EJN European Journal of Neuroscience FENS Federation of European Neuroscience Societies

Essential Role of Endogenous Calcitonin Gene-Related Peptide in Pain-associated Plasticity in the Central Amygdala

Kei Shinohara, Ayako M. Watabe, Masashi Nagase, Yuya Okutsu, Yukari Takahashi, Hiroki Kurihara and Fusao Kato

Review timeline:

Submission date:	10 April 2017
Editorial Decision:	15 May 2017
Revision received:	04 August 2017
Accepted:	14 August 2017

Editor: Masahiko Watanabe

1st Editorial Decision 15 May 2017

Dear Prof. Watabe,

Your manuscript was reviewed by external reviewers as well as by the Section Editor, Prof. Masahiko Watanabe, and ourselves.

The reviewers collectively indicated that your experiments generated new and important information. However, there are several important issues that need to be resolved before we can further consider your manuscript for publication. Most importantly, both reviewers pointed out that the weak correlation of nociceptive behavior and synaptic transmission needs to be reported and discussed more clearly. Accordingly, the revisions would need to resolve this and other important issues.

We also noted the following points:
- Please include a 'Data statement'
- Figures probably need to be of a higher resolution
- Fig 3b: please include data points as you have in the other bar charts
- Please indicate the total number of animals used

If you are able to respond fully to the points raised, we would be pleased to receive a revision of your paper within 12 weeks.

Thank you for submitting your work to EJN.

Kind regards,

Paul Bolam & John Foxe
co-Editors in Chief, EJN

Reviews:

Reviewer: 1 (Yuki Hashimotodani, University of Tokyo, Japan)

Comments to the Author
The paper by Shinohara et al. examines the roles of CGRP in the central amygdalar synaptic plasticity and the nociceptive behavior. To address these questions, the authors use electrophysiology and behavioral experiments. The evidence is shown that formalin-induced synaptic potentiation at PB-CeC synapses is attenuated in the CGRP KO mice. Furthermore, nociceptive behavior is reduced at specific period (20-25 min) of post-formalin injection in the CGRP KO mice. Finally, they show that bilateral tactile allodynia is decreased in the CGRP KO mice. Based on these findings, the authors conclude that endogenous CGRP contributes to pain-associated plasticity in the central amygdala.

In general, the experiments are well controlled, and the results are convincing. However, there are a few issues need to be addressed for their revision.

1. Stimulation intensity seems quite strong at the intensities showing significant differences (0.4-1 mA). Is there justification that this stimulation never recruits other inputs than PB?

2. The result showing no significant correlation between the synaptic potentiation and the nociceptive behavior (Fig 3C) is confusing, because the manuscript appears to mislead into the strong correlation between them. This evidence should be emphasized in the text that provide better understandings of this study.

3. P. 18, 4 lines from bottom: It is hard to understand this sentence. The authors just compare the relationship between the EPSC amplitude and the PPR. Why the authors can conclude like this.

4. The change in PPR is so small in the CGRP WT, even if there is statistically significant. Is it possible to test CV, strontium asynchronous release or mEPSCs? Multiple data will further clarify whether synaptic potentiation actually accompanies with presynaptic release probability.

5. Related to question #4, previous study (Han et al., 2010) demonstrated that exogenous application of CGRP potentiates PB-CeC EPSCs with no change in PPR. Did the authors also test the effects of exogenous application of CGRP? These experiments may indicate the different effects of CGRP on synaptic transmission: postsynaptic change by acute, while presynaptic change by long-term event.

Reviewer: 2 (Volker Neugebauer, Texas Tech University Health Sciences Center, USA)

Comments to the Author
This manuscript describes novel and important findings on the role of calcitonin gene-related peptide (CGRP) for synaptic plasticity in the amygdala and for behaviors in a mouse model of inflammatory pain (formalin). The results show that in brain slices from CGRP-knockout (KO) mice, excitatory synaptic transmission at the parabrachial input to the central nucleus of the amygdala is not increased 6 h after formalin injection whereas CGRP-wild type (WT) mice show increased transmission. Analysis of paired-pulse facilitation shows a decrease in brain slices from WT but only little change in KO mice. Nociceptive behaviors differ between WT and KO mice only in the early stage of the second phase. However, mechanical hypersensitivity 6 hours after formalin injection is decreased in KO compared to WT mice. The results show an important contribution of CGRP to synaptic plasticity in the amygdala and to pain behaviors. The data are of high quality and the analysis is sophisticated. In particular, the correlation between electrophysiology and behavior is important. I have some suggestions that may help improve the manuscript.
1) Conceptually, the KO model creates a global lack of CGRP, and CGPR also plays an important role in spinal nociceptive processing, which may explain the relatively weak correlation of nociceptive behavior in the formalin tests over 1 hour and synaptic transmission in the amygdala at the 6 hour time point. This should be reported and discussed more clearly. The correlation between synaptic transmission and nociceptive score in Fig. 3C would be more meaningful if done for mechanical sensitivity (Fig. 4).
2) Along those lines, it is important to explain the rationale for correlating brain slice physiology at 6 hours after formalin with “acute” nociceptive behavior in the formalin test for the first hour. This mismatch should be emphasized.
3) With regard to the analysis of paired-pulse facilitation, the data may also suggest a change in the CGRP KO model although not significant (Fig. 2 B2). The correlation analysis of paired pulse ratio and synaptic response is not quite clear. There seems to be a correlation (Fig. 2 C1) and there is a symbol indicating significance, but the text repeatedly states that no correlation was found. The data interpretation that “while an increase in presynaptic release probability is accompanied by synaptic potentiation, it is unlikely to play a predominant role in the pain-related plastic changes in PB-CeC transmission in the CGRP WT mice following formalin injection” (p. 18) is not clear and does not seem to be justified. For one, any change in paired pulse ratio indicates a presynaptic change, and since CGRP is released into the amygdala from extra-amygdala sources, removing CGRP could be reasonably expected to affect measurements of presynaptic mechanisms.
4) Methods state that male and female mice were used, but results are not reported for each sex. Some lack of significance might be due to sex differences and variability; analyzing these two sets of data separately may be needed.
5) A minor comment about the “sensitization index” (Fig. 4). I would suggest modifying the calculation such that an increased index correlates with increased pain behaviors.
6) Another minor issue are repeated references to this being the first study to address the role of endogenous CGRP in amygdala plasticity. Previous work cited in the manuscript used receptor antagonists to determine the role of CGRP, and it seems plausible that antagonist effects probe receptor activation by the endogenous ligand. Therefore, the authors’ statement and claim of novelty should be rephrased; it would not diminish the significance of their studies reported here.

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Authors' Response 04 August 2017

Your comments and those of the reviewers were highly insightful and enabled us to greatly improve the quality of our manuscript. Below, please find our **point-by-point responses** to each of the comments of the reviewers as well as your own comments.

Revisions in the text are shown in bold and red, with yellow highlighting to indicate additions. In accordance with the editorial and reviewers' suggestions, we have more thoroughly reported and discussed about the correlation between pain behaviors and synaptic potentiation in the Results (page 21, line 17; page 23, line 16; page 26, line 10) and Discussion sections (page 31, line 8) of the revised manuscript. In addition, we also have added new data to further support the presynaptic origin of the synaptic potentiation (Fig. 2C and D), and reanalyzed our previous data (Figure 4C, D and E). We hope that the revisions in the manuscript and our accompanying responses will be sufficient to make our manuscript suitable for publication in the *European Journal of Neuroscience*.
We look forward to hearing from you at your convenience.

Yours sincerely,

Ayako M. Watabe, Ph.D.

Responses to the Editorial comments

The reviewers collectively indicated that your experiments generated new and important information. However, there are several important issues that need to be resolved before we can further consider your manuscript for publication. Most importantly, both reviewers pointed out that the weak correlation of nociceptive behavior and synaptic transmission needs to be reported and discussed more clearly. Accordingly, the revisions would need to resolve this and other important issues.
We also noted the following points:
- Please include a 'Data statement'
- Figures probably need to be of a higher resolution
- Fig 3b: please include data points as you have in the other bar charts
- Please indicate the total number of animals used

Response: Thank you for these helpful comments and suggestions. We agree that the correlation between nociceptive behaviors and synaptic plasticity was not clearly described in the original manuscript. In accord with the editors' suggestion and reviewers' comments, we have substantially revised the original manuscripts to more thoroughly discuss this critical point in the Results (page 21, line 17; page 23, line 16; page 26, line 10) and Discussion section (page 31, line 8).

In addition, we reformatted the figures to increase the resolution (800 dpi), added individual data points to the bar chart in Fig. 3B, and indicated the total number of animals in the material and method section (page 9, line 10).

Responses to Reviewer 1 comments

1. Stimulation intensity seems quite strong at the intensities showing significant differences (0.4-1 mA). Is there justification that this stimulation never recruits other inputs than PB?

Response: We thank the reviewer for raising this important point. As the reviewer pointed out, no significant differences were observed in EPSC amplitude at 50 - 200 μA stimulation intensity between CGRP WT and KO groups. Because the afferent stimulation in this range gave rise to only small EPSC responses (amplitude smaller than 20 pA), it is likely that the stimulating electrode used in this study recruited only a few fibers with small intensity range, which was insufficient to cause significant EPSC amplitude difference

especially when the potentiation is likely to result from changes in release properties. We have revised the manuscript in Results section (page 18, line 7).
Also, we used conventional stimulating electrodes. As the reviewer insightfully pointed out, with this traditional technique, we cannot be certain that stimulation never recruits inputs other than PB, although this methodology is widely considered appropriate for examining PB-CeC synaptic transmission in many laboratories including ours (Neugebauer et al., 2003, 2004, 2005, 2010; Ikeda et al., 2007; Delaney et al., 2007; Cheng et al., 2011; Watabe et al., 2013; Sugimura et al., 2016). Importantly, several factors support the assumption that the method in the present study preferentially evoked PB-originating fibers; 1) The stimulating electrode was carefully positioned onto the fibers that run just ventromedial to the central amygdala (Fig. 1A), as described in a single fiber tracing study by Sarhan and colleagues who reported the precise characteristics of the PB-CeC synapses (Sarhan et al., 2005). 2) In a previous study, we employed an optogenetics approach to address this problem, by measuring light-evoked EPSCs which specifically and exclusively originated from the axon terminals of PB fibers (Sato et al., 2015; Sugimura et al., 2016). Importantly, the results demonstrated that light-evoked EPSCs were spatially restricted in their illumination sites, in accord with the notion that PB fiber bundles are fasciculated, rather than broadly innervating the CeC (Sugimura et al. 2016). Furthermore, we also demonstrated that light-evoked EPSCs are potentiated in formalin-induced inflammatory pain model rats, consistent with the observation obtained in the present study in WT mice treated with formalin. Nonetheless, to avoid any potential confusion, we revised the original manuscript to clarify that this method preferentially, rather than exclusively, evokes PB-CeC synaptic transmission (page 14, line 16).

2. The result showing no significant correlation between the synaptic potentiation and the nociceptive behavior (Fig 3C) is confusing, because the manuscript appears to mislead into the strong correlation between them. This evidence should be emphasized in the text that provide better understandings of this study.
Response: We thank the reviewer for pointing out this critical issue. We agree that our description of the correlation between synaptic potentiation and behaviors was not clear in the original manuscript. We have substantially revised the text to clearly distinguish acute nocifensive behavior, which occurred 1 h post injection and showed no correlation with synaptic potentiation, from mechanical allodynia, which occurred 6 h post injection and exhibited a strong correlation with the synaptic potentiation (page 21, line 17; page 23, line 16; page 26, line 10). We also reanalyzed the correlation in Figure 3C using the value of mean ESPC amplitude in each mouse, instead of each neuron, and revised Results section (page 23, line 17; Figure 3C).

3. P. 18, 4 lines from bottom: It is hard to understand this sentence. The authors just compare the relationship between the EPSC amplitude and the PPR. Why the authors can conclude like this.
Response: We agree with the reviewer that this sentence was confusing, and have removed it in the revised manuscript. Instead, we added a new analysis of the CV of synaptic currents (Figure 2C and D), which reflects presynaptic changes (Manabe et al., 1993). Our results further support the presynaptic origin of the synaptic potentiation induced in the formalin-induced inflammatory model. Accordingly, we have added new content to the Results, Discussion, and References in the revised manuscripts (page 20, line 15; page 29, line12).

4. The change in PPR is so small in the CGRP WT, even if there is statistically significant. Is it possible to test CV, strontium asynchronous release or mEPSCs? Multiple data will further clarify whether synaptic potentiation actually accompanies with presynaptic release probability.
Response: We thank the reviewer for raising this important point. According to the reviewer's suggestion, we added new content to the Results and Figures (Figure 2C, D) showing the CV analysis of EPSCs. These results, together with the PPR results, further support the notion that the synaptic potentiation induced in the formalin model was accompanied with changes in presynaptic release probability (page20, line 15).

5. Related to question #4, previous study (Han et al., 2010) demonstrated that exogenous application of CGRP potentiates PB-CeC EPSCs with no change in PPR. Did the authors also test the effects of exogenous

application of CGRP? These experiments may indicate the different effects of CGRP on synaptic transmission: postsynaptic change by acute, while presynaptic change by long-term event.
Response: We thank the reviewer for raising this interesting point. We did indeed test the effects of exogenous application of CGRP, and found that it induced postsynaptic changes (Okutsu et al., 2017). Thus, as the reviewer suggested, CGRP induces post synaptic changes by acute application, while presynaptic changes are induce by long-term events. We added this new reference to the revised discussion, and added a new paragraph regarding the site of action (page 29, line 12).

Responses to the Reviewer 2 comments

Comments to the Author

The data are of high quality and the analysis is sophisticated. In particular, the correlation between electrophysiology and behavior is important. I have some suggestions that may help improve the manuscript.

1) Conceptually, the KO model creates a global lack of CGRP, and CGPR also plays an important role in spinal nociceptive processing, which may explain the relatively weak correlation of nociceptive behavior in the formalin tests over 1 hour and synaptic transmission in the amygdala at the 6 hour time point. This should be reported and discussed more clearly. The correlation between synaptic transmission and nociceptive score in Fig. 3C would be more meaningful if done for mechanical sensitivity (Fig. 4).
Response: We thank the reviewer for raising this important issue. We agree that distinguishing events at 1 h and 6 h is critical in the manuscript. Thus, we revised the manuscript to report and discuss this point more clearly (page 21, line 17; page 23, line 16; page 26, line 10). We agree with the reviewer's point regarding the correlation between synaptic transmission and nociceptive score, and conduced a new analysis of the data in Fig. 4. Interestingly, we found a strong correlation between synaptic transmission and mechanical allodynia in the left paw, while no such correlation was observed in the right paw (Figure 4D). These results suggest that while formalin injection induces tactile allodynia bilaterally, the molecular mechanisms underlying ectopic hypersensitivity might differ from the allodynia observed on the injured side. Further studies are needed to explore the precise mechanisms, which will reveal the modulatory mechanisms of amygdala-mediated descending pain facilitation.

2) Along those lines, it is important to explain the rationale for correlating brain slice physiology at 6 hours after formalin with "acute" nociceptive behavior in the formalin test for the first hour. This mismatch should be emphasized.
Response: This helpful suggestion is related to comment 1), and also to Reviewer #1's comment 2). We agree that text of the original manuscript contained confusion regarding acute and long-term events. In accord with your suggestion, we substantially revised the manuscript to clarify these points, and have emphasized this mismatch in the revised Results and Discussion sections (page 21, line 17; page 23, line 16; page 26, line 10; page 31, line 8).

3) With regard to the analysis of paired-pulse facilitation, the data may also suggest a change in the CGRP KO model although not significant (Fig. 2 B2). The correlation analysis of paired pulse ratio and synaptic response is not quite clear. There seems to be a correlation (Fig. 2 C1) and there is a symbol indicating significance, but the text repeatedly states that no correlation was found. The data interpretation that "while an increase in presynaptic release probability is accompanied by synaptic potentiation, it is unlikely to play a predominant role in the pain-related plastic changes in PB-CeC transmission in the CGRP WT mice following formalin injection" (p. 18) is not clear and does not seem to be justified. For one, any change in paired pulse ratio indicates a presynaptic change, and since CGRP is released into the amygdala from extra-amygdala sources, removing CGRP could be reasonably expected to affect measurements of presynaptic mechanisms.
Response: We agree with the reviewer that our original analysis of the correlation between PPR and synaptic response was unclear. In the revised manuscript, we included new results that further support the presynaptic nature of the synaptic potentiation, with a CV analysis of EPSCs (Fig. 2C, D). Therefore, we

deleted the relevant sentence from p. 18, line 18, rewrote the Results section and modified the Discussion section in accord with the reviewer's suggestion (page 20, line 15; page29, line12).

4) Methods state that male and female mice were used, but results are not reported for each sex. Some lack of significance might be due to sex differences and variability; analyzing these two sets of data separately may be needed.

Response: We thank the reviewer for raising this issue. We attempted to analyze each sex separately as shown below. Although analyses of electrophysiological experiments and behavioral experiments revealed similar tendencies in both male and female mice, the statistical significance was relatively weak when the animals were grouped by sex (Figures S1 and S2). One possibility is that the smaller sample size in each group weakened the statistical effects (page 9, line 10). Another possibility is that hormonal cycle variation in female mice affected the results. Because the overall trends were similar, we pooled the data of both groups.

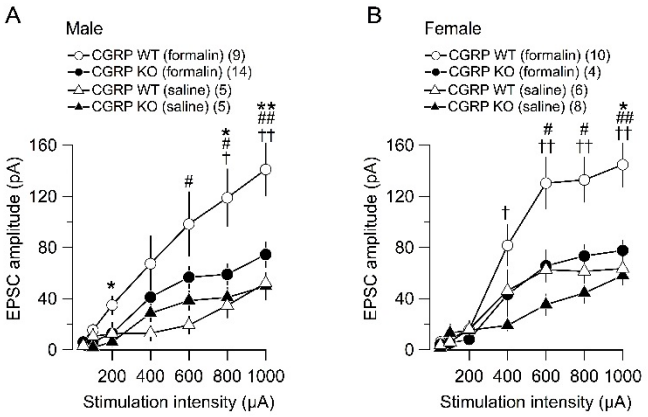

Figure S1

Figure S1; (A) Relationship between stimulation intensity and evoked EPSC amplitude in male (CGRP WT formalin, n = 9 neurons from 6 mice; CGRP KO formalin, n = 14 neurons from 6 mice; CGRP WT saline, n = 5 neurons from 2 mice; CGRP KO saline, n = 5 neurons from 2 mice). ** $P < 0.01$ and * $P < 0.05$ (vs. CGRP KO formalin); ## $P < 0.01$, # $P < 0.05$ (vs. CGRP WT saline); †† $P < 0.01$ (vs. CGRP KO saline), ANOVA followed by *post hoc* Gabriel's test. (B) Relationship between stimulation intensity and evoked EPSC amplitude in female (CGRP WT formalin, n = 10 neurons from 5 mice; CGRP KO formalin, n = 4 neurons from 4 mice; CGRP WT saline, n = 6 neurons from 3 mice; CGRP KO saline, n = 8 neurons from 3 mice). * $P < 0.05$ (vs. CGRP KO formalin); ## $P < 0.01$, # $P < 0.05$ (vs. CGRP WT saline); †† $P < 0.01$ and † $P < 0.05$ (vs. CGRP KO saline), ANOVA followed by *post hoc* Gabriel's test.

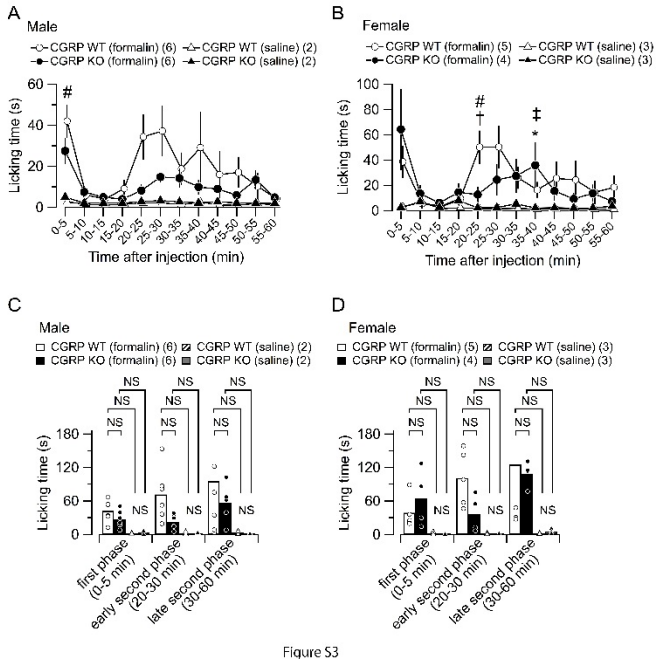

Figure S3

Figure S3; (A) The licking behavior in male mice (CGRP WT formalin, n = 6; CGRP KO formalin, n = 6); CGRP WT saline, n = 2; CGRP KO saline, n = 2). # $P < 0.05$ (CGRP WT formalin vs. CGRP WT saline). (B) The licking behavior in female mice (CGRP WT formalin, n = 5; CGRP KO formalin, n = 4); CGRP WT saline, n = 3; CGRP KO saline, n = 3). ## $P < 0.05$ (CGRP WT formalin vs. CGRP WT saline); † $P < 0.05$ (CGRP KO formalin vs. CGRP KO saline); * $P < 0.05$ (CGRP WT formalin vs. CGRP KO formalin); ‡$P < 0.05$ (CGRP KO formalin vs. CGRP KO saline).

5) A minor comment about the “sensitization index” (Fig. 4). I would suggest modifying the calculation such that an increased index correlates with increased pain behaviors.
Response: We thank the reviewer for this insightful comment. In the revised manuscript, we modified the definition of the sensitization index so that increased pain behaviors were correlated with increased index values (Fig. 4 C).

6) Another minor issue are repeated references to this being the first study to address the role of endogenous CGRP in amygdala plasticity. Previous work cited in the manuscript used receptor antagonists to determine the role of CGRP, and it seems plausible that antagonist effects probe receptor activation by the endogenous ligand. Therefore, the authors’ statement and claim of novelty should be rephrased; it would not diminish the significance of their studies reported here.
Response: We thank the reviewer for raising this issue. We agree with the reviewer’s comment, and have modified the manuscript accordingly (page 3, line 10; page7, line8; page 29, line 9).