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UNIVERSITY OF CALIFORNIA, SAN DIEGO

**Time Course of Brainstem Glia Activation in Rat Respiratory Centers Following
Exposure to Chronic Sustained Hypoxia**

A Thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Biology

by

Tara Arbogast

Committee in charge:

Professor Frank Powell, Chair
Professor Brenda Bloodgood, Co-Chair
Professor Randolph Hampton

2016

The Thesis of Tara Arbogast is approved and it is accepted in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2016

DEDICATION

This is dedicated to my family. To my mom, brother, and dad, for their constant support and encouragement to chase my dreams.

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Lastly, I would like to thank all members of the Powell lab: Esteban, Tatum, Erica, Zhenxing, Steve, Meredith, and Maria for your support and guidance.

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ABSTRACT OF THESIS

Time Course of Brainstem Glia Activation in Rat Respiratory Centers Following
Exposure to Chronic Sustained Hypoxia

by

Tara Arbogast

Master of Science in Biology

University of California, San Diego, 2016

Professor Frank Powell, Chair

Professor Brenda Bloodgood, Co-Chair

Neuron-glia communication in the central nervous system is emerging as an important component of neuronal signaling in respiratory control centers of the brainstem. To investigate glial cell contributions to ventilatory acclimatization to hypoxia, I measured the time course of activation of microglia and astrocytes' neural circuits that control breathing following different lengths of exposure to sustained hypoxia (10% O₂). Rats were exposed to sustained hypoxia at the following time points: 30 minutes, 1-, 4-, and 24-hours, 4- and 7-days. Activation of glial cells was measured in two respiratory control centers: the nucleus tractus solitarius (NTS), a sensory integrative center known to be important for ventilatory acclimatization to hypoxia, and the

hypoglossal (XII) motor nucleus, a respiratory motor nucleus adjacent to the NTS.

Microglia (Iba-1 positive cells) show an active morphology (shorter and fewer branches measured with image analysis, IMARIS) after 1 hour of hypoxia in the NTS and XII and return to the resting ramified state (longer filaments and extensive branching) after 4 hours of hypoxia. The number of microglia stayed constant throughout activation.

Astrocytes (glial fibrillary acidic protein, GFAP positive cells) increased GFAP antibody immunofluorescent intensity after 4 hours of sustained hypoxia in the NTS but not until 7 days in XII. Astrocyte proliferation was only observed after 7 days of sustained hypoxia in the hypoglossal motor nucleus. These results show that glial activation with sustained hypoxia occurs sequentially with microglia activation preceding astrocyte activation and a time course that is unique in different medullary respiratory centers. This indicates an important role for glia in neural plasticity with sustained hypoxia and ventilatory acclimatization to hypoxia.

INTRODUCTION

Physiological response to hypoxia

Hypoxic conditions resulting from high altitude or in certain diseases of the heart and lung elicit a physiological increase in ventilation that facilitates acclimatization (Powell et al. 1998). Acclimatization is the body's way of maintaining adequate oxygen levels for effective function as the environment changes. The animal response to high altitude or hypoxia is tissue oxygen deprivation. Following hypoxia, ventilation, or breathing, is increased in a time-dependent manner. Breathing reaches an initial elevation after only a few minutes of hypoxia, followed by a slight decline in ventilation levels. However, within a day or two of hypoxia, ventilation approaches a second steady elevation called the ventilatory acclimatization to hypoxia (VAH) (Weil, 1986; Bisgard and Neubauer, 1995; Powell et al. 1998). The phenomenon of increased ventilation persists for days even after return to normal oxygen levels. The mechanisms behind the time-dependent increase in ventilation are not completely clear, but they involve neural plasticity in carotid bodies and the central nervous system (CNS) (Kumar and Prabhakar, 2012; Pamenter and Powell, 2016). Such plasticity may involve glia as well as neurons, considering that glia cells in the brainstem respiratory centers are involved in the signaling to increase ventilation (Gourine et al. 2010).

In response to carotid-body oxygen-sensing cells in the peripheral nervous system, neurons are activated in the central nervous system. In the central nervous system, neuromodulators released by stimulated neurons will activate neighboring glial cells to further propagate the signal by an unknown mechanism that increases ventilation

and allows the body to acclimatize (Bezzi and Volterra 2001; Croft et al. 2015). Recent findings show that glia cells are necessary for the acclimatization to hypoxia, as pharmacological inhibition of microglia blocked ventilatory acclimatization (Tadmouri et al. 2014). Understanding the neuronal and glial signaling involved for the time-dependent increase in ventilation following chronic sustained hypoxia will aid in the diagnosis of respiratory diseases and give insight to the time points of pharmacological treatment.

In rats, ventilatory acclimatization occurs after 48 hours of hypoxic exposure and remains elevated at 7 days of hypoxia (Dwinell et al. 2000). To understand the neuronal and glial signaling behind the increase in ventilation, it is necessary to recognize that the signaling starts with chemosensitive neurons from the peripheral nervous system. These neurons, located in the carotid body (a very small organ with high blood flow and chemosensitive neurons located at the bifurcation of the carotid artery), synapse with neurons in the central nervous system at the nucleus tractus solitarius (NTS) (Pamenter and Powell, 2016). Hence, the NTS is a known respiratory center in the brainstem that acts as a relay station to convey signals for breathing to other respiratory centers that ultimately signal motor neurons to increase ventilation (Figure 1). Another important respiratory center is the PreBötzinger complex, which is responsible for generating the central respiratory rhythm. Projections from these respiratory control centers innervate the phrenic motor nucleus in the cervical spinal cord, which innervates the diaphragm, causing it to contract and initiate inspiration (McCrimmon et al. 1995).

Cell types involved in acclimatization to hypoxia

Glia

Glia is a broad term identifying microglia, astrocytes, oligodendrocytes and ependymal cells that share the common function of maintaining homeostasis in the central nervous system (Parronchi and Verkhratsky 2012). The specific functions of microglia (surveyors of the environment) and astrocytes (homeostasis), give clues to their probable roles in regulating ventilation. Since glia tend to surround neuronal synapses and adjacent blood vessels in respiratory control centers, there is strong reason to believe they can affect breathing (Bezzi and Volterra 2001). Processing information by responding to and releasing neuromodulators would allow glia to make large adjustments in breathing (Dall'Arcangelo et al. 2013). Studies that inhibit microglia in the NTS result in a lack of the ability of the animal to acclimatize to hypoxia (Tadmouri et al., 2014), proving they are necessary for acclimatization. In fact, microglia activation with 24 hours of sustained hypoxia in the NTS correlates with the increase in ventilation with ventilatory acclimatization to hypoxia (Tadmouri et al. 2014)

Microglia are known as scavengers that clean up extracellular space and release cytokines during an inflammatory response (Bezzi and Volterra 2001). They normally exist in a resting state and scan the central nervous system environment for immune and pathological signals. Once activated, microglia gradually morph to an amoeboid, phagocytic form that aims to restore the normal function of the central nervous system (Biber et al. 2007). This morphology shift from ramified (extended) to reactive

(amoeboid) can be visualized using immunolabeling techniques and quantified with image analysis.

Similarly, astrocytes play a role in the increase in ventilation following hypoxia. The role of astrocytes in ventilatory signaling may be through relaying important hypoxic signals from the surrounding vasculature (e.g. low PO_2 , high PCO_2) to neighboring astrocytes, microglia or neurons. Astrocytes are a heterogeneous population, with a variety of different functions. Some astrocytes provide nutrients to neurons or uptake remaining neurotransmitters in the synaptic cleft released by neurons; others release proinflammatory molecules that aid in the hypoxic ventilatory response (Funk et al. 2015). One type of astrocyte is chemosensitive, responding to physiological decreases in pH by releasing ATP. As it senses high PCO_2 and/or low pH, the astrocyte experiences a rise in its intracellular calcium stores that initiates the release of more ATP. The release of ATP activates surrounding astrocytes and respiratory neurons (Gourine et al. 2010). Based on an astrocyte's location in respiratory control centers and their role in acclimatization, it is hypothesized that astrocyte plasticity plays a role in information processing during hypoxia. Astrocytes may be processing the information they receive from the surrounding hypoxic blood flow more efficiently through their mechanism of plasticity, allowing for acclimatization to hypoxia (Croft et al. 2015).

Neuron to glia signaling

Following hypoxia, the primary afferents of chemosensitive neurons in the carotid body terminate in the NTS causing neuronal release of neurotransmitters (Pamenter and

Powell, 2016), which potentially could activate glia. Microglia are also present in the NTS and the crosstalk that exists between neurons and glia is necessary for the acclimatization to chronic sustained hypoxia (Tadmouri et al. 2014). Neuromodulators released by neurons include glutamate, adenosine and noradrenaline (Bezzi and Volterra 2001). Glutamate can bind and activate astrocytic metabotropic glutamate receptors on astrocytes provoking a rise in intracellular Ca^{2+} concentrations (Bezzi and Volterra 2001). Glia can also be activated by dopamine, acetylcholine and GABA, a precursor to glutamate, released by postsynaptic terminals of neurons and all of these neuromodulators are found in respiratory centers (Stornetta 2008).

Glia to glia signaling

Once activated, glia release gliotransmitters such as Ca^{2+} , glutamate, ATP, prostaglandins or nitric oxide that propagate the signal to activate surrounding glia (Bezzi and Volterra 2001). Communication between glia via gliotransmitters allows for amplification of a signal such as hypoxia. If microglia are activated they may be relaying the signal to astrocytes as well as having a positive feedback mechanism to activate more microglia. Small amounts of ATP released from activated microglia can activate astrocytes and additional microglia (Pascual et al. 2012).

During hypoxia, an inflammatory response contributes to acclimatization as demonstrated by nonsteroidal anti-inflammatory drugs (e.g. ibuprofen) blocking ventilatory acclimatization in rats (Popa et al. 2011) and blunting it in humans (Basaran et al. 2016). During an inflammatory response, activated microglia also communicate

with astrocytes by releasing inflammatory cytokines: tumor necrosis factor- α (TNF- α) and interleukins (IL)-1 β and IL-6 (Bezzi and Volterra 2001). In response to IL-1 β , for example, a greater number of astrocytes show increased calcium waves indicating excitation and therefore increased neuromodulators being released by the astrocytes (Bezzi and Volterra 2001). Therefore, the inflammatory cytokines act to amplify the hypoxia signal to increase the ventilatory response.

Glial cell activation in the brainstem following hypoxic exposure

It is known that hypoxia plays a role in modulating inflammatory gene expression and initiating the release of protective cytokines from microglia to defend against cell damage from lack of oxygen; however, the role that microglia and astrocytes in the brainstem play in acclimatization is not yet established (Smith et al. 2013). Microglia in the respiratory control centers of the brainstem mediate acclimatization to hypoxia (Tadmouri et al. 2014; Smith et al. 2013). Preliminary work from our lab using a microglia inhibitor, minocycline, shows that microglial activation is necessary for ventilatory acclimatization to hypoxia. Additionally, astrocyte activation was also inhibited at both 24 hours and 7 days of hypoxia when microglia were inhibited (unpublished lab results). Therefore, astrocyte activation is dependent on microglia activation indicating communication occurs between the two cell types. This supports other findings of microglial activation modulating astrocyte activation (Pascual et al. 2012). These results led us to address the timeline of activation of glia cells in respiratory centers of the brainstem. Understanding the glial activation timeline will determine the

order of activation and the amount of hypoxic exposure necessary for glial activation to persist. Neuronal and glial signaling mechanisms behind the time-dependent increase in ventilation following chronic sustained hypoxia will give rise to possible new treatments of respiratory diseases that can target time specific microglial signaling pathways.

MATERIALS AND METHODS

Animals

Animal experiments were conducted according to the protocols established by the Institutional Animal Care and Use Committee of the University of California, San Diego. Male, Sprague Dawley, rats were purchased from Harlan at 225-250 grams and were housed two per cage in a vivarium maintaining a 12-hour light/dark cycle with lights on at 6am. Experiments of 4 hours and shorter were carried out during the light cycle, 12 hours was carried out primarily in the dark cycle, and experiments of 24 hours and greater were carried out in the vivarium's 12-hour light/dark cycle. For experiments of 12 hours and greater, animals received food and water ad libitum.

Hypoxic Exposure

Rats were exposed to hypoxic conditions using two types of chambers depending on the length of time exposed. Time points of hypoxia exposure include: 30 minutes, 1 hour, 4 hours, 12 hours, 24 hours, 4 days, and 7 days. For shorter time points of four hours or less, rats were placed in a chamber with constant air flow maintained at 10% O₂. Longer time points of 12 hours or more required a hypoxic hypobaric chamber with 10% O₂, 0.5 atm (PO₂ = 80 Torr). Animals were weighed at the start of each experiment and at the end of experimental time points of 12 hours or greater. Animals were sacrificed at the end of each time point.

Perfusion and Tissue Fixation

At the end of each time point, rats were anesthetized with 0.2 mL of Fatal-plus sodium pentobarbital solution and transcardially perfused with 0.9% saline/ 0.004% heparin followed by 4% paraformaldehyde (PFA). The brainstem was extracted and postfixed in 4% PFA overnight and then transferred to 30% sucrose prior to sectioning. Brainstems were mounted with OCT using a flash-frozen technique. Frozen brainstems were then sectioned transversely in 40 μm sections on a Leica cryostat. Brainstem sections were kept in antifreeze cryoprotectant solution until immunofluorescent staining.

Immunofluorescent staining

Free floating 40 μm brainstem sections were incubated in a block of 5% goat phosphate buffered saline (PBS) with 0.1% Triton (X-100). A double fluorescent label was done by incubating tissue at 4°C for 48 hours using antibodies against GFAP (generated in chicken 1:2000, Life Technologies) and Iba-1 (generated in rabbit 1:2000, Wako) to visualize astrocytes and microglia, respectively. Sections were incubated for one hour at room temperature in Cy3 goat anti rabbit (1:2000) and 488 goat anti chicken (1:2000) secondary antibodies. The sections were mounted on glass slides and cover slipped using ProLong Gold Anti-fade with DAPI.

Confocal

A Leica SP5 confocal system was used to image the brainstem sections. An objective lens with a magnification of 20x was used to image all slides used for quantification of glial activation in the NTS and hypoglossal (XII) motor nucleus (Figure

4). A z-stack image of the middle 15 μm was acquired. All images that were compared to each other were imaged on the same day. All settings were the same (e.g. laser intensity and gain).

Astrocyte Quantification

Astrocyte quantification was done using ImageJ (FIJI) software v. 2.0.0-rc-48/1.50i freely available by the NIH. The groups were coded during quantification in order to maintain integrity of the results. Astrocyte activation is quantified by an increase in mean glial fibrillary acidic protein (GFAP) immunofluorescent intensity, this is based on previous work showing astrocytes upregulate GFAP protein during activation (Eng and Ghirnikar 1994; Anon n.d.). Fluorescence intensity was measured using the Analyze-Measure tool in FIJI to analyze the mean fluorescence intensity of the GFAP positive area within a selected region. All images were background corrected based on their own fluorescent staining by subtracting the individual image's background region from the GFAP positive selection. Areas assessed were the NTS and the hypoglossal (XII) motor nucleus region. Based on the preliminary results of time points from the NTS, selected time points of interest where microglia or astrocyte activation could occur were analyzed in the hypoglossal (XII) motor nucleus. To address the fact that astrocytes may be proliferating, an astrocyte count was performed.

Microglia Quantification

IMARIS software, by Bitplane, Oxford Instruments, v. 8.2, was used to quantify the microglia morphology. Using the IMARIS Filament Tracer package, the microglia filaments were traced through the three-dimensional plane of the z-stack image creating a

red skeleton overlaying the blue immunofluorescent staining. The NTS or hypoglossal regions were selected and parameters to assess microglia activation included amount of branching (measured by quantifying the average number of endpoints per microglia) and the average length of branches per microglia.

Statistics

All statistics are based on raw data values and data is presented as group mean \pm SEM. All data are analyzed with a one-way analysis of variance (ANOVA) and a Dunnett's post hoc test. Each group contains 3-4 animals and p values are as follows for all data: * $p < 0.05$; ** $p < 0.01$. All analyses were completed using Prism statistical software, CA, USA.

RESULTS

My results show astrocytes increase their mean GFAP immunofluorescent intensity in the NTS region following 4 and 24 hours of sustained hypoxia but the number of astrocytes did not change (Figure 6). In our original set of measurements, astrocyte activity decreased after 12 hours of hypoxia but this data was collected with a different protocol in which rats were normoxic in the daytime and returned to the vivarium for hypoxic exposure overnight (Figure 7). When we repeated this experiment with hypoxia for 12 hours during the day in the laboratory, astrocytes showed increased activation also (Figure 7), as predicted for the increases measured at 4 and 24 hours (Figure 6). To compare the NTS with other regions of the brainstem that participate in the control of ventilation, the hypoglossal motor nucleus (XII) was also assessed for glial activation. In the hypoglossal, the methods we used to quantify astrocyte activation show statistically significant increase in mean GFAP fluorescent intensity after 7 days of sustained hypoxia exposure (Figure 8). Interestingly, astrocytes proliferated in the hypoglossal region at the longest time point studied of 7 days of hypoxia (Figure 9). This means that the increase in mean GFAP intensity observed at 7 days can be attributed to a greater number of astrocytes comprising a larger mean GFAP intensity, and therefore, astrocytes did not activate after 7 days of sustained hypoxia in the NTS.

Under normal physiologic conditions, microglia extend their processes to survey their environment. When they become activated by a sufficient hypoxia stimulus, their morphology changes into an amoeboid, phagocytic form. Activated microglia have shorter branch length and fewer branch endpoints (less branching) than resting microglia

(Morrison and Filosa 2013). Our results show microglia with an active morphology in the NTS after 1 hour of sustained hypoxia exposure (Figure 9). The number of microglia stayed relatively constant throughout all time points of exposure in both the NTS and the hypoglossal (XII) motor nucleus indicating that no proliferation occurred (Figure 9 and 10). In the hypoglossal motor nucleus region, microglia activation also occurred after 1 hour of sustained hypoxia (Figure 10). Interestingly, the region of the hypoglossal appears to have less microglia but each individual microglia looks extensively ramified, with long branches and many end points.

DISCUSSION

Respiratory control centers in the brainstem participate in neuronal signaling involving communication with glia. The increase in ventilation following chronic sustained hypoxia, which is necessary for acclimatization, involves neural plasticity which we hypothesize is supported by the activation of microglia and astrocytes. Since the exact mechanisms and neural circuitry of ventilatory acclimatization are still unknown, determining the order and time course of glial activation provides a foundation for further study of this problem.

Following 1 hour of sustained hypoxia, the results show a morphological shift of microglia into an amoeboid reactive form indicative of activation (Figure 8 and Figure 9). Microglia activation is characterized by a gradual morphology shift from long dendritic processes into the clumped, phagocytic active form and is an accepted criterion to evaluate activation (Morrison et al. 2013). Ramified microglia with extended filaments in their morphological resting state survey the environment by endocytosis of extracellular CNS fluid. During normoxia, the results show microglia existing in this ramified state able to retract and extend their processes in a local circumscribed area to sense the area around them (Thomas 1992). In their activated conformation, the microglia may function as macrophages in order to phagocytose dying neurons, or rewire synaptic connections by neuronal pruning (Tremblay 2011). After 1 hour of sustained hypoxia, microglia may be phagocytosing axon terminals and dendritic spines of neurons in order to relay the hypoxic signal in a more efficient manner to allow for ventilatory acclimatization.

After a 24-hour time point of sustained hypoxia, microglia appear to revert back into a highly ramified resting state with long processes and extensive branching. Since microglia aid in the immune response to hypoxia, it is possible that after 24 hours of hypoxia they may be releasing cytokines such as IL-1 and IL-6 (Liu et al. 2003). Although not significant, it appears that microglia extend their processes out even further than in normoxia, perhaps to efficiently secrete the cytokines and other possible neuromodulators such as ATP (Thomas 1992). One effect of IL-1 on astrocytes is the regulation of mitosis that leads to astrocytic proliferation. Following 7 days of sustained hypoxia in the hypoglossal (XII) motor nucleus, astrocytic proliferation occurred. It is possible that 7 days of hypoxia is a sufficient amount of time to allow microglial release of IL-1 and cause astrocytic proliferation. In the NTS, however, proliferation of glia did not occur at the time points studied. It may be that glial proliferation occurs in the NTS but that the longest time point in this study of 7 days is insufficient to induce proliferation.

One question that remains unanswered is what is the signal for the initiation of the glial response to hypoxia. Glia may receive the hypoxic stimulus directly, from neuronal release of neuromodulators, or from sensing their local chemical surroundings. The chemosensitive neurons in the carotid body of the peripheral nervous system terminate in the central nervous system at the NTS (Pamenter and Powell 2016). Since microglia activation occurs after just 1 hour of hypoxia and there is no knowledge of a chemosensitive form of microglia, there is reason to believe they are directly responding to the neuronal release of neuromodulators. In contrast, astrocytes are active at 4 hours of sustained hypoxia after microglial activation providing evidence that they could be

receiving the stimulus from microglial gliotransmitters release. It is also possible that there are chemosensitive astrocytes in the NTS that receive the hypoxic stimulus from sensing hypoxic blood flow through the nearby vasculature. Astrocytes may combine the stimuli they receive from gliotransmitters released by microglia and the hypoxic vasculature in order to become reactive and aid in the ventilatory acclimatization to hypoxia.

The data show that microglia are activated before astrocytes. This contrast with published findings in mice showing astrocytes are the first to be activated sometime between 1 and 6 hours of sustained hypoxia, followed by microglia sometime between 6 and 24 hours of sustained hypoxia (Tadmouri et al. 2014). However, that study did not use image analysis to quantify microglial activation while the IMARIS software used in my results quantifies a complete shape change of microglia that can be linked to function. I believe this method provides a more accurate determinant of microglial activation. In addition, compared to the Tadmouri hypoxia time points, the additional time points of sustained hypoxia exposure I studied provide a comprehensive view of the time course of glial activation.

In order to uncover additional information on respiratory signaling involved in sustained hypoxia, glial activation was measured in a second respiratory center of the brainstem, ventral to the NTS: the hypoglossal motor nucleus (XII). The PreBötzinger, which is responsible for generating the respiratory rhythm, projects to the hypoglossal (XII) motor nucleus as well as the phrenic motor nucleus that drives ventilation via the diaphragm. The hypoglossal nerve (cranial nerve XII) innervates the tongue and fires

with the inspiratory phase of breathing, just before the phrenic nerve that innervates the diaphragm (reviewed in Figure 11). Hence, hypoglossal nerve firing stabilizes the tongue and other muscles in the throat and neck to keep the airway unobstructed during inspiration; failure of this mechanism may result in obstructive sleep apnea (Smith et al. 2009). Since my results show astrocytes are activated after 7 days of hypoxia in the hypoglossal region, it may be that they are acting to stabilize ventilation and prevent airway obstruction. A possible mechanism by which astrocytes in the hypoglossal may keep ventilation elevated is by receiving the hypoxic signal from neuromodulators released by microglia and then maintaining the signal by making local hypoglossal neurons hyper-excitable by constant excitatory neuromodulator release by astrocytes (Gourine et al. 2010).

Determining the time points of microglia and astrocyte activation provides a basis for looking at specific time points in future glial inhibition studies. Additional studies including carotid body denervation at the time points where glia activate may confirm whether glia are activated via neurons or from their local chemical surroundings. These results showing a sequential order of glial activation demonstrate glia a target for treatments in respiratory ailments.

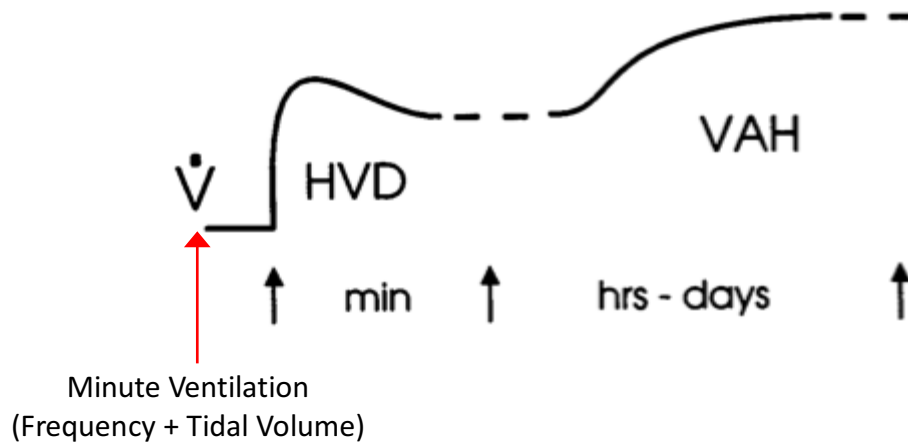


Figure 1. Ventilatory response of vertebrates following sustained hypoxic exposure. Following the first initial increase in ventilation during sustained hypoxia is a hypoxic ventilatory decline (HVD). A second increase in ventilation occurs, termed ventilatory acclimatization to hypoxia (VAH) that persists after return to normoxia. This figure is adapted from (Powell 2006).

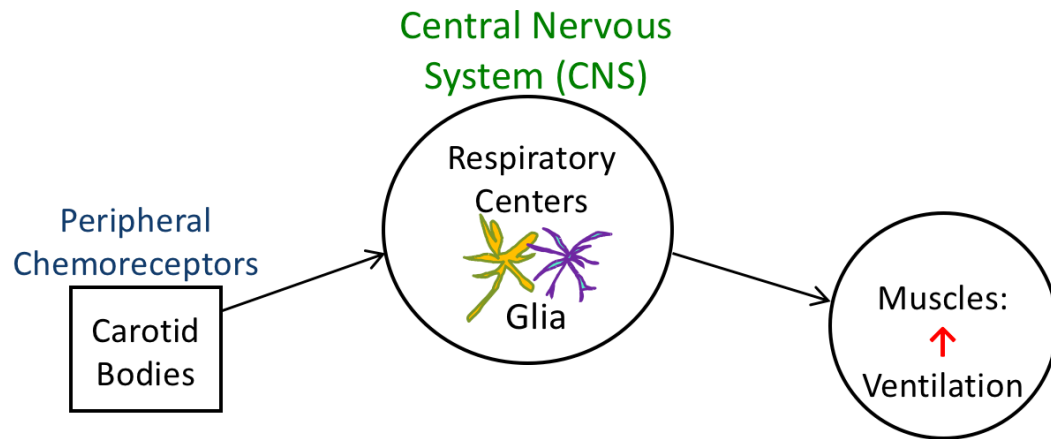


Figure 2: Peripheral chemoreceptor neurons in the carotid bodies sense decreases in arterial PO_2 and synapse at respiratory control centers in the central nervous system. Respiratory control centers relay the signal to motor neurons to increase the components of ventilation.

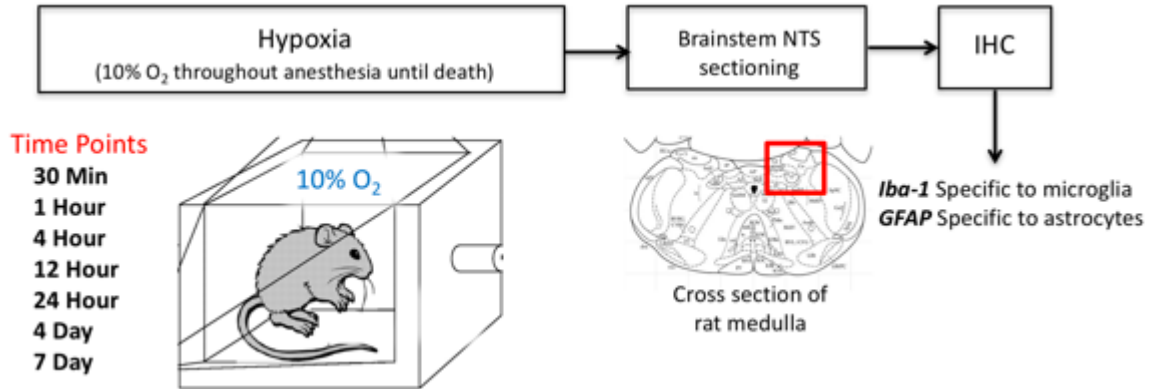


Figure 3. Animals were exposed to 10% O₂ in a positive pressure constant air flow chamber for the time points listed. The brainstems were sectioned to include the NTS region and immunofluorescent antibody staining was performed using Iba-1, specific to microglia, and GFAP, specific to astrocytes in order to visualize glial activation.

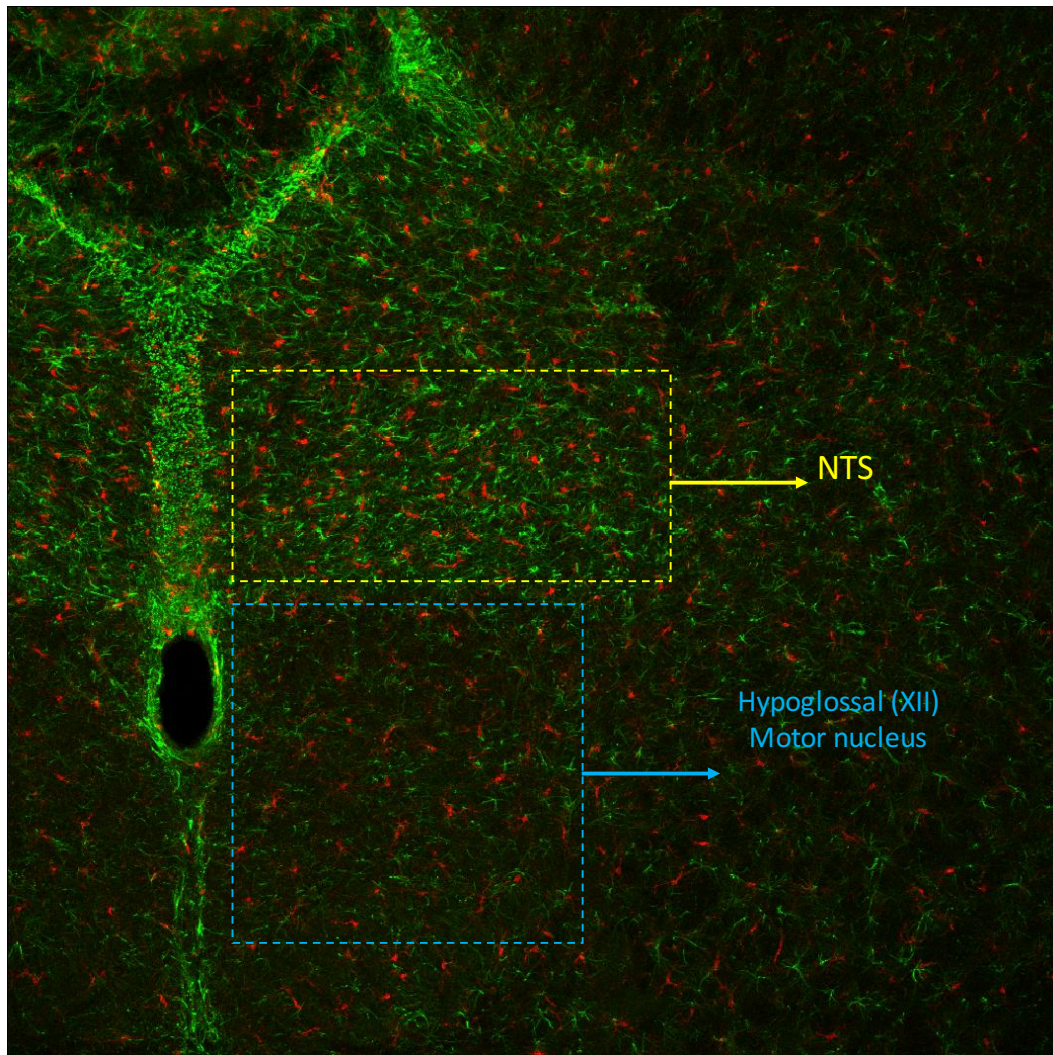


Figure 4. Confocal 10x image showing the two areas of interest for glial quantification. The NTS region was cropped from higher resolution images at 20x and quantification was performed on the cropped images. The hypoglossal (XII) motor nucleus was cropped from 20x images and quantification analysis was completed on the selected area.

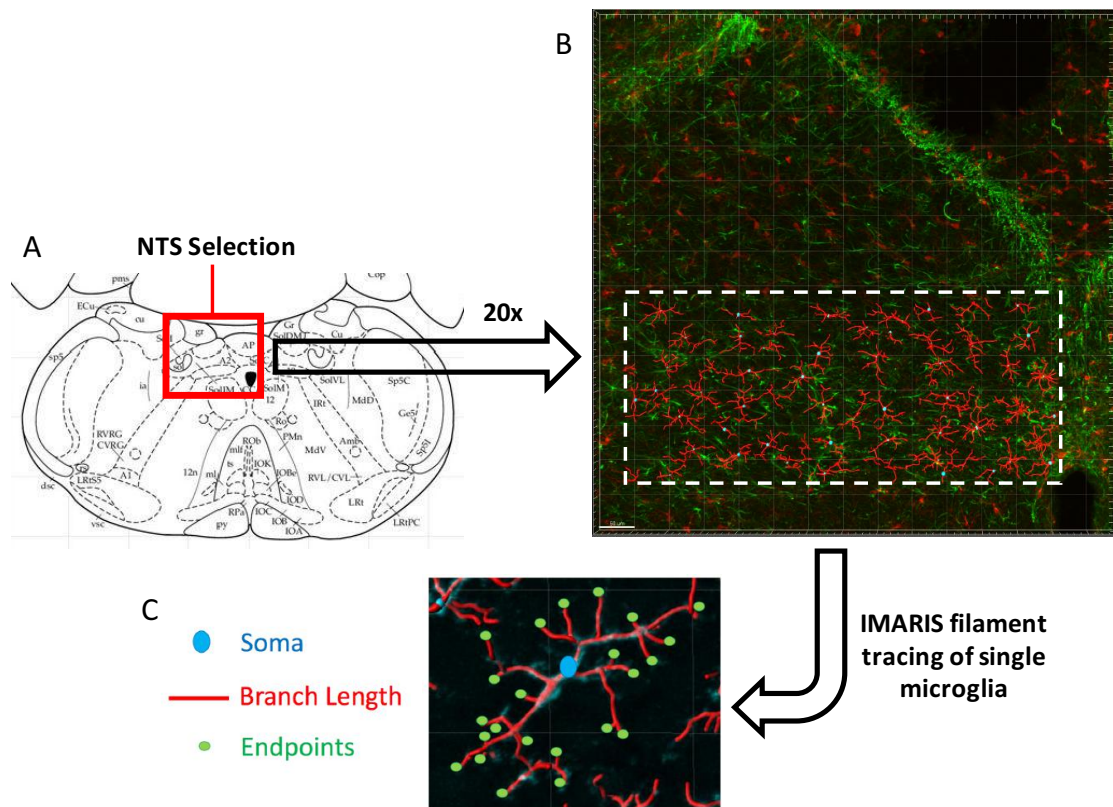


Figure 5. Microglia filament tracing using IMARIS software. A. Cross section of the rat brainstem with a selection of the NTS region. B. IMARIS software was used to trace a skeleton of microglia filaments through the three-dimensional plane and measure parameters (average branch length and average endpoint number per individual microglia) to assess activation. Parameters were assessed on the IMARIS filament tracing of the NTS immunofluorescent stained region selected in white. Scale bar is 50 microns in size. C. Close up of one fluorescently stained microglia with red IMARIS skeleton overlay.

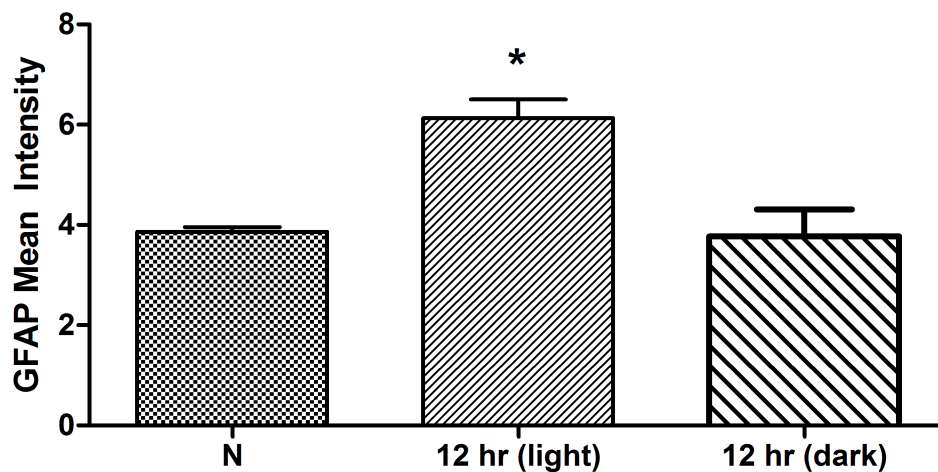


Figure 6. Astrocyte activation after 12 hours of daytime hypoxia in the NTS. One group of rats were exposed to 10% O₂ for 12 hours during the daytime in a chamber with constant air flow. The other group of rats were placed in a hypoxic hypobaric chamber with 10% O₂, 0.5 atm (PO₂ = 80 Torr) during the night. This study was performed to address the intermediate time point between 4 and 24 hours of hypoxia exposure on the astrocyte activation profile. The effects of daytime hypoxia and nighttime hypoxia were also compared. The mean GFAP intensity significantly increased compared to normoxic animals.

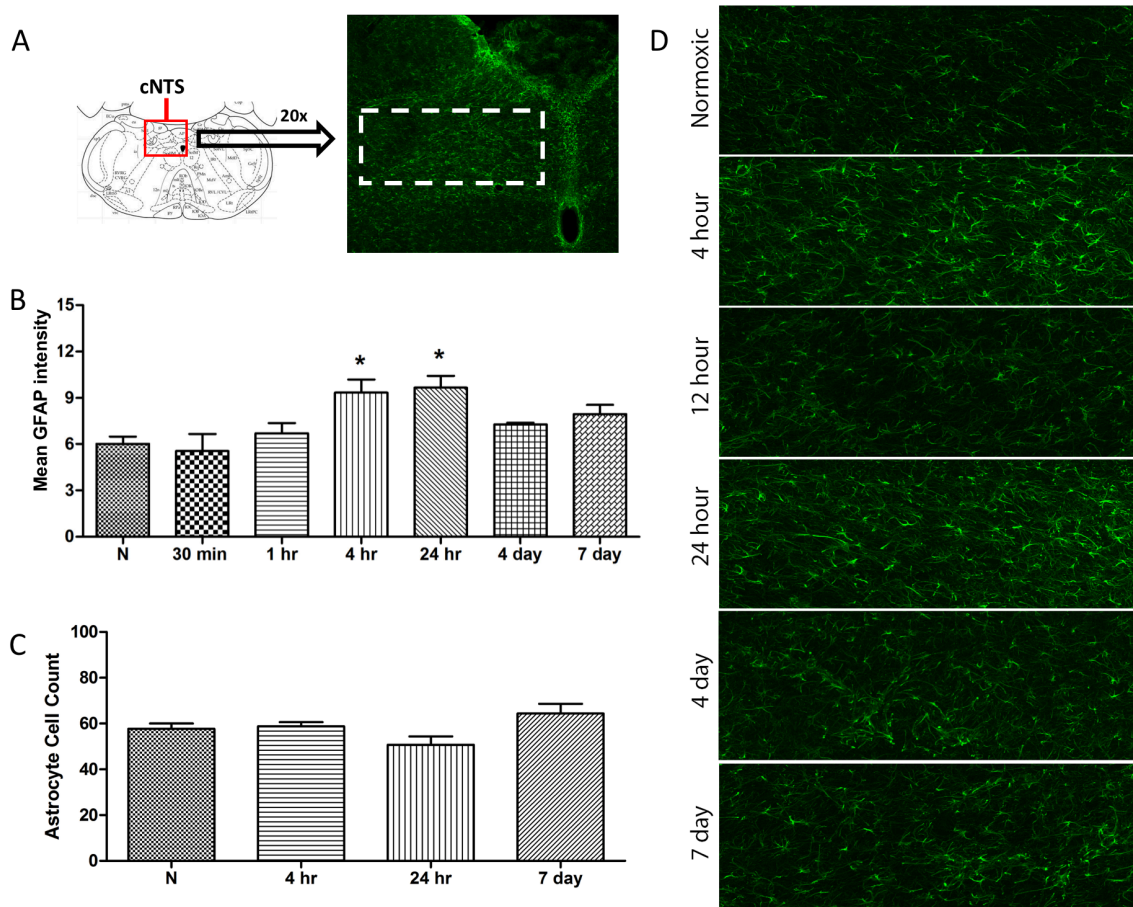


Figure 7. Astrocyte activation observed in the NTS after 4 hours and 24 hours of sustained hypoxia exposure (10% O₂). Rats were exposed to 10% O₂ for a range of the time points displayed followed by immunofluorescent antibody staining of astrocytes in green (GFAP). A. Rat brainstem selection of the NTS. Images were cropped to include the NTS region outlined by the white dashed line rectangle. B. The mean GFAP antibody intensity increased after 4 hours and 24 hours of sustained hypoxia exposure. C. The number of astrocytes did not change throughout selected timepoints of interest. D. Confocal images of the cropped NTS region showing an increase in GFAP antibody intensity at 4 and 24 hours of sustained hypoxia. (n=3-4 animals/group; *p<0.05)

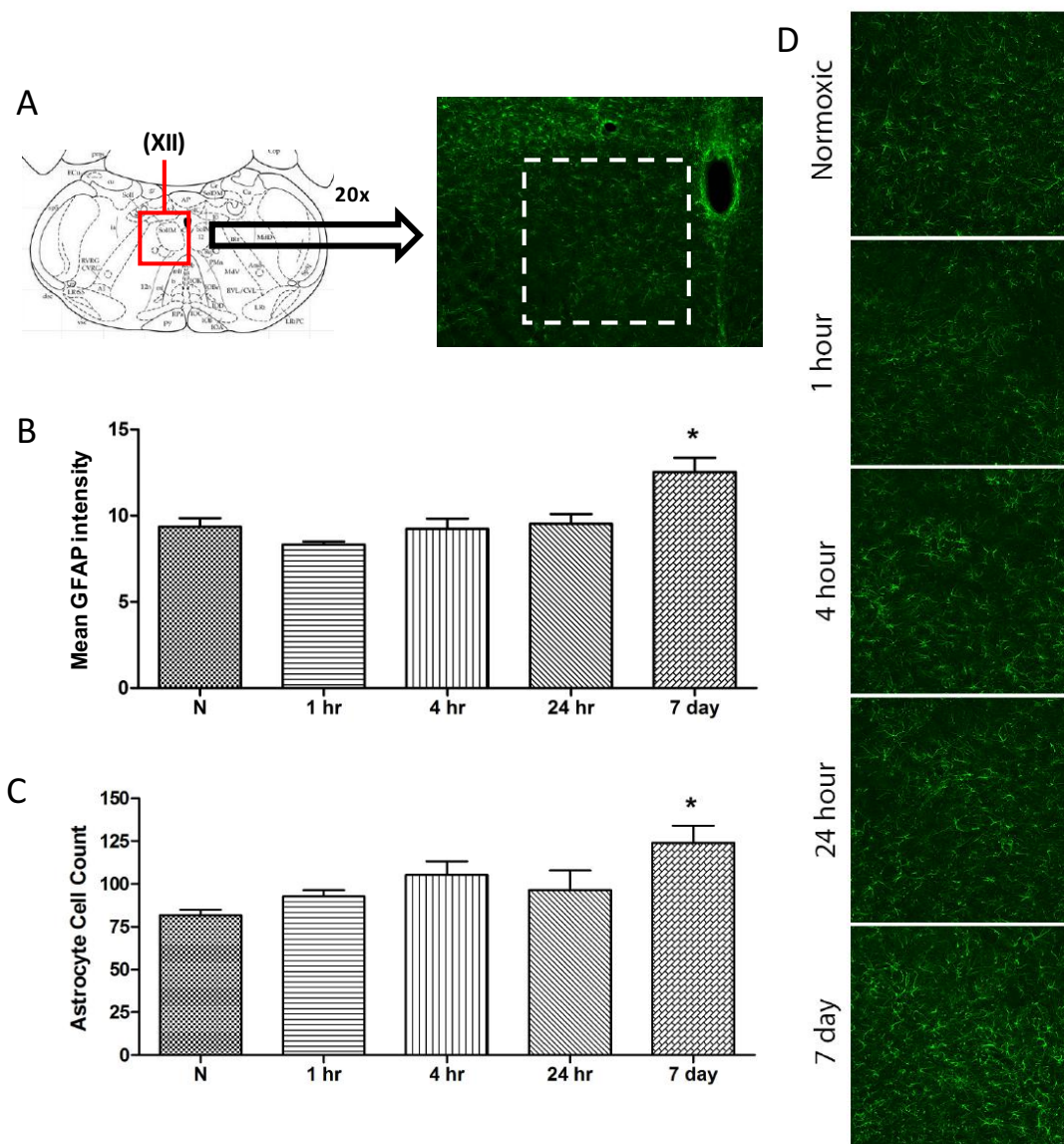
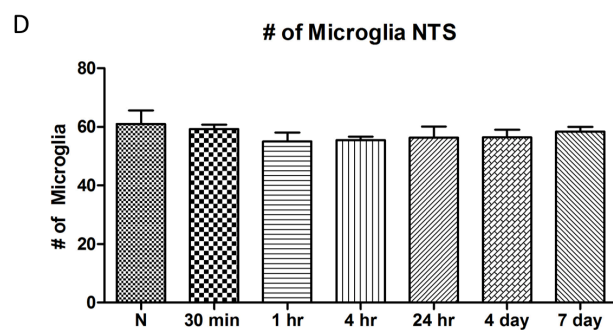
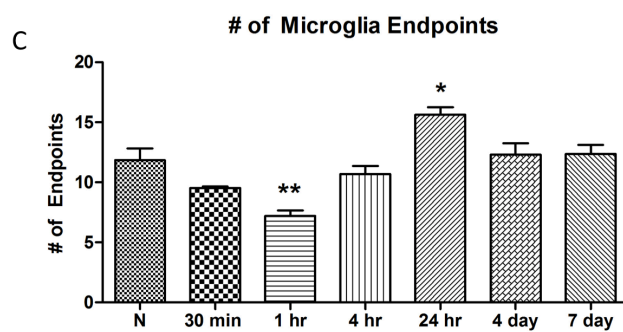
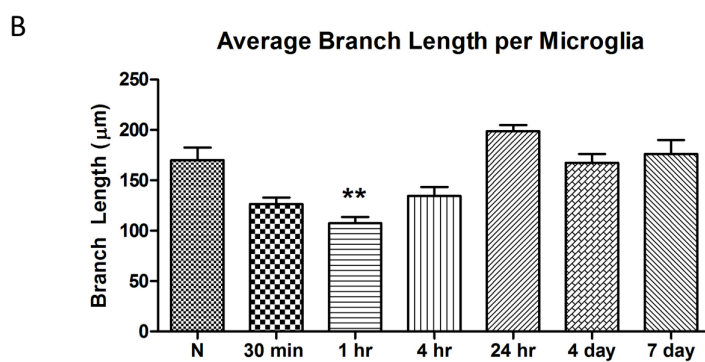
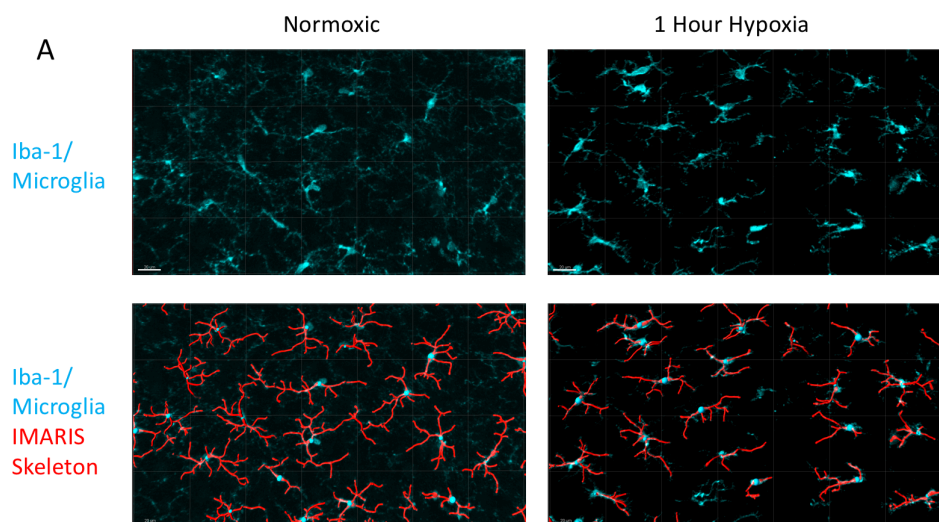


Figure 8. Astrocyte activation in the hypoglossal (XII) motor nucleus after 7 days of sustained hypoxia exposure (10% O₂). Rats were exposed to 10% O₂ for a range of the time points displayed followed by immunofluorescent antibody staining of astrocytes in green (GFAP). A. Rat brainstem images were cropped to include the hypoglossal region outlined by the white dashed line rectangle. B. The mean GFAP antibody intensity increased after 7 days of sustained hypoxia exposure. C. The number of astrocytes increased at 7 days of sustained hypoxia. D. Confocal images of the cropped hypoglossal region showing an increase in GFAP antibody intensity at 7 days of sustained hypoxia. (n=3-4 animals/group; *p< 0.05)

Figure 9. Microglia morphology shift quantified using IMARIS software in the rat NTS following sustained hypoxia. Rats were exposed to 10% O₂ at the time points displayed followed by immunofluorescent staining of microglia in blue (Iba-1). A. Microglia undergo a morphology shift upon activation to decrease their branching and display shorter overall branches. The top two images show blue immunofluorescent staining of microglia (Iba-1). The bottom two images show an IMARIS red skeleton overlaying the blue fluorescent staining. B/C. After 1 hour of hypoxia the microglia have shorter branches and show less branching (decrease in endpoints) and as quantified by IMARIS software. D. The number of microglia remained constant throughout the time points of hypoxia exposure. (n=3-4 animals/group; *p< 0.05; **p<0.01)



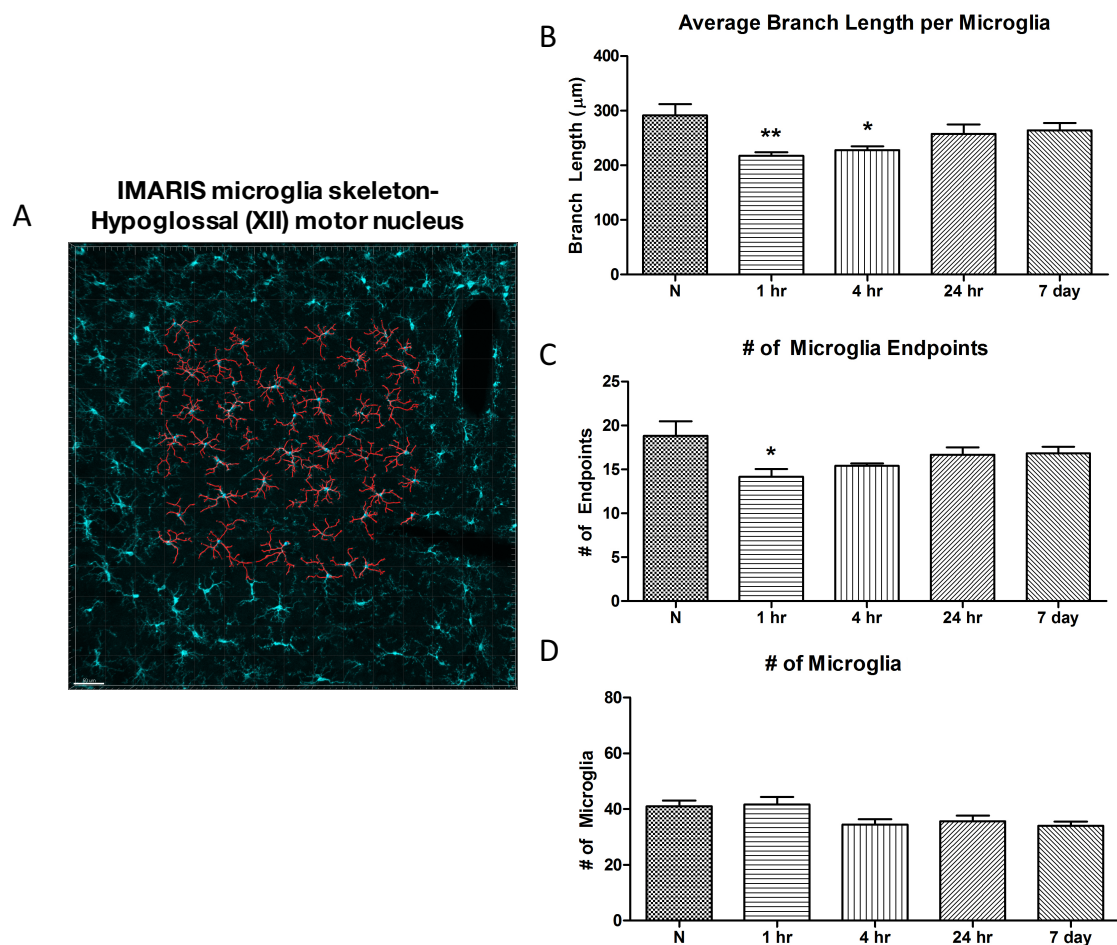


Figure 10. Microglia morphology shift quantified using IMARIS software in the rat hypoglossal (XII) motor nucleus following sustained hypoxia. A. Z-stack confocal image with blue immunofluorescent staining of microglia showing IMARIS skeleton overlay in red of the hypoglossal region. Scale bar reads 50 μm . B/C. After 1 hour of sustained hypoxia, microglia activate by undergoing a change in their morphology showing less branching (decrease in endpoints) and shorter branches as quantified by IMARIS software. D. The number of microglia remained constant throughout the time points of hypoxia exposure. (n=3-4 animals/group; * $p < 0.05$; ** $p < 0.01$)

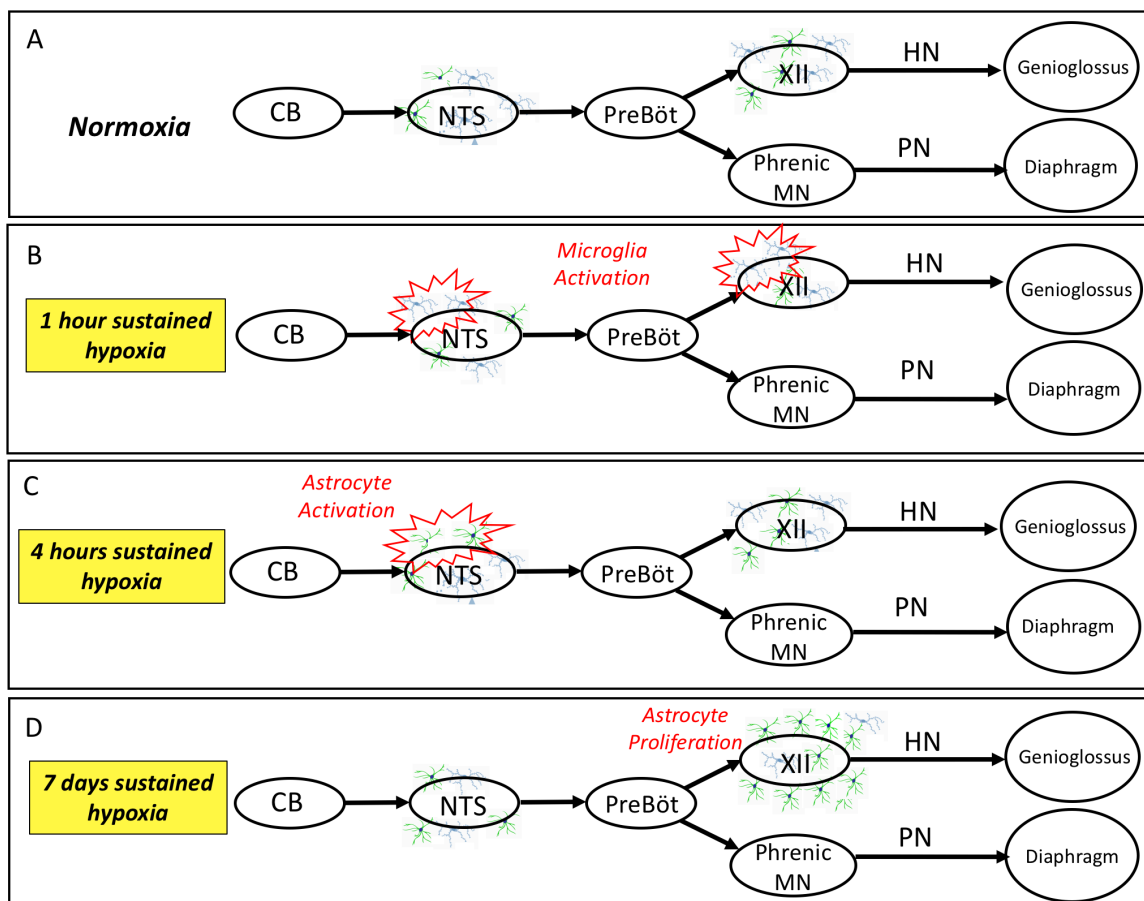


Figure 11. Schematic of relevant respiratory circuitry and glial activation in the rat brainstem incorporating study results. A. The carotid body (CB) innervates both the nucleus tractus solitarius (NTS) and the PreBötzing complex (PreBöt) (not shown). The NTS relays information to the PreBöt. The hypoglossal nerve (HN) originates in the hypoglossal region and innervates the genioglossus, or tongue muscles. The PreBöt is a respiratory rhythm generator and innervates the phrenic motor nucleus (Phrenic MN), which innervates the diaphragm via the phrenic nerve (PN). B. Activation of microglia, in blue, occurs after 1 hour of sustained hypoxia in the NTS and XII. C. Astrocytes, in green, activate after 4 hours of sustained hypoxia in the NTS. D. Astrocytes proliferate during long term sustained hypoxia in the hypoglossal motor nucleus (XII) (Takakura et al. 2006; King 2007; Nattie and Li 2012).

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