Microglia Activation and IL-1β Expression in Select Ventilatory Control Regions Following Exposure to Chronic Sustained Hypoxia

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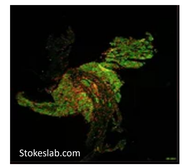
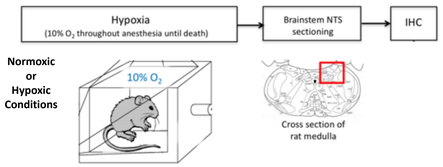


Abstract and Background

Abstract: Ventilatory acclimatization to hypoxia (VAH) is defined as the time-dependent increase in ventilation which occurs with chronic sustained hypoxia (CSH) of several hours to months. Previous research has shown that astrocytes and microglia become activated upon exposure to hypoxic conditions and may contribute to VAH. Understanding when and how the different cell types in respiratory control regions are activated is pertinent to understanding ventilatory control during hypoxic conditions. There are a number of ventilatory control regions in the brainstem, but the two regions of recent interest in our lab are the nucleus tractus solitarius (NTS) and the Pre-Bötzinger Complex (PBC). During CSH, the NTS is activated by stimuli from the peripheral chemoreceptors in the carotid body causing changes in ventilation to maintain homeostasis, while the PBC is thought to generate the normal breathing rhythm in mammals. The first part of this study aimed to optimize the sholl analysis method to detect morphological changes in microglia. Two different microglia antibodies, Iba-1 and CD11b[Ox42], were compared using sholl analysis to determine which antibody best represents the branching pattern of the individual microglia. Analysis indicated that neither antibody was statistically different from the other in terms of microglia branching ($p < 0.05$), so CD11b[Ox42] was chosen to simplify the immunohistochemistry protocol. The second part of this study assessed the activation of microglia in the NTS and PBC following CSH exposure. Based on previous research, we hypothesized that microglia in the NTS and PBC would be activated, as assessed via a morphology shift to a more amoeboid state, following CSH exposure. To address this hypothesis, rats were exposed to either normoxic, 60-minutes of CSH, or 12-hours of CSH. Microglia morphology was assessed in perfused brainstem tissue via immunohistochemistry, confocal imaging, and image analysis. In the NTS, microglia branching analysis revealed a trend towards a more amoeboid morphology at the 60-minute CSH time point, but only one of the sholl analysis brackets, 21-30μM, was statistically significant ($p<0.05$). In the PBC, microglia branching analysis also revealed a trend towards a more amoeboid morphology at the 60-minute CSH time point with statistically significant ($p<0.05$) branch patterns at the 11-20μM, 21-30μM, and 31-40 μM sholl brackets. A morphology shift of microglia to a more amoeboid state could indicate their localized response to neurotransmitters or cytokines. In the second study we investigated the expression if IL-1β in the NTS region using immunofluorescent labeling. Rat brainstem tissue from normoxic conditions, 15-minute CSH, and 60-minute CSH was labeled with antibodies against IL-1β, GFAP, and Cd11b[Ox42]. IL-1β positive cells were counted in the NTS region. This preliminary set of cell counts (n=2) suggests that there is an increase in IL-1β expression with CHS exposure. Taken together these data provide a great starting point assessing the activation profiles of glial cells and cytokine activity in select respiratory control regions.

Methods

Animals: Rats were exposed to experimental conditions and tissue was perfused and fixed at the University of California San Diego lab of Dr. Frank Powell (Division of Physiology). All animal experiments were carried out according to protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Diego (under the Guide for Care and Use of Laboratory Animals, National Institutes of Health publication 85-23, Bethesda, MD, USA).

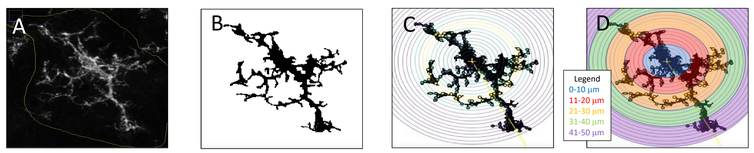


Study 1 – Microglia Morphology:
Microglia – Cd11b[Ox42] and Iba-1
Neurons (for Pre-Bötzinger Complex location only) – NK1R

Study 2 – IL-1β and glial cell localization:
IL-1β
Astrocytes – GFAP
Microglia – Cd11b[Ox42]

Immunohistochemistry: Primary antibodies for microglia used in were Cd11b[Ox42] at 1:1000 (Abcam) and Iba-1 at 1:1000 (Wako), and astrocytes were visualized with GFAP at 1:000 (Abcam), and IL-1β at 1:500 (AbCam). Images were obtained using a Leica SP5 Confocal Microscope analyzed using ImageJ (FIJI).

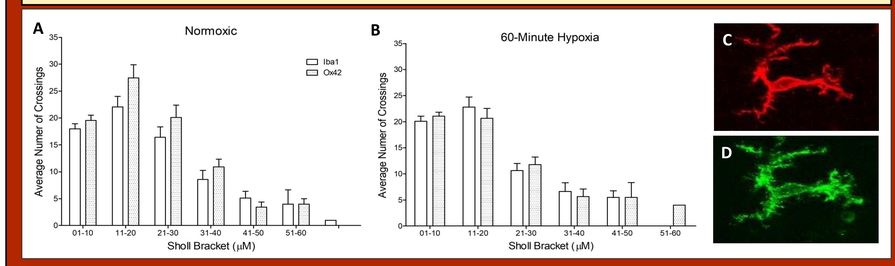
Microglia Morphology Quantification: Microglia activation was quantified using ImageJ (FIJI) software version 2.0.0. The extent of activation was measured by analyzing the change in branch proliferation and length using the sholl analysis plugin in FIJI to assess morphological changes. Individual microglia in the region of interest were isolated (A), cleaned (B) and then analyzed using the sholl analysis plug-in (C-D). 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 (D).



IL-1βb positive cell counting: IL-1β positive cells were counted in the NTS region. Only clearly defined cell-like regions of IL-1β positive fluorescent expression were counted.

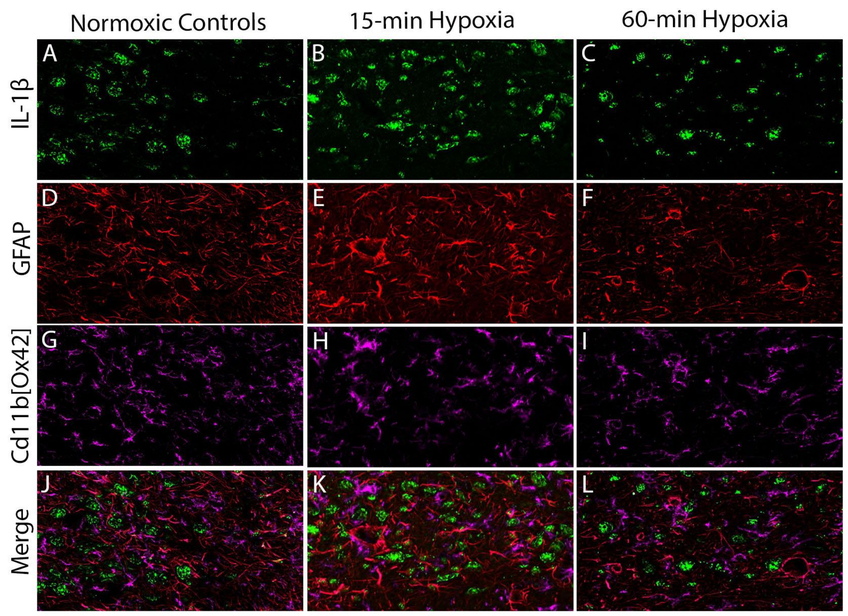
Results

Figure 1: Comparison of microglia morphology using Iba-1 and Cd11b[Ox42] antibodies. Microglia branching was assessed using sholl analysis and the average number of branch crossings were calculated per sholl bracket (A, B). There was no significant difference in the branching patterns between the two antibodies as defined by sholl analysis, assessed by a 2-way ANOVA ($p > 0.05$). Confocal images of the same microglia visualized with Iba-1 (C) and Cd11b[Ox42] (D) antibodies are shown. This study suggests that Iba-1 and Cd11b[Ox42] are labeling the same microglia and yielding similar branching patterns both in normoxic (A) and hypoxic conditions (B). All data presented as mean ± SEM; n=2-3 animals/group.



A morphology shift of microglia to a more amoeboid state could indicate their localized response to neurotransmitters or cytokines. In the figures below we investigate the expression if IL-1β in the NTS region.

Figure 3: Immunofluorescent imaging of IL-1β, astrocytes, and microglia in the NTS region. Rat brainstem tissue from normoxic conditions, 15-minute CSH, and 60-minute CSH was labeled with antibodies against IL-1β (A-C), GFAP (D-F), and Cd11b[Ox42] (G-I). Images were obtained using a Leica SP5 confocal microscope with a 40x oil immersion objective, and a 10uM Z-stack with images every 0.2uM. The image below is a Z-projection of the entire image stack (by ImageJ). The merged images (J-L) do not suggest overlap of the secondary fluorophores, therefore a colocalization analysis was not performed.



Future Studies

- Additional section analysis which will increase the n for each of the conditions, as well as analysis of microglia cell body size and endpoint number.
- Inclusion of antibodies for neurons (NeuN, Map2)
- Additional cytokines (TNF-α, IL-6) will also be visualized.

Figure 2: Sholl analysis of microglia in the NTS and PBC. The average number of branch crossings were calculated for each treatment group (normoxic, 60-minute hypoxia, and 12-hour hypoxia) across five different sholl brackets: 0-10 μM, 11-20 μM, 21-30 μM, 31-40 μM, and 40+ μM (not shown). In the NTS (A), sholl bracket 21-30 μM shows a significant decrease in branch crossings for the 60-minute hypoxia group compared to the normoxic (N) group ($p < 0.05$). In the PBC (B), sholl brackets 11-20 μM, 21-30 μM, and 31-40 μM show a significant decrease in branch crossings for the 60-minute hypoxia group compared to the normoxic (N) group ($p < 0.05$). All data presented as mean ± SEM; n=2-3 animals/group; 1-way ANOVA with Dunnett's post-hoc test; *$p < 0.05$.

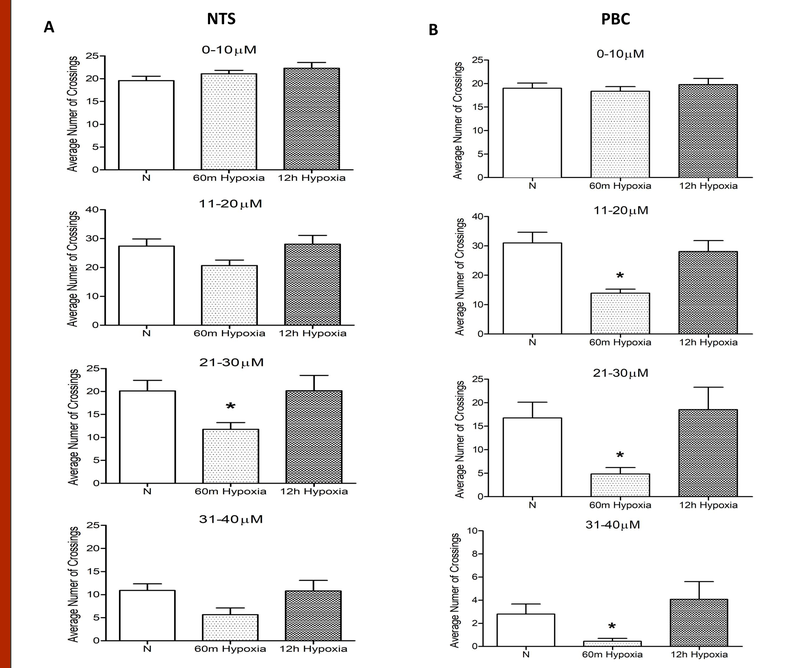
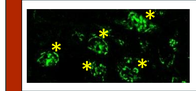
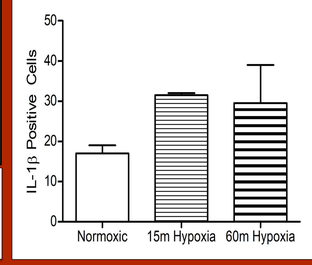


Figure 4: Preliminary data of IL-1β positive cell counts in the NTS region. IL-1β positive cells were counted in the NTS region. Only clearly defined cell-like regions of IL-1β positive fluorescent expression were counted. Normoxic, 15-minute, and 60-minute CSH tissue was assessed. This preliminary set of cell counts (n=2) suggests that there is an increase in IL-1β expression with CHS exposure.



Cells identified by a yellow asterisk (left) are examples of the clearly defined cell-life regions that would be included in the IL-1β positive cell count.



Conclusions

- The first study (Figure 1) indicates that both Iba-1 and CD11b[Ox42] antibodies can be used to visualize microglia and assess branching patterns.
- The second study (Figure 2) suggests a morphology shift of microglia during hypoxic exposure in two different central respiratory control regions during an early CSH time point of 60 minutes. These data support previous findings from our lab (Stokes et al. 2017).
- Cytokine IL-1β is expressed in the NTS region (Figure 3) and a trend of an increase in IL-1β protein expression was observed with hypoxia (Figure 4).

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