Repetitive Acute Intermittent Hypoxia Does Not Promote Generalized Inflammatory Gene Expression in the Rat CNS

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Abstract

Modest protocols of repetitive acute intermittent hypoxia (rAIH) enhance motor function in patients with chronic incomplete spinal injury. Since chronic intermittent hypoxia (CIH) elicits neuroinflammation, there is potential for rAIH to have similar effects. Thus, we tested the hypothesis that rAIH has minimal impact on microglial inflammatory gene expression, but up-regulates key neurotrophic factor expression in a CNS region-specific manner. Using real time PCR, we evaluated mRNA levels of inflammatory and neurotrophic factors in immunomagnetically-isolated microglia from rat frontal cortex, brainstem and upper and lower cervical spinal cord following rAIH (ten, 5-minute episodes, thrice weekly, 4 weeks). In agreement with our hypothesis, rAIH had no significant impact on microglial inflammatory gene expression in any region studied. On the other hand, neurotrophic factor expression was altered in a gene-and region-specific pattern. These results have important implications for the safety of rAIH as a potential therapy to enhance neuroplasticity and motor function in patients with spinal injury or other neurologic disorders.

INTRODUCTION

Intermittent hypoxia (IH) can have both detrimental and beneficial effects, depending on the frequency of cycles per day, the severity of hypoxic episodes and the duration of exposure (i.e. the "dose" of IH; (Navarrete-Opazo and Mitchell, 2014; Xi and Serebrovskaya, 2012). For example, IH protocols simulating that experienced during sleep apnea (hundreds of hypoxic episodes per night for many nights; chronic intermittent hypoxia, CIH) is associated with maladaptive changes, including insomnia, cardiovascular disease, hypertension, metabolic syndrome and cognitive impairments (Brooks et al., 1997; Gozal et al., 2001; Lesske et al., 1997; Perry et al., 2008; Savransky et al., 2007). Cognitive impairments arise from neuronal apoptosis in brain regions necessary for learning and memory, including the hippocampus and frontal cortex (Gozal et al., 2001; Row et al., 2003). CIH-induced

hippocampal apoptosis involves neuroinflammation and oxidative stress (Li et al., 2003; Li et al., 2004; Shan et al., 2007). In contrast, exposure to 3, 5-minute bouts of hypoxia (acute intermittent hypoxia; AIH), elicits neuroplasticity that persists for hours post-hypoxia (Bach and Mitchell, 1996; Terada and Mitchell, 2011). Regimens of repetitive acute intermittent hypoxia (rAIH) consisting of either daily (dAIH) or 3 times weekly (3×W AIH) exposure to 10, 5-minute hypoxic episodes also promote neuroplasticity (Lovett-Barr et al., 2012; MacFarlane, 2010; Satriotomo et al., 2012; Vinit, 2010; Wilkerson and Mitchell, 2009) and restore motor function in clinical disorders that compromise movements (e.g. spinal injury and ALS) such as breathing (Lovett-Barr et al., 2012; Nichols et al., 2013) or limb function (Hayes et al., 2014; Lovett-Barr et al., 2012; Trumbower et al., 2012).

rAIH is thought to promote neuroplasticity by up-regulating key proteins critical for neuroplasticity, including brain-derived neurotrophic factor (BDNF) and its receptor TrkB (Lovett-Barr et al., 2012; Satriotomo et al., 2012). Importantly, dAIH promotes neuroplasticity in the absence of hypertension or neuronal apoptosis typically associated with CIH (Lovett-Barr et al., 2012; Wilkerson and Mitchell, 2009), suggesting that it does not induce pathologies characteristic of CIH. In a cervical hemisection model of spinal cord injury, dAIH nearly completely restores the capacity to increase tidal volume during maximal chemoreceptor stimulation, and strengthens spontaneous and evoked electrical potentials in the phrenic nerve below the site of hemisection (Golder and Mitchell, 2005). Repetitive AIH also improved lower extremity strength and walking in humans with incomplete, chronic spinal cord injuries (Hayes et al., 2014; Trumbower et al., 2012), indicating that even mild paradigms of intermittent hypoxia may have clinical benefit.

Multiple CNS cell types produce inflammatory mediators, although microglia (resident CNS immune cells) are most often implicated. Microglia respond to CIH with increased inflammatory gene expression in a region- and time-dependent manner (Smith et al., 2013). However, the impact of rAIH on microglial or CNS inflammatory gene expression has not yet been investigated. Thus, a major goal of the present study was to examine regional CNS differences in inflammatory gene expression in microglia and CNS tissue homogenates (that include all CNS cell types) after AIH delivered 3 times per week, for 4 weeks (3×W AIH). After 3×W AIH, respiratory motor plasticity is enhanced in rats (MacFarlane, 2010). Since microglial neurotrophic factor production promotes neuronal survival and plasticity (Lalancette-Hebert et al., 2007; Parkhurst et al., 2013), we hypothesized that 3×W AIH would also increase microglial and tissue homogenate neurotrophic, but not inflammatory, gene expression in a region-specific manner.

We evaluated the expression of 10 genes (Table 1) in isolated microglia and regional CNS tissue homogenates. As "markers" of inflammation, we chose to analyze the expression of the pro-inflammatory cytokines interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)α, and the enzyme cyclooxygenase (COX)-2, that inducibly produces prostaglandins. All inflammatory genes chosen are well-known to contribute to neuronal death in multiple models of CNS injury (Iadecola et al., 2001; Merrill, 1992; Munoz-Fernandez and Fresno, 1998; Spooren et al., 2011; Tzeng et al., 2005), and we have previously shown that CIH upregulates the expression of inducible nitric oxide synthase (iNOS), COX-2, IL-1β and IL-6 in microglia, depending on the CNS region evaluated (Smith et al., 2013). Also,

important in the context of CIH, are the observations that inhibiting COX-2 protects neurons and reduces behavioral deficits induced by CIH exposure (Li et al., 2003). We included evaluation of toll-like receptor (TLR)4 as well, since we have previously reported that CIH upregulates its expression in microglia (Smith et al., 2013), and increased TLR4 activities are associated with neuroinflammation and neurodegeneration (Lehnardt et al., 2008; Rivest, 2009). We evaluated the expression of 4 neurotrophic factors, each of which have been shown in other models of CNS injury and pain to be upregulated specifically in microglia [e.g. insulin-like growth factor (IGF)-1 (Lalancette-Hebert et al., 2007) and BDNF (Coull et al., 2005)], and/or that are known to promote various forms of neuronal plasticity or neuroprotection [e.g. vascular endothelial growth factor (VEGF; Dale-Nagle et al., 2011; Storkebaum et al., 2004), BDNF (Numakawa, 2014; Parkhurst et al., 2013), IGF-1 (Benarroch, 2012; Deak and Sonntag, 2012), and adrenomedullin (ADM)-2 (Cheyuo et al., 2012)]. ADM-2 and VEGF are also highly hypoxia-responsive in other systems (via HIF activation) (Berra et al., 2000; Kaur et al., 2005).

As predicted, 3×W AIH did not increase inflammatory gene expression in microglia from frontal cortex, brainstem, upper cervical spinal cord (C1–C3) or lower cervical spinal cord (C4–C6). However, there were region- and gene-specific effects of 3×W AIH on certain inflammatory genes in brainstem and frontal cortex homogenates, although there were no detectable effects on inflammatory gene expression in cervical spinal homogenates. Contrary to our hypothesis, 3×W AIH did not increase microglial expression of the growth/trophic factors examined here, although there were gene-specific increases in brainstem tissue homogenates. Collectively, our results suggest that 3×W AIH does not promote microglial inflammation, nor does it augment their expression of growth/trophic factors that are critical for neuroplasticity. Other cell types do increase neurotrophic factor expression in some CNS regions, although this appears to only occur in CNS regions in which increased inflammatory gene expression was observed in tissue homogenates.

METHODS

Drugs/Materials

Anti-PE magnetic beads and MACS MS columns were purchased from Miltenyi Biotech (Germany). PE-conjugated mouse anti-rat CD11b was purchased from BD Biosciences (San Jose, CA). Papain was purchased from Sigma-Aldrich (St. Louis, MO) and DNaseI was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Hank's Buffered Salt Solution (HBSS) was purchased from Cellgro (Herndon, VA). TRI-Reagent was purchased from Sigma Aldrich (St. Louis, MO). Glycoblue reagent was purchased from Ambion (Austin, TX). MMLV reverse transcriptase was purchased from Invitrogen (Carlsbad, CA). Oligo dT, random primers, and RNAse inhibitor were purchased from Promega (Madison, WI). Primers were designed using Primer 3 software and were purchased from Integrated DNA Technologies (Coralville, IA). Power SYBR green was purchased from Applied Biosystems (Foster City, CA).

Animals

Adult male Sprague Dawley rats (3–4 months old) were housed in an AAALAC-accredited facility in 12hr:12hr light-dark conditions and were provided with food and water ad libitum. All surgical and experimental procedures were approved by the Animal Care and Use Committee at the University of Wisconsin. All procedures conformed to policies enumerated in the NIH *Guide for the Care and Use of Laboratory Animals.*

Rats were divided into two groups (n=8 each). The experimental group (3×W AIH) received ten, 5-minute episodes of 10.5% O_2, alternating with 5-minute intervals of room air (21% O_2), three days/week for 4 weeks, as described previously (Satriotomo et al., 2012). The control group received room air for 95 minutes, three times weekly for four weeks (normoxia; Nx). Both groups were placed in custom-made Plexiglas cylinders measuring 4.5 × 12 in, where oxygen and carbon dioxide levels were continuously monitored and regulated by a computer controlled using a specific script written in LabVIEW Software (National Instruments, New Delhi, India). Appropriate admixtures of O_2 and N_2 were piped into the chambers at a speed of 4 liters per minute. Control animals were exposed to room air only. Twenty-four hours following the last exposure, rats were euthanized by an overdose of isoflurane. Spleen samples were collected, and rats were perfused with ice-cold intraaortic phosphate-buffered saline to remove circulating myeloid cells before harvesting the following central nervous system regions: frontal cortex, brainstem, upper cervical spinal cord (C1–C3) and lower cervical spinal cord (C4–C6).

Microglial and CNS tissue homogenate sample preparation

Microglia were isolated from each CNS region using immunomagnetic separation, as previously described (Crain et al., 2009) with the exception that the tissue was dissociated with papain (0.7 mg/mL) and DNAse I (2 mg/mL) in HBSS for 24 minutes at 37C. Myelin was removed with high-speed centrifugation at 850g in 27% Percoll solution, and remaining cells were resuspended in buffer (PBS, BSA, EDTA). This suspension was incubated with PE-conjugated anti-CD11b^{+} antibodies, followed by incubation with an anti-PE antibody conjugated to a magnetic bead. Samples were loaded onto MACS MS columns, washed in a magnetic field, and CD11b^{+} cells were eluted. Prior to the anti-CD11b^{+} antibody incubation step, a sample was taken for CNS homogenate tissue analysis. As previously shown, this method of immunomagnetic isolation results in >97% of CD11b^{+}/CD45^{low} cells (Nikodemova and Watters, 2012). We subsequently refer to these CD11b^{+} cells as "microglia."

RNA isolation and quantitative RT-PCR

Total RNA from CD11b^{+} cells, spleen and CNS region tissue homogenates was isolated using TriReagent and Glycoblue reagent according to the manufacturers' instructions. Complementary DNA (cDNA) was synthesized from total RNA using MMLV reverse transcriptase and Oligo dT, random primers, and RNAse inhibitor as we have previously described (Crain et al., 2009). Based on RNA abundance, 150 ng, 300 ng and 1 µg of total RNA from isolated microglia, CNS tissue homogenates and spleen, respectively, were used in the cDNA synthesis reactions. qPCR was performed using PowerSYBR green PCR master mix on an ABI 7500 Fast system. Standard curves (Rutledge and Cote, 2003) were

used to determine the relative expression of each gene in microglial and homogenate tissue samples using the average of duplicate, interpolated C_T values, which were normalized to the expression of 18s ribosomal RNA. The delta-delta C_T method was employed to determine relative expression of splenic genes relative to 18s ribosomal RNA (Livak and Schmittgen, 2001). The primer sequences used for qRT-PCR are shown in Table 1. Primers were designed using Primer 3 software and were purchased from Integrated DNA Technologies (Coralville, IA). Primers were designed to span introns wherever possible, and primer efficiency was assessed by use of standard curves, as we have previously reported (Crain et al., 2009). Gene transcripts were considered undetectable and not included in statistical analyses if their C_T values fell outside of the linear range of the standard curve for that primer set; for all genes assessed here, this value was 34 cycles. If 50% or more of the animals in a treatment group had C_T values that were undetectable or that fell outside the linear range of the primer set, that gene was considered below assay detection limits and data were excluded.

Statistical Analyses

For CNS tissues, analyses were performed on normalized, interpolated C_T values from standard curves for each gene, as previously described (Smith et al., 2013). Data from spleen were similarly analyzed using fold change of each gene in question relative to ribosomal RNA. C_T values were identified and discarded as outliers if they fell outside of 1.96 z-scores from the mean, as evaluated by the Grubb's test. The number of outliers per sample varied from zero to three and is indicated in the Results. Student t-tests were conducted using Sigma Plot 11.0 software (San Jose CA). Data sets failing normality were logarithmically transformed prior to analysis with the Whitney-Mann Rank Sum Test. Statistical significance was indicated by a p-value <0.05.

RESULTS

3×W AIH does not elicit cortical microglial inflammation, but induces gene-specific effects in CNS homogenates

Cortical microglial expression of inflammatory and neurotrophic genes was unchanged by 3×W AIH (Fig. 1A), although there was a non-significant trend towards decreased *Cox-2* gene expression (~50%; p=0.169). The only significant change detected in cortical homogenates following 3×W AIH was increased *Cox-2* expression (>70%; p=0.042; Fig. 1B), suggesting that the relevant *Cox-2* was in cells other than microglia.

3×W AIH differentially affects gene expression in brainstem microglia versus homogenates

3×W AIH did not change inflammatory or neurotrophic factor gene expression in brainstem microglia (Fig. 2A), although there was a non-significant trend towards decreased *Bdnf* mRNA levels (~50%; p=0.084). In contrast, there were selective increases in pro-inflammatory gene expression in brainstem homogenates (Fig. 2B). Specifically, *Tlr4* gene expression increased >2.5 fold (p=0.017). With regard to neurotrophic factor expression, *Vegf* mRNA levels increased >50% in brainstem homogenates (p= 0.018).

3×W AIH gene-specifically decreases expression of microglial neurotrophic factors in the upper cervical spinal cord

Inflammatory gene expression in microglia from the upper cervical spinal cord was unchanged by 3×W AIH (Fig. 3A). However, in this region, trophic genes demonstrated a varied response. Specifically, microglial *Adm-2* (p=0.015) and *Vegf* (p=0.021) expression decreased by nearly 50% (Fig. 3B).

3×W AIH has no detectable effects on microglial or homogenate inflammatory or neurotrophic factor gene expression in the lower cervical spinal cord

Neither pro-inflammatory nor trophic factor gene expression was altered by 3×W AIH in C4–C6 microglia (Fig. 4A) or homogenates (Fig. 4B).

3×W AIH does not promote observable inflammation in the spleen

We evaluated the expression of 6 key pro-inflammatory and 2 anti-inflammatory genes in the spleens of rats exposed to 3×W AIH. We were unable to detect mRNA levels of the cytokine interleukin-4 following 3×W AIH (data not shown), and minimal change was observed in IL-10 mRNA, providing no evidence for anti-inflammatory (M2) macrophage activation. Although basal *Cox-2*, *Ifn-g*, *Il-1β*, *Il-6*, *Il-17*, and *iNo*s expression could be detected, no significant changes were observed after 3×W AIH (Fig. 5), suggesting that 3×W AIH does not promote systemic inflammation.

DISCUSSION

Modest protocols of repetitive acute intermittent hypoxia (rAIH) have therapeutic potential to treat clinical disorders that impair movement, inducing spinal motor plasticity, without the attendant pathologies of neuronal loss, reactive gliosis, or systemic hypertension (Dale et al., 2014; Navarrete-Opazo and Mitchell, 2014; Satriotomo et al., 2012). Microglial inflammatory activities likely contribute in part to neuroinflammation and neuronal loss resulting from "high dose" pathologic intermittent hypoxia [i.e. CIH; (Yang et al., 2013; Zhang et al., 2012)]. However, the effects of rAIH on neuroinflammation, and microglial inflammatory gene expression have not been previously examined. Here we show that a protocol of rAIH known to elicit functional benefits in rodent injury models [i.e. 3×W AIH; (MacFarlane, 2010)] does not increase microglial inflammatory gene expression in any CNS region examined here, although the expression of two pro-inflammatory genes increased in homogenates, suggesting a cellular source other than microglia (e.g. astrocytes or neurons). Likewise, we did not detect evidence of peripheral (splenic) inflammation based on the expression of 8 classical pro- and anti-inflammatory genes examined after 3×W AIH.

Given the beneficial effects of rAIH paradigms in rodent injury models and patients (Golder and Mitchell, 2005; Hayes et al., 2014; Trumbower et al., 2012), and known neuroprotective effects of microglial neurotrophic factor production (Lalancette-Hebert et al., 2007; Parkhurst et al., 2013), we also expected rAIH-induced upregulation of microglial expression of key growth factors important for neuronal plasticity and/or survival. However, we did not observe increases in any microglial neurotrophic/growth factor expression examined, although there were region- and gene-specific increases in tissue homogenates.

Together, these results suggest that, unlike CIH (Smith et al., 2013), 4 weeks of 3×W AIH does not promote CNS neuroinflammation, nor are there changes in the expression of any of the 10 inflammatory or neurotrophic genes evaluated here in microglia. Microglial and tissue homogenate expression of iNOS was also analyzed, and showed no statistically significant differences upon 3×W AIH exposure, regardless of the CNS region studied (data not shown). Thus, these results contribute to accumulating evidence that rAIH is a safe and effective therapeutic approach to treat motor impairment (Dale et al., 2014; Navarrete-Opazo and Mitchell, 2014).

Because of its importance to rAIH-induced respiratory motor plasticity and metaplasticity (Dale et al., 2014), we were especially interested in modulation of gene expression in the region of the phrenic motor nucleus (i.e. C4–C6). Similar rAIH protocols increase expression of proteins critical for phrenic motor plasticity (Satriotomo et al., 2012; Wilkerson and Mitchell, 2009), leading to our hypothesis that rAIH would increase mRNA levels of these same genes. However, no significant changes in microglial or homogenate gene expression of either neurotrophic factors or inflammatory markers were observed in this region. Growth/trophic factor upregulation within phrenic motor neurons (Satriotomo et al., 2012) is difficult to detect in tissue homogenates due to the extensive dilution of ~600 phrenic motor neurons in the large volume of the cervical ventral horn. From another perspective, the absence of cervical spinal inflammation is meaningful because systemic inflammation induces cervical spinal inflammatory gene expression and undermines AIH-induced spinal, respiratory motor plasticity (Huxtable et al., 2013). Therefore, it is encouraging that this rAIH protocol, which may be therapeutic in spinal injury patients, does not cause inflammation in the region of the phrenic motor nucleus that may undermine its therapeutic efficacy.

Significant increases in the expression of two pro-inflammatory genes were detected in tissue homogenates: *Cox*-2 in the cortex and *Tlr4* in the brainstem. Since corresponding changes in microglial expression of these genes were not observed, other cell types are most likely responsible for these changes in tissue homogenate mRNA levels (e.g. astrocytes or neurons). The functional significance of these observations is not yet known. Since COX-2 plays critical roles in synaptic plasticity (Gruol, 2014; Yang and Chen, 2008), and TLR4 signaling can promote neuroprotection (Chen et al., 2012; Marsh et al., 2009; Vartanian et al., 2011), increased expression of these genes, which are typically thought of as pro-inflammatory, may play roles other than neuroinflammatory/pathologic in this situation.

3×W AIH effects on microglial trophic factor gene expression varied by CNS region. Although we had hypothesized that this rAIH protocol would up-regulate microglial expression of genes important for neuroprotection and neuroplasticity, we found no detectable increases in the microglial expression of any of the gene studied here. However, there were limited region-specific decreases in microglial expression of certain genes. For example, C1–C3 cervical microglia demonstrated a reduced expression of *Adm-2* and *Vegf* - genes whose products exert neuroprotective effects (Cheyuo et al., 2012; Rosenstein et al., 2010) and are directly upregulated by the hypoxia-sensitive transcription factor HIF-1 (hypoxia-inducible factor 1) (Shibuya, 2013; Zudaire et al., 2006). In contrast, in brainstem homogenates, *Vegf* mRNA levels increased. Thus, it is important to understand cell- and

region-specific responses to 3×W AIH since microglial and CNS homogenate responses sometimes differ depending on the CNS region and the specific gene in question.

Leconte and colleagues (Leconte et al., 2012) demonstrated that a similar intermittent hypoxia protocol induced statistically significant increases in the expression of *Adm-2* and *Vegf* genes in mouse whole brain homogenates after 41 days of AIH exposure. However, there are multiple, important variables that differ between that study and ours, aside from species. First, the expression of genes in all cell types from whole brain will differ from results obtained in microglia and tissue homogenates from individual CNS regions as in our study. Further, their hypoxic exposures consisted of 8% O_2 for 1 hour per day, 3 days per week for 6 weeks; our exposures consisted of 10, 5 min episodes of 10.5% O2, 3 days per week for 4 weeks. It is well-established that the CNS responds differently to stimuli delivered in massed versus spaced patterns (Baker and Mitchell, 2000; Glanzman et al., 1990).

Brain-derived neurotrophic factor (BDNF) is important during development for neuronal survival and differentiation (Xie and Yung, 2012), motor learning (Parkhurst et al., 2013) including AIH-induced phrenic long-term facilitation (Baker-Herman et al., 2004), and hippocampal synaptic plasticity (Pang et al., 2004). Because AIH-induced phrenic long-term facilitation relies on increased BDNF synthesis (Baker-Herman et al., 2004; Wilkerson and Mitchell, 2009), and because rAIH elicits respiratory metaplasticity (Lovett-Barr et al., 2012; MacFarlane et al., 2008; MacFarlane, 2010; Vinit et al., 2009; Vinit, 2010; Wilkerson and Mitchell, 2009), we had hypothesized that BDNF expression in at least some CNS regions, would be up-regulated by 3×W AIH. In agreement, increased *Bdnf* expression has been reported in the pons, but not medulla, after 3 brief bouts of hypoxia in mice (Vermehren-Schmaedick et al., 2012). However, of the four CNS regions evaluated after 3×W AIH in our study, we detected BDNF mRNA only in cortical tissue homogenates and in brainstem microglia. Yet, despite trends, there were no significant changes after exposure to 3×W AIH. Interpretation of this finding must be made with caution however, since we do not consider three important factors in this study: 1) increased BDNF protein expression may occur via increased translation from pre-existing mRNA versus new transcriptional upregulation; 2) persistent changes in protein and mRNA levels within limited populations of cells (e.g. motor neurons; Satriotomo et al., 2012; Lovett-Barr et al., 2012) may be obscured by dilution in a large volume constituting the tissue homogenate; or 3) increased BDNF mRNA levels may be transient, and are no longer evident after 4 weeks of 3×W AIH.

In this regard, it is important to acknowledge the limitations of the present study. We utilized an immunomagnetic cell isolation approach to isolate microglia from different regions of the CNS. This method does not allow distinction of heterogeneous microglial inflammatory responses from cell populations that are low in frequency. Thus, it is feasible that there are small populations of microglia that respond differently to 3×W AIH that are too few in number to influence the overall means of the inflammatory gene expression measured here. However, the functional significance of such small populations is also unclear at this time, as are the mechanisms underlying their heterogeneic responses. In addition, inflammatory gene and growth factor mRNAs, tend to have very short half-lives due to the presence of adenine uridine rich elements within their 3′ untranslated regions

(Caput et al., 1986; Hamilton et al., 2012; Shaw and Kamen, 1986), so the timing of gene analysis performed here (1 day after the last AIH exposure for 4 weeks) may be too late to detect changes in inflammatory gene mRNA levels.

To assess the possibility of systemic inflammation induced by rAIH, we evaluated splenic expression of 7 prototypical (M1) pro-inflammatory cytokines – cyclooxygenase-2, interferon gamma, inducible nitric oxide synthase, interleukin-1β, interleukin-4, interleukin-6, and interleukin-17, as well as the canonical anti-inflammatory (M2) cytokine interleukin-10. We found no evidence for pro- or anti-inflammatory gene expression in the spleen after 3×W AIH, although there were insignificant increases in mRNA levels of some inflammatory genes. We conclude that 4 weeks of 3×W AIH does not initiate or maintain inflammation in the spleen, a key peripheral immune organ rich in B and T lymphocytes.

Modest intermittent hypoxia protocols are being investigated as a means to treat neuromotor disorders (Dale et al., 2014; Navarrete-Opazo et al., 2015). However, it is important to analyze the ability of such intermittent hypoxia therapy to cause pathologies such as untoward neural and systemic inflammation. The results presented here directly evaluate inflammatory gene expression in microglia and tissue homogenates from various CNS regions after a therapeutically applicable protocol of repetitive acute intermittent hypoxia. Although we found little evidence for appreciable inflammation after 4 weeks of rAIH, it would be useful to evaluate other time points in future investigations since it remains possible that immediate or long term rAIH could elicit pathologic inflammatory responses.

Acknowledgments

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Highlights

- The objective is proposing an evolutionary model which accepts knowledge as intensely active and dynamic and evolving in maturity from the early stages of research.

- But this is possible only if the research team adopt a clear, clean and joint process of disciplinary integration and transdisciplinary integration of the produced and discovered knowledge.

- In this way, the results of research will have a greater influence on society and they also will be adopted by society.

A.

B.
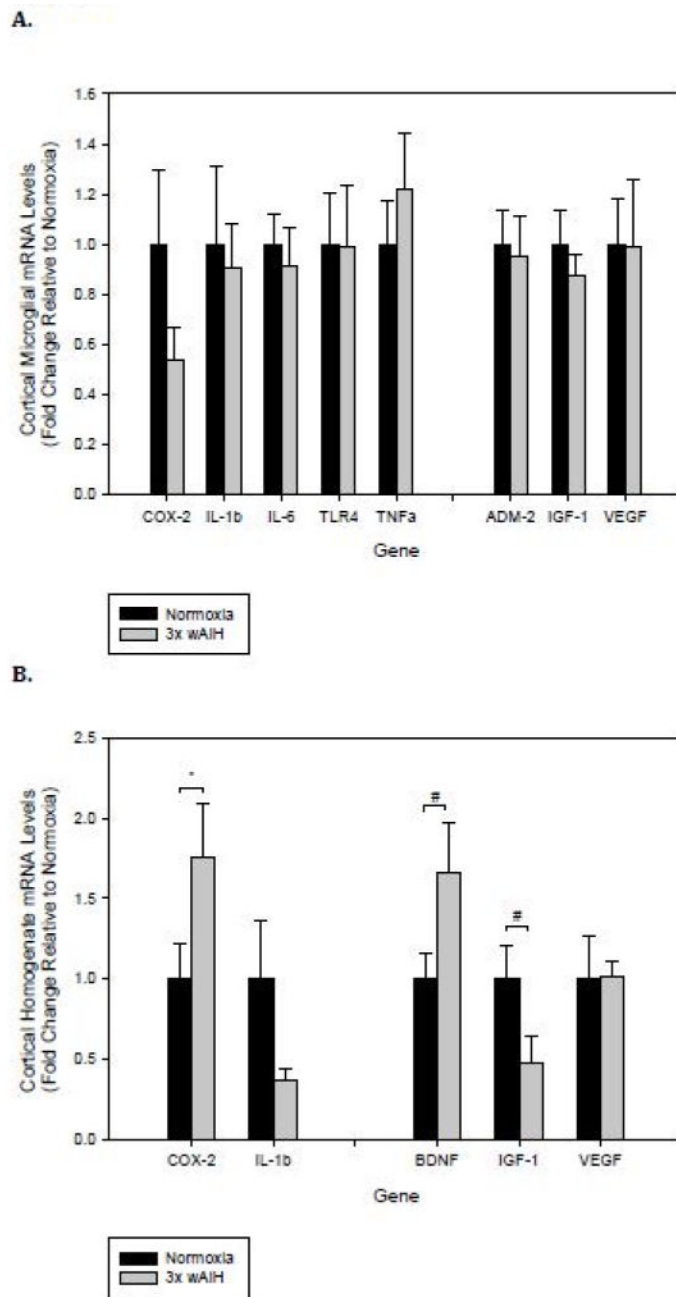
Figure 1. 3×W AIH does not promote inflammatory gene expression in rat cortical microglia, but it has gene-specific effects in cortical homogenates

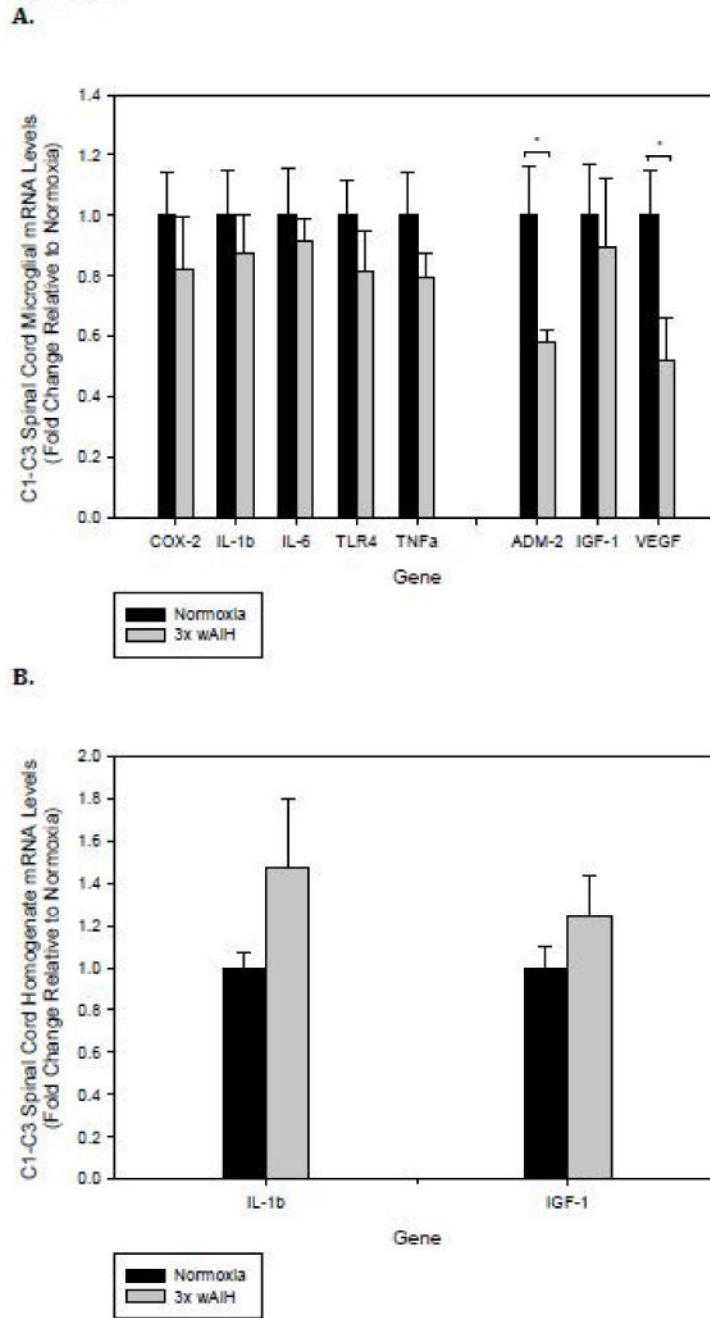Gene expression was assessed by qRT-PCR in immunomagnetically-isolated microglia (A), and tissue homogenates from the frontal cortex (B) following 4 weeks of 3×W AIH. Treatment did not alter the expression of any gene evaluated in microglia, but there were gene-specific effects in tissue homogenates. Data shown are means ± 1 SEM of n=5–8. *p<0.05 and #0.05<p<0.1 versus normoxia.

A.

B.
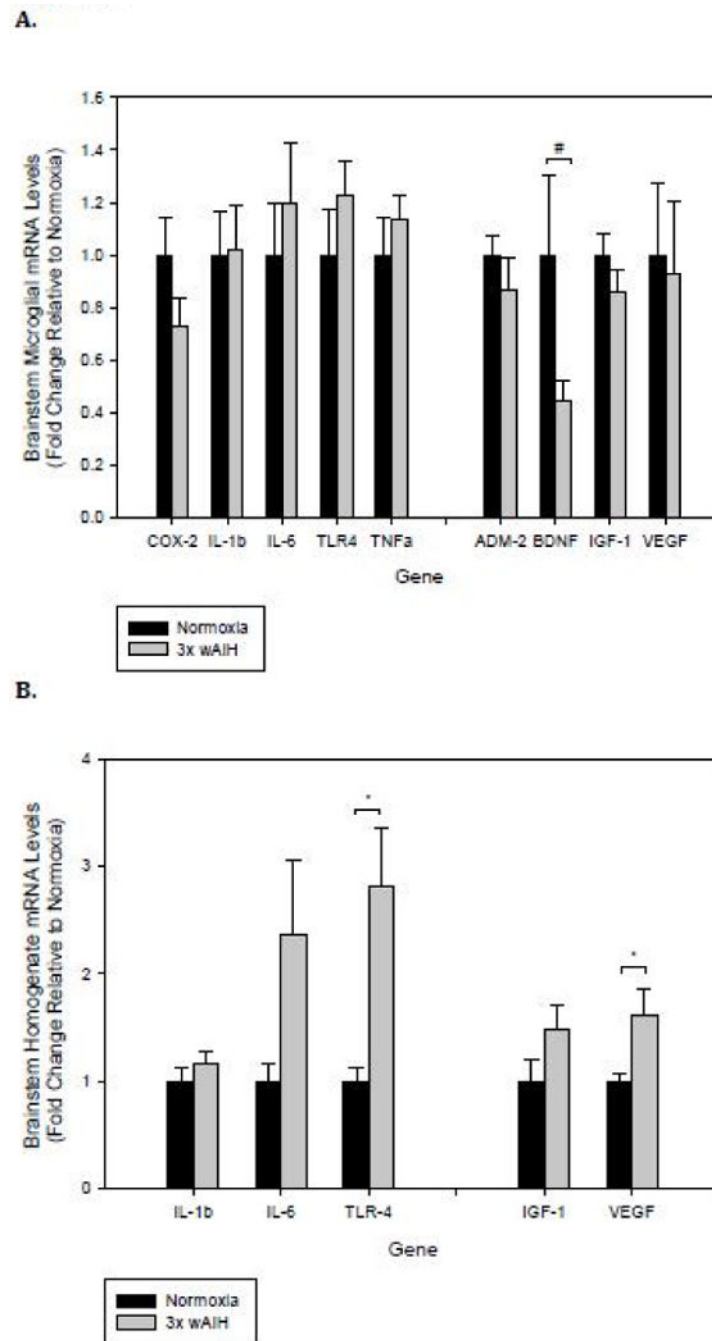
Figure 2. 3×W AIH does not promote inflammatory gene expression in rat brainstem microglia, but it increases expression of *Tlr4* and *Vegf* in brainstem homogenates

Gene expression was assessed by qRT-PCR in immunomagnetically-isolated microglia (A), and tissue homogenates from the brainstem (B) following 4 weeks of 3×W AIH. Treatment did not alter the expression of any inflammatory gene evaluated in microglia, though there was a non-significant reduction in *Bdnf* mRNA levels. 3×W AIH increased *Tlr4* and *Vegf* gene expression in tissue homogenates. Data shown are means ± 1 SEM of n=5–8. *$p < 0.05$ and #$0.05 < p < 0.1$ versus normoxia.

Figure 3. C1–C3 spinal microglial inflammatory gene expression was unaltered following 3×W AIH exposure, but *Adm-2* and *Vegf* mRNA levels were down-regulated

Gene expression was assessed by qRT-PCR in immunomagnetically-isolated microglia (A), and tissue homogenates from C1–C3 cervical spinal segments (B) following 4 weeks of 3×W AIH. Treatment did not alter the expression of any inflammatory gene evaluated in microglia, but it reduced *Adm-2* and *Vegf* mRNA levels. 3×W AIH had no effects on the expression of any gene evaluated in C1–C3 tissue homogenates. Data shown are means ± 1 SEM of n=6–8. *p<0.05 versus normoxia.

A.

B.
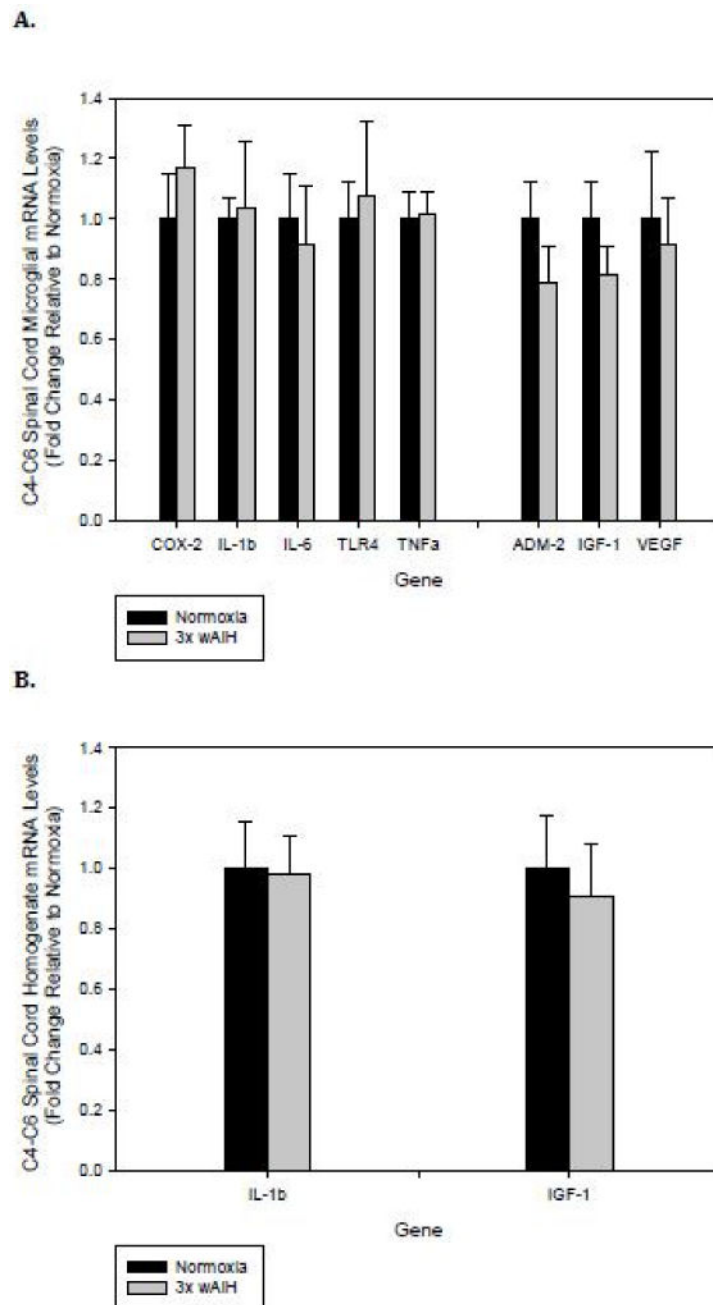
Figure 4. 3×W AIH had no effect on C4–C6 spinal microglial or tissue homogenate gene expression

Gene expression was assessed by qRT-PCR in immunomagnetically-isolated microglia (A), and tissue homogenates from C4–C6 cervical spinal segments (B) following 4 weeks of 3×W AIH. Treatment did not alter the expression of any inflammatory or trophic factor gene evaluated in either microglia or C4–C6 tissue homogenates. Data shown are means ± 1 SEM of n=5–8.
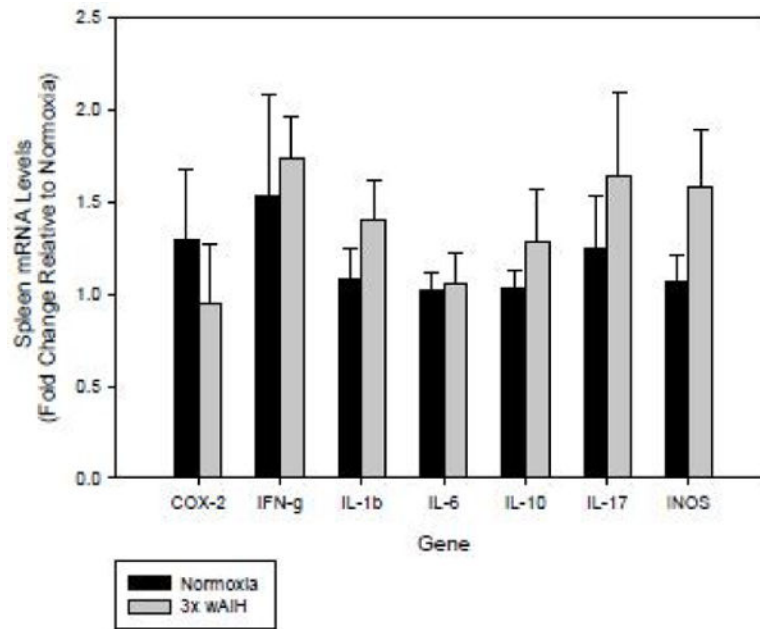
Figure 5. 3×W AIH had no effect on the expression of any gene evaluated in the spleen
Gene expression was assessed by qRT-PCR in the spleen following 4 weeks of 3×W AIH. Treatment did not alter the expression of any pro- or anti-inflammatory gene evaluated. Data shown are means ± 1 SEM of n=7–8.

TABLE 1

Gene	Forward (5′→3′)	Reverse (5′→3′)
18s	CGG GTG CTC TTA GCT GAG TGT CCC G	CTC GGG CCT GCT TTG AAC AC
BDNF	CCC ATC ACA ATC TCA CGG TAT TC	TGC GGA GGG TCT CCT ATG AA
COX-2	TGT TCC AAC CCA TGT CAA AA	CGT AGA ATC CAG TCC GGG TA
IGF-1	CAG TTC GTG TGT GGA CCA AG	TCT TGG GCA TGT CAG TGT G
IFN-γ	CAT GGA TGC TAT GGA AGG AAA GA	GGC TCT CAA GTA TTT TCG TGT TAC C
IL-lβ	CTG CAG ATG CAA TGG AAA GA	TTG CTT CCA AGG CAG ACT TT
IL-4	GCT TCC AGG GTG CTT CGC AAA T	ACG GTG CAG CTT CTC AGT GAG TT
IL-6	GTG GCT AAG GAC CAA GAC CA	GGT TTG CCG AGT AGA CCT CA
IL-10	AAG TGA TGC CCC AGG CAG AGA A	AAA TCG ATG ACA GCG TCG CAG C
IL-17	GGG AAG TTG GAC CAC CAC CT	TTC TCC ACC CGG AAA GTG AA
iNOS	AGG GAG TGT TGT TCC AGG TG	TCT GCA GGA TGT CTT GAA CG
TLR4	AGG CAG CAG GTG GAA TTG TAT C	TCG AGG CTT TTC CAT CCA ATA G
TNFα	TGC CAC TTC ATA CCA GGA GA	CCG GAC TCC GTG ATG TCT A
VEGF	TTG AGA CCC TGG TGG ACA TCT	CAC ACA GGA CGG CTT GAA GA