**Author's response to reviews**

**Title:** Expression levels of the hypothalamic AMPK gene determine the responsiveness of the rats to electroacupuncture-induced analgesia

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**Author's response to reviews:** see over
Dear Editors in *BMC Complementary & Alternative Medicine*,

Thank you very much for giving us the opportunity to revise our manuscript to be published in *BMC Complementary & Alternative Medicine*. We revised our manuscript according to your suggestion and reviewers' helpful comments. All changes in this revision are highlighted (blue text) in the revised manuscript. The detailed responses to reviewers’ comments are shown below.

**Reviewer #1**
Reviewer's report:
1] Background#Line 3#“stimulation to enhance the analgesic effects of acupuncture; Do you think that the analgesic effect of EAS is superior to that of manual acupuncture in clinical practice? Could you provide enough evidence (both clinical and experimental) for verifying this point?

[Response] According to the reviewer’s comment, the two references for verifying this issue (one is clinical [Schliessbach et al., 2011, *Pain Med*] and the other [Kim et al., 2005, *Exp Neurol*] is experimental) were cited in this revision.


2] Discussion: only 586 words’ discussion seems too simple, without elucidating the limitation of results of the present study: including 1) only 4 animals in each group, 2) experiments conducted in normal rats rather than in pathological pain model animal. 3) Since AMPAK is an intracellular proteinase, it involves several signaling transduction pathways and not merely the ‘energy balance’?? The present manuscript should give the readers much more information.

[Response] According to this (#1) and other (#3) reviewers’ comments, 1) we increased the number of animals (n=8/group) in this revised manuscript. 2) We also added the following sentences in the Discussion: “It would be interesting to examine the analgesic effects of EA on pathological pain, such as neuropathic pain [Kim et al., 2013, *eCAM*], the mechanism of
which is somewhat different from acute pain (e.g. TFL test). Although the individual differences in the sensitivity of acute nociceptive and chronic neuropathic pain to EA were known to be maintained in rats [Kim et al., 2007, Brain Res Bull], we believe that studies using pathological pain models could provide a better understanding of EA-induced analgesia and its responsiveness.”

3) In this revision, we added more information in the Discussion as follows: “Several studies also suggested that AMPK activation plays a significant role in important neuronal processes, including the regulation of neuronal plasticity and long-term potentiation, and the protection of neurons from neurodegenerative diseases [31]”…“It is currently unclear how the hypothalamic AMPK plays a role in EA-induced analgesia as shown in this study. One possible explanation is that AMPK might regulate EA analgesia-related neuropeptides that released in the hypothalamus. AMPK activation in the hypothalamus is positively correlated with neuropeptide Y (NPY) expressions [33] and this hypothalamic NPY has a significant antinociceptive effect [34]. Interestingly, several reports demonstrated that acupuncture or EA stimulation at ST36 decreases NPY levels in the hypothalamus [35, 36]. Thus, we cautiously assumed that the responder rats with high AMPK levels, but not non-responders, might maintain sufficient NPY levels in the hypothalamus to be involved in antinociception, although EA stimulation decreased NPY expressions. In addition to this, further studies to explore the relationship between the AMPK and beta-endorphin in the hypothalamus, a well-known EA analgesia mediator, are required.”

3] Since the standards for responder and non-responder were set to >30% and <20%, do you think that statistical analysis of the TEL is necessary? It seems that such statistical analysis may violate the basic principles of statistics.

[Response] We agree with the reviewer’s opinion. Actually we did the statistical analysis of the TFLs between the responder and non-responder rats, because such analysis was asked in our previously published papers by some referees. In this revision, we deleted such graph (Figure 2 in the previous version of the manuscript) and related descriptions in the Results.

4] On page 10 “Effects of adenoviral gene transfer of AMPK into the hypothalamus on EA induced analgesia” In the sentence: “There were no significant differences in TFL between the WT virus-injected and DN virus injected rats during 2-week experimental period”. Please complete the related data and Figure ?, P >0.05 labeling ?.

[Response] According to the reviewer’s advice, we showed the related data in Figure 3 of the revised manuscript.
5. Incorrect language expression: Figure 1 (A): Representative photograph of the Nissl staining showing the injection position (X40) not “the microinjection of 2 µl viral suspension”. 40x; please use an arrow-head to mark the injection site validated after your experiment.

[Response] Thanks to the reviewer’s kind comment, we replaced the incorrect expression in Figure 1 legend by the correct phrase as the reviewer suggested. In addition, we marked the injection site in Figure 1 by using an arrowhead.

- Minor Essential Revisions
  1. English language error words:
     a) Page 8: in the line 10 to the bottom of page 8: the rat’s head were fixed.
     b) Page 9: Statistical analysis and graphic works were, is the ‘works’ right? …
     c) Page 10: stimulation at -1d, 3d, 7d and 14d ……: the word “at” should be instead of “on the 1st, 3rd, 7th and 14th day, or on day 1, 3, 7 and 14” at 14 days?
     d) Page 10: “Comparison of the TFL increase ratio “show” a significant” …should be “shows” …

[Response] Thanks to the reviewer’s kind comments, we corrected all the English errors in this revision as the reviewer suggested.

2] In Figure 1, it would be much better if the authors draw a pane on photo A to mark the range of GFP fluorescence in the ARC in photo B.

[Response] According to the reviewer’s suggestion, we indicated the ARC region in Figure 1.

Reviewer #2
Reviewer’s report:
Minor Essential Revisions
1. In materials and methods, a pair of needles was used to stimulate ST36. Clarify how the needles were electrically stimulated at ST36 acupoint, i.e., positive electrode in left ST36, and which depth was inserted.

[Response] According to the reviewer’s comment, we described the method of EA stimulation in more detail in this revised manuscript as follows;

“For EA stimulation, a pair of stainless steel acupuncture needles (0.25 mm in diameter and 3 cm long) was inserted (5 mm in depth) into the “Zusanli” acupoint (ST36), ~”

“An electrical stimulator was connected to the two acupuncture needles (cathode to ST36 and
2. In discussion, authors need to describe how acupuncture produces more analgesic effects in the rat with higher AMPK than the rats expressing lower AMPK.
3. In discussion, since Ref #17, 18, 30 show AMPK activation in peripheral & spinal cord, they do not support your results that AMPK expression in hypothalamus plays role in EA-analgesia. It should be clarified how expression of AMPK in hypothalamus correlates with thermal pain.

[Response] In this revision, we addressed these two comments by adding several sentences in the Discussion as follows;

“It is currently unclear how the hypothalamic AMPK plays a role in EA-induced analgesia as shown in this study. One possible explanation is that AMPK might regulate EA analgesia-related neuropeptides that released in the hypothalamus. AMPK activation in the hypothalamus is positively correlated with neuropeptide Y (NPY) expressions [33] and this hypothalamic NPY has a significant antinociceptive effect [34]. Interestingly, several reports demonstrated that acupuncture or EA stimulation at ST36 decreases NPY levels in the hypothalamus [35, 36]. Thus, we cautiously assumed that the responder rats with high AMPK levels, but not non-responders, might maintain sufficient NPY levels in the hypothalamus to be involved in antinociception, although EA stimulation decreased NPY expressions. In addition to this, further studies to explore the relationship between the AMPK and beta-endorphin in the hypothalamus, a well-known EA analgesia mediator, are required.”

Reviewer #3
Reviewer's report:
- Major Compulsory Revisions

1 Methods, Acute thermal pain behavior, rats were divided into three types by TFL change. Authors defined the rats which TFL increase after EA stimulation was greater than 30% as responders, and less than 20% as non-responders. But it is not clearly what is the type of the rats TLF increase between 20% and 30% and why the subjects were discarded.

[Response] We determined three successive TFL before and after EA stimulation. The averaged values became the pre-EA TFL and post-EA TFL, respectively. Empirically, the rats showing TFL increase between 20% and 30% are ambiguous for statistical significance depending on the variation of raw data. In order to use more clear “responders” and “non-
responders” for further experiments and analyses, we have discarded those rats in previously published papers and the current manuscript. We briefly described this in the Methods of the revised manuscript as follows;

“Since the other subjects (20-30% TFL increase after EA) are ambiguous for a clear classification, those rats were discarded [15].”

2 Same part, the testing of TFL should be corrected. The thermal stimulation should be given by a definite intensity, and 1-min interval time was too short to eliminate the pain memory of rat.

[Response] Once the intensity of the light bulb was set such that the basal TFL was 3.0±0.5 sec in pre-test period, we used the fixed intensity for TFL test in each rats over the whole experimental periods. In order to make the readers not confusing, we replaced “baseline TFL” by “pre-EA TFL” and described more in the Methods of the revised manuscript as follows;

“For TFL test, the intensity of the light bulb was set such that the baseline reaction time was 3.0 ± 0.5 sec during the pre-test period. In the experimental period, three successive determinations of TFL using the same intensity of the light bulb that had been determined during the pre-test period were conducted at 1-min intervals with a cut-off time of 15 sec, and these values were averaged (pre-EA TFL). For EA stimulation, a pair of stainless steel acupuncture needles (0.25 mm in diameter and 3 cm long) was inserted (5 mm in depth) into the “Zusanli” acupoint (ST36), which is located in the anterior tibial muscle, 5 mm lateral and distal to the anterior tubercle of the tibia, and into the point 5 mm distal from the first needle. EA stimulation at this point is known to produce analgesia in rats [3, 8]. An electrical stimulator was connected to the two acupuncture needles (cathode to ST36 and anode to the other point), and train-pulses (2 Hz, 0.5 ms pulse duration, 0.2-0.3 mA) were then applied for 20 minutes. The average of three successive TFL determinations (post-EA TFL) was then recorded. The analgesic effects are expressed as percent changes from the pre-EA TFL.”

\[
\text{Acquired TFL change (\%)} = \frac{\text{Post-EA TFL} - \text{Pre-EA TFL}}{\text{Pre-EA TFL}} \times 100
\]

In addition, we did not stimulate the same spot of the rat tail, but stimulated the different spots (at least 1 cm apart from the other spots) every session of the total 3 test sessions. This
method has not induced sensitization or pain memory in our previous studies and the current manuscript.

3 It is not clear when the EA stimulation was given.

[Response] Every experimental day, pre-EA TFL value was first determined and then EA stimulation was performed. Thereafter, post-EA TFL value was determined. This was described more clearly in this revision. Please see the above response to the reviewer’s 2nd comment.

4 The description about the method of real-time PCR needs to be more detailed, some important information, such as amplification efficiency, standard curve, multiple poles and computing method are lacking.

[Response] According to the reviewer’s advice, we amended the method of real-time PCR in detail in the Methods of this revision as shown below. We performed real-time PCR using a LightCycler 480 and analyzed the data automatically with a relative quantification mode based on crossing point (Cp) values. Thus, a standard curve is not required in a relative quantification mode. We also checked the specificity of the amplified PCR product by performing a melting curve analysis.

The real-time PCR was conducted by a LightCycler 480 (Roche Applied Science, Indianapolis, IN) employing SYBR Green I as the dsDNA-specific binding dye for continuous fluorescence monitoring. The PCR protocol comprised 10 min at 95°C; 45 cycles of 10s at 95 ºC, 10s at 60ºC and 10s at 72ºC. After the cycles were finished, the signal of each temperature between 65 and 95 ºC was also detected to generate a dissociation curve. The sequences of the human primers were AMPK (forward 5’-tgaagccagagaacgttg-3’, reverse 5’- ataatttggcgatccacagc -3’) and GAPDH (forward 5’-tgccactcagaagactgtgg-3’, reverse 5’-ttcagctctgggatgacctt-3’). The mRNA levels of AMPK were compared by calculating the crossing point (Cp) value and normalized by the reference genes (GAPDH) using the LightCycler 480 Relative Quantification software (Roche).

5 The number of animals in each group need to be more. Now the data was lacking of convincing.

6 In Figure 4, the significant difference was only showed on post-14d. It is not supported the
[Response] In this revision, we addressed these two comments by increasing the number of animals (n=8/group). We made a new graph (Figure 4) showing the significant difference on post-7d and post-14d.

We also added 3 researchers (Heera Yoon, Ji Hwan Lee, Fu Shi Quan) as co-authors in this revision, because they contributed to the additional experiments and analyses. Each author has seen and approved the contents of the submitted manuscript. We declare that we have no competing interests.

We hope that this revised article fulfills all your suggestions and reviewers’ comments.

Yours sincerely,

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