1 h and final denaturation at 95°C for 5 min. Samples were subsequently diluted to 20 μL in RNase-free water and stored at -20°C. The expression of each hcrt gene was analyzed by qPCR using iQ™ SYBR® Green Supermix and the Bio-Rad iCycler iQ5 PCR Thermal Cycler (Bio-Rad, CA, USA). All reactions were performed in 25 μL reactions, in triplicate within the same PCR run. The most appropriate Tm of hcrt and β-actin was determined using the temperature gradient test. A temperature of 61°C was selected. PCR amplification efficiency was measured by serial dilution and PCR (Fig. 1). For each well 1.5 μL of cDNA from each sample was added to 23.5 μL of PCR reaction mix, which consisted of 12.5 μL of 2xSYBR® Green Supermix, 9 μL RNase free water and 2 μL of 1× gene expression assay primer-probe mix (Sangon, Shanghai, China). The PCR reactions were initiated with a 5-min incubation at 95°C, followed by 40 cycles of 95°C for 10 s, 61°C for 30 s and 72°C for 30 s, in accordance with the manufacturer’s recommendations.

The primers for hcrt and β-actin were:

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’-3’)</th>
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<tbody>
<tr>
<td>Hcrt_F1</td>
<td>tctgccccgtctcgaact</td>
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</table>
**Result:**
Between group differences were calculated using the LSD test. A $P < 0.05$ was regarded as a significant difference. Hcrt mRNA expression in the hypothalamus was significantly increased at 1 h post-laparotomy when compared with the normal group ($P < 0.05$). There was no difference between the model group and the EA 2/15 Hz group.

**Discussion**
Because of the report that hcrt mRNA is only expressed in the lateral and perifornical areas of the posterior hypothalamus [3], we measured hcrt mRNA levels in the hypothalamus. Results showed that hcrt mRNA increased significantly in the hypothalamus 1 h after surgery (Fig. 3), which indicates a demand for orexins after surgery. In our manuscript, we have shown that OXA levels in the hypothalamus, PAG and spinal cord decreased at 1 h in the model group. This may be because laparotomy inhibited the hydrolysis process of PPO to OXA. The decrease of OXA in the above areas may act as a negative feedback mechanism, which requires the hypothalamus to produce more hcrt. EA did not significantly regulate hcrt mRNA in the hypothalamus (Fig. 3), but increased OXA levels in the hypothalamus, PAG and spinal cord, which may suggest that the role of EA is to stimulate and accelerate the hydrolysis process and produce more orexins. This is most likely a fast cell reaction independent of gene regulation.

**References:**
PCR Amplification Efficiency. Amplification efficiencies for each hcrt or β-actin gene assay were calculated using the following formula: $E = \left(5 - \frac{1}{\text{slope}} - 1\right) \times 100$, using the slope of the plot of Ct versus log input of cDNA (5-fold dilution series). A threshold of 10% above and below 100% efficiency was applied. PCR amplification efficiency for each candidate gene is shown in Fig. 1A, B (hcrt) and C, D (β-actin).
Relative expression of hcrt mRNA in the rat hypothalamus. Relative mRNA expression was determined using the $2^{-\Delta\Delta C_T}$ method. Data are represented as the mean ± SEM. *$p < 0.05$ vs Normal group by LSD test.