EMBO Molecular Medicine

P2X4 receptor controls microglia activation and favours remyelination in autoimmune encephalitis

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Review timeline:

Submission date:	04 December 2017
Editorial Decision:	19 January 2018
Revision received:	04 May 2018
Editorial Decision:	25 May 2018
Revision received:	05 June 2018
Accepted:	08 June 2018

Editor: Céline Carret

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 19 January 2018

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the two referees whom we asked to evaluate your manuscript.

You will see that while they appreciated the study and translational relevance, they also would like to see more mechanism of action, better controls and further detailed explanations. I would like to encourage you to address these as requested.

We would welcome the submission of a revised version within three months for further consideration and would like to encourage you to address all the criticisms raised as suggested to improve conclusiveness and clarity. Please note that EMBO Molecular Medicine strongly supports a single round of revision and that, as acceptance or rejection of the manuscript will depend on another round of review, your responses should be as complete as possible.

EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

Please also contact us as soon as possible if similar work is published elsewhere. If other work is published we may not be able to extend the revision period beyond three months.

Please read below for important editorial formatting and consult our author's guidelines for proper formatting of your revised article for EMBO Molecular Medicine.

I look forward to receiving your revised manuscript.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

The authors use a wide range of appropriate and sophisticated techniques to address the question of the role of microglial P2X4R in demyelination.

Referee #1 (Remarks for Author):

The paper significantly improves understanding of the mechanisms of microglia responses during demyelination. Specifically, the study demonstrates a key role for purinergic receptor P2X4 (P2X4R) in microglia/macrophages during autoimmune inflammation. Importantly, the study shows that genetic or pharmacological blockade of P2X4R signaling exacerbates disease in EAE, the mouse model of MS. Furthermore, P2X4R regulated remyelination and repair. These important findings support the possibility that P2X4R may be a potential therapeutic target in MS

Some minor comments:
1) Levels of P2x4R expression were increased at the peak of the disease and remained elevated during the recovery phase of EAE (Fig. 1), which suggest against either a specific protective or destructive role for P2X4R, since they are elevated during both damage and repair? How does this square with the subsequent findings on the regulatory role of P2X4R?

For example, the gene expression (Fig. 4) indicates that blockade of P2X4R with TNPATP did not significantly alter anti-inflammatory gene expression, which is associated with repair, but did significantly increase pro-inflammatory gene expression, which is associated with the peak of EAE.

2) TNP-ATP was used to antagonise P2X4R, but TNP-ATP is a non-selective P2X antagonist. Although they also used the P2X4R KO to show the effects are mediated primarily through P2X4R, it would sill have been good to use some other more specific P2X4R antagonists. Also, what was the concentration in the brain?

3) Also with TNP-ATP, the data in figure 2 indicate that blockade of P2X4R decreases the neurological score, but the gene expression (Fig. 4) indicates that blockade of P2X4R with TNP-ATP significantly increase pro-inflammatory gene expression, which is associated with the peak of EAE and considered detrimental. Can this be explained?

Referee #2 (Comments on Novelty/Model System for Author):

This is a thorough study that might have important practical applications, especially because one of the drugs investigated, ivermectin, is already used or the treatment of human diseases. Clarity might be improved.

Referee #2 (Remarks for Author):

This MS highlights the role of the P2X4R as an anti-inflammatory receptor that helps to damp down inflammation in a model of experimental autoimmune encephalitis (EAE) and to promote remyelination. Processes involved in the pathogenesis of EAE are a matter of hot debate in view of their obvious relevance for multiple sclerosis, but unfortunately little progress has been made towards elucidation of molecular pathways involved. In this context, the P2X4R is attracting interest as one of the P2Rs whose expression is increased at sites of neurological damage in EAE. However, role of this receptor in EAE is unknown. The study by Zabala et al. aims at clarifying this issue. The main finding is that the P2X4R turns out to have an anti-inflammatory, protective, role in this disease. The study is thorough, informative and of potential therapeutic relevance. My main criticism relates to the lack of clear mechanistic explanation for the role of P2X4. The Authors show that P2X4 blockade with TNP-ATP reduces myelin endocytosis, pointing to phagocytosis modulation as a possible mechanism, yet this was not investigated in depth. For example, I wonder why no phagocytosis experiments were performed with microglia from P2X4-KO mice. Also, the finding that IVM treatment does not increase myelin endocytosis in anti-inflammatory microglia is

disturbing. In this regard, IVM should also be tested in the P2X4-KO mice: here no effect is anticipated. In addition, the Authors briefly addressed the potential role of BDNF. This is an interesting point since BDNF release is linked to P2X4 activation. However, this was not investigated in depth, and even basic controls in the P2X4-KO model were not performed. Additional minor points for the Authors to consider are detailed below.

1. Please, for the sake of the lay reader, explain what MOG35-55 is, and the rationale for its use, in the Result section.

2. Please, add "+" superscript to CD4 and CD8 in several places throughout the MS.

3. At pg 9, line 19, "is" should be "are".

4. It is kind of unusual that Dr Rassendren is thanked for the gift of the P2X4-KO mice when he is in fact a co-author.

5) A pg 24 (Pain assessment): I do not understand what the reference to "Ugo Basile" means.

6) Sentence at pg 29, lines 9-12, starting with "Naive..." should be amended.

7) Images in Fig. 2B are not really convincing. I urge the Authors to provide better images.

1st Revision - authors' response 04 May 2018

RESPONSE TO REVIEWERS

We appreciate all the points raised by the reviewers which helped us to improve our study. We have carefully addressed them as explained in detail below. In particular, we have performed new experiments to elucidate the mechanism by which P2X4R modulate microglia reaction. In addition to controlling BDNF release as previously described (Fig EV2), we have analyzed whether P2X4R could directly control lysosome function and thus modulate directly myelin phagocytosis. We indeed have observed that P2X4R potentiation with ivermectin induces lysosome acidification (Fig. EV6). Further analysis, out of the scope of the present study, are necessary to determine the mechanism by which P2X4R controls lysosome function.

Point by point response to reviewers

Referee #1

Some minor comments:

1) Levels of P2x4R expression were increased at the peak of the disease and remained elevated during the recovery phase of EAE (Fig. 1), which suggest against either a specific protective or destructive role for P2X4R, since they are elevated during both damage and repair? How does this square with the subsequent findings on the regulatory role of P2X4R?

For example, the gene expression (Fig. 4) indicates that blockade of P2X4R with TNPATP did not significantly alter anti-inflammatory gene expression, which is associated with repair, but did significantly increase pro-inflammatory gene expression, which is associated with the peak of EAE.

Response:

Blockage of P2X4R with TNP-ATP induced a significant increase in pro-inflammatory gene expression only at the recovery phase. At the peak of the disease there was no significant changes in pro-inflammatory gene expression (see new Fig EV2). The lack of effect of P2X4R blockage on the inflammatory reaction at EAE peak is in accordance with the lack of effect on immune priming after prolong treatment (from day 0 after immunization, Fig. 3) and points to a crucial role of microglial P2X4 receptor mainly at the recovery phase. Innate immune response is essential to phagocyte myelin, a key process crucial to proceed with remyelination. Our data (see Fig 8 and new Fig EV6) supports the idea that P2X4R activation increases myelin endocytosis and degradation at lysosomes, probably by inducing lysosome acidification (Fig EV6).

2) TNP-ATP was used to antagonise P2X4R, but TNP-ATP is a non-selective P2X antagonist. Although they also used the P2X4R KO to show the effects are mediated primarily through P2X4R,

it would sill have been good to use some other more specific P2X4R antagonists. Also, what was the concentration in the brain?

Response:
The specificity of the pharmacological tools has been demonstrated using the $P2X4^{-/-}$ mice. Thus, drugs have no effect on $P2X4^{-/-}$ mice (see Fig 2). Unfortunately, there is no a selective and potent antagonist of P2X4R with solubility in water. 5-BDBD works as a selective P2X4 receptor antagonist. However, the compound displays a very low water-solubility, which limits its application using systemic injection. An exception is the new compound NP-1815-PX (5-[3-(5-thioxo-4H-[1,2,4]oxadiazol-3-yl)phenyl]-1H-naphtho[1, 2-b][1,4]diazepine-2,4(3H,5H)-dione) which is a potent and selective antagonist of P2X4R (PMID:27576299). However, the compound is not commercial.

A previous study reported IVM brain accumulation after chronic IVM i.p. injection in mice (3 mg/kg; see Fig 2 in PMID:25004078). No previous study has analyzed the levels of TNP-ATP after i.p. injection and we did not have the methodological tools to analyze that. However, the severe CNS inflammation in EAE leads to BBB breakdown and thus increasing permeability to drugs.

3) Also with TNP-ATP, the data in figure 2 indicate that blockade of P2X4R decreases the neurological score, but the gene expression (Fig. 4) indicates that blockade of P2X4R with TNP-ATP significantly increase pro-inflammatory gene expression, which is associated with the peak of EAE and considered detrimental. Can this be explained?
Response:
Blockage of P2X4R with TNP-ATP exacerbates EAE.

Referee #2
This MS highlights the role of the P2X4R as an anti-inflammatory receptor that helps to damp down inflammation in a model of experimental autoimmune encephalitis (EAE) and to promote remyelination. Processes involved in the pathogenesis of EAE are a matter of hot debate in view of their obvious relevance for multiple sclerosis, but unfortunately little progress has been made towards elucidation of molecular pathways involved. In this context, the P2X4R is attracting interest as one of the P2Rs whose expression is increased at sites of neurological damage in EAE. However, role of this receptor in EAE is unknown. The study by Zabala et al. aims at clarifying this issue. The main finding is that the P2X4R turns out to have an anti-inflammatory, protective, role in this disease. The study is thorough, informative and of potential therapeutic relevance. My main criticism relates to the lack of clear mechanistic explanation for the role of P2X4. The Authors show that P2X4 blockade with TNP-ATP reduces myelin endocytosis, pointing to phagocytosis modulation as a possible mechanism, yet this was not investigated in depth. For example, I wonder why no phagocytosis experiments were performed with microglia from P2X4-KO mice. Also, the finding that IVM treatment does not increase myelin endocytosis in anti-inflammatory microglia is disturbing. In this regard, IVM should also be tested in the P2X4-KO mice: here no effect is anticipated. In addition, the Authors briefly addressed the potential role of BDNF. This is an interesting point since BDNF release is linked to P2X4 activation. However, this was not investigated in depth, and even basic controls in the P2X4-KO model were not performed. Additional minor points for the Authors to consider are detailed below.

Regarding BDNF, we have performed new control experiments to demonstrate that P2X4 receptor is linked to BDNF release. We demonstrated that P2X4R activation with IVM induces an increase in BDNF production in microglia from WT mice, an effect absent in $P2X4^{-/-}$ microglia. This information has been added to the new Fig EV4. In addition, we have added new data (Fig EV4) showing that *Bdnf* mRNA is reduced in control and EAE $P2X4^{-/-}$ mice *vs* wild type mice.

Regarding mechanism, two possibilities are discussed in the manuscript. One is that P2X4R modulate microglia polarization and indirectly, myelin phagocytosis. Indeed, P2X4R control microglia phenotype in the absence of any phagocytic stimulus. However, we have checked various signaling pathways controlling microglia polarization like CSF-1R, AKT and CREB and we have not detected any changes in their phosphorylation in the presence of P2X4R agonist/antagonists (data not shown). The other possibility is that P2X4R could control directly phagocytosis and by

doing that, could alter microglia inflammatory response. Indeed, P2X4R activation by IVM induces an increase in myelin endocytosis and degradation in WT mice (see new Fig EV6), which is absent in P2X4$^{-/-}$ mice. Since precious data on literature have observed the expression of P2X4R in lysosome, we have performed new experiments to determine whether P2X4R could directly influence lysosome function. Preliminary data showed that P2X4R stimulation with IVM induces endosome-lysosome fusion and pH acidification (see Fig EV6). These data suggest that P2X4R could potentially modulate myelin phagocytosis directly. This information and hypothesis have been added and discussed in the new version of the manuscript.

1. Please, for the sake of the lay reader, explain what MOG35-55 is, and the rationale for its use, in the Result section.
2. Please, add "+" superscript to CD4 and CD8 in several places throughout the MS.
3. At pg 9, line 19, "is" should be "are".
4. It is kind of unusual that Dr Rassendren is thanked for the gift of the P2X4-KO mice when he is in fact a co-author.
5. A pg 24 (Pain assessment): I do not understand what the reference to "Ugo Basile" means.
6. Sentence at pg 29, lines 9-12, starting with "Naive..." should be amended.
7. Images in Fig. 2B are not really convincing. I urge the Authors to provide better images.

All the minor points have been corrected. Ugo Basile is the company that produces the aesthesiometer. We have improved quality of images in Fig 2B and eliminate Hoechst staining to facilitate cell visualization.

2nd Editorial Decision 25 May 2018

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed report from the referee asked to re-assess it. As you will see the reviewer is now supportive and I am pleased to inform you that we will be able to accept your manuscript pending final editorial amendments.

Please submit your revised manuscript within two weeks.

I look forward to reading a new revised version of your manuscript as soon as possible.

***** Reviewer's comments *****

Referee #2 (Comments on Novelty/Model System for Author):

This is a well-written and experimentally sound study that might have an important impact in the development of novel treatments for neurodegenerative diseases.

Referee #2 (Remarks for Author):

I think that this MS deserves publication as a full report.

2nd Revision - authors' response 05 June 2018

The authors made the requested editorial changes.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Maria Domercq
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2017-08743

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical methods were used to predetermine sample size. Sample sizes were chosen based on previous experience and based upon similar studies from the literature.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	No statistical methods were used to predetermine sample size. Sample sizes were chosen based upon similar studies from the literature. For EAE experiments a minimum of 10 mice were used for each experimental group to minimize variability.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Age matched mice were used to minimize variability. For in vivo studies, mice that did not develop any EAE symptoms were excluded from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Mice were randomly allocated to the treatment and were equally divided to the different cages to exclude any cageing effect.
For animal studies, include a statement about randomization even if no randomization was used.	All mice were randomized before the immunization and before the appearance of EAE symptoms.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	For all in vitro analysis, all the samples have been included. For in vivo experiments, no exclusion has been made except mice that do not develop the disease. The investigators were not blinded during analysis of the data.
4.b. For animal studies, include a statement about blinding even if no blinding was done	For in vivo experiments, quantification of neurological score and recpording the latency was undertaken by readers blinded to the study.
5. For every figure, are statistical tests justified as appropriate?	Yes. Appropriate statistical tests are described in the manuscript
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normal distribution was corroborated using Graph Pad software and D'Agostino and Pearson omnibus normality test. Neurological score from EAE mice did not follow a normal distribution and it was analyzed by the U Mann-Whitney nonparametric test. In some experiments, the n is too small to the normality test and we assumed the normal distribution.
Is there an estimate of variation within each group of data?	Variation is included in all graphs.
Is the variance similar between the groups that are being statistically compared?	We did not determine whether the variance was similar between the different groups.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All antibody catalog numbers are provided.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Only primary cells were used and characterized.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Animal information details are included in the manuscript. Mice were kept under conventional housing conditions (22 ± 2ºC, 55 ± 10% humidity and 12-hour day/night cycle) at the University of the Basque Country animal facilities. Mice were provided by ENVIGO.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experiments were performed according to the procedures approved by the Ethics Committee of the University of the Basque Country (UPV/EHU). Animals were handled in accordance with the European Communities Council Directive.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We have consulted the ARRIVE Guidelines Checklist.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No