Author’s response to reviews

Title: Comparison of electroencephalographic changes in response to acute electrical and thermal stimuli with the tail flick and hot plate test in rats administered with opiorphin

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Reviewer reports:

Catherine Rougeot (Reviewer 1): REVIEWER COMMENTS:

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- Comparison of electroencephalographic changes in response to acute electrical and thermal painful stimuli with the tail flick and hot plate tests in rats administered with opiorphin -

by Preet Singh et al.
In this submission the authors look at the activity of systemically administered opiorphin, in comparison to morphine, by recording electroencephalogram (EEG) in anesthetized female rats subjected to noxious thermal and electrical stimuli. The interesting finding of this article lies to the fact that the authors demonstrated that median and spectral edge frequencies of EEG, which are indicators of nociception, were not affected by acute pain stimuli after IV administration of 2 mg/kg opiorphin, on the contrary to control-saline treated-animals. Moreover the anti-nociceptive effect of opiorphin, demonstrated in EEG responses, was reversed in the presence of the non-specific opioid receptor antagonist, naloxone. These data, suggesting a direct ad/or indirect specific effect of opiorphin on central nociceptive mechanisms, constitute an original finding which is important to bring to the attention of the scientific community.

In parallel, authors show that systemic administration of opiorphin attenuates the nociceptive response to spinally controlled thermally-induced acute pain in the tail flick model; these data have been already supported by other observations and publications (Tian XZ et al Peptides 2009; Rougeot et al J Physiol Pharmacol, 2010 in male mouse and rat models, respectively). Furthermore they use a different kind of thermal painful stimulus, the supraspinally-organized hot plate test in rat; in this standard behavioral pain model no analgesic opiorphin effect at 2mg/Kg IV was observed.

All together these results strongly suggest that the analgesic effect of opiorphin in acute pain tests mainly occurs at spinal level.

Since its discovery in 2006, human opiorphin molecular and functional characterization as well as mechanism of action have been described a number of times and found to be analgesic acting by increasing the levels of endogenous opioid peptides that results in activation of endogenous mu and delta-opioid pathways. Recent publication shows that the anti-hyperalgesic effect of STR-324, a stable analog of opiorphin, in the postoperative long-acting pain model is linked to its ability to reduce c-fos expression in the spinal nociceptive pathways (STR-324, a stable analog of opiorphin, causes analgesia in postoperative pain by activating endogenous opioid receptor-dependent pathways. Sitbon et al. Anesthesiology 2016, V 125, N°5). Thus, the present
study conducted by Preet Singh et al. strengthens previous observations using others tools or models.

In order to add clarity and precision and thus to improve the quality of the manuscript a number of following suggestions are proposed.

The manuscript must be more carefully referenced. As example, the particular data related to opiorphin QRFSR-peptide mechanism of action (line 53, page 3) have to be also referenced, with the Ref. 3 (Wisner et al, PNAS, 2006).

- Line 55, page 3, the sentence has to be also referenced with Ref. 1, in addition to Ref. 4.

- Line 60, page 3, authors have to add the following reference " Bogeas, A et al. Structure activity relationship study and function-based peptidomimetic design of human opiorphin with improved bioavailability property and unaltered analgesic activity. Biochem. Pharmol., 2013, 2: issue 3, 1000122, 1-11."

- Page 11, line 258 authors have to add Ref. 2 in addition to Ref. 5.

Introduction section:

Page 3

- Line 54 - add the following detail in bold: "endogenous opioid peptides"

- Line 63 - authors have to suppress "and" in the following sentence: "chemically-induced inflammation causing chronic pain and the formalin test"

- Line 64 - specify "… in chronic peripheral pain. "


- Line 66 - for clarity it is necessary to precise in bold "The hot plate test, that is a supraspinally controlled thermal acute pain test, has less ethical cost "

Results:

Page 9

- Chapter "EEG responses" 2nd paragraph: For clarity it is necessary to identify the Figure 6 in the text for the spectral edge frequency (F95) analyses (for example Line 217 at the end of the sentence);

Discussion:

Page 11

- Last paragraph, lines 266-270: for better clarify and for linearity in the reported discussion, may be the authors have to move the sentence " Wisner et al. … injected rats ", to line 263 after the first sentence related to supra-spinal analgesic models

- Page 12

- Line 272: it is important to specify in bold " endogenous opioid peptides, in particular enkephalins "

- Line 275: for clarity "The present report was the only study that used …"
Figure legends:

- Figure 3: sec instead of s

- Figure 5-6-7: It would be preferably to stipulate the following detail in bold "Least square means ....stimuli in anesthetized rats injected with ..."

Figures:

- Figure 7 must be rearranged, at the level of X axis legend which is not readable: may be use legend similar to that in figure 5 and 6

ANS: We agree with all the suggestions by reviewer 1, and have emended the manuscript accordingly.

Bernard Roques, Professor Emeritus (Reviewer 2): The aim of this study was to use electroencephalographic data as internal objective demonstration of analgesic responses to different types of pain in particular central pain elicited by the not plate test in rat.

Morphine was used as reference in all cases. Another more will recommend method to measure the relationship between a response to a pain stimulus and the produced antinociceptive effects was proposed by J.C. Willer (Benoist J.M. et al Eur. J. Pharmacol., 2002, 445(3), 201-210.) and this method is more relevant than the EEG use and therefore must be cited in the paper.
ANS: Other objective (experimental) methods such as measuring the changes in electromyographic reflex thresholds (Benoist et al.2002) and expression of the neural marker of nociception, c-fos (Harris, 1998; Le Guen et al.2002) may further delineate the neural basis of the antinociceptive effects of opiorphin.(Added in discussion).

Another disadvantage of this EEG method in the case of opiorphin is the population of rats which is reduced strictly to female due to priapism caused by the peptide to male rat ! Ref to this observation (n°15) is incomplete !

ANS: We have corrected the reference. This is not a disadvantage of the method (EEG) but of the drug (Opiorphin). The University ethics committee was worried about priapism due to administration of Opiorphin in male rats based on the findings in the Ref provided.

The introduction suffers greatly from clarity. This dual inhibition of NEP and APN two enkephalinases involved in enkephalins inactivation was proposed by Roques et al. well-cited in reference 1 but which must be also cited in place of reference 4.

ANS: Corrected

According to the unclear results reported in the paper (see further comments), it could be more interesting to measure the expression of cFos, a biomarker currently used to evaluate the potency of various compounds, including dual enkephalinase inhibition, to counteract the nociception (see Le Guen S. et al. Eur. J. Pharmacol., 2002, 441(3), 141-150).

ANS: We agree it would be interesting to measure changes in expression of cFos, but this is a very indirect measure of antinociception.
Results.

A - Pharmacology.

- Figure 3 shows clearly that the responses in the TF test are more impressive for morphine than for opiorphin only active at 5 min. This could be more valuable if this was expressed in % of max effect as usual.

ANS: We do not know the maximum possible effect since we applied a cut off to avoid tissue damage.

- The reversion by naloxone was rightly observed for both MO and OP.

- Figure 4 shows the absence of effect of OP in contrast to MO. Curiously if we look at the effects it seems that as compared to the base line responses an hyperalgesia was observed for the control and the OP including after Naloxone ! This must be discussed and interpreted.

ANS: A decrease in the treatment latencies as compared to the baseline values in the hot plate test in case of both the control and opiorphin injected rats could be due to hyperalgesia or learning effect which is common in the hot plate test. It seems to be learning effect rather than hyperalgesia, as there was no change in the latencies after administration of naloxone in the opiorphin group. We have already discussed disadvantages of hot plate test in our manuscript. With this limited data we cannot interpret whether the rats experienced hyperalgesia.

- EEG-responses : Figure 5 reports the results of increase in F50 induced by nociceptive stimuli (thermal and electrical) is which is which ?

ANS: It is now stated clearly in the figure captions.
- It is necessary to express the results in numerical values. Why the control group give a response higher than that observed in baseline? The observed effects in presence of naloxone is interesting but not discussed although this probably the most interesting result of this study.

ANS: There was non significant difference between post normal saline F50 and post naloxone F50 in the control group, both during thermal and electrical stimulus. F50 was significantly higher post treatments as compared to the baseline because baseline values were without any painful stimulus, F50 increases after a painful stimulus. Thus effect of naloxone in the control group (if any) cannot be interpreted from this data. For comparison of OP and MO after naloxone treatment, see Discussion paragraph 2.

- Figure 6 is entirely dis-informative since the representation in histograms give exactly the same results whateveryer the experiments!

ANS=: We have clarified the legend.

- Figure 7 includes a discussion about an effect not really significant after naloxone for an electrical stimulus. What happens after a thermal stimulus?

ANS: There was no significant difference in Ptot compared to baselines in any of the three groups. The Ptot in morphine group rats significantly decreased after an injection of naloxone. Superscripts represent significant differences.

Discussion.

This paragraph begins by a disastrous mention with two references (18 and 19) of a complete absence of changes in the EFG studies after administration of analgesic drugs!!

ANS: The EEG changes in response to noxious stimulus; analgesics reduce this change.

We did not mean "a complete absence of changes in the EEG indicators of nociception after analgesic administration." We found non-significant changes between baseline EEG and the post-stimulation (surgery or electrical stimulus) EEG due to analgesic drugs.
Therefore, we are concerned by the interest of the present study. The only interesting results concern the effect of naloxone which indirectly shows that the opioids receptors could become stimulated by endogenous enkephalins released by pain stimuli due to the protection of the endogenous opioids by oporphin. Several references of the literature must be added in this paragraph (discussion in ref 1).

The absence of effect of OP contrasting to that of MO is curious and cannot be explained by a lack of complete activation of opioid receptors. It is essential to do again a control by the claimed TP test with a thermal stimulation at 52-54°C.

ANS: We are not sure what the reviewer means by this.

The observed hyperalgesia in Figure 4 for the HP test must be imperatively discussed.

ANS: We suspect that this represents learning rather than hyperalgesia.

In conclusion of the study it should have been interesting to know the inhibitory potency against Neprilysin and aminopeptidase of oporphin because several dual inhibitors of both enzymes have been shown to be very potent to alleviate painful stimuli measured by TF, HP tests after several routes of administration and since some of them are now in clinical trials for instance to counteract neuropathic pain. Therefore the conclusion of the paper which is for me the most interesting part of the paper should include the reference to this study.

ANS: Rewritten conclusion

There are several dual enkephalinase inhibitors currently under clinical trials show promising results in their preclinical studies (Roques 2012); especially orally administered P37 (Tesfaye 2016) which has potential to replace opioid pain management protocols in diabetic neuropathy.

Further research is required to enable use of oporphin for clinical pain management. There is a need to conduct a proper pharmacokinetic study to know the half-life, distribution and
elimination of opiorphin in the target species. The rapid breakdown of opiorphin peptide could be a hindrance in testing its analgesic efficacy. A different drug delivery approach may be required to make it a viable candidate for a new class of analgesic drugs.