Author's response to reviews

Title: COX-2, CB2 and P2X7-immunoreactivities are increased in activated microglial cells/macrophages of multiple sclerosis and amyotrophic lateral sclerosis spinal cord

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Author's response to reviews: see over
Response to reviewers' comments:
The authors would like to thank the reviewers for their positive comments and helpful suggestions. We have taken all comments into consideration and accordingly have made revisions to improve the manuscript. We attach two versions of the revised manuscript one of which has revised text highlighted in italic bold.

Reviewer #1 Noel G Carlson:

MAJOR REVISIONS (revised text is highlighted in bold and italic)

Point 1) Controls for expression of these antigens are described in graphs but are never shown in IHC.

Response: A new figure has now been added to show immunostaining for antigens in control tissues and figure legends updated accordingly. Text in Results page 10 has been updated to include the following:

Control spinal cord

Antibodies to COX-2, CB2 and P2X7 were immunoreactive with scattered, small, nucleated cells some showing fine short processes typical of microglial cells/macrophages (Fig 1A - C). Similar staining was obtained with antibodies to CD68 (Fig. 1D) which is known to be a marker of microglial cells/macrophages.

Point 2 No western blots are shown. A representative western blot should be shown for each protein.

Response: Because of limited spinal cord tissue and extracts, it was only possible to perform Western blots for COX-2. A representative Western blot of COX-2 in control and MS spinal cord extracts, together with mouse macrophage control extract, (BD Biosciences, Oxford,UK) is now shown in the revised Figure 6 and its legend has been updated accordingly.

Point 3) In the western blots it is not clear how the samples are normalized for differences in loading. It is noted on page 8 in the methods that the inter gel variation
was corrected by comparison of the OD of the positive control in each blot and adjusted to the OD reading accordingly. What was the positive control? Was actin or some other standard used? This should be spelled out in the methods and an example western blot shown with the standard along with the protein being assessed.

**Response:** The paragraph below has now been added to the Western blot methods section and a figure of the COX-2 Western blot in controls and MS cord is shown in Figure 6 (see below).

*To ensure that similar amounts of protein were loaded onto gels, protein extracts (20 µg; measured by the Bradford dye-binding protein assay, Bio-Rad laboratories, Hertfordshire, UK) were loaded and protein bands visualised by staining membranes with 0.1% (w/v) Ponceau S in 1% (v/v) acetic acid. Only lanes that had similar protein profiles were used for subsequent Western blot analysis. Positive control for the Western blots was mouse macrophage (BD Biosciences, Oxford, UK) extract which also reacted to give a strong 70 kDa band.*

**Point 4)** Image analysis in not adequately described. What magnification(s) was used? Also, the authors only present the % of the image area of a particular antigen relative to the area of the field scanned. However, in images such as Figs 10 and 12 where portions of the field don not contain tissue, this is misleading. The % reactive area should be assessed relative to the tissue area in the field.

**Response:** All image analysis was performed at x40 objective magnification as shown in the lower panels of Figs 10 and 12 (new Figures 11E and F and Figure13E and F). The text has been updated to clarify this and the sentence - ‘Images were captured via video link to an Olympus BX50 microscope and scanned by the computer’ now reads as ‘Images were captured via video link to an Olympus BX50 microscope *at x40 objective magnification so that tissue fully occupied each field*, and scanned by the computer’.

**Point 5)** There is essentially no information describing the tissues used in the study. For instance, what types of MS plaques were examined? (Were the plaques classified according to Lassmann et al. 1998 J Neuroimmunology?). In several instances in the
paper the authors refer to expression near active plaques. How do they identify whether the plaques were active with respect to demyelination? Were the plaques stained with LFB or some other myelin stain to assess demyelination? This should be described for the MS tissues. Also, more information describing the ALS tissues needs to be included, particularly since the findings in this study are different from those of Almer et al regarding COX-2 expression in neurons.

Response: A full description of the tissues used and their classification (indeed based on the paper cited by the referee above, but with more recent references cited) has been incorporated into the Methods section under ‘Spinal cord’ as follows:

‘Segments of deep frozen human spinal cord were obtained from the rapid autopsy system of the Netherlands Brain Bank (NBB) and MRC Brain Bank, King’s College London, from a total pool of controls (n = 12), ALS (n = 9) and MS (n = 19), for study by immunocytochemistry, Western blotting and autoradiography for peripheral benzodiazepine binding sites. All MS tissues were classified according to the following criteria: 1. preactive lesions - expression of CD68, CD45 on clusters of perivascular microglial cells in white matter with no loss of myelin, 2. active demyelinating lesions, characterized by the presence of macrophages with luxol fast blue or myelin basic protein (MBP) - positive inclusions, and GFAP-positive reactive astrocytes with long processes, in demyelinating regions 3. chronic active - hypocellular demyelinated centre of the lesions containing a small number of CD 68-positive macrophages, with reactive astrocytes localized mainly at the edge of the lesion, or 4. chronic non-active lesions - hypocellular demyelinated lesion with gliosis (as described previously, validated by MRI-guided sampling) (DeGroot 2001; Nijeholt 2001). ALS lumbar spinal cord was obtained post-mortem from patients with clinically and pathologically confirmed sporadic ALS (mean disease duration was 34 months, ranging from 7–84 months; mean age 67 years, range 51–76 years). The post-mortem delay for ALS patients ranged from 4 to 24 h, and for control subjects from 14 to 26 h’.

More information regarding COX-2 expression in neurons is provided in the Discussion, as also requested by Reviewer #2 (see below).
MINOR REVISIONS - (revised text is highlighted in bold and italic)

**Point 1)** Many of the figures are confusing. Perhaps labeling the panels with the antigens examined could help (eg. Fig 1, A—PK1195, B- CD68, E-COX-2, etc)

**Response:** Figures 1, 2, 4, 8, 10, 11, 13, and 15 have been re-labelled as suggested for clarification.

**Point 2)** Figure 3 is confusing. What is stained red and what is stained black? What tissue is presented in E and F?

**Response:** All preparations except those with double immunostaining were counterstained red/pink to highlight nuclei. Text in Methods has been revised as follows:

‘Sites of primary antibody attachment were revealed using avidin-biotin peroxidase method (Vector Labs UK – ABC - black product) and nuclei were counterstained in 0.1% w/v Neutral Red to give contrasting pink/red nuclei but not in double labelling experiments to avoid confusing colours.’

**Point 3)** Labeling “MND” in Figs 4, 11, 13 is not defined in the figure legends.

**Response:** “MND” in the figures has been changed to ALS to be consistent with the legends.

**Point 4)** The results presented in Fig 9A are different than described in the text and Fig legend. Not all the COX-2 co-localizes with Ferritin (as seen left side of Figure).

**Response:** Text in Co-localisation studies page 12 has been revised as follows:

‘Immunostaining with a mixture of antibodies to ferritin and COX-2 or P2X7 showed that the majority of cells with red (P2X7 – Fig 10A; COX-2 – Fig 10B) immunoproduct also contained black immunoproduct (ferritin), indicating the presence of P2X7 and/or COX-2 in microglia/macrophages.’
Point 5) The writing in the paper could also be improved. The paper needs a stronger rationale for why each experiment was done.

Response: We have revised a considerable amount of redundant text and data which should contribute to the overall reading and writing clarity. We have also introduced a brief rationale per experiment.

The overall rationale is stated in the Abstract: “Therefore, three key molecules known to be expressed in activated microglial cells/macrophages, COX-2, CB2 and P2X7, which plays a role in inflammatory cascades, were studied in MS and ALS post-mortem human spinal cord” The relationship of changes of these potential therapeutic target was therefore studied in these tissues.

Point 6) In many cases the data are presented both in the figures and in the text (eg page 14, CD68 section and Fig 11). In other examples such as on pages 12-13 (CB2 data) should be summarized in a graph or table.

Response: In addition to the above revisions and to improve clarity, repeated data, already shown as figures/graphs, have been removed from the text as follows:

Immunocytochemistry COX-2 pp10 -

‘This was confirmed by overall image analysis. Control spinal cord, n=7, % area, median and range, 0.79 (0.33-1.55), MS spinal cord, n=10, 1.545 (0.51-4.27), P = 0.025, Mann Whitney U test (Fig 2)’

Immunocytochemistry CB2 pp11-

‘control spinal cord, n = 8, % area, median and range, 0.225 (0.11-0.75), MS spinal cord with no apparent lesions, n = 11, 0.19 (0.10-0.52), MS spinal cord with lesions, n = 8, 2.645 (0.51-6.46)’

Immunocytochemistry results CB2 pp12-14
‘dorsolateral white matter % area, 0.159 (0.012-1.116) n = 8; grey matter of the ventral horn % area 0.149 (0.076-1.446) n = 8, P = 0.798’

**P2X7 pp12-**

‘compared to controls: control spinal cord, n=13, % area of immunoreactivity, median and range, 0.2 (0.06-0.96), MS spinal cord, n=6, 2.7 (0.01-4.9)’

**COX-2 pp13-**

‘Control spinal cord % area of immunoreactivity, median and range, 0.79 (0.30-1.55) n = 7, ALS spinal cord 1.73 (1.25-2.28) n =9, P = 0.0012, Mann Whitney U test  In ALS cord the intensity and frequency of the COX-2 staining was significantly greater in the dorsolateral white matter compared to the grey matter or dorsal columns, white matter % area of immunoreactivity, 1.035 (0.70-2.14) n =6; grey matter % area of immunoreactivity 0.375 (0.26-0.85) n=6, P = 0.0087’

‘white matter % area of immunoreactivity, 0.64 (0.25-1.36) n = 7; grey matter % area of immunoreactivity 0.69 (0.22-1.06) n = 7, P = 0.80’

‘control spinal cord in white matter % area, median and range, 0.159 (0.012-1.116) n = 8, ALS spinal cord lesion (dorsolateral white matter) 1.254 (0.154-5.75) n = 8’

‘Mann Whitney U test. Within the ALS cords the CB2 staining was significantly greater in the dorsolateral white matter compared to the dorsal columns and grey matter: dorsolateral white matter % area, 1.254 (0.154-5.75) n =8, dorsal columns 0.096 (0.034-0.34) n = 8, P = 0.0011 and grey matter of the ventral horn % area 0.082 (0.02-1.124) n = 8, P = 0.0059’

**pp14-**

‘In all ALS cord very intense microglial cell/macrophage-like staining associated with long processes were seen with the antibody to CD68 at a titre of 1:750. Staining was almost exclusively localized to the dorsolateral white matter (Fig 4E and F):
dorsolateral white matter lesion % area 8.587 (3.76-13.8) n = 8, ventral horn % area 2.146 (0.825-5.46) n = 7, P = 0.0012. In control spinal cord there was a small significant difference between the two regions, white matter % area 2.979 (1.934-3.824) n = 8, ventral horn % area 2.273 (1.002-3.206) n = 8, P = 0.0499. There was a significant increase of CD68 in the white matter of ALS cord compared to controls: ALS 8.587 (3.76-13.8) n = 8, controls, 2.979 (1.934-3.824) n = 8, P = 0.0006. There was a significant increase of CD68 in the white matter of ALS cord compared to controls, (Spearman r = 0.81, P = 0.0218, XY pairs = 8)

CD68 pp14-

‘White matter % area of immunoreactivity 8.97 (6.65-9.19) n = 6, vs. grey matter % area of immunoreactivity 0.665 (0.44-1.43), n = 6, P = 0.0022. In control spinal cord there was no difference between the two layers, white matter % area of immunoreactivity 1.43 (0.74-1.76) n = 7, vs. grey matter % area of immunoreactivity 1.55 (1.25-2.54) n = 7, P = 0.21. There was a significant increase of CD68 in the white matter of ALS cord compared to controls, ALS, 8.97 (6.65-9.19) n = 6, controls, 1.43 (0.74-1.76) n = 7, P = 0.0012 and a significant decrease in the grey matter, ALS, 0.665 (0.44-1.43) n = 6, controls, 1.55 (1.25-2.54) n = 7, P = 0.0047’

P2X7 pp 14-

‘There was a significant increase of P2X7 in the white matter of ALS cord compared to controls, ALS, 2.4 (0.6-5.5) n = 6, controls, 0.2 (0.06-0.96) n = 12, P = 0.0001’
Reviewer #2 Hermann Schluesener:

MINOR REVISIONS - (revised text is highlighted in bold and italic)

Point 1) A sound neuropathological description of the MS lesions………..

Response: This point has been covered in Referee #1 Point 5 above as follows:
A full description of the tissues used and their classification has been incorporated into the Methods section under ‘Spinal cord’ as follows:

‘Segments of deep frozen human spinal cord were obtained from the rapid autopsy system of the Netherlands Brain Bank (NBB) and MRC Brain Bank, King’s College London, from a total pool of controls (n = 12), ALS (n = 9) and MS (n = 19), for study by immunocytochemistry, Western blotting and autoradiography for peripheral benzodiazepine binding sites. All MS tissues were classified according to the following criteria: 1. preactive lesions - expression of CD68, CD 45 on clusters of perivascular microglial cells in white matter with no loss of myelin, 2. active demyelinating lesions, characterized by the presence of macrophages with luxol fast blue or myelin basic protein (MBP) - positive inclusions, and GFAP-positive reactive astrocytes with long processes, in demyelinating regions 3. Chronic active - hypocellular demyelinated centre of the lesions containing a small number of CD 68-positive macrophages, with reactive astrocytes localized mainly at the edge of the lesion, or 4. Chronic non-active lesions - hypocellular demyelinated lesion with gliosis (as described previously, validated by MRI-guided sampling) (DeGroot 2001; Nijeholt 2001). ALS lumbar spinal cord was obtained post-mortem from patients with clinically and pathologically confirmed sporadic ALS (mean disease duration was 34 months, ranging from 7–84 months; mean age 67 years, range 51–76 years). The post-mortem delay for ALS patients ranged from 4 to 24 h, and for control subjects from 14 to 26 h’.

Point 2) Throughout the manuscript, the term “microglial cells/macrophages” should be used, as a clear-cut separation of these populations in the inflammatory lesions is not possible and the use of morphological criteria might be misleading.
Response: All references to microglia have been replaced by ‘microglial cells/macrophages’ throughout the revised manuscript.

Point 3) There are some differences to reports from other groups and these should be discussed: Notably, expression of markers in the endothelia and in neuronal cells. This is of course important to the rather bold process of image analysis. Staining by peroxidase is non-linear. In addition, other cell populations might contribute. Further, information on cell-populations with different staining intensity will lost during this process.

Response: Differences and new references (34 and 36) to other reports regarding neuronal and endothelial localisation are included in the 2nd and 3rd paragraphs of the discussion page 15 as follows:

‘COX-2 over-expression in the spinal cord of patients with ALS has also been shown to be present in neurons and glial cells of the CNS,(34- Maihöfner et al 2003) and some reports have shown that COX-2 immunoreactivity in normal rats is localised to neurons of laminae II-III, motoneurons of lamina IX and glial cells [6, 32]. In support of this we have found COX-2 immunoreactivity in our studies of human and rat peripheral nerves using the same methods and antibodies as the present study (36 - Durrenberger et al 2004). We did not detect neuronal associated COX-2 immunoreactivity in any of the spinal cord specimens studied including the control spinal cords, in which only a few microglial cells/macrophages with few processes were COX-2 -immunoreactive. These contrasting results between laboratories may reflect differences in methods and/or reagents’.

Point 4) Western blotting: The authors state that: “Similar results were obtained on two separate occasions”. This is a somehow strange statement. Were there more “occasions” with different results? The authors should rephrase this sentence appropriately.

Response: There were not any more occasions of different results. The following statement has been added to the Methods in the revised manuscript: ‘Because of
loading limitations due to the number of wells on the comb it was necessary to perform Western blots on more than one occasion in order to increase number of patient extracts for statistical validity. Our results and controls were reproducible on each occasion.

**Point 5**) The concluding wake-up call for ‘urgent evaluation……..

**Response:** "urgent" has been removed from concluding sentence.