UNIVERSITY OF CALIFORNIA SAN DIEGO

Time Course of Brainstem Glia Activation in Mouse Nucleus Tractus Solitarius During Exposure to Chronic Hypoxia

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

Master of Science

in

Biology

by

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Professor Gulcin Pekkurnaz

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

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Co-Chair

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Chair

University of California San Diego

2020

DEDICATION

This is dedicated to my parents. To my mom, dad, step mom, and step dad, for their unconditional love and support. Also, my little sister for being by my side through every moment.

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ACKNOWLEDGEMENTS

I would like to thank Dr. Frank Powell, for believing in me and giving me the opportunity to explore the field of hypoxia and pursue a Masters in his lab. I appreciate his kindness, support, and skill of teaching. I am grateful to have had Frank as my advisor.

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ABSTRACT OF THE THESIS

Time Course of Astrocyte Activation in the Mouse Nucleus Tractus Solitarius During Exposure to Hypoxia

by

Jacqueline Stenger Howe

Master of Science in Biology

University of California San Diego, 2020

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

Chronic sustained hypoxia (CH) occurs in populations living at high altitude and in patients with chronic pulmonary disease. Exposure to CH produces ventilatory acclimatization to hypoxia (VAH) and increases the hypoxic ventilatory response (HVR) by mechanisms that involve areas of the brainstem that control breathing. The nucleus tractus solitarius (NTS) is a sensory integrative center in the medulla receiving carotid body afferents and known to be crucial for VAH. Our previous results demonstrated that glia cells in the rat NTS contribute to VAH but these mechanisms have not been studied in mice exposed to CH beyond 24 hours. We hypothesized that CH produces an early transient activation of astrocytes and microglia in the mouse brainstem as observed in rats. To study the activation of glial cells with CH, we exposed mice to normobaric hypoxia (10% FiO_2) for 0.5, 1 and 4 hours and 1 and 7 days. Mice were

perfused with 4% paraformaldehyde and brainstem sections were obtained. We quantified astrocyte activation by measuring glial fibrillary acidic protein (GFAP) intensity with immunofluorescence, and microglial activation using image analysis to measure changes in branch morphology. Exposure to CH significantly increased GFAP intensity after 30 minutes and 1day but it returned to normoxic control levels after 1 week of CH. Measurements of microglia during 7 days of CH have not been completed. The results differ from those in rats (Stokes et al., 2017) but suggest that astrocyte activation in the NTS may be an initial step in VAH.

INTRODUCTION

Oxygen is one of the most important elements for basic survival and the function of aerobic metabolism in animals, which use oxygen to generate ATP as a source of energy for the body. When a low supply of oxygen, which is known as hypoxia, is presented to the mammalian body, then generation of ATP and energy decline. Consequently, mammals, including humans, have developed physiological responses that insure O_2 homeostasis (West, 1990). Examples of hypoxic conditions stimulating these responses in humans include exposure to high altitude and chronic lung disease in patients (Powell et al., 1998). Therefore, the study of the mechanisms of response to hypoxia is important to understand the physiology of humans in extreme environments, but also, to develop treatments for pathological conditions with hypoxia.

When the mammalian body experiences hypoxia the first, and arguably most important, form of compensation is the increase of pulmonary ventilation. Pulmonary ventilation is the breathing of air into and out of the lungs, which rapidly exchange gases with the blood surrounding their alveoli. Arterialized blood is pumped by the heart to circulate throughout the body. Arterial blood is well-saturated with oxygen (hemoglobin-O_2 saturation > 95%) in healthy people breathing air at sea level (i.e. normoxia), and this oxygen enriched blood perfuses every organ and tissue within the body (West, 1990). During exposure to hypoxia, the decreased levels of O_2 in the blood (concurrently with a decrease in O_2 saturation) produce an increase in ventilation due to activation of a chemoreflex response initiated in the carotid body (Gonzalez et al., 1994; Kumar & Prabhakar, 2012). The carotid body is a small sensory organ located along both sides of the neck where the carotid artery bifurcates. This sensory organ receives the highest blood flow of any organ in the body so it is well-equipped to sense the level of oxygen within the blood. Changes in the arterial levels of O_2 are detected by the carotid body chemoreceptors, that

send neural information to the brainstem activating a reflex response that increases activity of the diaphragm and increases ventilation (Kumar & Prabhakar, 2012). This increase in ventilation is known as the hypoxic ventilatory response (HVR) (Teppema & Dahan, 2010).

The HVR is the first physiological reaction that the mammalian body experiences when exposed to hypoxia and it is caused by decreased oxygen partial pressure (P_{O_2}) in the arterial blood (Teppema & Dahan, 2010). However, the HVR can change with neural plasticity in arterial chemoreceptors and central nervous system (CNS) respiratory centers when the body is exposed to hypoxia for different durations (Pamenter and Powell, 2016). Understanding the mechanisms for such different time domains of the HVR has been an intense area of study in the field of pulmonary physiology. One of the important examples of plasticity in the HVR studied in our laboratory is Ventilatory Acclimatization to Hypoxia (VAH). VAH is a time-dependent increase in ventilation and the HVR during chronic hypoxia and it is how the body attempts to maintain homeostasis in the presence of the extended challenge of chronic sustained hypoxia (Powell et al., 2000). VAH is observed after a day or two of sustained hypoxia (depending on the species) and involves increases in the acute HVR that is responding to hypoxia within seconds.

Mechanisms of plasticity in the CNS respiratory centers explaining VAH are not completely understood but they involve changes in neurotransmitters, expression of O_2-sensitive transcription factors, inflammatory signals and activation of glial cells, all in the nucleus tractus solitarius (NTS) (reviewed by Pamenter & Powell, 2016). The NTS is significant for the HVR and VAH as the site of the first synapse from the carotid body chemoreceptors in the CNS. We know from previous investigations that glutamate is the excitatory neurotransmitter for the HVR in the NTS and our lab demonstrated that plasticity in glutamatergic receptors in the NTS play a role in the VAH (Pamenter et al., 2014). It is also known that Hypoxia inducible factor-1α (HIF-

1α) , which alters gene expression to maintain oxygen homeostasis (Semenza, 2011) in the NTS is important for VAH (Moya et al., 2020). HIF-1α controls the expression of inflammatory cytokines, erythropoietin, catecholamines, nitric oxide and heme oxygenase, all of which have been shown to play a role in the plasticity of the HVR (Pamenter & Powell, 2016). Another factor involved in VAH appears to be glial cells, which are activated by hypoxia in the NTS of rats and mice as discussed below (Stokes et al., 2017; Tadmouri et al. 2014). Recent evidence has shown that reactive glial cells play important roles in regulating CNS inflammation (Sofroniew, 2010) and inflammation is a byproduct of exposure to hypoxia (Cummins & Taylor, 2017), so glia may be involved in the inflammatory signals for VAH.

Throughout the mammalian brain, there are many types of cells that help maintain homeostasis and glia is one of these. Glia surround and interact with neurons to facilitate brain signaling. In the glial cell family there are microglia, astrocytes, oligodendrocytes, and ependymal cells. Microglia are considered the immune cells of the central nervous system and are constantly surveying the brain for any abnormal processes such as injury, decreased blood flow, low oxygen etc. (Nimmerjahn et al., 2005). Astrocytes are another important glial cell that are known to be a large component of neuronal-glial communication and essential for basic brain function (Nedergaard et al., 2003). Both astrocytes and microglia respond to sustained hypoxia during VAH (Stokes et al., 2017; Tadmouri et al. 2014). Specifically, Stokes et al. (2017) found microglia in the NTS of rats were activated by sustained hypoxia after only 1 hr but returned to control levels after 24 hr; in contrast, astrocytes were not activated until 4 hr and returned to control levels after 4 days of sustained hypoxia. All of these changes were blocked by minocycline, which also blocked VAH (Stokes et al., 2016) and increased expression of

inflammatory cytokines in the NTS (Hocker et al., 2017) suggesting that microglia may be involved in the inflammatory signals for VAH.

Tadmouri et al. (2017) also explored the time-dependent activation of glial cells in the NTS of mice within the first 24 hours of exposure to hypoxia. They found astrocytes had increased GFAP levels after 1-6 hr that returned to normal after 24 hr, and minocycline blocked the increase at 6 but not 1 hr. Microglia showed increased CD11b levels only after 6 to 24 hr. Minocycline blocked the changes in glia and also the increase in ventilation or VAH after 24 hr hypoxia.

All of these results are consistent with a role for glia in VAH but it appears that the time course of glial activation may differ between species. Tadmouri et al. (2014) found astrocytes to be the first responders to hypoxia in mice while Stokes et al. (2017) found microglial activation before astrocytes in rats. However, species were not all that was different between the studies. Both studies used GFAP immunofluorescence as indicators of astroglia "activation". However, activation can refer to a variety of important astrocyte functions that could affect plasticity in the ventilatory chemoreflex pathway, and at least some of these may change independently of GFAP expression (see Discussion). Similarly, microglia activation can refer to a variety of processes and different methods have been used to estimate microglia activity in the studies to date. Moreover, Tadmouri et al. (2014) studying mice used co-expression of immunofluorescence for CD11b, which generally indicates microglia activation, and Iba-1, which identifies microglia. Stokes et al. (2017) used image analysis of microglia stained with Iba-1 to quantify changes in microglia morphology with activation in rats. To determine if there are important differences between rats and mice in glial activation during chronic hypoxia, or if differences reported to

date may involve methods or other factors, we repeated the studies done in our laboratory on rats using the same techniques on mice.

Another motivation for determining the time course of glial activation during chronic hypoxia in mice is that we eventually want to test hypotheses about glia and VAH with experiments using transgenic mice. Recently, we used conditional deletion of the gene for HIF-1α in NTS neurons of mice to demonstrate it is necessary for VAH (Moya et al., 2020). Ultimately, we would like to use a similar transgenic approach to alter cytokine expression and other functions in specific types of glia in the NTS to understand their role in VAH. However, first we need to establish the normal time course of glial activation in mice.

MATERIALS AND METHODS

Animals

All animals and experiments were managed according to the protocols established by the Institution Animal Care and Use Committee of the University of California San Diego. Our experiments used adult male mice between 9 and 12 weeks old. The mice were housed in a vivarium with a 12-hour light/dark cycle with the lights on at 6 AM. Mice were weighed at the beginning of each experiment.

Hypoxia Exposure

Mice were exposed to hypoxia (10% O_2) in manually controlled chambers during 30 min, 1hr and 4hr exposures during the day and in a BioSpherix OxyCycler for 24 hour and 7-day exposures with the 12-hour light/dark cycle. After chronic hypoxia in the BioSpherix chambers, mice were moved to the smaller manually controlled chambers in the laboratory for an hour before tissue collection during the day, so final hypoxia exposure was the same for all time points. At the desired time point of hypoxia exposure, mice were anesthetized with Isoflurane gas administered during the final 5 min of hypoxia exposure. After that the animals were removed from the chambers for fixation.

Perfusion and Tissue Fixation

Once anesthetized the mice were perfused through the left ventricle with 60ml of NaCl saline solution and cut at the right atrium to allow the outflow of saline. After that they were perfused with 60ml of 4% paraformaldehyde (PFA) in a phosphate buffered saline (PBS). The Brainstem was then dissected and post-fixed in 4% PFA overnight at 4° C. Then the brainstems were cryoprotected by immersion in 16% sucrose in PBS overnight at 4 degrees Celsius.

The brainstems were then mounted by inmersion in optimal cutting temperature compound (OCT) and frozen in isopentane kept cold with liquid nitrogen at -80° C until ready for sectioning. Brainstem tissues were cut using a cryostat LEICA CM1520 in slices of 30 µm thickness and then storaged in floating sections in antifreeze solution at 4° C.

Immunohistochemistry

Using 12-well plates, the 30 micron brainstem sections were washed for 30 minutes in phosphate buffered saline (PBS), followed by another 30 min wash in 0.5% Triton (X-100) in to build blocks that will be further sliced. Tissues embedded in OCT were kept in - PBS. The tissues were then immersed in 0.5% goat serum in PBS for at least 1 hr, to attach all the proteins eliminating excessive background. Primary antibody is then incubated overnight at 4° C to visualize the astrocytes and microglia using GFAP (generated in chicken 1:2000) and Iba-1 (generated in rabbit 1:2000). After the primary antibody, tissues were washed in PBS for 30 min before administering the secondary antibody. The tissues were in the secondary antibody Cy3 goat anti rabbit (1:1000) and 488 goat anti chicken (1/500) for 1 hr at room temperature. The sections were plated on glass slides and covered with ProLong anti-fade DAPI and a small microscope cover glass.

Confocal Microscopy

A Leica SP8 confocal fluorescent microscope was used to visualize the brainstem sections. We used a magnification of 20x for all slides to image the glial activation in the NTS. A z-stack of 10 images covering 10 µm was taken for a section from each experimental animal. Laser intensity and gain remained constant throughout every photo.

Astrocyte Quantification

We used GFAP fluorescence to visualize astrocyte activation on the sections from the dorsal region of the brainstem that have the NTS. The images were taken on the Leica8 with the same parameters for every slide. Using Image-J the mean grey value was used to quantify fluorescent intensity. The mean grey value measures the relative brightness of pixels within the region of choice. For each image we selected the region of interest using the same exact area for each image via Image-J macros.

Microglia Quantification

Microglia were visualized with Iba-1 immunofluorescence and a z-stack of 10 images from each animal was saved from the confocal florescent microscope as described for astrocytes. Using the IMARIS Filament Tracer package by Bitplane, Oxford Instruments v. 8.2, we traced the microglia morphology through a three-dimensional plane in the z-stack to construct skeletons for the microglia. This allowed measurements of branch lengths, number of endpoints of branches and cell counts. These measurements were taken in the same region of interest as the astrocytes in the NTS.

Statistics

Statistic are taken from legitimate data and analyzed with a one-way analysis of variance (ANOVA) and post-hoc test. Analyses were done with the Prism software, CA, USA. Each time point contained 6-10 animals.

RESULTS

Figure 1 shows changes in GFAP in astrocytes, as a measure of their activation, after different lengths of exposure to sustained hypoxia. Fig 1a shows the area of the medulla where we focused our analysis, which includes the nucleus tractus solitarus (NTS), and also parts of the dorsal motor nucleus of the vagus. Together these areas are commonly referred to as the solitary complex. GFAP fluorescence intensity significantly increased at 30 min and 24 hr of hypoxia. GFAP tended to be greater than control at intermediate time points between 30 min and 24 hr also, although this was not significant. However, after 7 days, GFAP decreased significantly compared to 1 day and was not different from control.

Figure 2 shows pilot data for the effects of chronic hypoxia on microglial branching, as a measure of activation, from the same region of the brain. In normoxic control conditions, the dendrites of microglia are extended as they survey their environment. With activation , they change their shape and size to an amoeboid, phagocytic form, shortening and decreasing the number of their branches (Morrison and Filosa, 2013). We observed such changes in shape with sustained hypoxia, as demonstrated by a significant decrease in the number of endpoints and average branch length (Fig. 2c). The number of microglia is not changed by hypoxia (Fig. 2c), consistent with the idea that changes in these measurements reflect changes in microglia morphology and not recruitment or elimination of specific microglial populations. As described above, further studies are planned to quantify the effects of 30 min, 1 and 4 hrs, 1 and 7 days of hypoxia on microglial morphology, but they are completed for reporting here.

DISCUSSION

Chronic sustained hypoxia (CH) increased GFAP levels in astrocytes in medullary respiratory centers of mice, consistent with glial activation that is hypothesized to be involved in ventilatory acclimatization to hypoxia (VAH). GFAP levels increased significantly after 30 min and 24 hr of hypoxia and tended to be greater than control levels at 1 and 4 hr of CH too. GFAP levels significantly decreased by 7 days, returning to control levels. The possible roles of astrocytes in VAH and limitations of these experiments for inferring such roles are considered below. Future experiments to determine the order of glial activation, specific changes in glial function during activation, and the role of astrocytes in VAH are discussed also.

Comparison with the literature

Tadmouri et al. (2014) also measured changes in GFAP levels during after 1, 4, 6 and 24 hours of CH and found significant increases localized to the NTS during 1 to 6 hr of CH, similar to our results (Fig. 1). However, their GFAP levels decreased after 24 hr of CH to levels not significantly greater than control, while we did not find this decrease until 7 days (Fig. 1). Both their study and ours found no change in the number of GFAP expressing cells during CH, indicating increased GFAP expression versus astrocyte proliferation in CH.

Stokes et al. (2017) also studied changes in GFAP during CH in rats and found results similar to ours. They found significant increases in GFAP in the NTS after 1 and 4 hrs of CH but not as early as we did in mice at 30min and 1 hr (Fig. 1). They also found GFAP levels returning to control levels after 4 and 7 days of CH, similar to our results (Fig. 1). The number of astrocytes did not change so increased GFAP levels were interpreted as increased expression of GFAP.

Hence, despite some minor differences in the time course between studies and species, the findings to date support increased expression of GFAP between 1 and 24 hours of CH, which returns to normoxic control levels after 1 7 days of CH. As discussed in the Appendix, we do not think different anesthesia between the two studies explain the differences. Tadmouri and c0-workers (2014) used pentobarbital but we used isoflurane, since we found it easier to maintain consistent hypoxic exposure to short timepoints (e.g. 30 minutes) by administering gas anesthesia directly in the hypoxia chambers.

Other studies measuring GFAP during hypoxia have focused on ischemia or stroke, or different time courses than the ones considered here that are relevant for VAH. Such other studies generally find non-reversible changes resulting in astrogliosis or scar formation following increases in GFAP, as discussed below (reviewed by Sofroniews and Vinters, 2010). The effects of systemic hypoxia we are studying here are reversible and scarring or cell death is not observed, indicating that oxygen supply to the brain is still adequate in our model.

GFAP, astrocytes and VAH

Here we consider how changes in GFAP relate to changes in astrocyte activation and function, which may ultimately affect the plasticity underlying VAH. Glial fibrillary acidic protein (GFAP) is an intermediate filament that is important for the cytoskeletal framework of astrocytes (Pekny et al., 1999). GFAP is also responsible for managing the glial relationship with nearby neurons, blood brain barrier, and astrocyte strength (Eng et al., 2000). Astrocytes are one of the most abundant cells in the CNS and can respond to all types of activity that occurs within the CNS with what has been termed as reactive astrogliosis. According to Sofroniew and Vinters (2010), reactive astrogliosis in general is much more than scar formation associated with disease and includes four main features: (1) a spectrum of changes in astrocytes in response to different

severity of perturbations, (2) specific to the nature and severity of the insult, (3) regulated in a context-specific manner, and (4) and involving both gain and loss of function for beneficial or harmful effect.

In our experiments, GFAP is a useful marker for early astrocyte activation, which we hypothesize to be involved in beneficial effects of neural plasticity resulting in VAH for several reasons. Again, as reviewed by Sofroniew and Vinters (2010), GFAP is the prototypical marker for astrocytes in the CNS, although it may not be detected with immunohistochemistry in all CNS astrocytes under normal healthy conditions. Although GFAP can be expressed in other cell types in the CNS, these are not common in the medullary respiratory centers we are investigating. Our global sustained hypoxia is apparently causing what Sofroniew and Vinters (2010) term "mild to moderate reactive astrogliosis", which upregulates GFAP and other genes without profound structural changes to astrocytes. Such mild astrogliosis is associated with non-penetrating or non-contusive insults, or innate immune activation, and resolves with astrocytes returning to normal, healthy appearance (Sofroniew and Vinters, 2010).

Sofroniew and Vinters (2010) reviewed several molecular triggers for mild reactive astrogliosis that could also occur with hypoxia in our experiments, including cytokines (possibly from microglia, as discussed later), glutamate (from tonic sensory input from carotid body chemoreceptors) or reactive oxygen species. Such triggers could be either reduced during extended exposure to hypoxia, or may not lead to further triggers for more severe forms of astrogliosis and instead could promote beneficial versus harmful effects. Another example of an astrocytic response to hypoxia that could involve similar mechanisms has been described \n the medullary respiratory rhythm generator (Pre-Botzinger complex), which respond directly to

physiological decreases in P_{O_2} via increased ROS levels in mitochondria that cause increased intracellular calcium (Angelova et al., 2015).

The physiological role of astrocytes in healthy brains, which could contribute to plasticity and VAH, include calcium induced neurotransmitter release and uptake, release of cytokines and growth factors, regulation of cerebral blood flow and fluid, ion homeostasis through ion and water channels and regulation of the blood-brain barrier (reviewed by Sofroniew and Vinters, 2010). The effect of decreased P_{O_2} to increase intracellular calcium in Pre-Botzinger complex astrocytes described above, causes vesicular release of ATP to excite adjacent neurons and stimulate breathing (Angelova et al., 2015). Inflammatory cytokines have been hypothesized to signal VAH, based on experiments blocking inflammatory signals and VAH with non-steroidal anti-inflammatory drugs or inhibitors of microglial activation as discussed below. Hence, there are multiple aspects of normal astrocyte function that could contribute to plasticity in VAH. However, it remains to be determined if such normal astrocyte functions need to be sustained to maintain VAH during chronic hypoxia, or if they are only an early step necessary to initiate VAH, as suggested by the transient increase in GFAP expression.

Microglia and VAH

Microglia are known to be involved in synaptic plasticity (Helmut et al., 2011) so we are interested in their role in VAH and intended to study them as part of this project. However, we were unable to complete these experiments when the laboratory shut down for COVID-19. Microglia are known as the immune cells of the CNS and generally survey the CNS for insult, damage and signs of inflammation (Nimmerjahn et al., 2005). When a threat is sensed, the microglia change shape by contracting their extensions and becoming more amoeboid and migrating towards neurons that are threatened (Nimmerjahn et al., 2005). However, similar to

astrocytes and astrogliosis, microglial activation is not a single process and may include many stages, including beneficial steps contributing to neural plasticity (Hocker et al. 2016)

Evidence for a role of microglia in VAH has been published for both mice and rats. In mice, Tadmouri et al (2014) quantified the percentage of microglia in the NTS that were activated by 1 to 24 hr of hypoxia using cells co-labeled for the inflammatory marker CD11b plus the microglial marker Iba-1 in mice. They found microglial activation at 6 and 24 hr and this was blocked by minocycline, which is an antibiotic and microglial inhibitor. Note that activation of microglia was detected after astrocytes (6 vs. 1 hr) in these experiments (Tadmouri et al., 2014). In rats, Stokes et al. (2017) also found hypoxic activation of microglia in the NTS using a different method, namely image analysis to detect shape changes in microglia stained with Iba-1. Specifically, they found the number of microglia branch endpoints and the average length of branches was decreased significantly by 1 hr of sustained hypoxia, with a tendency to decrease at 30 min and 4 hr, and then a return to normoxic control values after 1, 4 and 7 days of CH. Minocycline blocked microglia activation in these studies also but note that microglia activation occurred before astrocytes in the rats. Reasons for this difference in ordering of glial responses between these two experiments remain to be explained but we speculate that the Cd11b marker Tadmouri's group used to measure microglia activation could respond more slowly to hypoxia than the changes in shape measured by Stokes' group. Of course, species differences may be involved also.

Interestingly, inhibition of microglia with minocycline also blocked increases in GFAP, indicative of astrocyte activation, as well as normal VAH in both mice and rats (Tadmouri et al., 2014; Stokes et al., 2017). Moreover, Stokes et al. (2017) found that administering minocycline after VAH had been initiated, by waiting until day 4 of 7 days of CH to start treatment, blunted

but did not block VAH in rats. Hence, they concluded that microglial activation was an early and necessary step in VAH and activation of astrocytes followed microglia, contributing to later phases of VAH.

A possible mechanism for an early signal for plasticity from microglia could be the increased expression of cytokines. Microglia in the spinal cord and brain stem of rats express inflammatory cytokines in response to intermittent hypoxia or chronic hypoxia increases their expression in the NTS of rats (Smith et al., 2013; Popa et al., 2010; Hocker et al., 2016). Evidence for this includes minocycline and anti-inflammatory drugs blocking increased cytokine expression in the rat NTS, in addition to blocking normal VAH (Hocker et al., 2016; Popa et al, 2011). However, more experiments are necessary to determine if similar changes occur in mice, and if microglia are the source of increased cytokine expression in the CNS respiratory centers.

Future directions

It is clear that more studies need to be performed to understand the contribution of glial cells, both microglia and astrocytes, to the CH-induced neuroplasticity occurring in the brainstem and resulting in VAH. There is the challenge of determining the order of glial activation, as well as the value of measuring GFAP expression for astrocytes and morphology or CD-11b expression for microglia activation to understand mechanisms of VAH.

One possible experiment to solve the question of order of activation would be to culture microglia and astrocytes extracted from NTS biopsies of mice. We could compare measures of activation in cultures from normoxic control versus chronically hypoxic mice, as well as exposing cultured glia to different periods of hypoxia under controlled conditions *in vitro*. Activation by hypoxia *in vitro* could also be compared with other well-known methods of glial activation such as LPS. Culture media from one type of glia could be applied to the other type to

see if there is a chemical signal for activation between the two types of glia and co-cultures of astrocytes and microglia could be used to study their interactions. In all cases, we could measure the expression of cytokines and other gene expression patterns (e.g. with RNAseq) to identify putative mechanisms of plasticity with glial activation by hypoxia.

In order to study the specific contribution of astrocytes to VAH *in vivo*, we might inhibit astrocytes with NTS-targeted microinjections of astrocyte activity inhibitors such as fluorocitrate or arundic acid and measure the effects on VAH, as well as glial activation at different time points. These experiments would basically mirror those done with minocycline to inhibit microglia activation, as discussed above.

Further experiments focusing on astrocytes may be important considering reports of direct effects of decreased O_2 on astrocytes in the Pre-Botzinger Complex discussed above. We have some indirect evidence for this in pilot experiments showing microglia activation by chronic hypoxia persists in the NTS of rats after carotid body denervation, which removes the increase in synaptic input to the NTS (Moya, unpublished observations).

Finally, our laboratory has new evidence that a potassium channel (Kir4.1) in astrocytes is critical for VAH. Conditional deletion of Kir4.1 potassium (K^+) channels in astrocytes of transgenic mice causes exaggerated increases in ventilation when normoxia is restored after chronic hypoxia, which is the first experimental treatment we have found to enhance versus blunt or block VAH (Moya et al., 2017). This suggests that astrocytes have an inhibitory effect during VAH. Djukic et al. (2007) demonstrated a similar result in the hippocampus, which was explained by decreased glutamate uptake from synapses by astrocytes when K^+ channels are blocked. Usually, K^+ is increases in the synapse when glutamate is released during presynaptic depolarization, and high K^+ in the synaptic space produces an inward K^+ current in astrocytes

that depolarizes them and activates glutamate uptake. Astrocyte-targeted conditional deletion of Kir4.1 channels produced a marked impairment on both K^+ and glutamate uptake (Djukic et al., 2007). We know that astrocytes in the NTS regulate glutamate levels through excitatory amino acid transporter 2 (EAAT2) and 7 days of CH increased the expression of EAAT2 in the NTS (Mattot et al., 2020). Hence, changes in glutamate uptake may be involved in VAH and we hypothesize that the functional changes in astrocytes accompanying increased GFAP during chronic hypoxia include changes in glutamate uptake that permit higher levels of synaptic activity but protect against excitatory amino acid toxicity. Further *in vivo* animal experiments will be necessary to test this hypothesis. Knowing the time course and physiological significance for markers of glial activation as measured in this study will be important for interpreting the results in such future studies.

APPENDIX 1

Isoflurane vs. Pentobarbital Anesthetics

When exposing a mouse to chronic hypoxia it is important that sustained 10% oxygen is maintained. Eventually the animal is anaesthetized for proper collection of the brain stem and spinal cord. In the past pentobarbital was injected as the anesthetic for rats. This method deems more difficult for mice, and requires the mouse being exposed to normoxic 21% oxygen. We had concerns that his temporary time of O_2 fluctuation could confound our results of glia cell activation. The question of Isoflurane was hypothesized because the mouse could be anaesthetized within its chambers while continuously receiving the hypoxic air. Since the isoflurane is a respiratory anesthetic, there was concern that perhaps it would alter our results for glia activation in the NTS region of the brain.

Materials and Methods

Initially an intraperitoneal dose of pentobarbital 390mg/kg was injected into the mice's inner thigh during hypoxic exposure as the anesthetic for euthanizing in these particular experiments. However, we found it to be challenging to physically inject the mice while they were in their chamber of 10% oxygen for short periods of time, without exposing them to normoxic air. Isoflourane proved to be an easier anesthetic because we could administer it directly into the chamber without exposure of normoxia. We conducted an experiment testing the difference in glia cell activation when anaesthetized with pentobarbital and isofluorane with n=3 for both treatments.

Results and Conclusion

When anaesthetizing the mice, it is important for us to know if our anesthetic is affecting our results. We found no significant differences in our quantitative measures astrocytes (Fig. 3) or microglia (Fig. 4) in the 3 mice anesthetized with pentobarbital versus isoflurane. However, not demonstrating a significant difference is not the same as finding a significant difference and this study is underpowered with n=3 to say with confidence (> 95%) that the anesthetics do not produce different effects. Hence, we decided to do all of the future experiments using isoflurane for all hypoxia timepoints to avoid any confounding effects.

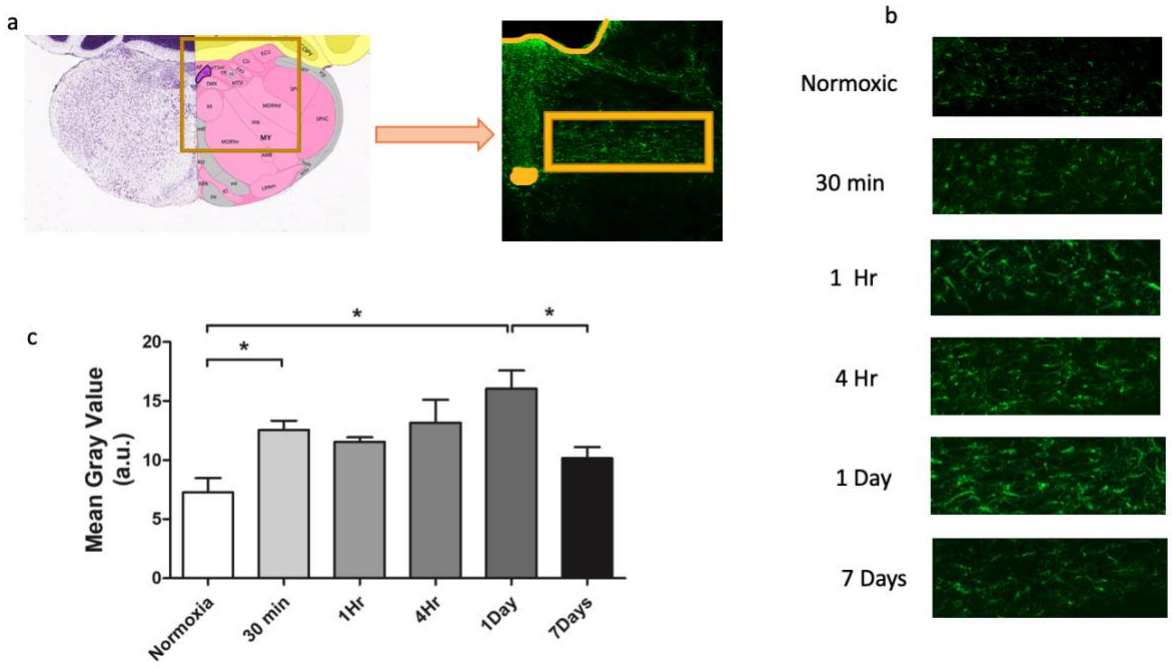

Fig 1. **Quantification of Astrocyte Activation in the Nucleus Tractus Solitarus Region of the HIF-1 knockout Mouse Brainstem When Exposed to Chronic Hypoxia (10% Oxygen) for specific time points of 30min, 1hr, 4hr, 1 Day, and 7 Days.** a. Example of the region of the brainstem sliced, immunohistochemically stained, and imaged. The orange boxed region of the illustration is the location of the nucleus tractus solitarius region of the brainstem that was imaged. c-b. A series of images taken of the nucleus tractus solitarius region of the brainstem in order of time points. Images are stained with GFAP Astrocyte marker via immunohistochemistry and quantified by fluorescent intensity (mean grey value). High fluorescence indicates activation. The * illustrates significant differences between certain time points.

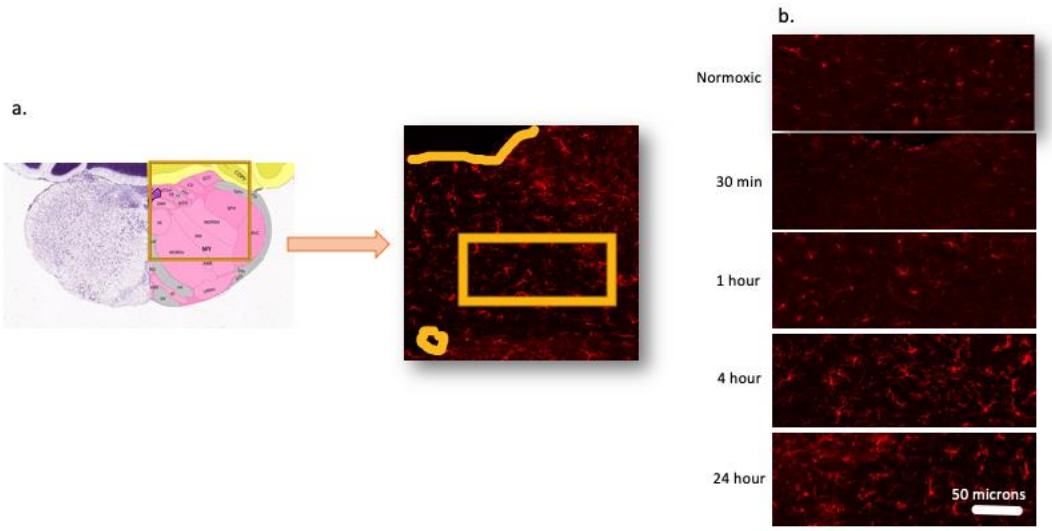

Fig 2. **Quantification of Microglia Activation in the Nucleus Tractus Solitarus Region of the HIF-1 knockout Mouse Brainstem When Exposed to Chronic Hypoxia (10% Oxygen) for specific time points of 30min, 1hr, 4hr, and 1 Day.** a. Example of the region of the brainstem sliced, immunohistochemically stained, and imaged. The orange boxed region of the illustration is the location of the nucleus tractus solitarus region of the brainstem that was imaged. b. A series of images taken of the nucleus tractus solitarus region of the brainstem in order of time points. Images are stained with Iba-1 microglia marker via immunohistochemistry.

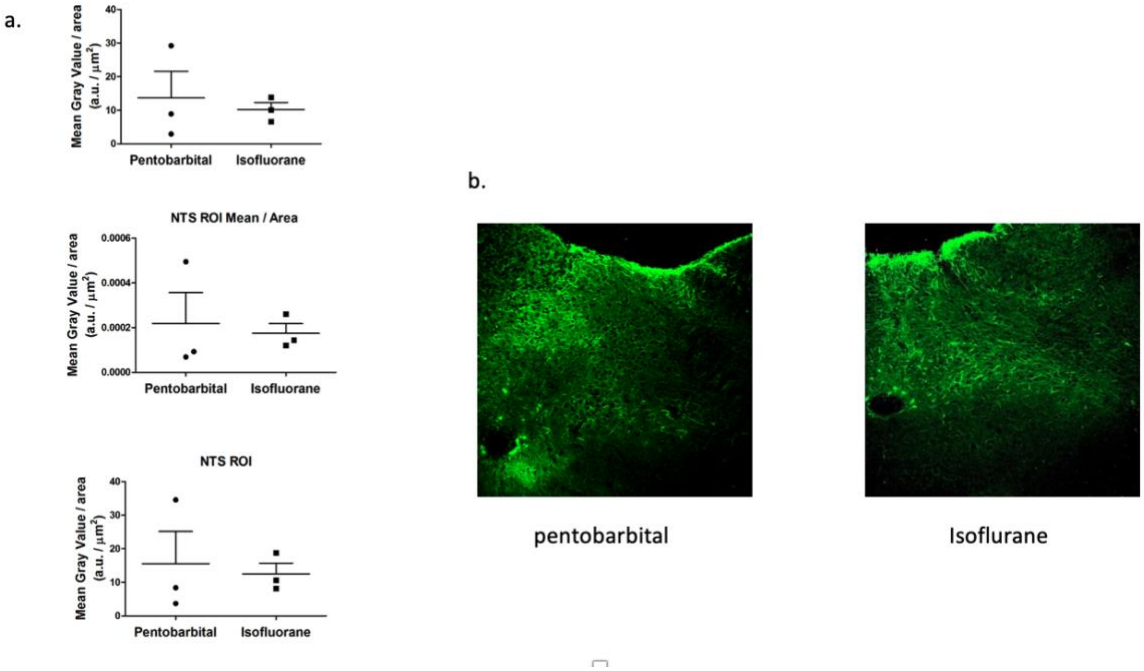

Fig 3. **Pentobarbital vs. Isoflurane anesthetic and the activation of astrocytes in the NTS.** a. Quantification of GFAP intensity via mean grey value of astrocytes in1 hour hypoxic mice anesthetized with isoflurane vs. pentobarbital in the NTS region of the brainstem b. Images taken on Leica microscope with 10x magnification showing the difference between astrocytes quantification in isoflurane vs. pentobarbital.

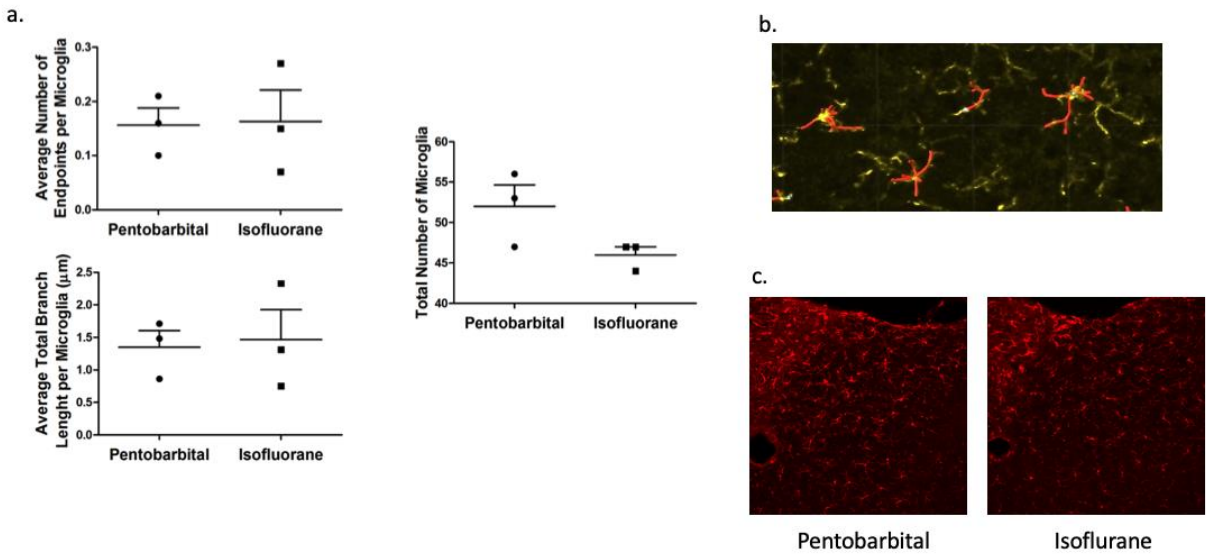

Fig 4. **Pentobarbital vs. Isoflurane anesthetic and the activation of microglia in the NTS.** a. Quantification of microglia Iba-1 after 1 hour of hypoxia and anesthetized with pentobarbital or isoflurane, microglia show activity by morphology change with decreased branching and end points, and no significant change in cell count. b. IMARIS method of measurements and quantification of microglia. c. Images taken on Leica5 at 10x magnification of NTS region of brainstem.

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