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Cerebral nitric oxide represses choroid plexus NF B-dependent gateway activity for leukocyte trafficking

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Review timeline:

Submission date:	29 October 2014
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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 15 December 2014

Thank you for submitting your manuscript to The EMBO Journal. Two good experts have now reviewed your study and their comments are provided below.

As you can see both referees find the analysis interesting, but they also find it too preliminary. While referee #2 would like to see the Nf-kB part better developed, referee #1 wants more analysis using wild-type and AD mice. Both referees are not able to recommend publication here at this stage. I see that the Nf-kB part can probably be strengthened within a reasonable timeframe. However, the issues raised by referee #1 are more challenging to address, but nevertheless valid. Given that it is not clear if the raised issues can be addressed, I unfortunately can't offer to consider a revised version at this stage. However, given the potential interest in the paper, I can offer to consider a resubmission should you be able to strengthen the in vivo part of the study as well as the NF-kB part. I should point out that for resubmission we consider novelty at time of resubmission and if needed might involve new referee(s).

I am sorry that I can't be more positive on this occasion, but I hope that you find the referee comments helpful.

REFEREE REPORTS

Referee #1:

In the manuscript ´Cerebroventricular nitric oxide represses choroid plexus NFκB activation for leukocyte trafficking´, Baruch et al. describe that nitric oxide (NO) inhibits TNFalpha-induced NF-κB signaling and expression of leukocyte trafficking molecules in cultured choroid plexus epithelial

cells. Moreover, they find that epithelial cells isolated from the choroid plexus of AD transgenic mice show reduced TNFalpha-induced NF-κB signaling. Treatment of AD transgenic mice with the NO scavenger Rutin partially decreased NO levels, and normalized TNFalpha-induced NF-κB signaling.

The findings are interesting, but very preliminary. Cell culture experiments are helpful for pharmacological studies, but they do not reflect the complexity of the NO system in the brain and choroid plexus. NO synthase (NOS) is expressed in choroid epithelial cells, but also in choroidal vessels. There are different isoforms of NOS, which are known to play different roles in neuroinflammation. The authors need to address these issues in more detail in wild type and AD transgenic mice, including the use of cell-specific genetic targeting of NOS. Peripheral administration of NO scavengers, like Rutin, will affect all cell types: what are the alterations in blood flow, leukocyte infiltration and inflammation in the brain? In the case of AD transgenic mice, how does this affect plaque deposition and Abeta clearance in relation to leukocyte infiltration via the choroid plexus? The findings may be complemented by data from cultured choroid plexus epithelial cells from 5XFAD mice (to be compared with the data from wild type choroid plexus epithelial cells presented in Figs. 1-3).

Referee #2:

The author present data on a possible mechanism how a central inflammatory event, in this case a TNFα injection, causes repression of NfκB thereby facilitating leukocyte entry.
I think that the study is well conducted but lacks compelling mechanistic evidence. NO has a plethora of targets. Therefore, the suggested target (NfκB) has to be more thoroughly investigated.

Major comments:
Figure 1B/D: Could it be, that part of the CD45+ cells are microglia, which obviously have not entered the CNS via the plexus choriodius but were just activated by the treatment?

Page 5, 2nd paragraph: Please cite a relevant paper for the involvement of NO in AD (e.g. Nathan et al., JEM 2005, Kummer et al, Neuron 2011, Fernández-Vizarra et al., Neurobiol. Dis. 2004)

Figure 2: It seems necessary to demonstrate that the TNFα challenge causes a nuclear translocation of NFκB/p65 while the cells are still in an undissociated state by e.g. immunocytochemistry and confocal microscopy.

Page 6, 1st paragraph: NO has many effectors in the cell. I have no doubts that the measured effects of NO donors and inflammatory markers are valid, but I would suggest that the effect of NO on p65 should be demonstrated in the cell culture model used as in shown Kelleher et al, JBC, 2007.

Page 9, 2nd paragraph: A more balanced discussion about the positive and negative effects of infiltrating leukocytes during neurodegeneration is advisable.
In addition, in AD it still remains a matter of debate whether leukocytes play any role at all.

Minor comments:
Figure 1 and 3: It is the Newmann-Keuls post hoc test.

Figure 3: The explanations for the treatment abbreviations (M/M etc.) are missing. They are in the legend of figure 2, but it is inconvenient for the reader to move forth and back, thus please add them.

Resubmission 08 March 2015

Referee #1:

In the manuscript ´Cerebroventricular nitric oxide represses choroid plexus NFκB activation for leukocyte trafficking´, Baruch et al. describe that nitric oxide (NO) inhibits TNFalpha-induced NF-κB signaling and expression of leukocyte trafficking molecules in cultured choroid plexus epithelial cells. Moreover, they find that epithelial cells isolated from the choroid plexus of AD transgenic

mice show reduced TNFalpha-induced NF-κB signaling. Treatment of AD transgenic mice with the NO scavenger Rutin partially decreased NO levels, and normalized TNFalpha-induced NF-κB signaling.

The findings are interesting, but very preliminary. Cell culture experiments are helpful for pharmacological studies, but they do not reflect the complexity of the NO system in the brain and choroid plexus. NO synthase (NOS) is expressed in choroid epithelial cells, but also in choroidal vessels. There are different isoforms of NOS, which are known to play different roles in neuroinflammation. The authors need to address these issues in more detail in wild type and AD transgenic mice, including the use of cell-specific genetic targeting of NOS.

Authors: We appreciate the referee's request to further probe the involvement of NOS in our model. It is clear to us that the effects of nitric oxide in the brain are complex, but the strength of our data is in demonstrating the downstream consequences of nitric oxide in choroid plexus function. Importantly, we show that regardless of the extracellular source of nitric oxide, and due to its ability to freely diffuse across cell membranes, its cytoplasmic accumulation in CP epithelial cells inhibits NFκB/p65 subunit translocation (Fig. 2B-G; Fig. 4A-B, F-G). Moreover, we now show in the revised manuscript (addressing a comment raised by Referee #2), that NO acts by S-nitrosylation of protein residues in CP epithelial cells (Fig. 2D, E), further substantiating the involvement of NO in affecting CP epithelial intracellular pathways; this effect is evident regardless of the source of the NO.

Peripheral administration of NO scavengers, like Rutin, will affect all cell types: what are the alterations in blood flow, leukocyte infiltration and inflammation in the brain? In the case of AD transgenic mice, how does this affect plaque deposition and Abeta clearance in relation to leukocyte infiltration via the choroid plexus? The findings may be complemented by data from cultured choroid plexus epithelial cells from 5XFAD mice (to be compared with the data from wild type choroid plexus epithelial cells presented in Figs. 1-3).

Authors: As requested by this referee, in the revised manuscript we further examined the functional in-vivo effects of Rutin administration on disease pathology and CP function in AD-Tg mice. We found that the effect of nitric oxide scavenger in AD-Tg mice was associated with a clear effect on disease pathology – clearance of Ab plaques (Fig. 4C, D) and reduced cerebral gliosis (Fig. 4C, E), which was associated with reduction of local-CP NO levels (Fig. 4F), p65 nuclear translocation in the CP of the Rutin-treated AD-Tg mice (Fig. 4G), upregulation of leukocyte trafficking gene expression by the CP (Fig. 4H), and enhanced monocyte-derived macrophage accumulation in the CNS (Fig. 4I).

Referee #2:

The author present data on a possible mechanism how a central inflammatory event, in this case a TNFα injection, causes repression of NfκB thereby facilitating leukocyte entry.
I think that the study is well conducted but lacks compelling mechanistic evidence. NO has a plethora of targets. Therefore, the suggested target (NfκB) has to be more thoroughly investigated.

Major comments:

Figure 1B/D: Could it be, that part of the CD45+ cells are microglia, which obviously have not entered the CNS via the plexus choriodius but were just activated by the treatment?

Authors: Figure 1B/D shows cells in the cerebrospinal fluid (CSF). In the CSF, both in steady-state and following injurious conditions, there are no microglia. Structurally, the CSF is secluded from the CNS parenchyma by the ependymal layer of the brain ventricles, and microglia are located only in its sub-ventricular zone and the brain parenchyma itself. Thus, the cerebrospinal fluid that we aspirated in the experiments presented in figure 1B-E, contains no microglia.

Page 5, 2nd paragraph: Please cite a relevant paper for the involvement of NO in AD (e.g. Nathan et al., JEM 2005, Kummer et al, Neuron 2011, Fernández-Vizarra et al., Neurobiol. Dis. 2004)

Authors: The citations were added, as suggested.

Figure 2: It seems necessary to demonstrate that the TNFα challenge causes a nuclear translocation of NFκB/p65 while the cells are still in an undissociated state by e.g. immunocytochemistry and confocal microscopy.

Authors: As requested by the referee, we performed this experiment, and added the findings to the manuscript (supplementary figure 2). Indeed, we found that both at the single-cell level (Fig. 2A-E) and in undissociated tissues (supplementary figure 2), exposure of the CP to NO could repress the NFκB/p65 signaling pathway.

Page 6, 1st paragraph: NO has many effectors in the cell. I have no doubts that the measured effects of NO donors and inflammatory markers are valid, but I would suggest that the effect of NO on p65 should be demonstrated in the cell culture model used as in shown Kelleher et al, JBC, 2007.

Authors: We thank this reviewer for this constructive suggestion. During the revision process we performed experiments using the biotin-switch protein S-nitrosylation assay, as suggested, and we now show (Fig. 2D, E) the effect of NO on protein S-nitrosylation in CP epithelial cells. Notably, we originally attempted to tackle this issue using the particular method suggested by the referee (Kelleher et al.) but encountered technical difficulties due to the overall limited amount of protein that we could extract from the CP cultures (we are dealing with a very small tissue in the mouse, and satisfactory protein extraction to enable western-blotting would mean pooling the CPs of many mice; from each mouse we can extract roughly 200,000 CP epithelial cells, which do not proliferate in culture).

Page 9, 2nd paragraph: A more balanced discussion about the positive and negative effects of infiltrating leukocytes during neurodegeneration is advisable.
In addition, in AD it still remains a matter of debate whether leukocytes play any role at all.

Authors: We revised the paragraph according to this referee's comment.

Minor comments:

Figure 1 and 3: It is the Newmann-Keuls post hoc test.

Authors: Thank you, this typo was corrected.

Figure 3: The explanations for the treatment abbreviations (M/M etc.) are missing. They are in the legend of figure 2, but it is inconvenient for the reader to move forth and back, thus please add them.

Authors: Thank you, this is now corrected.

Accepted 31 March 2015

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by referee #2. Referee #1 was not available to review the revised version.

As you can see below, the referee appreciates the introduced changes and supports publication here. I am therefore very pleased to accept the manuscript for publication here.

REFEREE REPORT

Referee #2:

The authors answered all my points.

