Astrocyte and Microglia Activation in the Pre-Bötzinger Complex

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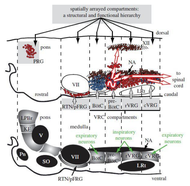
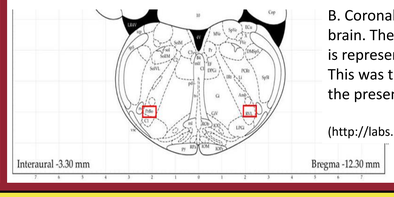
Background

Abstract: *Ventilatory acclimatization to hypoxia (VAH) is defined as the time-dependent increase in ventilation which occurs with sustained chronic hypoxic exposures of several hours to months. Through previous research findings, it has been determined that astrocytes and microglia become activated upon exposure to hypoxic conditions and may be responsible for VAH. The pre-Bötzinger complex is hypothesized to generate the normal breathing rhythm in mammals. Through use of the neurokinin-1 receptors localized to the pre-Bötzinger complex in the brainstem, this region of interest can be identified. Additionally, microglia and astrocytes up-regulate expression of Cd11-b and glial fibrillary acidic protein upon activation, respectively, therefore both can be used a marker for identification through the use of immunohistochemistry. Microglia have also been shown to undergo a morphology change with activation, characterized by shorter branch lengths and fewer branch points. In order to assess activation of astrocytes and microglia in the pre-Bötzinger complex, 2-3 rat brain tissue samples were obtained from rats exposed to normoxic conditions, hypoxic conditions for 60 minutes, and hypoxic conditions for 12 hours. Each tissue sample was stained with antibodies against NK1, GFAP, and CD11b (Ox42). Secondary antibodies were used to visualize the neurons containing the NK1 Receptors, which was used to identify the pre-Bötzinger complex, as well as the astrocytes and microglia within the region. Pixel intensity quantification and the Sholl analysis were used to assess astrocyte and microglia activation. The pixel intensity quantification method did not suggest a significant amount of activation of both astrocytes and microglia within the pre-Bötzinger complex; however, the Sholl analysis did show that activation of microglia had occurred in the groups exposed to hypoxic conditions for 60 minutes when compared to the groups exposed to normoxic conditions and to those exposed to hypoxic conditions for 12 hours. Taken together with other research, activation of microglia in this region suggests that they could be releasing cytokines, such as prostaglandin E2 and interleukin-1β, which have been shown to regulate respiratory rhythm and activate neighboring astrocytes, which are also important for generating the respiratory rhythm in the pre-Bötzinger complex.*



A. Respiratory control network and signaling pathways.

(Smith et al., 2009)



B. Coronal section of the rat brain. The pre-Bötzinger complex is represented by the red boxes. This was the region targeted in the present study.

(http://labs.gaidi.ca/rat-brain-atlas/)

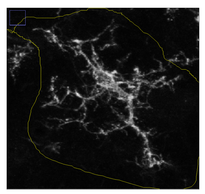
Methods

Immunohistochemistry: Tissue sections were each stained with primary antibodies against NK1, GFAP, and CD11b (Ox42). Secondary antibodies against these primary antibodies were then used to visualize neurons, astrocytes, and microglia within each section, respectively.

Region Acquisition: The region of the tissue section with the heaviest amount of stained neurons, due to the presence of the NK1 receptors, was used to identify the pre-Bötzinger complex.

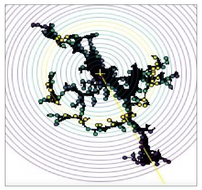
Astrocyte Quantification: Activation was assessed by measuring the mean GFAP intensity within the isolated region, as astrocytes upregulate GFAP upon activation using FIJI by ImageJ software version 2.0.0. The Analyze-Measure tool in FIJI was used to analyze the mean positive area GFAP intensity within the region of interest.

Microglia Quantification: Microglia activation was quantified using ImageJ (FIJI) software version 2.0.0. The extent of activation was measured by analyzing the change in branch proliferation and length using the Sholl analysis plugin in FIJI to assess morphological changes. The number of crossings by each branch was assessed using a starting radius of 2 mm with a step size of 2 mm. The extent of branching at different time points was compared by analyzing the number of crossings at different step intervals. Additionally, the pixel intensity quantification method used to analyze mean GFAP intensity was also used to analyze mean Ox42 intensity within the cropped region of interest.

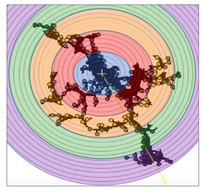


A. Microglia captured using ImageJ (FIJI) software version 2.0.0 from a cross section of rat brain tissue.

B. Microglia image with background and non-specific staining removed.

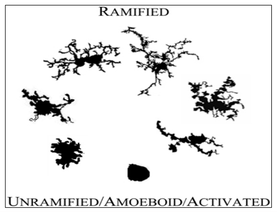


C. Microglia image with Sholl analysis applied.

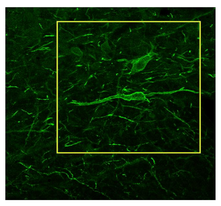


D. Color coding of Sholl brackets.

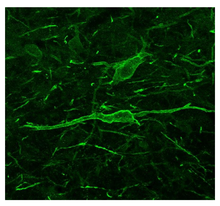
Legend
0-10 μm
11-20 μm
21-30 μm
31-40 μm
41-50 μm



E. Morphological changes of microglia.
(Karperien et al., 2013)

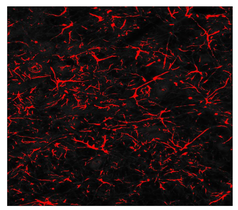


A. Neurons stained with antibodies against the NK1 receptor. The region identified as the pre-Bötzinger complex is within the yellow box.



B. Cropped region of interest from A.

A. Astrocytes stained with antibodies against GFAP.



B. Pixels analyzed using the area analysis protocol. The image was adjusted to exclude excessive background and non-specific staining from the analysis.

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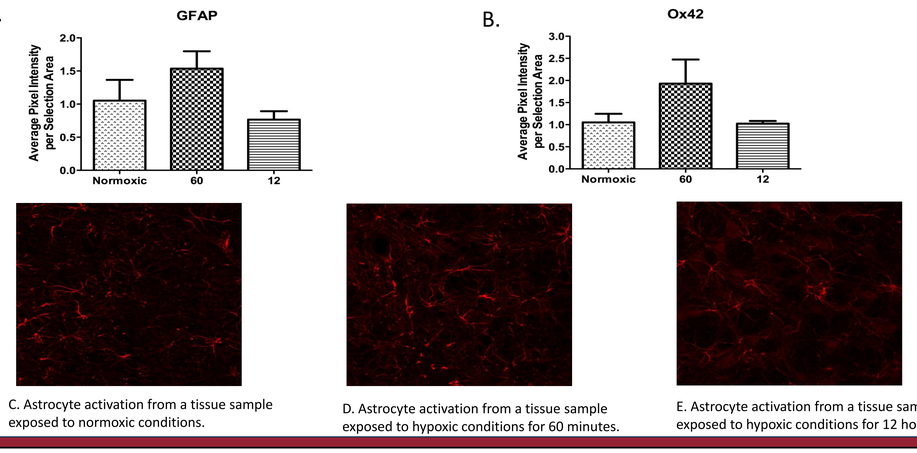
Results

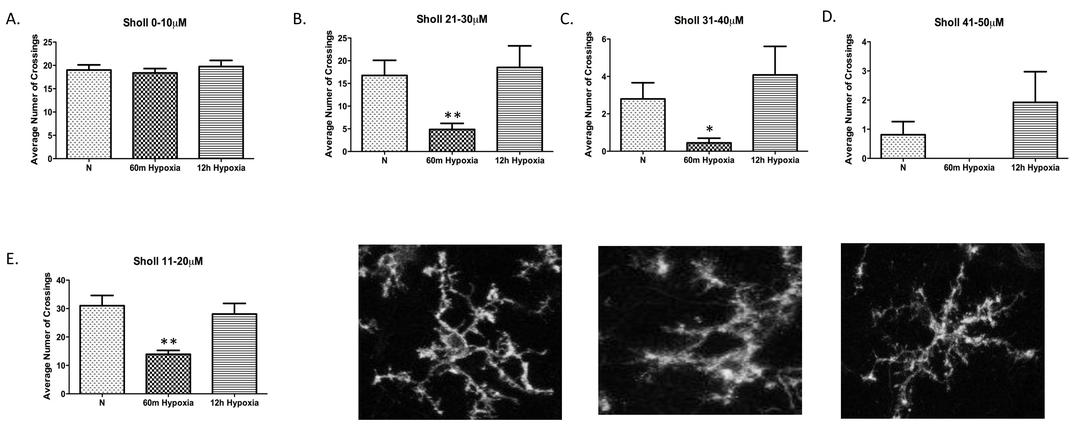


C. Astrocyte activation from a tissue sample exposed to normoxic conditions.

D. Astrocyte activation from a tissue sample exposed to hypoxic conditions for 60 minutes.

E. Astrocyte activation from a tissue sample exposed to hypoxic conditions for 12 hours.

Figure 1: *Pixel intensity quantification of astrocytes (GFAP) and microglia (Ox42).* Although the results do not suggest a significant amount of activation for either cell type, there is a trend towards activation amongst the 60 minute hypoxia groups. A. Quantification of GFAP intensity, p = 0.2495. B. Quantification of Ox42 intensity, p = 0.2520. C,D, E. Images of GFAP fluorescence amongst the three treatments. All data presented as mean ± SEM (1-way ANOVA, n=2-3).



F. Microglia from a tissue sample exposed to normoxic conditions.

G. Microglia from a tissue sample exposed to hypoxic conditions for 60 minutes.

H. Microglia from a tissue sample exposed to hypoxic conditions for 12 hours.

Figure 2: *Sholl analysis of microglia.* A, B, C, D, E. Average number of crossings counted for each treatment group amongst five different step intervals. The results for the intervals 0-10 μm and 41-50 μm do not suggest significant activation of the microglia, $p > 0.05$; however the results for the intervals 11-20 μm, 21-30 μm, and 31-40 μm show a significant decrease in branch end points and branch length for the 60 minute hypoxia group, $p < 0.001$. The Dunnett's post hoc test revealed a significant decrease in branching for the 60 minute hypoxia group when compared to the normoxic group, $^{**}p < 0.01$; the Dunnett's post hoc test revealed a significant decrease in branching for the 60 minute hypoxia group when compared to the 12 hour hypoxia group, $^{*}p < 0.05$. F, G, H. Images of microglia from each of the treatment groups. All data presented as mean ± SEM (1-way ANOVA, n=2-3).

Conclusions

- The results of the pixel intensity quantification do not definitively support activation of microglia or astrocytes; however, there is a trend towards activation.
- The results of the Sholl analysis support microglia activation during the 60 minute hypoxic conditions.
- Additional studies to increase the group number, optimize the pixel intensity method, and the addition of other hypoxic time points would provide more definitive details of the role of non-neuronal cells in the pre-Bötzinger complex during hypoxia.