Author's response to reviews

Title: Mechanisms of Escape Phenomenon of Spinal Cord and Brainstem in Human Rabies

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Author's response to reviews: see over
Dear Biomedcentral Editorial Team,

**In response to Prof. Anthony Fooks’ comments.**

1. Tables 2 and 3 are not user-friendly to the reader and could be improved. Where there is discrepancy between rabies antigen and cytochrome c positive cells in brainstem (midbrain, pons, medulla) and spinal cord (cervical, thoracic, lumbar and sacrum), the numbers will be shown in bold and italic.

2. The description of antigen positive and apoptotic cells requires additional description to avoid using a scale measurement (0-4), which is a bias. I suggest that stained cells are included to indicate apoptotic staining and antigen distribution.
   Scale measurements of 0 – 4 were based on the followings:
   - 0: no antigen positive neuron in all fields
   - 1: 1 – 25% antigen positive neuron(s) in the whole section
   - 2: 26 – 50% antigen positive neurons in the whole section.
   - 3: 51 – 75% antigen positive neurons in the whole section.
   - 4: 76 – 100% antigen positive neurons in the whole section.
   This clarification was also inserted in the text.

3. The authors need to clarify how cells are shown to be neuronal and not another cell type. Which specific cell markers were used? We serially sectioned the specimen and had them stained as follows: H&E, rabies antigen, cytochrome c, TUNEL, neurofilament protein (as a neuronal marker). Number or density of neurons in each slide was examined by H&E and was found to be correlated with neurofilament immunostaining technique.

**In response to Prof. Bernhard Dietzschold’s comments.**

1. Only double staining of brain sections for RV antigen and apoptosis markers would allow a direct comparison of RV infection and apoptosis in neurons. It is not clear from the description of the methods and results whether double staining was actually performed.
   Double labeling was not performed since we were using immuno-histochemistry-based technique (not immunofluorescence). Cytoplasm of many neurons in regions of interest (brainstem and spinal cord) contained massive amount of rabies antigen, thus, precluding us from visualizing other label, i.e., cytochrome c. TUNEL staining revealed that almost all neurons in all CNS regions were apoptotic.
   The way we can solve this is to serially section the specimen and having them stained as follows: H&E, rabies antigen, cytochrome c, TUNEL, neurofilament protein (as a neuronal marker). Number or density of neurons in each slide was examined by H&E and was found to be correlated with neurofilament protein (NF) immunostaining technique. As the density of neurons in H&E section parallels that of the neurofilament
protein-stained section, we assume that slides stained with other antibodies, which were in between the H&E and NF sections, would have the same density of neurons. In brainstem and spinal cord regions, rabies antigen positive neurons were numerous (mostly 3 or 4+). This was in contrast with cytochrome c positive neurons in the same region (0 or 1+). We then had the conclusion that many of the rabies positive neurons were actually cytochrome c negative.

2. The paper would greatly benefit from the presentation of figures showing immunostained sections obtained from representative regions of the CNS. Figures of rabies positive-, cytochrome c positive- neurons and TUNEL staining were included.

3. Instead of the subjective plus/minus scoring of stained section shown tables 1 and 2, a morphometric analysis(e.g., cell counts.mm2 plus statistical analysis) would be more appropriated to support the conclusions drawn in this paper.
   We did not perform a morphometric analysis.
   Scale measurements of 0 – 4 were based on the followings:
   0- no antigen positive neuron in all fields
   1- 1 – 25 % antigen positive neuron(s) in the whole section
   2- 26 – 50% antigen positive neurons in the whole section.
   3- 51 – 75% antigen positive neurons in the whole section.
   4- 76 – 100 % antigen positive neurons in the whole section.
   Although we realized that this measurement might not be of highest accuracy, the results obtained were striking in terms of density of cytochrome c and rabies antigen positive neurons in brainstem and spinal cord, thus, should be sufficient to draw the conclusion.

All inserted contents were underlined in the revised manuscript.

We greatly appreciate your kind consideration and useful comments of both reviewers.

With our warmest regards,

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