Morphological Changes in Microglia not Evident at Various Time Points

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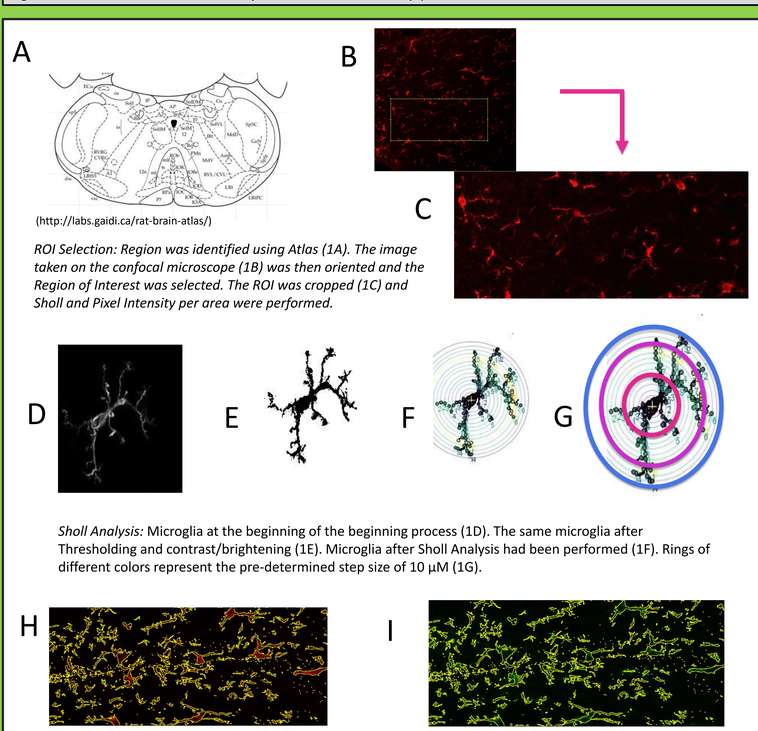
Abstract

The NTS receives and integrates visceral afferent fibers from many systems in the body such as: gustatory, gastrointestinal, pulmonary, and cardiovascular. The NTS is primarily activated when the body is not in homeostasis, specifically while in hypoxic conditions due to increased peripheral chemoreceptor input. In terms of ventilation, while in sustained hypoxia (where the PaO_2 drops below threshold of about 60mmHg), the NTS is activated by stimuli from the peripheral chemoreceptors in the carotid body causing changes in ventilation to maintain homeostasis. (Vassilakopoulos, T., 2011). Understanding when and how the different cell types in this region are activated is pertinent to understanding ventilatory control in hypoxic conditions. Microglia are thought to be upstream of astrocytes so that upon activation, ATP is released, activating nearby astrocytes that proceed to amplify the signal (Pascual et al. 2012). We hypothesize that both astrocytes and microglia will undergo morphological changes due to detection of neurotransmitters and cytokines released from primary afferent neuron of the carotid body. To address this hypothesis, brain tissue of rats exposed to 60-minutes and 12 hours of hypoxia were assessed for glial cell activation in the NTS region. There were no significant data to suggest that the microglia within the tissue sections we observed were activated. This contradicts previous works.

Methods

Rats were exposed to hypoxic conditions and CNS tissue fixed at the University of California San Diego lab of Dr. Frank Powell (Department of Physiology). Microglial cells were visualized using immunohistochemistry. A 5% goat blocking serum was applied for 1 hour to inhibit non-specific antibody binding. These tissues were stained with Iba-1 (rabbit) and Ox42 (mouse), 1° antibodies for 48 hours at a 1:1000 concentration in 1% goat PBS-TX diluent. These tissues were washed in 0.1% PBS-TX 6 x 5 minutes. 2° antibodies, 594 goat anti rabbit and 488 goat anti-mouse, were added at a 1:500 concentration in 1% goat PBS-TX diluent for one hour. Tissue was mounted on slides and let dry. Prolong-Diamond with DAPI nuclear stain was used as the mounting media. Images were taken at LSU-HSC and analyzed using FIJI/ImageJ. To analyze the images, Sholl analysis and fluorescent intensity per pixel area were performed, as depicted below.

***Figure 1**: ROI Selection, Sholl Analysis, and Pixel Intensity per area*



ROI Selection: Region was identified using Atlas (1A). The image taken on the confocal microscope (1B) was then oriented and the Region of Interest was selected. The ROI was cropped (1C) and Sholl and Pixel Intensity per area were performed.

Sholl Analysis: Microglia at the beginning of the beginning process (1D). The same microglia after Thresholding and contrast/brightening (1E). Microglia after Sholl Analysis had been performed (1F). Rings of different colors represent the pre-determined step size of 10 µM (1G).

Pixel Intensity per area: The ROI was cropped. Using a FIJI/ ImageJ tool, the region was threshholded and a mask was made (1H). The mask was then put on a duplicated image of the region (1I) and analysis was performed.

Results

***Figure 2:** Average Branch Crossings of Microglia stained with Iba-1 and Ox42..*
Graphs showing rats exposed to normoxic conditions (2C), 60-minute hypoxia (2F), and 12-hour hypoxia (2I). All data presented as mean ± SEM. (n=2-3 animals/group; all p>0.05, 2-way ANOVA). There was no significant difference in the branching patterns between the two antibodies as defined by Sholl Analysis and the 2-way ANOVA performed. Figures 2A and 2B are the same normoxic condition microglia, 2D and 2E are the same 60 minute hypoxic condition microglia, and 2G and 2H are the same 12 hour hypoxic condition microglia, but visualized with different antibodies: Iba-1 and CD11b [Ox42] as labeled on the image. Taken together, this suggests that Iba-1 and Ox42 are staining the same cell and can be used for the same morphological analysis.

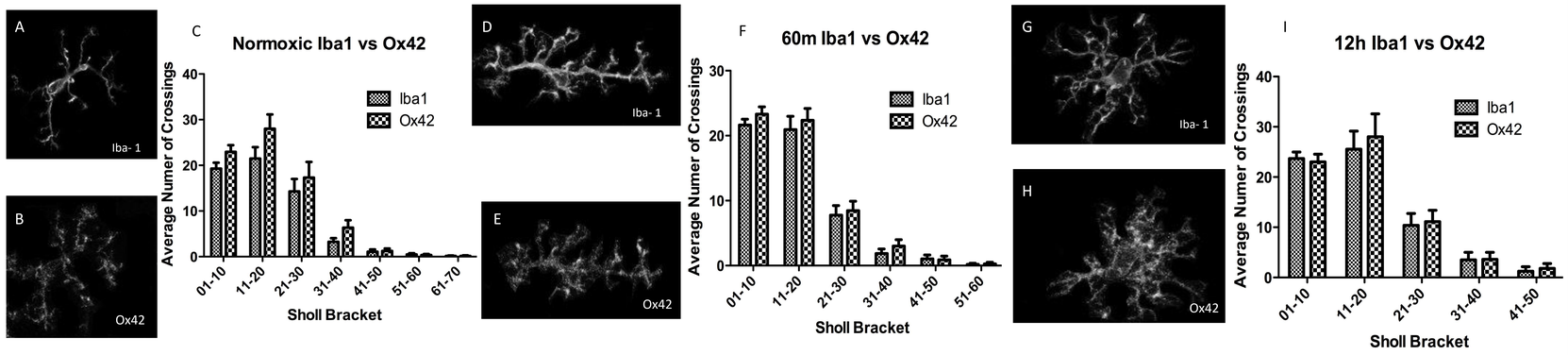
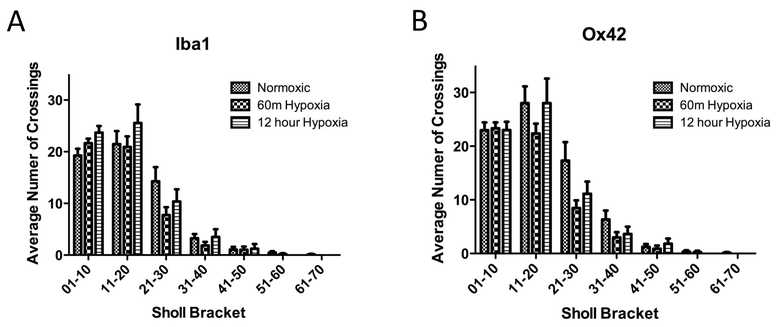


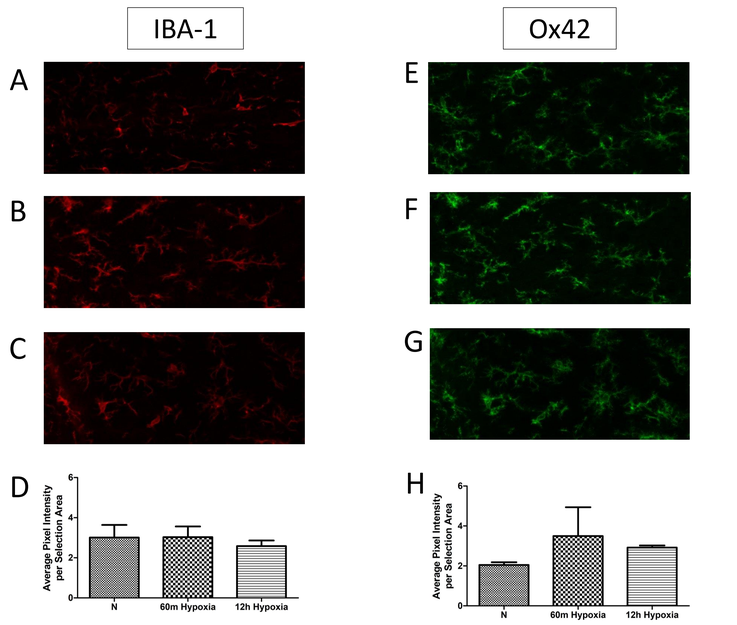
Figure 3: *Sholl Analysis Comparing Treatments Showing Iba-1 and Ox42.*
Figure 3A shows the average branch crossings of microglia in tissue showing Iba-1 in normoxic conditions, exposed to 60-min hypoxia, and exposed to 12-hour hypoxia. Figure 3B shows the average branch crossings of microglia in tissue showing CD11b [Ox42] in normoxic conditions, 60-min hypoxia, and 12-hour hypoxia. All data presented as mean ± SEM. (n=2-3 animals/group; all p>0.05, 2-way ANOVA). The lack of morphological changes observed indicate that the microglia did not shift to the more amoeboid shape with hypoxic exposure.



Conclusions

- Both Iba-1 and CD11b [Ox42] antibodies can be used to visualize microglia and assess branching patterns.
- The lack of morphological changes observed indicate that the microglia did not change from the ramified to a more amoeboid state during the chosen hypoxic time points.
- In the Area Intensity Analysis, CD11b [Ox42] showed increase in fluorescent signal at 60-minute Hypoxia indicating increase protein production.

Figure 4: Fluorescent intensity per pixel area
Images show tissue stained with Iba-1 during normoxic conditions (Figure 4A), 60-min hypoxia (Figure 4B), and 12-hour hypoxia (Figure 4C). Figure 4E, Figure 4F, and Figure 4G show tissue stained with Ox42 at the same time points, respectively. The graphs show the average pixel intensity normalized to the average area for tissue stained with Iba-1 (4D) and Ox42 (4H). All data presented as mean ± SEM. (n=2-3 animals/group; all p>0.05, 1-way ANOVA). There were no significant increases or decreases in pixel intensity as seen by use of one-way ANOVA. This indicates that there was little to no increase in protein production.



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