Author’s response to reviews

Title: TENS Attenuates CFA-induced Hyperalgesia and Inhibits Spinal ERK1/2-COX-2 Pathway Activation in Rats

Authors:

Junfan Fang (fangjunfan0223@163.com)
Yi Liang (liangyiwww@126.com)
Junying Du (dujunying0706@163.com)
Jianqiao Fang (fangjianqiao7532@163.com)

Version: 3 Date: 8 April 2013

Author’s response to reviews: see over
COVER LETTER

April 8, 2013

Dear Dr. Tom Rowles

Thank you very much for your hard-work in processing our manuscript entitled “TENS Attenuates CFA-induced Hyperalgesia and Inhibits Spinal ERK1/2-COX-2 Pathway Activation in Rats” (MS Number: 2167240758853412)

We are very pleased to be asked to submit a revision, and we found the comments and suggestions of the reviewers to be extremely helpful. New experiments had been supplemented and some grammar and spelling errors in the original manuscript had also been corrected. Furthermore, the relevant regulations had been made in the original manuscript according to the comments of reviewers, and the major revised portions were marked in red. We also responded point by point to each reviewer comments as list below, along with a clear indication of the location of the revision.

We believe their comments and suggestions have significantly improved the quality of manuscript and made it publishable.

Thanks again for your reconsideration of our manuscript for publication in your journal. We look forward to your favorable decision.

Yours sincerely

Jianqiao Fang.
RESPONSE TO REVIEWERS’ COMMENTS

MS Number: 2167240758853412 Title: TENS Attenuates CFA-induced Hyperalgesia and Inhibits Spinal ERK1/2-COX-2 Pathway Activation in Rats

Thank reviewers for constructive suggestions and comments. Our responses are below:

Comments from Reviewer 1 (Jean-Xavier Mazoit):

1. This study shows that TENS decreases ERK phosphorylation and COX-2 stimulation in CFA injected rats. RT-PCR (COX-2 expression) and PGE_2 measurement (ELISA or WB) are lacking. Nevertheless, the message is interesting.

Response: It is really an interesting discussion and a grateful suggestion. Real-time PCR for COX-2 mRNA and ELISA for PGE_2 protein have been supplemented in our study now. The methods and results of real-time PCR and ELISA is described in the Methods (page 9-10) and Results (page 13) section in our paper. In brief, the expression of COX-2 mRNA was increased both at 5 h and 6 h after CFA injection at the ipsilateral side of SCDH. However, PGE_2 levels in the SCDH of model group were only significantly increased at 6 h after CFA injection. TENS decreased the both COX-2 mRNA expression and PGE_2 level after once administration. We also discussed these new results. Since p-ERK1/2, COX-2 mRNA and protein were sequential up-regulation in SCDH by peripheral inflammation, we believed that
ERK1/2-COX-2 pathway contributed to the inflammatory pain hypersensitivity in SCIDH. Furthermore, besides p-ERK1/2 and COX-2, TENS also inhibited the high secretion of PGE2 in SCIDH after once administration. Thus, TENS may attenuate CFA-induced hyperalgesia by inhibiting spinal ERK1/2-COX-2 pathway activation in rats.

2. English needs to be corrected by a native speaker. Many abbreviations have been defined only in the abstract, not in the text.

Response: To be exact, indeed, as you mentioned, the English expression needs modification. Some grammar and spelling errors had also been corrected. All abbreviations used in the manuscript have also been defined in the Background or the first place where it appeared in the text. However, I still believe that English needs further correction.

3. The number of animals in each group needs to be more detailed.

Response: As for this comment, the number of animals that used in western blot, real-time PCR, ELISA and behavioral testing were label in the Figures section. We also changed the description of the number of animals in each group in Methods section (page 6). In total 90 adult Male Sprague-Dawley rats were used in this study. They were randomly divided into three groups: (a) the control group with saline injection (n=14); (b) the model group with CFA injection (n=38); (c) the TENS group with CFA injection and TENS treatment (n=38). Rats in each group were respectively
used for western blot, real-time PCR, ELISA. Among those rats, 10 rats in each group were randomly chosen for testing the PWTs and paw volumes.

4. Statistics. The two way-ANOVA is OK. The one-way ANOVA is not needed. In addition, the between groups comparison by one-way factorial ANOVA needs correction for multiple comparison. In fact, when the two-way ANOVA is significant with interaction, one perform a one-way ANOVA for repeated measure to test the evolution with time in each group (the authors did such an analysis), but it is not necessary to perform additional testing, time by time because of the risk of type II error.

Response: Thanks for your comments. For none different between model and TENS groups was indicated by the two way-ANOVA, we removed the one-way ANOVA test to the paw volumes of rats in each group. However, as we wrote in our manuscript, LSD tests indicated a significant difference between the model and TENS groups. So we used one-way ANOVA for PWTs. It may be our inaccurate description resulted in the misunderstanding. Only the PWTs at the same time point was compared by the one-way ANOVA test. In another word, we individually compared the PWTs of each groups at four different time points, before modeling, and 5 h, 6 h, 25 h after CFA injection by one-way ANOVA test. Thanks again for you helping our statistics. We have changed the description of results at page 11.
5. In the first paragraph of the results, the last sentence is difficult to understand \((p > 0.05 \text{ or } p < 0.05)\).

**Response:** Thanks for your careful reading and reviewing. We have changed the "\(p < 0.05\)" to "\(p > 0.05\)."

6. It is not clear when TENS was applied after CFA administration.

**Response:** Thanks for your reviewing. TENS was applied at 5 h and 24 h after CFA administration. The description of TENS stimulation time was changed, the last sentence of the TENS administration part in Methods section (page 8).

7. Last page of the results. Inhibition of COX-2. The authors did not test the expression of COX2 (by measuring the RNA), but measured the concentration of the enzyme.

**Response:** Thanks for your proposal. The expression of COX-2 mRNA is detected by real-time PCR in our study. The expression of COX-2 mRNA was increased both at 5 h and 6 h after CFA injection in the ipsilateral side of SCDH. TENS decreased the COX-2 mRNA expression in the ipsilateral side of SCDH at 6 h after injection (Fig. 3 A). TENS did inhibit COX-2 mRNA expression in SCDH after once administration.

8. Next page, line 5. The authors speculate on transcriptional regulation. They did not measure RNA, but only the protein.

**Response:** Thanks for your proposal. As the comment, only measuring the protein is
not enough to speculate on transcriptional regulation. So the expression of COX-2 mRNA is detected by real-time PCR in our supplemental study. The expression of COX-2 mRNA was increased both at 5 h and 6 h after CFA injection in the ipsilateral side of SCDH (Fig.3 A). TENS decreased the COX-2 mRNA expression in the ipsilateral side of SCDH at 6 h after injection. So we believed that ERK1/2 activation regulated COX-2 expression via transcriptional regulation and TENS produce analgesic effect on CFA rat by inhibiting ERK1/2-COX-2 pathway activation.

9. *It is not possible to cite a paper "to be published".*

**Response:** The paper has been published now. It is the No.17 reference in this report.

10. *The references section is very long (51 references).*

**Response:** References cited in the paper were carefully chosen. The references section is shorter than the original manuscript (44 references)
Comments from Reviewer 2 (zhao jun):

The evidence is not strong enough to support their findings. Fig. 1, Fig. 2 and Fig. 3 are not complete to show all the time points. The data from 6 h to 25 h after CFA injection in model and TENS groups should be supplemented.

Response: Thanks for your carefully reviewing and grateful suggestion. To be exact, indeed, as you mentioned, there is no strong evidence supporting the notion that ERK1/2 involves in the central pain mechanisms of TENS analgesia for inflammatory pain. So we supplemented the effect of TENS on the expression of COX-2 mRNA and PGE2 protein in SCDH in CFA rat models. The results showed that the expression of COX-2 mRNA was increased both at 5 h and 6 h after CFA injection at the ipsilateral side of SCDH. However, PGE$_2$ levels in the SCDH of model group were only significantly increased at 6 h after CFA injection. TENS both decreased the COX-2 mRNA and PGE$_2$ level after once administration.

For two reasons that we did not detect the COX-2 mRNA and protein expression at 25h in present study: firstly, we focused on the contribution of TENS to regulate the activation of ERK1/2 pathway in SCDH, and we had found that p-ERK1/2 were high expressed at 5 h and 6 h after CFA injection and recovered at 25 h after CFA injection; secondly, TENS only regulated p-ERK1/2 expression at 6 h, but not 25 h after modeling. Thus, it is less interesting to detect the downstream substance of ERK1/2, for instance COX-2, at 25 h after CFA injection. On the other hand, the expression of COX-2 mRNA in SCDH from 6 h to 49 h after CFA injection was carefully measured.
in our another experiment. The results indicated that COX-2 mRNA increased at 6 h, and declined to normal level in SCDH at 25 h after CFA injection. (Because the results were used in another paper which has been submitted, we couldn’t provide the data. We are very sorry for that). Thanks again for you helping us promote the quality of our study.