The Dual Role of Microglia in Activity-Dependent Respiratory Plasticity

by

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Table of Contents

Acknowledgements ii

Abstract iii

I. Introduction 1

II. Spinal TNFα initiates compensatory plasticity in response to prolonged respiratory neural activity deprivation and requires microglia 19

Figures 42

III. Microglial Depletion Rescues LPS-Induced Impairment of Compensatory Response to Recurrent Reductions in Respiratory Neural Activity 46

Figures 67

IV. Maternal intermittent hypoxia during pregnancy causes cardiorespiratory deficits and gut dysbiosis in adult male, but not female, offspring 72

Figures 91

V. Recurrent hypoxia in a model of sleep apnea during pregnancy leads to microglia-dependent respiratory deficits and persistent neuroinflammation in adult male offspring 105

Figures 135

VI. Discussion 148

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Abstract

Plasticity is a component of the neural control of breathing that allows the system to adjust to persistent or abnormal stimuli, including indications of inadequate respiratory output. In response to reduced respiratory neural activity, a potentially deadly scenario, there is a proportional, compensatory increase in phrenic motor output called inactivity-induced inspiratory motor facilitation (iMF). The mechanisms underpinning iMF have received considerable attention, but the cell types involved in iMF expression remained unknown. Further, the impairment of iMF by relevant insults, such as inflammation, had not been investigated. The purpose of this dissertation was to advance our understanding of the role of microglia, the resident immune cells of the CNS, in iMF expression in the healthy CNS and in the context of neuroinflammation. In Chapter 2, I show that TNF-alpha-dependent iMF following a single, prolonged neural apnea is microglia-dependent, while retinoic-acid-dependent iMF following a series of recurrent neural apneas is cell autonomous. In Chapter 3, I demonstrate that microgila actually have the capacity to impair iMF induced by recurrent neural apneas following an inflammatory stimulus, revealing context-dependent effect of microglial influence on iMF expression. In Chapters 4 and 5, I describe the long-term, sex-specific deficits in microglial function and respiratory control induced by gestational exposure to intermittent hypoxia, a maternal model of sleep apnea during pregnancy. Collectively, these studies characterize the context-dependent effect of microglia on the expression of iMF and indicate inflammation as a potential target for intervention in diseases of respiratory motor control.

Chapter I

Introduction

Breathe in, breathe out: the generation of a breath seems like a simple process, but decades of research into the diverse mechanisms guiding respiration indicate that it is anything but. And that's a good thing. Indeed, if breathing were dictated by a rigid system, our respiratory efforts would be unable to adapt to life's many highs and lows. In sickness (coughing, sneezing) and in health (exercise, eating), for better (laughing), and for worse (crying), our respiratory rhythm persists with remarkable flexibility till death does us part. Understanding the precise mechanisms underlying breathing and its seemingly endless adaptability not only allows us to appreciate the astounding capabilities of our own brain and body, but also to understand the network dysfunction that underlies respiratory failure, and how we may overcome it.

One feature of the neural system controlling breathing that confers considerable flexibility is respiratory neuroplasticity (Fuller and Mitchell, 2017). Plasticity is a component of the healthy CNS that allows neural circuits to respond to abnormal or persistent stimuli (Mitchell and Johnson, 2003). In respiratory motor neurons, plasticity is well-documented following exposure to stimuli that indicate inadequate respiratory output, like hypoxia or reduced respiratory neural activity (Braegelmann et al., 2017). The respiratory control system is meant to fire consistently, from the moment we are born to the moment we die. Thus, interruptions in the neural drive to breathe represent a perturbation that is incompatible with life. Over the last decade, a form of plasticity elicited by reduced neural activity of the respiratory control system, called inactivity-induced inspiratory motor facilitation (iMF) has been characterized (Baker-Herman and Strey, 2011; Mahamed et al., 2011; Strey et al., 2012; Broytman et al., 2013; Streeter and Baker-Herman, 2014a, 2014b; Baertsch and Baker-Herman, 2015; Baertsch and Baker, 2017a, 2017b; Braegelmann et al., 2021). iMF manifests as an increase in phrenic motor output that is proportional to the level of activity reduction experienced (Braegelmann et al., 2017), and an expansion of the dynamic range of phrenic motor output (Baertsch and Baker-Herman, 2013; Baertsch and Baker, 2017b). iMF is hypothesized to be a form of homeostatic plasticity, or a

compensatory response to abnormal neural activity in order to return neural circuit output to a homeostatic “set-point” (Turrigiano, 2012; Strey et al., 2013; Braegelmann et al., 2017). Previous studies in our lab have described mechanisms underpinning diverse types of iMF, which are summarized later in this introduction. Despite a greater understanding of the pathways leading to iMF, the cell types involved in its expression remain unknown.

Recent advances in neuroscience research have garnered appreciation for the quad-partite synapse, that is, the dynamic relationship among neurons, microglia, and astrocytes in the healthy and diseased CNS (Schafer et al., 2013). Microglia are the innate immune cells of the CNS, previously appreciated for little more than their ability to mount an immune response in the presence of injury or disease, or “support” neurons in its absence. However, more recent advances have revealed that microglia are dynamic creatures that tenaciously survey the CNS, sampling the milieu and making critical synapse-level adjustments to tune network function, in addition to their responsibilities as immune sentinels (Nimmerjahn et al., 2005). Despite evidence that TNF-alpha, a cytokine primarily released by microglia, is critical for the expression of some forms of iMF (Broytman et al., 2013), and, on the flip side, that inflammation impairs other forms of respiratory motor plasticity (Vinit et al., 2011; Huxtable et al., 2013), we have relatively little knowledge of the role of microglia in respiratory neuroplasticity. **The goal of this dissertation was to advance our understanding of the role of microglia in iMF under homeostatic and pathophysiologic conditions.** We expanded on Kendra Braegelmann’s work characterizing the TNF-alpha pathway underlying one form of iMF by investigating the requirement for microglia in iMF in the healthy CNS (Chapter 2). Then, we tested the susceptibility of iMF to impairment by inflammation, and whether microglia play a role (Chapter 3). Finally, we investigated whether respiratory control, including iMF, is affected by sex-specific, epigenetic reprogramming of microglia that occurs in adult offspring of mothers exposed to gestational intermittent hypoxia, our rat model of sleep apnea during pregnancy

(Chapters 4 and 5). Some background on the generation of respiratory rhythm, expression of iMF, and the contexts in which we investigated the role of microglia is provided below.

Respiratory Rhythm Generation

Breathing under normal circumstances (eupnic breathing) consists of three phases: inspiration, post-inspiration, and expiration (Ramirez and Baertsch, 2018). According to the triple oscillator hypothesis, inspiration, which serves to generate negative air pressure to draw air into the lungs for gas exchange, is initiated by a microcircuit of rhythmically active neurons in the ventral medulla called the preBotzinger complex (preBotC) (Smith et al., 1991; Anderson and Ramirez, 2017). Post-inspiration, which increases airway resistance to prolong lung deflation and promote more thorough gas exchange, is orchestrated by the aptly named post-inspiratory complex (PiCo) (Anderson et al., 2016). PiCo and preBotC mutually inhibit each other, such that activation of PiCo delays the onset of the next inspiration and activation of preBotC inhibits PiCo to prevent elevations in airway resistance during inspiration (Anderson et al., 2016). Finally, expiration, which is passive at rest but becomes active with increased oxygen demand (Del Negro et al., 2018), is determined primarily by the lateral parafacial nucleus (pFL; (Janczewski and Feldman, 2006; Abdala et al., 2009; Pagliardini et al., 2011; Huckstepp et al., 2015, 2016). Like PiCo, the preBotC inhibits pFL neurons during inspiration to prevent coincidence of inspiratory and expiratory efforts (Del Negro et al., 2018). The preBotC also orchestrates other, more complex orofacial behaviors that coordinate with breathing such as swallowing (Pitts et al., 2013), sniffing (Kleinfeld et al., 2015), and coughing (Pitts et al., 2013). Thus, the preBotC is considered the “master clock” of respiratory rhythm generation (Moore et al., 2013).

PreBotC neurons project to premotor neurons in the rostral ventral respiratory group (rVRG) and dorsal respiratory group (DRG) that transmit respiratory rhythm to the inspiratory muscles to coordinate inspiration. Hypoglossal premotor neurons synapse on hypoglossal motor neurons in the ventral medulla, which upon activation strengthen the upper airway, beginning just prior to

inspiration, to withstand the negative pressure generated by expansion of the thoracic cavity (Fregosi, 2011). Premotor neurons of the phrenic and inspiratory intercostals travel down the ventrolateral funiculus, terminating primarily in the ipsilateral cervical (C3-C6 in the rat; Goshgarian and Rafols, 1981) and thoracic spinal cord, where they synapse onto phrenic and inspiratory intercostal motor neurons, respectively (Feldman et al., 1985; Lipski et al., 1994). There is evidence that some phrenic axons cross to terminate on contralateral motor neurons (Duffin and Li, 2006; Goshgarian, 2009). Simultaneous activation of the phrenic and inspiratory intercostal motor neurons leads to expansion of the chest cavity and generation of negative pressure to draw air into the lungs for gas exchange. Projections from the pFL terminate on expiratory premotor neurons in the caudal ventral respiratory group (cVRG), which innervate expiratory intercostal (Lane, 2011) and abdominal muscles (Butler, 2007) recruited for expiratory force when necessary.

Between the preBotC and the generation of a breath, there are many mechanisms that sculpt each breath to ensure adequate respiratory output based on moment-to-moment demands and the experience of the organism. Peripheral and central structures help adjust medullary rhythm and pattern generation, like the parabrachial complex (including the Kolliker Fuse) (Yang et al., 2020; Dutschmann et al., 2021), the retrotrapezoid nucleus (RTN; (Guyenet et al., 2019), locus coeruleus (Nattie and Li, 2012), and the nucleus tractus solitarius, which collates and integrates information from a number of peripheral sensory afferents including chemo- and mechano-sensory systems (Zoccal et al., 2014; Zyuzin and Jendzjowsky, 2022). There are also mechanisms sensing and responding to stimuli at the level of each inspiratory motor nucleus, including respiratory plasticity.

Inactivity-induced inspiratory motor facilitation (iMF)

Plasticity is a component of many neural systems, including the neural control of breathing, that allows for persistent changes in system performance based on experience (Mitchell and Johnson, 2003). Thus, it is thought that perturbations in the neural system controlling breathing induce plasticity to maintain adequate and dynamic respiratory output (Fuller and Mitchell, 2017). One disruption in respiratory control that could lead to fatal outcomes is reduction or cessation of respiratory neural activity. Indeed, the respiratory control system must fire reliably from birth until death, with minimal disruption from life's many challenges. Reductions in respiratory neural activity trigger one form of respiratory plasticity that results in an increase in inspiratory motor output termed inactivity-induced inspiratory motor facilitation (iMF).

The idea that reduced respiratory neural activity could induce compensatory plasticity was reported by Castro-Moure and Goshgarian, when they unilaterally reduced descending spinal activity for 4 hours by cooling the cord to the point of activity disruption (Castro-Moure and Goshgarian, 1996). After reversal of conduction block, they noted a robust increase in diaphragm EMG activity ipsilateral, but not contralateral, to activity disruption that was associated with morphological changes to phrenic motor neurons and the diaphragm. Thus, a hypothesis emerged that activity deprivation promoted changes in phrenic motor neurons or the diaphragm that resulted in increased respiratory output upon resumption of activity.

Mahamed et al. expanded on this idea in a more isolated preparation by measuring phrenic motor output from the cut, desheathed end of the phrenic nerve in anesthetized, paralyzed, and mechanically ventilated rats (Mahamed et al., 2011). Following prolonged neural apnea (or hypopnea) induced by high-frequency ventilation (via vagal afferent feedback), isoflurane, or reducing CO2 below the threshold for apnea (hypocapnia), persistent increases in phrenic motor output were recorded for up to 60 minutes, and inactivity-induced inspiratory motor facilitation (iMF) was born. Importantly, iMF was elicited in the absence of blood-gas changes,

since increased phrenic motor output was elicited in the absence of hypoxia and persisted despite maintenance of pCO2 at baseline levels.

Subsequent studies characterized pattern specificity of iMF expression, which is a feature of other forms of respiratory plasticity (Baker-Herman and Strey, 2011). iMF was successfully elicited following a single, prolonged (30 min) neural apnea or by a series of five, brief (~1.25 min) neural apneas separated by a resumption of activity, but not by a single amassed neural apnea of the cumulative duration of the brief neural apneas (~6.25 min) (Baertsch and Baker-Herman, 2013). CO_2

Importantly, iMF following prolonged versus intermittent neural apneas is initiated by distinct mechanisms. Following a single, prolonged neural apnea, iMF is dependent on TNF-alpha signaling and requires atypical, but not novel or classical, protein kinase C (aPKC) activity (Strey et al., 2012; Broytman et al., 2013). More specifically, iMF following prolonged neural apnea requires the cleavage of transmembrane TNF-alpha to its soluble form by TNF-alpha converting enzyme (TACE), followed by activation of TNF-alpha receptor 2 (TNFR2), while TNFR1 activation constrains expression of iMF (Chapter 2). Similarly, exogenous TNF-alpha application elicits dose-dependent phrenic motor facilitation (pMF) in an aPKC-dependent manner (Broytman et al., 2013). On the other hand, iMF elicited by exposure to brief, intermittent neural apneas is TNF-alpha independent, instead requiring synthesis of protein and retinoic acid, and activation of retinoic acid receptor alpha and aPKC (Baertsch and Baker-Herman, 2015; Baertsch and Baker, 2017a; Braegelmann et al., 2021). Similar to TNF-alpha-dependent plasticity, exogenous retinoic acid application induces pMF (Baertsch and Baker, 2017a) iMF following prolonged neural apnea does not require protein synthesis or retinoic acid (Baertsch and Baker, 2017a). Interestingly, both prolonged and intermittent neural apnea-induced iMF are constrained by NMDA-receptor activation (Streeter and Baker-Herman, 2014b;

Fields et al., 2019). Collectively, this indicates that iMF following prolonged or intermittent neural apnea(s) is initiated by distinct signaling cascades, eventually converging on a common, NMDAR constrained pathway to elicit a persistent increase in phrenic motor output.

Throughout the above studies, several additional features of iMF were uncovered. First, activity *cessation* is not necessary for induction of iMF (Streeter and Baker-Herman, 2014a). Indeed, iMF can be elicited in response to *reduced* neural respiratory activity, and the compensatory response is proportional to the degree of activity deprivation (Braegelmann et al., 2017). Second, sensation of, and response to, reduced activity of phrenic motor neurons occurs at the level of the phrenic motor pool. Reduction of descending neural respiratory activity induced by unilateral conduction block in the cervical spinal cord elicits phrenic iMF ipsilaterally, but not contralaterally (Streeter and Baker-Herman, 2014a). Further, local inhibition of critical signaling molecules like aPKC (Strey et al., 2012; Broytman et al., 2013; Streeter and Baker-Herman, 2014a, 2014b; Baertsch and Baker-Herman, 2015), retinoic acid (Baertsch and Baker, 2017a; Fields et al., 2019), or TNF-alpha (Broytman et al., 2013; Streeter and Baker-Herman, 2014a, 2014b) impairs its expression. Third, iMF proportionally expands the phrenic dynamic range. Inspiratory motor output operates along a dynamic range at which the minimum is the apneic threshold (AT), or the level of arterial CO2 at which respiratory drive ceases, and the maximum is the amplitude generated by exposure to extreme hypercapnia (high CO2). Expression of iMF is associated with a proportional decrease in the apneic threshold (Baertsch and Baker, 2017b). Following each recurrent neural apnea, the apneic threshold lowers, and the reduced AT persists for at least 60 minutes following resumption of neural activity. After a single prolonged neural apnea, the apneic threshold is similarly persistently lowered. On the other end of the dynamic range, the phrenic response to CO2 is proportionally increased in rats following iMF compared to rats that are not exposed to neural apnea (Streeter and Baker-Herman, 2014a).

Finally, expression of iMF has also been documented in hypoglossal and inspiratory intercostal motor neurons (Baker-Herman and Strey, 2011), indicating that iMF may be a core feature of inspiratory motor control.

The features of iMF described above support the hypothesis that the compensatory response to reduced respiratory neural activity is a form of homeostatic plasticity (Braegelmann et al., 2017). Homeostatic plasticity in the CNS describes the intrinsic mechanisms that are elicited when neural activity moves away from the "set-point" for that system, and serve to return activity to its targeted range and stabilize network output (Turrigiano, 2012). There are many systems of the body that operate around a certain physiological set-point, which, if not maintained, can lead to detrimental consequences (Cannon, 1932). Respiration is similar, in that reductions or extreme elevations of activity lead to pathophysiology or, worse, death. The fact that magnitude of iMF expression is proportional to activity deprivation, and that phrenic response to hypercapnia is increased to a similar degree, indicates a compensatory increase in phrenic network excitability that is finely tuned to the level of activity reduction experienced. Further, the progressive reduction in apneic threshold with each reduction in neural activity makes it more difficult for the next apnea to be elicited. These nuances of iMF expression are consistent with the idea that iMF is elicited as a compensatory response to a potentially life-threatening stimulus, and serves to maintain respiratory activity within the targeted range most compatible with life. Thus, we hypothesize that iMF may help maintain respiratory motor function following cessation of neural activity that occurs in diseases marked by reductions in respiratory neural activity, like spinal cord injury (Strakowski et al., 2007) or sleep apnea (Dempsey et al., 2010).

Individuals with sleep apnea suffer from repeated, pathological pauses in breathing, often due to recurrent reductions in inspiratory motor output (Dempsey et al., 2010). Central sleep apnea refers to failed respiratory output due to impaired generation, transmission, and/or execution of

respiratory motor output. On the other hand, purely obstructive sleep apnea derives from anatomical obstruction of the upper airway, leading to failed gas exchange. Most commonly, individuals with sleep disordered breathing suffer from “mixed” sleep apnea, or a combination of both obstructive and central events. Why, then, is iMF not effective in preventing recurrent neural apneas? Evidence from a recent study in our lab indicates that coinduction of recurrent neural apneas with moderate, but not mild, hypoxia, such as occurs during sleep apnea, impairs phrenic motor facilitation (Fields et al., 2019). Typically, acute exposure to intermittent hypoxia elicits its own form of respiratory plasticity, called phrenic long-term facilitation (pLTF; Bach and Mitchell, 1996). pLTF expression requires NMDAR signaling, which constrains iMF expression (McGuire et al., 2005; Streeter and Baker-Herman, 2014b; Fields et al., 2019). Indeed, inhibition of NMDAR prior to exposure to concurrent neural apnea with moderate hypoxia (hypoxic neural apnea) resulted in pMF expression. Interestingly, administration of retinoic acid also permitted pMF expression following recurrent hypoxic neural apnea. This elegant study suggests that iMF may not provide protection in individuals with moderate or severe sleep apnea, but that retinoic acid supplementation may provide a route by which iMF can be harnessed to stabilize respiratory output.

Microglia in Neuroplasticity in the Healthy CNS

Despite all that is known about the mechanisms underlying iMF expression, knowledge is considerably lacking regarding the respective duties of neurons, astrocytes, and microglia in the sensation and response to neural inactivity in the respiratory control system. Microglia are the resident immune cells of the CNS. In the healthy CNS, microglia play a critical role in network function by monitoring and maintaining normal synaptic activity (Nimmerjahn et al., 2005). They survey the CNS and respond to abnormalities, including abnormally high or low activity of neuronal circuits (Umpierre and Wu, 2021). TNF-alpha, which is released primarily by microglia in the CNS, plays critical roles in homeostatic plasticity in the hippocampus (Stellwagen and

Malenka, 2006). Nevertheless, investigations into microglial participation in homeostatic plasticity remain limited. **In Chapter 2 of this dissertation, we describe our investigation into the requirement of microglia for expression of iMF induced by a single, prolonged neural apnea or by brief, recurrent neural apneas.**

Microglia, Neuroinflammation, and Respiratory Plasticity

In the face of disease or injury, microglia become "activated" and orchestrate a powerful immune response characterized by the release of cytokines, chemokines, prostaglandins, and growth factors (Elmore et al., 2014), leading to neuroinflammation. Neuroinflammation is a common feature of many disorders characterized by inadequate respiratory output, like sleep apnea (Díaz-García et al., 2022), spinal cord injury (Pang et al., 2022), and neurodegenerative diseases (Han et al., 2022), thus the impact of neuroinflammation on respiratory motor plasticity has recently drawn interest. Peripheral inflammatory stimuli are used to induce neuroinflammation, which can be conferred to the CNS from the periphery via diverse mechanisms (Komaki et al., 1992; Banks et al., 1995; Maier et al., 1998; Laflamme et al., 1999; Litvin et al., 2020). Lipopolysaccharide (LPS), a component of gram negative bacterial cell wall (Poltorak et al., 1998), is a very commonly used immune stimulus that activates TLR4 receptors and induces an endogenous immune response, including microglial activation and neuroinflammation (Huxtable et al., 2011, 2013). Inflammation in the spinal cord following peripheral immune stimuli, including LPS, impairs phrenic long-term facilitation, another form of respiratory motor plasticity induced by acute exposure to intermittent hypoxia (AIH), but the impact on iMF remains unknown (Huxtable et al., 2011, 2015, 2018; Vinit et al., 2011; Hocker and Huxtable, 2019). **Our investigation into the impact of LPS-induced neuroinflammation, and the role of microglia, on iMF is described in Chapter 3.**

Another source of aberrant immune signaling in the CNS is epigenetic reprogramming of immune-related systems toward a pro-inflammatory state induced by an adverse in utero environment (Al-Haddad et al., 2019). Long-lasting neurological deficits can be precipitated by diverse prenatal stimuli, such as maternal infection, stress, or metabolic disease (Bilbo et al., 2018; Bergdolt and Dunaevsky, 2019). Increased neuroinflammation due to reprogramming of immune function may lead to these deficits by undermining critical neural processes like neuroplasticity (Rizzo et al., 2018). One increasingly prevalent prenatal insult is maternal sleep-disordered breathing (SDB) during pregnancy, which is increasing in parallel with the obesity epidemic (Pien et al., 2014; Lockhart et al., 2015). SDB during pregnancy exposes expectant mothers to recurrent episodes of hypoxia, a potent inflammatory stimulus (Lam et al., 2012), and is associated with adverse neonatal outcomes, including premature birth and NICU admission (Ding et al., 2014). However, the long-term effects on adult offspring have not been reported. SDB during pregnancy is modeled by gestational exposure to intermittent hypoxia (GIH), and is associated with sex-specific impairments in metabolism (Khalyfa et al., 2017; Cortese et al., 2021), cardiovascular function (Song et al., 2021), and behavioral deficits characteristic of human autism-spectrum disorder (Vanderplow et al., 2022). Whether GIH leads to changes in adult offspring immune function or respiratory control, including respiratory motor plasticity, have not been reported. **In Chapters 4 and 5, we report the widespread effect of GIH on adult male offspring respiratory control, cardiovascular outcomes, gut microbiome, and immune function, including the specific impact on microglia and their role in respiratory deficits.**

Summary

After a decade of research into the mechanisms underlying iMF, the purpose of this dissertation was to advance our understanding of the participation of microglia in iMF following different

patterns of reduced neural inactivity, in the context of the healthy (Chapter 2) and diseased (Chapters 3, 4, and 5) CNS. These studies illuminate the complex intercellular communication that facilitates, or impairs, iMF expression. Further, it informs us of context and risk factors, like neuroinflammation induced by illness or epigenetic mechanisms, that may impede the harnessing of iMF for treatment of diseases characterized by inadequate respiratory output, and potential mechanisms that can be targeted to overcome these limitations.

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Chapter II

Spinal TNFα initiates compensatory plasticity in response to prolonged respiratory neural activity deprivation and requires microglia

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ABSTRACT

Neurons generating breathing are active nearly continuously from birth until death. Reductions in respiratory neural activity elicit a proportional, chemoreflex-independent increase in inspiratory motor output, a form of plasticity known as inactivity-induced inspiratory motor facilitation (iMF). Mechanisms giving rise to iMF differ depending on the pattern of reduced respiratory neural activity. iMF elicited by prolonged reductions in respiratory neural activity require TNFα, but the precise role for TNFα or the cell types involved are unknown. We show that spinal inhibition of TNFα converting enzyme (TACE) prior to a 30-min reduction in respiratory neural activity blocks iMF in phrenic inspiratory output, suggesting that iMF requires cleavage of membrane bound TNFα (tmTNFα) to soluble TNFα (sTNFα). sTNFα is required to initiate, but not maintain, iMF since pretreatment with a TNFα inhibitor blocked phrenic iMF, whereas TNFα inhibitors had no effect if given after iMF had been induced. Our data suggest that sTNFα initiates iMF via cooperative signaling through TNF receptors 1 and 2 since siRNA targeting TNFR2 abolished iMF, whereas TNFR1 siRNA enhanced iMF. Microglial depletion uniquely abolished iMF following prolonged reductions in respiratory neural activity, demonstrating for the first time a role for microglia in respiratory neuroplasticity. Microglial depletion did not impact iMF induced by brief, recurrent reductions in respiratory neural activity, a TNFα-independent form of iMF. These data indicate that cleavage of tmTNFα into a soluble form initiates, but does not maintain, compensatory phrenic responses to prolonged central apnea through activation of TNFR2 in a microglia-dependent manner.

INTRODUCTION

Breathing is a complex neuromotor behavior that must remain finely tuned and responsive to physiological challenges throughout a lifetime. Brainstem neurons generate the basic respiratory pattern, which is then transmitted in a highly coordinated fashion to inspiratory motor pools in the brainstem and spinal cord that control upper airway diameter and thoracic cavity expansion. Plasticity is a fundamental feature of multiple respiratory neuron groups, including at the final neural output relay, inspiratory motor neurons. For example, local mechanisms operating near inspiratory motor neurons driving the diaphragm, known as phrenic motor neurons, sense respiratory-related synaptic inputs and initiate a form of plasticity known as inactivity-induced inspiratory motor facilitation (iMF) when that command to take a breath falls below a set-point, even in the absence of a change in blood gases (Baertsch & Baker 2017c, Braegelmann et al 2017, Broytman et al 2013, Fields et al 2019, Fields et al 2017, Mahamed et al 2011, Streeter & Baker-Herman 2014a, Strey et al 2012). iMF is reflected as a prolonged enhancement in phrenic inspiratory output, and is proportional to the magnitude of activity deprivation (Braegelmann et al 2017). Mechanisms whereby the phrenic motor pool senses or responds to reduced phrenic synaptic inputs are not well understood, although it is clear that different patterns of reduced respiratory neural activity trigger distinct cellular mechanisms to give rise to iMF. Indeed, prolonged reductions in respiratory neural activity elicits release of the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα) in or near the phrenic motor pool (Broytman et al 2013), which induces association of an atypical PKC isoform (PKCζ or PKCι) with the scaffolding molecule p62/SQSTM1 to stabilize transient increases in phrenic burst amplitude into a long-lasting iMF (Strey et al 2012). By contrast, very brief, recurrent reductions in respiratory neural activity initiate iMF via a TNFα-independent mechanism, which ultimately converges on atypical PKC activity (Baertsch & Baker 2017b, Baertsch & Baker-Herman 2013, Baertsch & Baker-Herman 2015, Braegelmann et al 2021).

In addition to a role in respiratory plasticity, TNFα is an important mediator of plasticity in response to activity deprivation in critical neural circuits in the hippocampus, visual cortex, and the spinal cord (Beattie et al 2002, Broytman et al 2013, Kaneko et al 2008, Stellwagen & Malenka 2006). TNFα can function in two different, physiologically active states: as a membrane-bound (tmTNFα) or a soluble, diffusible ligand (sTNFα) (MacEwan 2002). TNFα converting enzyme (TACE), is primarily responsible for the cleavage of tmTNFα into sTNFα (Black 2002, Bzowska et al 2004, Huovila et al 2005). Prolonged activity block in hippocampal cultures drives glia to release sTNFα into the culture media, which is sufficient to increase excitability when media is transferred to neuronal cultures that were not exposed to activity deprivation (Beattie et al 2002, Stellwagen & Malenka 2006). Thus, TACE sheddase activity is critical to initiate homeostatic, compensatory plasticity in response to prolonged activity deprivation in the hippocampus. The bound (tmTNFα) versus soluble (sTNFα) state of TNFα not only determines whether the signal can diffuse, but also influences the kinetics of TNF receptor activation (MacEwan 2002, Vandenabeele et al 1995). TNFα signals through two distinct receptors, TNF receptor 1 and 2 (TNFR1 and TNFR2, respectively), both of which are found in neurons and glia in the CNS, including in spinal motor neurons (Holmes et al 2004, Veglianese et al 2006). Although both sTNFα and tmTNFα can activate TNFR1 and TNFR2, tmTNFα is thought to primarily signal through TNFR2, while sTNFα exerts its activity through both receptors (Grell et al 1995, Horiuchi et al 2010, MacEwan 2002, Vandenabeele et al 1995). Evidence suggests that TNFα signals through TNFR1 to induce homeostatic plasticity in response to activity deprivation in the hippocampus (Pribiag & Stellwagen 2013, Pribiag & Stellwagen 2014, Stellwagen et al 2005), although other studies indicate an additional role for TNFR2 (Becker et al 2015).

As the resident immune cells of the CNS, microglia play a pivotal role in CNS cytokine expression. Microglia have been shown to play key roles in neuroplasticity (Eyo & Wu 2013, Umpierre et al 2020, Zhou et al 2019), including in response to neuronal activity deprivation (Liu et al 2019, Ma

et al 2020). Microglial processes routinely survey neuronal synapses, and the dynamics of microglial extension/retraction of their processes at a synapse are regulated by neuronal activity (Ferro et al 2021, Liu et al 2019, Ma et al 2020, Tremblay et al 2010, Umpierre et al 2020, Wake et al 2009a). Recent studies indicate that microglia are critical for some forms of motor learning (Parkhurst et al 2013), but to date, it is unknown whether microglia participate in plasticity in the respiratory control system. While general microglial modulators, such as fractalkine and minocycline, can change respiratory activity associated with respiratory plasticity *in vitro* (Camacho-Hernandez et al 2019), the role of specific cytokines or microglia in respiratory neuroplasticity *in vivo* has not been previously reported.

Here, we tested the hypothesis that soluble TNFα is required to initiate, but not maintain iMF through TNFR1 activation, and that microglia are necessary for TNFα-dependent iMF. Our results indicate that TACE activity is necessary for iMF, suggesting the cleavage of tmTNFα to sTNFα initiates iMF in response to respiratory neural activity deprivation. Contrary to our hypothesis, we found that TNFR2 on phrenic motor neurons is required for iMF, whereas TNFR1 activation constrains iMF magnitude; thus, both TNFα receptors 1 and 2 contribute to sculpting compensatory phrenic inspiratory plasticity in response to a prolonged reduction in respiratory-related neural activity. Pharmacologic depletion of microglia selectively impairs iMF triggered by prolonged reductions in respiratory neural activity, but does not impact iMF triggered by brief, recurrent episodes of respiratory neural activity deprivation. Collectively, our findings suggest that microglia may release TNFα to trigger a compensatory enhancement in phrenic inspiratory output in response to prolonged reductions in inspiratory-related synaptic inputs to phrenic motor neurons. Thus, we identify for the first time a critical role for microglia in mediating neuroplasticity in the control of breathing.

METHODS

Animals

Experiments were performed on 2- to 4-month old male and female Sprague-Dawley rats. All rats were housed in pairs in a controlled environment (12 h light/dark cycle) with food and water *ad libitum*. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison, WI, USA.

Surgical preparation

Anesthesia was induced with isoflurane in a closed chamber and maintained through a nose cone (2.5-5%) in 50% O2:N2 balance. The tail vein was catheterized for intravenous delivery of fluids (0.5-5.0 ml/hr; 1:5 of 8.4% sodium bicarbonate/lactated Ringer's solution) and I.V. drugs. Body temperature was monitored using a rectal probe and maintained between 36-38ºC with a custom-designed heated table. The trachea was cannulated for pump ventilation (tidal volume 2-4mL; Rodent Ventilator 683 Harvard Apparatus, Holliston, Massachusetts or VentElite 55-7040 Harvard Apparatus, Holliston, Massachusetts). A bilateral vagotomy was performed to prevent entrainment with the ventilator. To avoid unintended neural apnea, tracheal pressure and end-tidal CO_2 were monitored (Capnogard; Respironics, Murrysville, PA or Physiosuite; Kent Scientific, Torrington, CT) throughout the surgery and inspired CO_2 and/or ventilation rate were appropriately adjusted to ensure the continuous generation of respiratory efforts ($ETCO_2$ approximately 45-47mmHg). A catheter was placed into the femoral artery to monitor blood pressure and sample blood gases throughout the experiment (ABL800; Radiometer, Copenhagen, Denmark). Rats were converted to urethane anesthesia (1.4-1.7 mg/kg, IV) and slowly weaned from isoflurane. Following surgery, rats were paralyzed with Pancuronium bromide (1.0 mg/kg, IV). Lactated Ringer's solution was administered IV throughout the protocol to maintain fluid homeostasis. To ensure adequate depth of anesthesia, blood pressure response to toe-pinch was monitored and rats were supplemented with urethane as necessary. The left

phrenic nerve was isolated via a dorsal approach, cut distally, desheathed, and submerged in either mineral oil or saline for electrophysiological recordings using a bipolar or suction electrode, respectively. Compound action potentials were amplified (x10k), band-pass filtered (0.3 - 10kHz), and integrated (time constant, 50 ms). Raw signals were recorded at a sampling rate of 10,000 s-1, digitized and recorded with a PowerLab data acquisition system (LabChart 8.0; ADInstruments, New Zealand).

In two series of experiments involving intrathecal drug delivery (Experimental Series 1, Experimental Series 2), a C2 laminectomy was performed over the spinal midline and a small hole was cut in the dura. A silicone catheter (2 French; Access Technologies, Skokie, Illinois) connected to a 30ul Hamilton syringe containing an inhibitor or vehicle control was inserted into the intrathecal space and advanced caudally (~5 mm) to lie on the dorsal surface of spinal segment C4.

Pharmacological interventions

Intrathecal drugs

Experimental series 1 and 2 required delivery of drugs intrathecally. Compounds were dissolved in artificial CSF (aCSF; in mM: 120 NaCl, 3 KCl, 2 CaCl, 2 MgCl, 23 NaHCO3, 10 glucose, bubbled with 95% O2-5% CO2). Rats received TAPI-1 (1mM in 10% DMSO/aCSF, Calbiochem; Millipore), Marimastat (100µM in 1% DMSO/aCSF, Sigma Aldrich), or sTNFR1 (1.3ug; with 1% bovine serum albumin (BSA); R & D Systems). Vehicle control rats received aCSF with 1% or 10% DMSO or 1% BSA as appropriate. For all drug delivery, 10µL in total was delivered to the intrathecal space in 2uL boluses over 2min.

Intrapleural siRNA

In Experimental series 3, siRNAs were delivered to the intrapleural space for retrograde transport to phrenic motor neurons. We first confirmed that small interfering RNAs (siRNAs; Accell siRNA SMARTpool; GE Dharmacon, Lafayette, CO) targeting TNFR 1 and 2 (siTNFR1 and siTNFR2, respectively) reduced receptor expression. Primary mixed glial cultures were prepared from P3 Sprague-Dawley rat pup brains. Cells were cultured in a 37°C incubator at 5% CO2, in growth medium [DMEM containing supplemental 10% FBS (Hyclone, Defined) and 100 U/ml penicillin/streptomycin (Corning Cellgro)]. Cells were cultured as we have described previously (Sagar et al 2020), one brain being cultured per 24 well plate well until ~80% confluent. For siRNA transfection, the growth medium was removed from the cells and replaced with 100 ul of Accell Delivery Media (cat. no. 005000-500; GE Dharmacon, Lafayette, CO) containing a final concentration of 1 uM of a nontargeting control Accell siRNA SMART pool (cat. no. D-001910-10), or Accell siRNA SMARTpools against TNFR1 (Tnfrsf1a; cat. no. E-090517-00) or TNFR2 (Tnfrsf1b; cat. no. E-094191-01). Cells were incubated for 72 h before the delivery media was removed for cell analysis of gene knockdown. Cells were collected into TriReagent (Sigma), and gene knockdown was assessed by quantitative RT-PCR as we have previously described (Smith et al 2013). Total RNA (0.5 µg) was reverse transcribed to cDNA using Moloney murine leukemia virus MMLV reverse transcriptase, and quantitative PCR for TNFR1 and TNFR2 was performed with Sybr Green (Applied Biosystems, Foster City, CA) using the following primer sequences: TNFR1: forward, 5=-CTGACGTTCCCAGACCTTGT and reverse, 5=-CCTGATTTTTCTTGCAGTGGCTG; TNFR2: forward, 5=-AGTCCAGTGTGCTTTTTGCC, and reverse, 5=-CATGGTAACAACTGGGCTCCT. Gene expression analyses were performed using the ddCT method (Livak & Schmittgen 2001)

To reduce spinal TNF receptor expression in phrenic motor neurons, rats were injected with

TNFR1 or TNFR2 siRNA into the intrapleural space (100pg/side) (Dale et al 2017, Devinney et al 2015, Huxtable et al 2018, Mantilla et al 2009). siRNAs were given once daily for 3 consecutive days. A group of control rats received non-targeting siRNA (100pg/side). iMF was tested one day following the last injection of siRNA.

Microglial Depletion

Experimental Series 4 required depletion of microglia. Rats were treated with Pexidartinib (PLX3397) (MedKoo Biosciences, Morrisville, NC), an inhibitor of the colony-stimulating factor 1 receptor (CSF-1R) necessary for monocyte survival. PLX3397 was formulated in DMSO, 1% PS80 and 0.5% hydroxycellulose. Control rats received a vehicle treatment containing DMSO, 1% PS80 and hydroxycellulose. Rats were dosed by oral gavage (80 mg/kg) for 7 consecutive days, and the expression of iMF was tested one day following the last dose.

Electrophysiological protocols

Approximately 1h after conversion to urethane anesthesia, “baseline” phrenic neural activity was set using one of two methods. In some rats, apneic and recruitment thresholds for phrenic activity were determined by slowly increasing the ventilator rate and/or lowering the inspired CO_2 until rhythmic phrenic burst activity ceased (apneic threshold); the ventilator rate was then lowered and/or inspired CO_2 was increased until phrenic activity resumed (recruitment threshold). $ETCO_2$ was raised 2-3 mmHg above the recruitment threshold to establish baseline phrenic discharge. In other rats, apneic and recruitment thresholds for phrenic bursting were not determined in order to avoid exposure to a neural apnea prior to the protocol; instead, baseline phrenic nerve activity was set by raising/lowering the ventilator rate and/or inspired CO_2 until the phrenic burst frequency was between approximately 45 and 48 bursts/min. There were no statistically significant differences in iMF magnitude using either method for setting baseline, so rats were combined.

"Baseline" phrenic nerve activity was established when phrenic burst amplitude remained stable under isocapnic conditions for at least 15–20 min. One or two arterial blood samples were drawn to obtain temperature-corrected baseline Pco_2, Po_2, and pH measurements. Rats were then exposed to either a single prolonged (~25 min) reduction in respiratory neural activity ("neural apnea") or a series of brief, intermittent neural apneas (5, ~1 min, separated by 5 min) by reducing $ETCO_2$ ~2 mmHg below the threshold for phrenic inspiratory activity ("apneic threshold"). Rats continued to be ventilated during neural apnea(s), so hypoxia was not experienced. To control for time-dependent changes in phrenic inspiratory output due to the surgery or drug application alone, a separate cohort of rats in each experimental series received the same surgical preparation and drug treatment(s), but no neural apnea ("time controls"). Arterial blood samples were drawn 5, 15, 30, and 60 min following neural apnea(s) or the equivalent period in time controls to confirm Pco_2 was within 1.5 mmHg of baseline, and that baseline levels of Po_2 and pH were maintained. Animals were euthanized with a urethane overdose after the protocol.

Experimental Series 1: To test whether TACE activity is required for TNFα-dependent expression of iMF, rats were treated with intrathecal TAPI (n=7), Marimastat (n=7), or vehicle (n=7) ~20 minutes prior to exposure to prolonged neural apnea. Time controls received intrathecal TAPI or Marimastat (n=7). *Experimental Series 2:* To test whether TNFα is required for initiation or maintenance of iMF, rats were treated with sTNFr1 ~20 minutes prior to prolonged neural apnea (n=10), or with vehicle (n=10) or sTNFR1 (n=8) immediately prior to resumption of respiratory neural activity after neural apnea. Time controls received sTNFR1 (n=7). *Experimental Series 3:* Rats were treated with daily intrapleural injections of siRNA targeting TNFR1 (siTNFR1; n=7) or TNFR2 (siTNFR2; n=7), or non-targeting siRNA (siNT, n=8) for three days prior to prolonged neural apnea to examine the role of TNFR1 and TNFR2 in TNFα-dependent iMF expression. Time controls received siTNFR1, siTNFR2 or siNT (n=9). *Experimental Series 4:* To test the

requirement of microglia in TNFα-dependent and TNFα-independent forms of iMF, rats were treated with Pexidartinib (PLX3397) or vehicle daily *po* for 7 days prior to testing responses to either prolonged (Pro) or intermittent (Int) respiratory neural activity deprivation (Veh-Int, n=7; PLX-Int, n=8; Veh-Pro, n=7; PLX-Pro, n=7). Time control rats received PLX or vehicle (n=6).

Statistical Analysis

Integrated phrenic burst amplitude was averaged over 60 breath bins immediately preceding blood gas samples at baseline and 5, 15, 30, and 60 minutes following neural apnea or corresponding time points in time control experiments. Similar results were seen at all post-apnea time points, so only 60 minute values are shown for clarity. Integrated phrenic burst amplitude 60 minutes following the restoration of neural activity is expressed as percent change from baseline (%baseline). There were no statistical differences in phrenic burst amplitude detected in time controls receiving different treatments, so they were combined within each experimental series. Statistical differences between groups were determined using a one-way repeated measures analysis of variance (ANOVA) and Fisher's LSD post-hoc tests (Prism 7, GraphPad Software, La Jolla, California). iMF was considered to be expressed if phrenic burst amplitude 60 minutes following resumption of neural activity was significantly greater than the corresponding point in time control rats of the same experimental series.

Arterial pCO_2, pO_2, pH, and mean arterial pressure (MAP) were analyzed using a two-way repeated measures ANOVA and Fisher's LSD post-hoc tests (Prism 7, GraphPad Software) at baseline and 60 min following neural apnea or equivalent duration in time controls. Within each treatment group, 60 min blood-gas values were compared to baseline values. In addition, baseline and 60 minute blood-gas values in each group were compared to the corresponding point in time control rats of the same experimental series. Significance level was set at $p<0.05$. All data are presented as mean ± SD.

RESULTS

Conversion of tmTNFα into sTNFα is necessary for iMF triggered by prolonged neural apnea

To investigate whether tmTNFα or sTNFα is required for iMF, we pharmacologically inhibited the enzyme responsible for cleaving tmTNFα into sTNFα (TNFα converting enzyme; TACE) with two different matrix metalloprotease (MMP) inhibitors: Marimastat and TAPI-1. These inhibitors have varying specificity for off target MMPs, but they have overlapping high selectivity for TACE. Marimastat or TAPI-1 were delivered intrathecally over the phrenic motor pool approximately 20 minutes prior to prolonged reductions in respiratory neural activity (i.e., neural apnea). Since rats were ventilated during respiratory neural activity deprivation, no hypoxia or hypercapnia was experienced. Representative compressed phrenic neurograms are presented in Fig. 1A, depicting phrenic burst amplitude at baseline, during a ~25 minute neural apnea and for 60 minutes following neural apnea in rats that received intrathecal vehicle (aCSF/DMSO), TAPI-1 or Marimastat. A represented trace from a time control rat that received intrathecal TAPI-1 but no activity deprivation is also included. Average percent change in phrenic burst amplitude from baseline 60 minutes following neural apnea is compared to time control rats in Fig. 1B. In time control rats, average phrenic burst amplitude remained unchanged relative to baseline, indicating that intrathecal MMP inhibitors have no effect on baseline phrenic firing (60 min: 9 ± 11 %baseline; n = 7; Fig. 1A and 1B). As expected, rats receiving an intrathecal injection of vehicle prior to prolonged neural apnea expressed a significant increase in phrenic burst amplitude upon resumption of respiratory neural activity (for clarity, only 60 min is shown: 73 ± 26 % baseline; n = 7; $p<0.0001$ compared to time controls, Fig. 1A and B), indicating iMF. By contrast, phrenic burst amplitude in rats pre-treated with intrathecal Marimastat or TAPI-1 was not significantly different from time controls at any time point after the resumption of respiratory neural activity (for clarity, only 60 min shown: 5 ± 16 %baseline, n=7, p=0.67 and 2 ± 19 %baseline, n=7, p=0.5, respectively; Fig. 1A and 1B). These data suggest TACE activity is required to cleave tmTNFα to sTNFα for the expression of iMF.

Spinal TNFα initiates, but does not maintain, iMF initated by a prolonged neural apnea

To determine whether sTNFα is required to maintain iMF once it has been triggered by a prolonged reduction in respiratory neural activity, a series of experiments were performed to block TNFα binding to it's receptors (TNFR1 and TNFR2) using the TNFα scavenger, sTNFR1 (Gray et al 1990). sTNFR1 was delivered intrathecally over the phrenic motor pool ~20 min prior to a prolonged neural apnea, or immediately before respiratory neural activity was resumed after neural apnea. Representative compressed phrenic neurograms from a vehicle and two sTNFR1 treated rats are presented in Fig. 2A, depicting phrenic burst amplitude during baseline, a ~25 minute neural apnea and for 60 minutes following the resumption of respiratory neural activity. A recording of equal duration in a time control experiment receiving intrathecal sTNFR1 is also depicted. Average percent change from baseline in phrenic burst amplitude 60 minutes following neural apnea is presented in Fig. 2B. In time controls, there was no change in phrenic burst amplitude from baseline (60min: 8 ± 16% baseline, n=8; Fig. 2A and 2B), indicating intrathecal delivery of sTNFR1 did not impact phrenic burst amplitude. As expected, neural apnea triggered a significant increase in phrenic burst amplitude in rats receiving intrathecal vehicle (60min: 45 ± 25; % baseline, n=10, p=0.01 relative to time controls; Fig. 2A and B), indicating iMF. As previously demonstrated (Broytman et al 2013), rats receiving intrathecal sTNFR1 prior to neural apnea did not express significant increases in phrenic burst amplitude following neural apnea (60min: 20 ± 27 % baseline, n=10, p=0.4; Fig. 2A and B), indicating that TNFα signaling is necessary for expression of iMF. By contrast, rats that received intrathecal sTNFR1 immediately prior to the resumption of respiratory neural activity after prolonged neural apnea expressed significantly increased phrenic burst amplitude relative to time controls (60min: 53 ± 45 % baseline, n=8, p=0.005; Fig. 2A and B). These data indicate that spinal TNFα is required to initiate iMF, but it is not required to maintain iMF after it has already developed.

TNFR2 activation is necessary for, whereas TNFR1 activation constrains, iMF induced by prolonged neural apnea

To determine the TNF receptor subtype required for iMF, TNFR1 or TNFR2 expression was suppressed using small interfering RNA (siRNA), which were injected into the intrapleural space for retrograde transport to phrenic motor neurons. This technique has been widely used to reduce protein expression in the phrenic motor pool (Dale et al 2017, Devinney et al 2015, Huxtable et al 2018, Mantilla et al 2013, Mantilla et al 2009). We first verified that siRNAs directed against TNFR1 (siTNFR1) and TNFR2 (siTNFR2) effectively reduced receptor expression in rat primary cell cultures (Fig. 3C). siTNFR1 significantly reduced TNFR1 expression (0.55 ± 0.27, n=4, p=0.015) relative to non-targeting siRNA controls (siNT, n=4). Similarly, siTNFR2 significantly reduced TNFR2 expression (0.29 ± 0.16%, n=4, p=0.0001) relative to non-targeting siRNA controls (siNT, n=4).

siTNFR1 or siTNFR2 were then injected into the intrapleural space daily for three days prior to testing responses to respiratory neural activity deprivation. Representative compressed phrenic neurograms are depicted in Fig. 3A, illustrating phrenic burst amplitude during baseline, a ~25-30 minute neural apnea, and 60 minutes following restoration of phrenic neural activity. A recording of equal duration is presented from a time control rat that received 3 daily injections of siRNA but was not exposed to a reduction in respiratory neural activity. Average change in phrenic burst amplitude 60 minutes following activity deprivation is shown in Fig. 3B. There was no change in phrenic burst amplitude from baseline in time controls (60min 2 ± 22 % baseline, n=9, p=0.74; Fig. 3A and 3B). As expected, rats injected with siNT expressed a significant increase in phrenic burst amplitude following neural apnea compared to time controls (60min: 38 ± 20 % baseline, n=8, p=0.0025; Fig. 3A and 3B). Contrary to our hypothesis that iMF requires TNFR1 activation, prolonged neural apnea elicited a significant increase in phrenic inspiratory activity in rats treated with siTNFR1 (60min: 73 ± 27 % baseline, n=7, p<0.0001; Fig. 3A and 3B), indicating TNFR1 is

not required for iMF. Further, in rats injected with siTNFR1, iMF was significantly larger in magnitude than in control rats injected with siNT (p=0.004, Fig. 3C), indicating that TNFR1 activation constrains iMF magnitude. By contrast, in rats pretreated with siRNA targeting TNFR2, prolonged neural apnea did not trigger a significant increase in phrenic burst amplitude compared to time controls (60min 4 ± 18 % baseline, n=7, p=0.87; Fig. 3A and 3B), indicating that impairment of TNFR2 signaling abolishes iMF (Fig. 3C). Together, these data suggest prolonged activity deprivation stimulates phrenic motor plasticity through TNFR2 activation, while activation of TNFR1 constrains the magnitude of plasticity.

Microglial depletion abolishes iMF triggered by prolonged, but not brief intermittent, respiratory neural activity deprivation

Since microglia are the primary source of TNFα in the CNS, we next investigated whether microglia are required for TNFα-dependent or TNFα-independent forms of iMF. Rats were treated with vehicle or PLX3397 for 7 days prior to testing responses to respiratory neural activity deprivation. Representative compressed phrenic neurograms from vehicle- or PLX3397-treated rats are presented in Fig. 4A, depicting phrenic burst amplitude at baseline, during a single prolonged (~30 min), or a series of brief intermittent (5, ~1 min) reduction(s) in respiratory neural activity, and for 60 minutes following activity deprivation. A representative recording is also presented from a time control rat treated with PLX3397 but not exposed to reduced respiratory neural activity. Average change in phrenic burst amplitude 60 minutes following neural apnea (or equivalent duration in time controls) is presented in Figure 4B. In response to intermittent reductions in respiratory neural activity, both vehicle- and PLX-treated rats expressed a significant increase in phrenic burst amplitude compared to time controls (Veh - 60 min: 67 ± 41 % baseline, n=7, p=0.03; PLX - 60 min: 63 ± 35 %baseline, n=8, p=0.04), indicating that microglia do not play a role in this (non-TNFa dependent) form of iMF. By contrast, in response to prolonged reductions in respiratory neural activity, only vehicle treated rats were able to express iMF (60min: 78 ± 42

% baseline, n=7, p=0.008; Fig. 4A and 4B); indeed, in rats treated with PLX, prolonged reductions in respiratory neural activity did not trigger an increase in phrenic inspiratory activity (60min: 22 ± 15 % baseline, n=7, p=0.8, Fig. 4A and 4B). Collectively, these data indicate that microglia are required for TNFa-dependent expression of iMF following a prolonged neural apnea, but not TNFa-independent iMF following intermittent neural apnea.

Physiological Variables

Table 1 lists average physiological parameters (mean arterial pressure (MAP), paCO2, paO2) at baseline and 60 minutes following neural apnea, or the equivalent in time controls. Although small but significant changes were noted in both between-treatment and within-treatment analyses in each experimental series, all values remained within normal physiological parameters, were not associated with expression of iMF, and were consistent with previous experiments conducted with this anesthetized rat preparation (Baertsch & Baker 2017a, Baertsch & Baker-Herman 2015, Dale-Nagle et al 2011, Streeter & Baker-Herman 2014b). Importantly, in all experiments, $paCO_2$ remained within 1.0 mmHg of baseline and pO_2 was > 200 mmHg throughout the protocol.

DISCUSSION

Here, we show that spinal processing of tmTNFα to sTNFα by TACE is required to initiate, but not maintain, iMF triggered by prolonged reductions in respiratory neural activity. sTNFα activates spinal TNFα receptors TNFR1 and TNFR2 to sculpt phrenic inspiratory plasticity since siRNAs targeting TNFR2 abolished, while siRNAs targeting TNFR1 enhanced, iMF. Additionally, pharmacologic depletion of microglia impaired the capacity for prolonged reductions in respiratory neural activity to induce iMF, but did not impact iMF triggered by brief, recurrent episodes of reduced respiratory neural activity, a form of iMF that is not TNFα-dependent (Baertsch & Baker 2017a, Baertsch & Baker-Herman 2015, Braegelmann et al 2021). These results show for the first time that microglia are critically involved in eliciting a unique form of activity-dependent

respiratory plasticity that may prevent, or compensate for, prolonged reductions in phrenic inspiratory motor output.

Inspiratory motor pools driving the respiratory muscles that dilate the upper airway and expand the thoracic cavity are active from birth until death, yet mechanisms that regulate that activity are poorly understood. Although chemoreflex feedback is an important mechanism to adjust breathing to maintain blood gas homeostasis, respiratory neural activity itself is monitored by the respiratory control system, even without a change in blood gases. Indeed, the respiratory control system is exquisitely sensitive to reductions in respiratory neural activity, and initiates mechanisms of plasticity that proportionally enhance inspiratory motor output, a form of plasticity known as inactivity-induced inspiratory motor facilitation (iMF). Complete phrenic "inactivity" need not be achieved to elicit iMF, as iMF is also induced by neuronal hypopnea (Braegelmann et al 2017) or only a partial blockade of phrenic synaptic inputs (Streeter & Baker-Herman 2014a).

The results presented here support our previous observations that mechanisms underlying iMF differ depending on the pattern of reduced respiratory neural activity (Baertsch & Baker 2017a, Baertsch & Baker 2017b, Baertsch & Baker-Herman 2015, Broytman et al 2013, Strey et al 2012). Prolonged reductions in respiratory-related synaptic inputs to the phrenic motor pool result in local (spinal) release of soluble TNFα, and initiate iMF via activation of TNFR2 on phrenic motor neurons. At the same time, TNFα also activates TNFR1, which constrains iMF expression, suggesting that the balance between TNFR2/TNFR1 activation sculpts phrenic neuroplastic responses to activity deprivation.

In the spinal cord, both TNFR1 and TNFR2 have been identified on ventral horn motor neurons and glia (Holmes et al 2004, Veglianese et al 2006). In the hippocampus, experiments utilizing genetic deletion of TNFRs and TNFR-neutralizing and -activating antibodies found that TNFR1,

not TNFR2, is required for compensatory increases in excitability following activity deprivation (Pribiag & Stellwagen 2013, Pribiag & Stellwagen 2014, Stellwagen et al 2005). However, in a model of entorhinal cortex denervation, either TNFR1 or TNFR2 activation was sufficient to elicit an increase in mEPSC amplitude (Becker et al 2015), suggesting the role of TNFR1 and TNFR2 in compensatory responses to neuronal activity deprivation may be context specific. Differential requirements for TNFR1 *versus* TNFR2 may also depend upon how rapidly TNF receptors are cleaved, internalized, or inserted into the membrane in response to stimuli. The acute, reversible respiratory neural activity deprivation that elicits iMF is brief (30 minute) compared to activity deprivation in other models, which require longer pharmacological deprivation, nerve injury, or denervation to induce plasticity. The difference in timescale may be due to respiratory neurons being "tuned" for more rapid responses to activity deprivation, since the neurons that generate breathing are active nearly continuously throughout life. Additionally, *in vivo* studies evaluate intact neural circuits versus *in vitro* cell culture/slice preparations that may have altered signaling pathways at baseline. On the other hand, the experimental paradigm used to investigate iMF likely causes some level of systemic inflammation that could rapidly alter the distribution and function of TNF receptors on phrenic motor neurons (Yan et al 2003), thereby leading to more rapid responses.

Although the cell type that releases the TNFα necessary for this form of iMF is not known, microglia are implicated. Indeed, microglia are the primary source for TNFα in the CNS, and recent evidence indicates that microglia are poised to be sentinels of neuronal activity (Eyo & Wu 2019, Ferro et al 2021). In the resting state, microglial processes dynamically extend and retract their processes at neuronal synapses, as they survey the local environment (Eyo & Wu 2019, Nimmerjahn et al 2005, Tremblay et al 2010, Wake et al 2009b). Strikingly, microglia respond within minutes of an acute change in neuronal activity – either up or down – by increasing the rate to which they sample the synapse (Liu et al 2019, Umpierre et al 2020). These findings are

consistent with a role for microglia in monitoring activity at nearby synapses, and responding to neuronal activity deprivation by inducing compensatory forms of plasticity to maintain circuit homeostasis. Indeed, we found that depletion of microglia abolished the capacity for prolonged reductions in respiratory neural activity to trigger iMF. By contrast, iMF triggered by brief, intermittent reductions in respiratory neural activity was unaffected by microglial depletion. This finding is not necessarily surprising since iMF triggered by brief, intermittent reductions in respiratory neural activity is TNFα-independent, and instead requires local retinoic acid synthesis and RARα activation to initiate (Baertsch & Baker 2017b, Braegelmann et al 2021, Fields et al 2019). Mechanisms whereby microglia selectively respond to prolonged neuronal activity deprivation (*versus* brief, but intermittent) are unknown. Nevertheless, taken together, these data implicate for the first time a direct role for microglia-respiratory neuron communication in respiratory neuroplasticity. Insight into the signaling cascades required for iMF may elucidate avenues for therapeutic intervention in circumstances of compromised breathing while minimizing extraneous activation of the many other cellular processes initiated by TNFα.

Although TNFα can mediate respiratory neuroplasticity, TNFα is a hallmark of inflammation that can also contribute to neurodegeneration (Brenner et al 2015, Sriram & O'Callaghan 2007). Whether neural plasticity initiated by TNFα is protective or ultimately destabilizes neural circuits and the behavior they control is unknown. Mechanisms that distinguish neurodegenerative versus neuroprotective properties of TNFα are ambiguous, but the expression profile of TNFα, TNF receptor subtype activation, and the type of cells activated by TNFα all likely contribute to its contrasting physiological roles (Sriram & O'Callaghan 2007). While it is unclear whether TNFα-induced neural plasticity protects neuronal function or causes aberrant signaling in hippocampal, visual or spinal systems, TNFα- induced plasticity in the control of breathing may have a more interpretable physiological impact. Plasticity that defends firing strength in response to reduced neural activity is likely a protective function in the respiratory system, at least initially to defend

and maintain the critical physiological function of breathing. Indeed, with rare exceptions, mammals must necessarily generate stable and reliable inspiratory neural drive to the respiratory muscles every few minutes, or death will result. Further investigation into TNFα signaling will bring clarity to the varied responses to this cytokine and illuminate the potential for treatment options to optimize endogenous protective mechanisms and mitigate destructive signaling propagated by TNFα. TNF receptors may pose an ideal site for intervention to promote recovery and breathing stability for patients that suffer from reductions in respiratory drive. Ventilatory failure is a major cause of death associated with multiple neuroinflammatory or neurodegenerative diseases, and understanding mechanisms whereby proper respiratory motor neuron function can be restored is critical for identifying new treatments.

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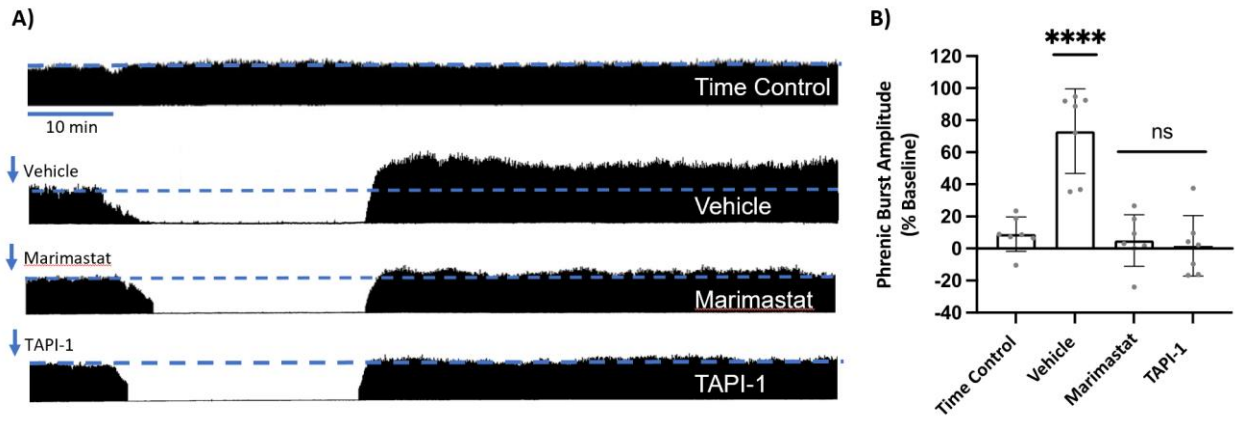

Figure 1 - Conversion of tmTNFα into sTNFα is necessary for iMF following prolonged neural activity deprivation

A) Representative compressed phrenic neurograms depicting phrenic burst amplitude before, during, and for 60 minutes following prolonged neural apnea (~25 minutes) or the equivalent duration in a rat not receiving neural apnea (Time Control). 20 minutes prior to respiratory neural activity deprivation, rats received intrathecal injections of the MMP inhibitors TAPI-1 or Marimastat, or intrathecal Vehicle. **B)** Average change in phrenic burst amplitude relative to baseline 60 min after the resumption of respiratory neural activity, or the equivalent in Time Controls (n=7). Rats treated with intrathecal vehicle expressed a significant increase in phrenic inspiratory output in response to prolonged reductions in respiratory neural activity (n=7, $p<0.0001$), indicating iMF. Prolonged reductions in respiratory neural activity did not result in an increase in phrenic inspiratory output in rats receiving intrathecal marimastat (n=7, $p=0.70$) or TAPI-1 (n=7, $p=0.47$), suggesting that TACE activity is required for iMF triggered by a prolonged neural apnea. ****$p<0.0001$ relative to Time Controls.

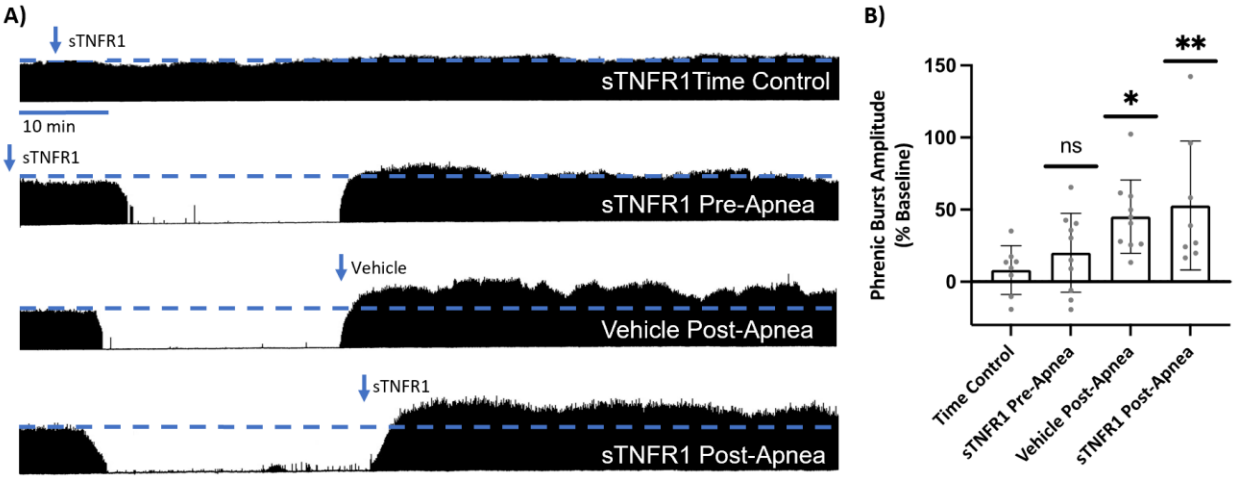

Figure 2 – Spinal TNFα initiates, but does not maintain, iMF elicited by a prolonged reduction in respiratory neural activity

A) Representative compressed phrenic neurograms depicting phrenic burst amplitude before, during, and for 60 minutes following prolonged neural apnea (~25 minutes) or the equivalent duration in a rat not receiving neural apnea (Time Control). Rats received a TNFα scavenger, sTNFR1, intrathecally to block TNFα signaling though it's receptors (TNFR1 and TNFR2) near the phrenic motor pool. Intrathecal sTNFR1 was delivered 20 min prior to neural apnea (sTNFR1 Pre-Apnea), or immediately prior to the resumption of respiratory neural activity after neural apnea (sTNFR1 Post-Apnea). **B)** Average change in phrenic burst amplitude relative to baseline 60 min after the resumption of respiratory neural activity, or the equivalent in Time Controls (n=8). Prolonged reductions in respiratory neural activity elicited significant increases in phrenic inspiratory output in rats receiving intrathecal vehicle (n=10, p=0.01), indicating iMF. Prolonged reductions in respiratory neural activity did not elicit a significant increase in phrenic inspiratory output in rats pre-treated with intrathecal sTNFR1 (sTNFR1 pre-apnea; n=10; p=0.40), indicating that TNFR activation is required for iMF. By contrast, in rats that received sTNFR1 immediately before the resumption of respiratory activity (sTNFR1 post-apnea), prolonged reductions in respiratory neural activity continued to elicit a significant increase in phrenic inspiratory output (sTNFR1 post-apnea; n=8, p=0.0052), indicating that TNFR activation is not required to maintain iMF once it has been initiated by a prolonged reduction in respiratory neural activity. *p<0.05, **p<0.01 relative to Time Controls.

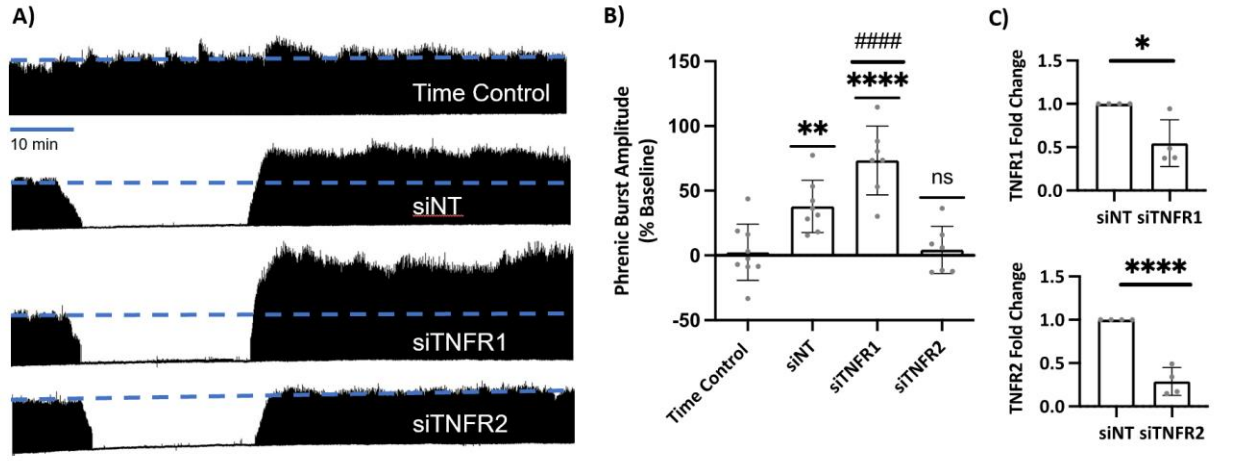

Figure 3 – TNFR2 is required for iMF following prolonged neural inactivity
A) Representative phrenic neurograms depicting phrenic burst amplitude before, during, and for 60 minutes following prolonged neural apnea (~25 minutes) or the equivalent duration in rats not receiving neural apnea (Time Control). Rats were treated for 3 days with intrapleural non-targeting siRNAs (siNT), or siRNAs against the TNFa receptors, TNFR1 (siTNFR1) or TNFR2 (siTNFR2), prior to testing responses to respiratory neural activity deprivation. **B)** Average change in phrenic burst amplitude relative to baseline 60 minutes after the resumption of respiratory neural activity, or the equivalent in Time Controls (n=9). In rats receiving siTNFR1, prolonged reductions in respiratory neural activity elicited a significant increase in phrenic inspiratory output (n=7, $p<0.0001$), which was significantly greater ($p<0001$) than that observed in control rats receiving siNT (n=8, $p=0.0025$). In rats receiving siTNFR2, a prolonged neural apnea did not trigger increases in phrenic inspiratory output (n=7, $p=0.87$). These data suggest that TNFR2 activation is required, whereas TNFR1 activation constrains, iMF in response to prolonged neural apnea. **$p<0.01$, ****$p<0.0001$ relative to time controls; ####$p<0.0001$ relative to siNT **C)** rt-PCR data from cultured cells confirming that siTNFR1 and siTNFR2 significantly reduced expression of targeted receptor. *$p<0.05$, ****$p<0.0001$ relative to siNT expression of targeted receptor.

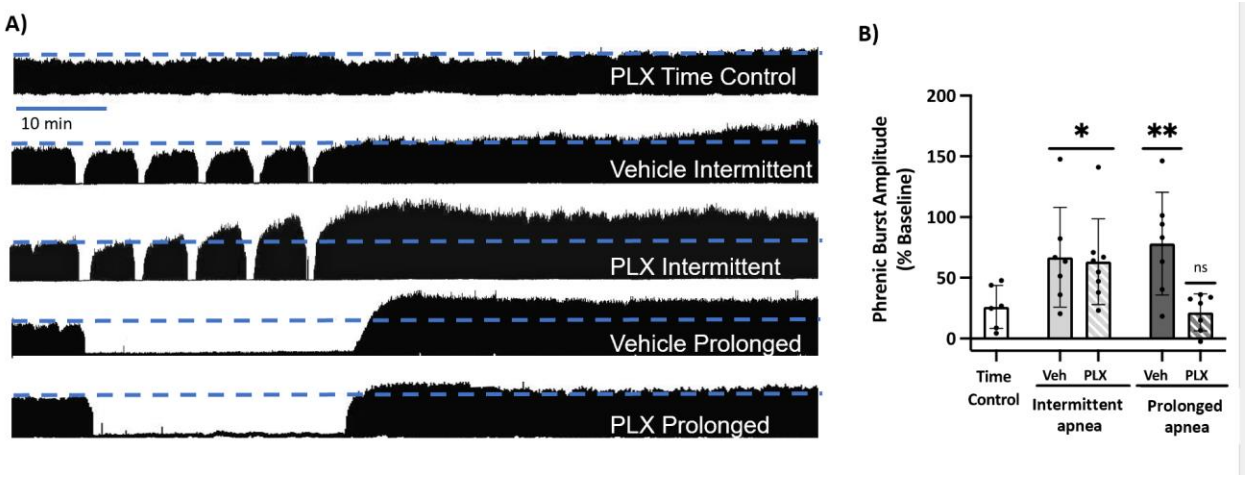

Figure 4 – Microglia are required for iMF following prolonged, but not brief intermittent, reductions in respiratory neural activity

A) Representative compressed phrenic neurograms depicting phrenic burst amplitude before, during, and for 60 minutes following a series of intermittent (5, ~1 min) or a single prolonged (~25 min) neural apnea, or the equivalent duration in a rat not receiving neural apnea (Time Control). To deplete microglia, rats were treated with the CSFR1 inhibitor PLX-3397 (80 mg/kg, p.o.) for 7 days prior to testing responses to respiratory neural activity deprivation. Control rats received vehicle for 7 days. **B)** Average change in phrenic burst amplitude relative to baseline 60 min after the resumption of respiratory neural activity, or equivalent in Time Controls (n=6). In vehicle- (n=7, p=0.03) and PLX-treated (n=8, p=0.04) rats, brief intermittent reductions in respiratory neural activity triggered a long-lasting increase in phrenic inspiratory output, suggesting that this form of iMF is not microglia-dependent. By contrast, prolonged reductions in respiratory neural activity was only able to elicit an increase in phrenic inspiratory output in vehicle-treated rats (n=7, p=0.008), but not in PLX-treated rats (n=7, p=0.81), suggesting iMF following prolonged neural apnea is microglia dependent. *p<0.05, **p<0.01 relative to Time Controls.

Chapter III

Microglial Depletion Rescues LPS-Induced Impairment of Compensatory Response to Recurrent Reductions in Respiratory Neural Activity

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In preparation

Abstract

Respiratory plasticity is an integral component of the control of breathing. Perturbations in respiratory control lead to persistent changes in system performance to maintain adequate and dynamic respiratory output. Inactivity-induced inspiratory motor facilitation (iMF) is one form of respiratory plasticity that results in persistent increase in phrenic motor output following reductions in respiratory neural activity, and is hypothesized to be a homeostatic response to prevent subsequent, potentially pathological, reductions in neural respiratory activity. The impact of inflammation on some types of respiratory plasticity has been characterized, but here we show for the first time that systemic inflammation induced by low-dose (0.1 mg/kg) lipopolysaccharide (LPS) impairs iMF the day after injection. Rats injected with LPS were unable to elicit a compensatory increase in phrenic motor output in response to a series of five brief (~1 min) central apneas separated by 5 minutes of activity, indicative of impaired iMF. To test the hypothesis that microglia, the resident immune cells of the CNS, are required for inflammation-induced impairment of iMF, we pharmacologically depleted microglia using CSF1R inhibitor Pexidartinib 3397 (PLX3397; 80 mg/kg; 7 days p.o.) prior to LPS injection. Microglial depletion restored the capacity to elicit iMF in LPS-treated rats. These studies show for the first time that inflammation impairs activity-dependent plasticity in the respiratory control system. Further, this is the first report of microglial participation in inflammation-induced impairment of any type of respiratory plasticity. These results implicate neuroinflammation and microglia as a target in diseases of inadequate respiratory output, including sleep apnea.

Introduction

The respiratory control system must fire reliably from birth until death despite acute or chronic challenges to breathing such as ascending to altitude, exercise, aging, pregnancy, or disease. Plasticity is a component of many neural systems, including the neural control of breathing, that allows for persistent changes in system performance based on experience (Mitchell and Johnson 2003). Thus, it is thought that perturbations in the neural system controlling breathing induce plasticity to maintain adequate and dynamic respiratory output (Fuller and Mitchell 2017). Reductions in respiratory neural activity, which could be fatal if left unchecked, trigger persistent augmentation of respiratory motor output and lower the CO_2 threshold for breathing, a response called inactivity-induced inspiratory motor facilitation (iMF; Mahamed et al. 2011; K. A. Strey, Baertsch, and Baker-Herman 2013; Kristi A. Strey et al. 2012). iMF is pattern sensitive, in that a single, prolonged (~25 min) neural apnea elicits iMF in a TNFα-, microglia-dependent manner (Broytman et al. 2013; Braegelmann et al., 2022 - in review). However, iMF triggered by a series of brief, intermittent neural apneas requires signaling through retinoic acid, independent of microglia (Baertsch and Baker 2017; Braegelmann et al., 2022 - in review). Both mechanisms require activation of PKCζ, indicating eventual convergence of these two pathways (Kristi A. Strey et al. 2012). It is hypothesized that iMF is a form of homeostatic plasticity (K. M. Braegelmann et al. 2017; K. A. Strey, Baertsch, and Baker-Herman 2013) that, together with chemoreceptor activation, protects and stabilizes breathing following pauses in neural respiratory activity that can occur in disease states like sleep apnea (recurrent neural apneas; Dempsey et al. 2010) and spinal cord injury (prolonged neural apnea; Strakowski et al. 2007; K. M. Braegelmann et al. 2017).

Many diseases with compromised respiratory control, such as sleep apnea (Díaz-García et al. 2022), spinal cord injury (Pang et al. 2022), and neurodegenerative diseases (Han et al. 2022), are accompanied by neuroinflammation, but the effects of inflammation on diverse types of

respiratory plasticity remain poorly understood. Neuroinflammation can occur as a result of disease or injury originating in the central nervous system (CNS), or can be conferred to the CNS from the periphery. Lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria that activates TLR4 and induces an endogenous immune response (Poltorak et al. 1998), is a commonly used insult to investigate the effects of inflammation (Huxtable et al. 2011). LPS cannot cross the blood-brain barrier (BBB) itself (Singh and Jiang 2004), but peripheral administration of LPS leads to expression of cytokines and prostaglandins that can cross and/or interact with the BBB, or signal through the vagus nerve, to activate microglia and induce an immune response in the CNS (Maier et al. 1998; A. G. Huxtable et al. 2011; Hocker et al. 2017; A. G. Huxtable et al. 2013). Microglia are the primary actors in the inflammatory response of the CNS and have the capacity to promote (Du et al. 2022) and impair (Raghuraman et al. 2019) neuroplasticity (Cornell et al. 2022). Neuroinflammation induced by LPS, prolonged intermittent hypoxia, or a viral mimetic impairs some mechanisms of respiratory plasticity induced by intermittent hypoxia (Huxtable et al. 2018; Huxtable et al. 2013; Hocker and Huxtable 2019; Hocker et al. 2017; Huxtable et al. 2015; Tadjalli et al. 2021; Agosto-Marlin et al. 2017; Vinit et al. 2011). However, whether activity-dependent respiratory plasticity is similarly affected by inflammation has not been studied. Further, the role of microglia in inflammation-induced impairment of respiratory plasticity has not been established.

In the present study we present evidence that low-dose LPS (0.1 mg/kg) impairs phrenic iMF the day after injection. Further, we show that LPS-induced impairment of iMF is microglia-dependent. Pharmacologic depletion of microglia prior to LPS injection rescues the capacity to induce iMF. These studies indicate for the first time that microglia play a critical role in LPS-induced impairment of respiratory plasticity and may present a target for intervention in inflammatory diseases complicated by ventilatory instability.

Methods:

Animals

Experiments were performed on 8-16 week-old male and female Sprague Dawley rats (n=65). Rats were pair-housed under standard conditions with 12:12 hr light cycles and food and water *ad libitum*. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison and were in accordance with the Guide for the Care and Use of Laboratory animals defined by the National Institutes of Health.

LPS Treatment

To assess the effect of low-dose systemic inflammation on iMF, rats were intraperitoneally injected with lipopolysaccharide (*E. coli* O111:B4; 500,000 EU/mg; Sigma–Aldrich, St. Louis, MO, USA**;** LPS; 0.1 mg/kg) or saline vehicle (Sal) ~20 hours prior to electrophysiology recording. LPS was vigorously vortexed prior to intraperitoneal administration to disrupt micelles.

PLX3397 Treatment

To probe the role of microglia in iMF expression following LPS, microglia were pharmacologically depleted using PLX3397 (MedKoo Biosciences, Morrisville, NC, USA)**.** PLX 3397 inhibits the tyrosine kinase of the CSF1 receptor that is critical to microglia survival.(Elmore et al. 2014) Rats were dosed with PLX3397 or vehicle (Veh) for 7 consecutive days (80 mg/kg, p.o.). Drugs were formulated in DMSO, 1% PS80, and 2.5% hydroxycellulose.

On the 7th day of PLX/Veh treatment, rats were injected with LPS (PLX-LPS, Veh-LPS) or saline (PLX-Sal, Veh-Sal) ~20 hours prior to electrophysiology recording. Differences in electrophysiology outcomes were not detected in LPS vs Veh-LPS groups or in Sal vs Veh-Sal

vs PLX-Sal, thus these groups were combined to minimize animal use and are referred to as “LPS” and “Vehicle”, respectively.

Immunohistochemistry (IHC) and Imaging

In adult rats, the expression of microglia, astrocytes, and neurons were quantified using IHC following 7 days of PLX3397 treatment and injection of Lipopolysaccharide (LPS) or saline. Male and female rats were transcardially perfused with 4% paraformaldehyde (PFA) in 1X phosphate buffered saline (PBS; pH 7.4). Cervical spinal cords were collected and post-fixed in 4% PFA for 24 hours before being cryoprotected in 20% sucrose solution in 1X PBS followed by 30% sucrose solution in 1X PBS. Cervical spinal cords were frozen in sectioning solution (1/3 O.C.T., 2/3 30% sucrose in 1X PBS) and stored at -80°C. Coronal sections 20mm thick were cut using a cryostat (CM3050S, Leica Biosystems, Deer Park, IL, USA) in the C3-C5 spinal regions. Spinal slices (n=2-4 per rat) were incubated with primary antibodies to IBA1 (1:1000, anti-rabbit, 019-19741, Wako Chemicals, Richmond, VA, USA), GFAP (1: 250, anti-rabbit, ab5804, EMD MilliporeSigma, Burlington, MA, USA), and NeuN (1: 500, anti-mouse, MAV377, EMD MilliporeSigma, Burlington, MA, USA) followed by secondary antibodies conjugated to Alexa Fluor fluorescent dyes (Invitrogen, Waltham, MA, USA) to identify the expression of microglia, astrocytes, and neurons, respectively. Images of ventral horn at 20X magnification were taken bilaterally for each slice with a fluorescence microscope (THUNDER Imager EM Cryo CLEM, Leica Microsystems Inc., Buffalo Grove, IL, USA). IBA1+, NeuN+, and GFAP+ cells were hand counted by two blind scorers using FIJI cell counter (ImageJ, public domain).

Assessment of Sickness Behavior and Neuroinflammation

To assess sickness behavior and inflammation in respiratory control regions following PLX/Veh and LPS/Sal treatments, a separate cohort of rats were dosed with PLX or Veh and LPS or Sal as described above. Body weight was monitored from the time of injection to harvest (20 hours), at which point rectal temperatures were collected to assess febrility. Rats were briefly exposed

to isoflurane to disorient them (<25 s in drop box) prior to decapitation, and the brainstem and cervical spinal segments C4-C5 were dissected and flash frozen for assessment of cytokine protein levels using Western Blot.

Analysis of protein levels of inflammatory cytokines IL-1B, TNF-alpha, and IL-6 are currently underway.

Surgical Preparation

Animals were induced with 5% isoflurane in a flow-through chamber and maintained on 2.5% isoflurane on a nose cone/tracheal tube. Rats were tracheotomized, mechanically ventilated (2-4.5 mL tidal volume; VentElite Small Animal Ventilator, Harvard Apparatus, Holliston, MA, USA), and bilaterally vagotomized to prevent entrainment with the ventilator. A tail vein catheter was placed for administration of urethane anesthesia and fluids (20% sodium bicarbonate in Lactated Ringer's Solution). Rats were slowly converted from isoflurane to urethane anesthesia (1.4-1.7 mg/kg i.v.) over thirty minutes, and paralyzed using Pancronium bromide (1.0 mg/kg i.v.). Body temperature was maintained between 36-38°C throughout surgical preparation and recording. Pressor response was monitored following paralysis to ensure adequate depth of anesthesia. A femoral arterial catheter was placed to monitor blood pressure and blood-gas parameters (pCO_2, pO_2, SBE, pH), which were assessed periodically using ABL90 Flex (Radiometer, Brea, CA, USA). The phrenic nerve was dissected via a dorsal approach, desheathed, and submerged in saline or mineral oil for recording with a suction or bipolar electrode, respectively. Raw phrenic nerve signals were recorded and digitized with PowerLab data acquisition system (LabChart 8.0, ADInstruments, Colorado Springs, CO, USA). Compound action potentials were amplified (x10k), band-pass filtered (0.3-10 kHz) and integrated (time constant 50 ms).

Electrophysiology Recording

To rapidly induce apnea during the protocol, rats were slightly hyperventilated and a small amount of CO_2 was added to the inspired gas line such that $ETCO_2$ ~45 mmHg. After isoflurane washout (>1hr), the apneic threshold was determined by removing inspired CO_2, and increasing ventilator frequency if necessary (up to 99 breaths/min), until phrenic activity ceased (apneic threshold). Inspired CO_2 was slowly increased until phrenic activity resumed (recruitment threshold), and then $ETCO_2$ was maintained 2-3 mmHg above the recruitment threshold. After 15-20 minutes of stable phrenic nerve activity ("baseline"), two blood samples were taken to establish baseline pCO_2. To elicit iMF, a neural apnea was induced by removing inspired CO_2 to reduce pCO_2 below the apneic threshold for ~1 min of activity cessation, then rapidly returned to baseline $ETCO_2$ for 5 minutes. This was repeated for a total of 5 recurrent neural apneas, after which phrenic nerve activity was recorded for 60 minutes. Importantly, rats remained ventilated throughout the neural apneas, ensuring that hypoxia was never experienced. Blood samples were taken 5, 15, 30, and 60 minutes following the last neural apnea. Phrenic burst amplitude 60 min following neural apneas was compared to baseline phrenic burst amplitude to determine the magnitude of iMF (expressed as percent change from baseline). Physiological parameters were maintained as follows: pCO_2 within 1.5 mmHg of average baseline pCO_2, pO_2 > 120 mmHg, SBE -3.0>0>3.0, MAP within 30 mmHg of baseline (**Table 1**). Following recording, rats were euthanized with a urethane overdose. CO_2

Statistical Analysis

For all electrophysiology experiments, phrenic nerve burst amplitude is expressed as a percent change from baseline. Phrenic amplitude was measured at baseline, 15, 30, and 60 min after the last induced neural apnea. Changes in apneic threshold were calculated as the difference between the first and first central apneas. Changes in body weight are expressed as a percentage of each rat's weight at the time of injection. Statistical differences between groups

were determined using one-way ANOVA and Tukey's *post hoc* test (α=0.05). Outliers were determined using Grubbs' Test (α=0.05), and subsequently removed.

Results

PLX Depletes Microglia in the Cervical Spinal Cord

Analysis of microglia depletion is still underway for this exact study. However, previous studies in our lab have shown that PLX depletes microglia in the cervical spinal cord at this same dose and duration. (**Figure 1**)

LPS Impairs Compensatory Response to Recurrent Central Apneas, which Is Rescued with Microglial Depletion

To test whether LPS impairs compensatory responses to recurrent central apnea, the capacity to respond to five, brief recurrent neural apneas was tested ~20 hours after intraperitoneal injection with LPS (0.1 mg/kg) or saline vehicle (Sal). A separate cohort of rats ("Time Controls", n=5) underwent the same treatments and surgical procedure, but were not exposed to recurrent neural apneas, and therefore did not express facilitation of phrenic motor output after 60 minutes (9.3±7.5% of baseline). Representative phrenic neurograms show phrenic activity at baseline, during, and for 60 minutes following exposure to intermittent neural apneas, or equivalent time in time controls **(Figure 2a)**. Vehicle-treated rats were able to elicit iMF following intermittent neural apneas (n=13; 54.0±3.8% of baseline, p=0.001 vs time controls), which was abolished with LPS treatment (n=7, 23.2±7.6% of baseline, p=0.59 vs time controls, p=0.004 vs Veh). **(Figure 2b)**

To test whether PLX rescues expression of iMF after LPS injection, iMF was tested after 7 days of PLX/Veh and ~20 hours after intraperitoneal injection with LPS or vehicle. Representative recordings show phrenic activity at baseline, during, and for 60 minutes following exposure to intermittent neural apneas (**Figure 2a).** Microglia depletion with PLX rescued the capacity to

elicit iMF in rats injected with LPS (n=8, 48.2±7.7 % of baseline, p=0.01 compared to time controls, p=0.88 compared to Veh, p=0.04 compared to LPS) **(Figure 2b)**. Collectively, these data indicate that microglia play a critical role in impairment of iMF by low-dose systemic inflammation.

Consistent with prior reports (cit), recurrent central apnea did not elicit frequency plasticity. Vehicle-treated rats exhibited a small increase in phrenic inspiratory frequency 60 minutes following recurrent apnea that was not significantly different from time controls (TC: 1.1±1.8% change from baseline; Veh: 5.2±1.4% change from baseline; p=0.48). Similarly, LPS (3.9±0.86% change from baseline) and PLX-LPS (3.8±2.6% change from baseline) treatments also did not impact phrenic inspiratory burst frequency 60 min following recurrent central apnea (all p>0.05).

Interestingly, LPS had a trending, but not significant, effect on recurrent central apnea-induced changes in the CO2 threshold for apnea threshold. In vehicle treated rats, recurrent central apnea resulted in a significant (p=0.0045) lowering of the apneic threshold from the first to the fifth apnea (-3.1±0.85 mmHg), which was not apparent in in LPS-treated rats (0.86±0.55 mmHg; p=0.11;**Figure 3)**. By contrast, central apnea-induced lowering of the apneic threshold appeared to be partially rescued in LPS rats pre-treated with PLX (-2.75±0.56 mmHg; p=0.94 vs Veh, p=0.23 vs LPS; **Figure 3)**. Collectively, these data indicate that microglia may play a role in inflammation-induced changes in apneic threshold associated with iMF expression.

Microglia Depletion Attenuates Weight Loss Following LPS

To test whether microglia depletion by PLX3397 prevents LPS-induced sickness behavior, body temperature and weight loss were assessed following treatment with PLX/Veh and LPS/Sal. Minor differences were detected in body temperature between vehicle-injected (Veh-Sal: 37.0±0.26, PLX-Sal: 36.7±0.12) and LPS-injected groups (Veh-LPS: 37.5±0.12, PLX-

LPS:37.1±0.1), with statistical significance only present between PLX-Sal and Veh-LPS groups (p=0.02).

LPS injection in Veh-treated rats resulted in a statistically significant reduction in body weight compared to Veh-Sal controls (Veh-LPS:-5.1±0.8; Veh-Sal:+0.89±0.6; p<0.0001; **Figure 4**). Pretreatment with PLX diminished weight loss in LPS-injected animals (PLX-LPS:-2.5±0.5; p=0.02 compared to Veh-LPS**; Figure 4**), but did not completely rescue it (p=0.02 compared to Veh-Sal). These data indicate that microglial depletion may reduce sickness behavior induced by low-dose systemic inflammation.

Regulation of Physiological Variables

Table 1 lists average PaCO, PaO_2, pH, mean arterial pressure (MAP) values at baseline and 60 minutes after treatments. No statistical differences were determined within or between treatment groups.

Discussion

Inflammation is a major component of most disease states, and characterizing its impact on plasticity is critical to advance our understanding of respiratory instability and the hurdles that may interfere with harnessing respiratory plasticity for disease treatment. Further, understanding the cell types involved in impairment of iMF illuminates intercellular communication that may play a role in expression or inhibition of respiratory plasticity. We found that intraperitoneal LPS impaired compensatory responses to recurrent central apnea. Following microglial depletion, expression of activity-dependent plasticity was rescued. As we have shown previously (Braegelmann et al., 2022 - in review), microglial depletion did not affect expression of intermittent apnea-induced iMF in vehicle-treated controls. While the effect of inflammation on other forms of respiratory plasticity have been investigated for years, this is the first report of the

impact of inflammation on iMF and the first documentation of microglial participation in impairment of any type of respiratory plasticity.

iMF is a form of activity-dependent plasticity that occurs within or near inspiratory motor pools in response to reductions in respiratory-related synaptic inputs (Baker-Herman and Strey 2011; Mahamed et al. 2011; Strey et al. 2012; Broytman et al. 2013; Streeter and Baker-Herman 2014; Streeter and Baker-Herman 2014; Baertsch and Baker-Herman 2015; Baertsch and Baker 2017; Baertsch and Baker 2017; Braegelmann et al. 2021). Most work on iMF mechanisms has used phrenic inspiratory motor neurons as a model. Evidence indicates that phrenic iMF is induced locally in the spinal cord, specifically within the activity-deprived phrenic motor neuron pool (Streeter and Baker-Herman 2014). iMF is also associated with a lowering of the CO_2 threshold for breathing(Baertsch and Baker 2017) and an elevation in phrenic dynamic range (Baertsch and Baker-Herman 2013). The magnitude of response to reduced respiratory neural activity is proportional to the level of activity deprivation, such that a reduction in neural respiratory activity (hypopnea) leads to a more modest compensatory response than that which follows a complete cessation (apnea) of neural respiratory activity (Braegelmann et al. 2017). iMF is elicited following diverse patterns of reduced neural respiratory activity, but follows distinct mechanisms. Following a single, prolonged neural apnea, soluble TNFα activates TNFR2 and elicits an increase in phrenic motor output in a microglia-dependent TNFα manner (Broytman et al. 2013; Braegelmann et al., 2022 - in review). Exogenous TNFα delivery to the region of the phrenic motor pool results in phrenic motor facilitation (pMF) that is phenotypically similar to that following a prolonged neural apnea (Broytman et al. 2013). On the other hand, recurrent reductions in neural respiratory activity, mimicking recurrent central apneas experienced by individuals with sleep apnea (Dempsey et al. 2010), elicits iMF independent of TNFα and microglia (Baertsch and Baker-Herman 2015; Braegelmann et al. 2022 - in review). This cell-autonomous form of iMF activates retinoic acid-receptor alpha (RARα) on or near

phrenic motor neurons and elicits a persistent increase in phrenic motor output, which can also be achieved by exogenous delivery of retinoic acid (Braegelmann et al. 2021; Baertsch and Baker 2017). Interestingly, when recurrent central apneas are induced simultaneously with moderate (but not mild) acute intermittent hypoxia, which typically elicits a distinct form of hypoxia-induced plasticity (Devinney et al. 2013), the two competing forms of plasticity inhibit one another and fail to induce any change in phrenic motor output (Fields et al. 2019). Though microglia are not required for expression of iMF following recurrent neural apneas (Braegelmann et al., 2022 - in review), here we present that they have the capacity to impair expression of iMF following low-dose systemic inflammation. Similarly, inflammation had a trending effect on the change in apneic threshold associated with iMF, which was returned to control levels with microglial depletion.

Peripheral administration of lipopolysaccharide (LPS) is a well-established model to induce systemic inflammation (Huxtable et al. 2011; Huxtable et al. 2013). LPS is a ligand for toll-like receptor 4 (TLR4) (Poltorak et al. 1998), which activates the transcription factor NFκB (Kawai and Akira 2007), leading to increased expression of inflammatory genes like interleukins (IL-1β, IL-6, IL-18), tumor necrosis factor alpha (TNFα), and cyclooxygenase-2 (COX-2), which catalyzes conversion of arachidonic acid to pro-inflammatory prostaglandin-2 (PGE2) (Kawai and Akira 2007). Inflammatory cytokines in the periphery can induce inflammation in the CNS via afferent nerves, such as the vagus (Maier et al. 1998; Goehler et al. 1997; Watkins et al. 1995; Roth and De Souza 2001), by activating receptors on endothelial cells of the BBB (Laflamme et al. 1999), by crossing the BBB via cytokine transporters (Banks et al. 1995), or by interacting with receptors in the circumventricular organs that confer the immune response to the CNS (Komaki et al. 1992; Litvin et al. 2020). Additionally, some immune states induce breakdown of the BBB, allowing infiltration of peripheral immune cells into the CNS (Wispelwey et al. 1988). Regardless of the mechanism, it is well-documented that systemic inflammation

results in activation of microglia (Elmore et al. 2014; Henry et al. 2009; Huxtable et al. 2013), the resident immune cells of the CNS that elicit their own inflammatory response characterized by the release of pro-inflammatory cytokines, nitric oxide, and prostaglandins (Kreutzberg 1996; Graeber and Streit 2010).

Microglia play diverse roles in neuroplasticity throughout the CNS. Under resting conditions, microglia are necessary for induction of hippocampal LTP (Wang et al. 2018; Raghuraman et al. 2019). After activation with an inflammatory stimulus, microglia can inhibit (Raghuraman et al. 2019) or enhance (Du et al. 2022) hippocampal LTP. Further, activated microglia are required for spinal plasticity in the development of pain sensitization (Milligan et al. 2004). iMF seems to have a similar differential requirement for microglial activity, in that prolonged apnea-induced, TNFα-dependent iMF requires microglia (Braegelmann et al., 2022 - in review), while activated microglia impair iMF elicited by recurrent neural apneas. Thus, there is considerable work to be done to further characterize the role of microglia and neuroinflammation in neuroplasticity in the healthy and diseased CNS.

Cytokine signaling actually promotes plasticity of respiratory control at multiple levels, including pattern and rhythm generation (Jacono et al. 2011; Hsieh et al. 2020), chemosensory plasticity (Popa et al. 2011), and motor plasticity (Broytman et al. 2013; Braegelmann et al., 2022 - in review), with a newly appreciated role for microglia. Interestingly, ventilatory acclimatization to chronic sustained hypoxia is a form of chemosensory plasticity that is associated with inflammation in the nucleus tractus solitarius (NTS) (Popa et al. 2011), a region critical to the coordination of respiratory activity (Zoccal et al. 2014). Administration of minocycline, a non-specific microglia inhibitor, reduces microglial activation and inflammatory cytokine (IL-1β, IL-6, TNFα) expression in the NTS, and impairs the VAH response, indicating a critical role for microglia in this form of respiratory plasticity (Stokes et al. 2017). Further, systemic inflammation reduces respiratory frequency in neonates, which can be reversed with microglial inhibition by

minocycline (Lorea-Hernández et al. 2016). Finally, as discussed previously, iMF induced by a prolonged neural apnea requires TNFα signaling and is impaired by microglial depletion. Thus, the requirement of inflammation, and more specifically microglia, in promoting certain forms of respiratory plasticity is beginning to be uncovered.

On the other hand, neuroinflammation can impair respiratory motor plasticity. Previous studies using another model of respiratory plasticity, phrenic long-term facilitation (pLTF) induced by acute intermittent hypoxia (AIH), have shown impairment by inflammation in some, but not all, forms of pLTF. pLTF can be induced by moderate or severe exposure to AIH (mAIH, sAIH, respectively). Interestingly, neuroinflammation induced by LPS (A. G. Huxtable et al. 2011; Vinit, Windelborn, and Mitchell 2011), viral mimetic polyinosinic:polycytidylic acid (polyI:C) (Hocker and Huxtable 2019), or prolonged intermittent hypoxia (Adrianne G. Huxtable et al. 2015, 2018) impairs mAIH-induced pLTF, while pLTF induced by sAIH appears to be inflammation-resistant (Agosto-Marlin, Nichols, and Mitchell 2017; Hocker and Huxtable 2019). Further, neonatal systemic inflammation induced by LPS abolishes pLTF expression in adulthood, indicating a long-lasting impact of early-life inflammation on respiratory motor control (Hocker et al. 2019). Despite consistent impairment of mAIH-induced pLTF following various inflammatory stimuli, the inflammatory molecules that are required for this impairment remain unclear.

Inducible nitric oxide synthase (iNOS) and COX-2, but not TNFα or IL-1β, displayed increased expression in homogenates and microglia from the cervical spinal cord 3 hours after LPS injection, but returned to baseline levels after 24 hours, despite persistent impairment of pLTF at 24 hours following injection (Huxtable et al. 2013). Following prolonged IH exposure, iNOS expression was upregulated in cervical spinal homogenates at the same time point of pLTF impairment, while IL-1β expression was upregulated in microglia isolated from the spinal cord (Huxtable et al. 2015). Finally, after polyI:C, COX-2 expression was upregulated in homogenates from the ventral cervical spinal cord 3 hours, but not 24 hours, after

administration, despite impairment of pLTF at the 24 hour, but not 3 hour, timepoint (Hocker and Huxtable 2019). Other studies showed that IL-1 receptor activation at the level of the phrenic motor pool is necessary, but not sufficient, for LPS-induced impairment of pLTF (Hocker and Huxtable 2018), and that COX-2 is not required for impairment of pLTF after prolonged intermittent hypoxia exposure (Huxtable et al. 2018). Further work demonstrated that inhibition of p38 MAPK (Huxtable et al. 2015) or okadaic acid-sensitive serine/threonine phosphatases (Tadjalli et al. 2021), which are activated by the inflammatory response, restores pLTF after LPS. These studies demonstrate that neuroinflammation induced by systemic immune stimuli is a dynamic insult that consistently impairs mAIH-induced pLTF, but that further work is required to identify the specific inflammatory pathways that lead to impairment of respiratory plasticity. Assessment of microglial inflammatory expression unsurprisingly indicates that microglia contribute to spinal inflammation, and are therefore hypothesized to participate in inflammation-induced impairment of pLTF, but a specific role for microglia in impairment of pLTF has not been delineated. We found that impairment of iMF by LPS-induced inflammation is dependent on microglia, demonstrating for the first time a critical role for microglia and intercellular communication in inflammation-induced impairment of respiratory plasticity.

We report neuroinflammation-induced impairment of iMF is rescued via pharmacologic depletion of microglia, but the precise mechanism by which microglia-dependent neuroinflammation undermines iMF remains unknown. Metaplasticity, or biochemical changes in the ability of a system to express plasticity (Abraham and Bear 1996), may underlie inflammation-induced impairment of iMF. Inflammation near the phrenic motor pool may promote some forms of respiratory plasticity that compete with iMF. For example, upregulation of pro-inflammatory cytokine TNFα as a part of the LPS response could activate TNFR2 on phrenic motor neurons and promote phrenic motor facilitation. It is unknown whether TNFα-dependent pMF participates in cross-talk inhibition with iMF induced by intermittent neural apneas, but remains a distinct

possibility given the prevalence of cross-talk inhibition between multiple other types of respiratory plasticity (Devinney et al. 2013; Fields et al. 2019). Since microglia are required for TNFα-dependent phrenic motor facilitation, microglial depletion would remove this constraint on iMF expression.

Another possibility is that inflammation may promote expression of inflammation-resistant phrenic motor facilitation (pMF). Inflammation causes extracellular accumulation of adenosine (Ribeiro, Sebastiao, and de Mendonca 2003), a product of ATP metabolism that can activate cellular pathways leading to pMF, in the absence of hypoxia exposure, via similar pathways as those induced by sAIH (Seven et al. 2018). Activation of A2A receptors on phrenic motor neurons promotes pMF,(Seven et al. 2018) which eventually leads to NMDAR activation and long-term augmentation of phrenic motor output (Golder 2009). iMF is constrained by NMDAR activation (Streeter and Baker-Herman 2014), and accordingly, coinduction of pLTF with iMF results in a failure to express any phrenic motor plasticity (Fields et al. 2019). Interestingly, preliminary evidence indicates that microglia are required for A2A-receptor mediated pMF (Tadjalli et al., 2021). Restoration of LPS-impaired iMF by phamarcologic depletion of microglia is consistent with the hypothesis that LPS may promote adenosine accumulation, leading to microglia- and adenosine-dependent pLTF and cross-talk inhibition of iMF.

Finally, microglial catabolism of retinoic acid may impair compensatory responses to intermittent neural apnea. Retinoic acid is a key signaling molecule in many types of homeostatic plasticity (Chen, Lau, and Sarti 2014), in which reductions in intracellular calcium trigger activation of retinoic acid, upregulating post-synaptic AMPA receptor translocation and eliciting activity-dependent plasticity (Chen, Lau, and Sarti 2014). Indeed, iMF following intermittent neural apeas requires activation of all-trans retinoic acid receptor alpha on or near phrenic motor neurons (Kendra M. Braegelmann et al. 2021). Further, exogenous delivery of retinoic acid elicits persistent increases in phrenic motor output. Activated microglia produce cytochromes

that increase catabolism of retinoic acid (Hellmann-Regen et al. 2013). Though retinoic acid signaling in iMF is presumed to be contained within the post-synaptic membrane (Baertsch and Baker 2017), RA is lipophilic and may exert extracellular effects that could be impaired by cytochromes derived from activated microglia. Alternatively, cytochromes could cross into nearby neurons and elicit an effect intracellularly. Thus, microglia depletion could rescue iMF by reducing catabolism of retinoic acid.

Recent studies have made it clear that microglia have the capacity to potently influence neuroplasticity in the healthy and inflamed CNS. However, whether microglia participate in impairment of respiratory neuroplasticity by inflammation remained an open question. In the present study we demonstrate that neuroinflammation induced by low-dose, peripheral LPS administration impairs activity-dependent respiratory motor plasticity (iMF) the day after administration, which can be rescued by microglial depletion prior to inflammatory insult. Interestingly, we also found that microglial depletion reduced weight loss following LPS exposure, indicating a role for microglia in anhedonia resulting from systemic inflammation. iMF is hypothesized to protect the respiratory control system from pathological reductions in respiratory neural activity. However, it seems that this compensatory mechanism may be absent in individuals with sleep apnea, which is characterized by many apneic events per hour (Dempsey et al. 2010). Importantly, individuals with sleep apnea also exhibit neuroinflammation (Patel et al. 2012). Impairment of iMF by inflammation may explain why humans with sleep apnea are unable to compensate for these recurrent reductions in respiratory neural activity, and may implicate neuroinflammation and microglia as potential targets for pharmacologic intervention.

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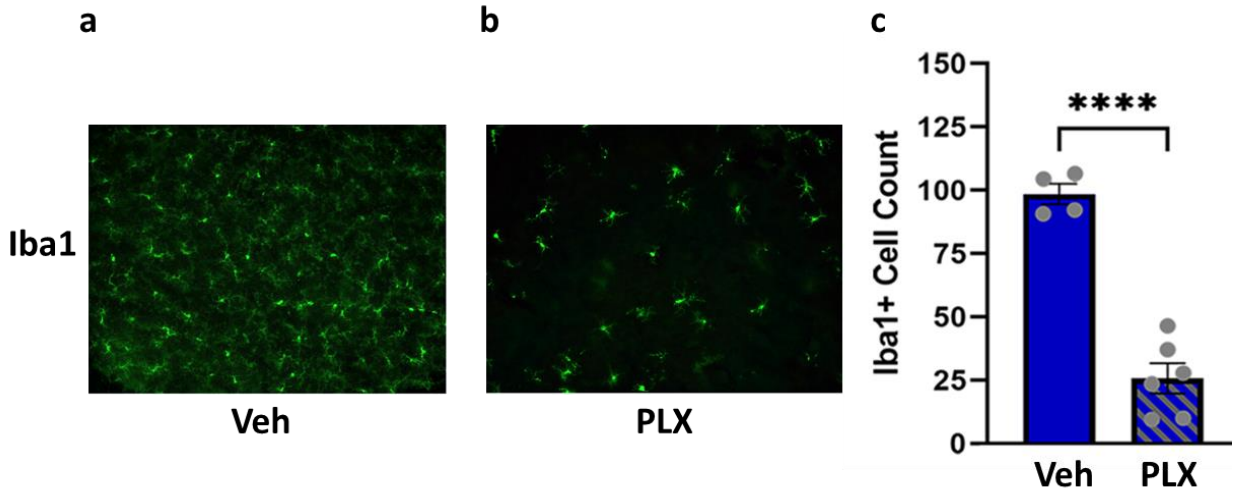
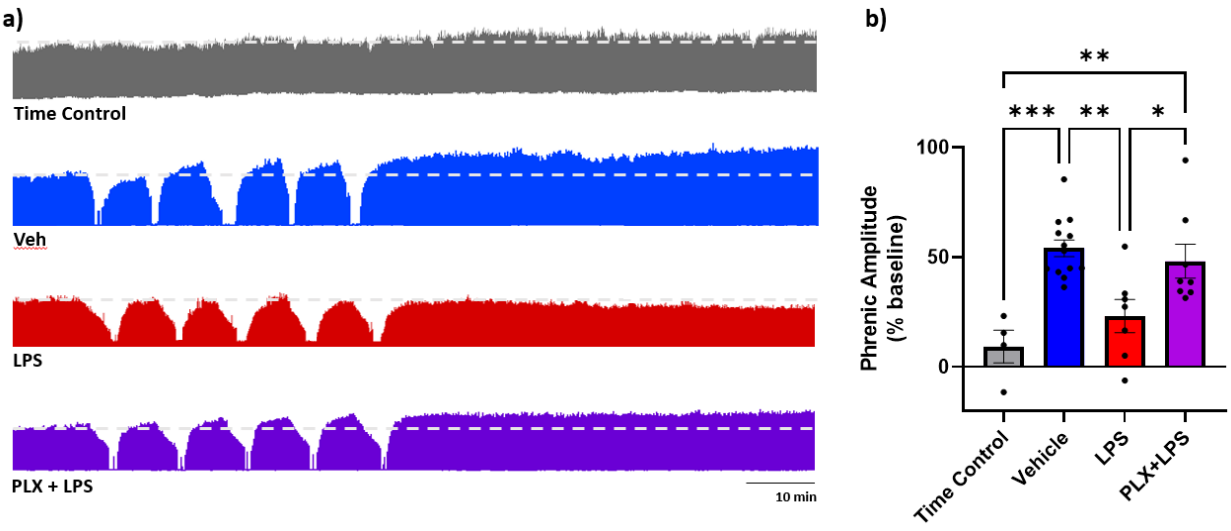
Figure 1: PLX3397 Depletes Microglia in the Cervical Spinal Cord



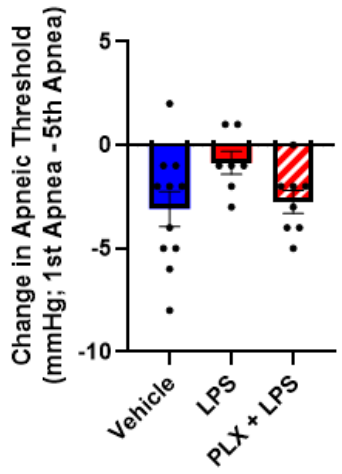
(a, b) Cervical spinal cord sections from PLX- and Veh-treated rats were immunostained for Iba-1 (microglia) Scale bar = 100um. (c) Cell count analysis indicated significant (~73%) depletion of Iba+ cells in PLX-treated offspring.

Figure 2: Microglia Depletion Rescues LPS-Induced Impairment of Compensatory Responses to Intermittent Reductions in Respiratory Neural Activity



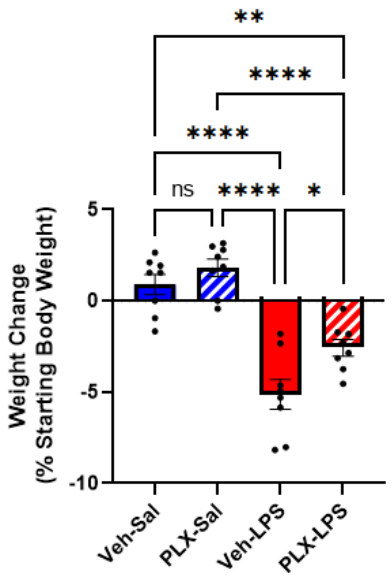
(a) Representative compressed phrenic neurograms depicting phrenic burst amplitude before, during, and for 60 minutes following exposure to a series of 5, (~1 min) brief reductions in respiratory neural activity, or the equivalent duration in a rat not receiving central apnea (time control). Dotted line represents baseline amplitude. (b) Average percent change (± SEM) in phrenic burst amplitude from baseline at 60 minutes following the fifth neural apnea. Vehicle treated rats elicited a significant increase in phrenic inspiratory burst amplitude 60 min following the fifth neural apnea compared to time controls (n=13; 54.0±3.8% from baseline, p=0.001 vs. time controls, n=5), which was abolished with LPS treatment (n=7, 23.2±7.6% from baseline, p=0.59 vs. time controls, p=0.004 vs Veh). Microglial depletion with PLX rescued expression of iMF after LPS administration (n=8, 48.2±7.7% from baseline, p=0.01 vs. time controls, p=0.88 vs. Veh, p=0.04 vs. LPS). These data demonstrate that LPS impairs expression of compensatory responses to intermittent neural apneas, and that microglia play a critical role in this impairment. ***p<0.001, **p<0.01, *p<0.05

Figure 3: Inflammation Has Trending Effect on Change in Apneic Threshold



Average change (mmHg; ±SEM) in apenic threshold from first to fifth apnea. Vehicle-treated rats exhibited a decrease in apneic threshold (-3.1±0.85 mmHg), which was reduced slightly, but not significantly, reduced with LPS (0.86±0.55 mmHg; p=0.11 vs vehicle). Microglial depletion with PLX prior to LPS increased the change in apneic threshold to values comparable to vehicle-treated rats (-2.75±0.56 mmHg; p=0.94 vs vehicle; p=0.23 vs LPS). These data indicate that LPS has a moderate effect on change in apneic threshold associated with iMF expression, and that microglia may participate.

Figure 4: Microglial Depletion Attenuates Weight Loss Following LPS



Average change in body weight as a percentage of starting body weight (±SEM). Rats treated with LPS exhibited a significant decrease in body weight ~20 hours after injection, compared to Veh-Sal controls (Veh-LPS: -5.1±0.8; Veh-Sal: 0.89±0.6; $p<0.0001$). Microglial depletion prior to LPS diminished weight loss (PLX-LPS: -2.5±0.5; $p=0.02$ vs Veh-LPS) but not to the level of Veh-Sal controls ($p=0.02$ vs Veh-Sal), indicating that microglia may play a role in weight loss induced by systemic inflammation.

Table 1: Metabolic Values

	Time Control		Vehicle		LPS		PLX + LPS	
	Baseline	60 min	Baseine	60 min	Baseline	60 min	Baseline	60 min
paCO2 (mmHg)	44.7 ± 1.0	45.4 ± 1.3	52.8 ± 2.7	53.1 ± 2.7	49.8 ± 2.7	49.4 ± 2.7	46.5 ± 1.5	46.7 ± 1.3
paO2 (mmHg)	206.8 ± 12.5	224.8 ± 13.7	240.0 ± 10.1	222.6 ± 9.3	230.5 ± 18.1	220.1 ± 23.4	235.3 ± 9.4	230.9 ± 9.9
pH	7.36 ± 0.01	7.36 ± 0.02	7.30 ± 0.02	7.30 ± 0.02	7.34 ± 0.02	7.33 ± 0.02	7.35 ± 0.01	7.36 ± 0.01
MAP (mmHg)	133 ± 13.3	114 ± 14.5	142.9 ± 5.5	131.6 ± 4.4	146.1 ± 10.1	133.2 ± 11.1	146.8 ± 4.0	148.7 ± 3.7

Arterial pCO_2, pO_2, pH, and mean arterial pressure are expressed as mean ± SEM at baseline and 60 minutes following intermittent neural apneas. No significance was detected within or between groups.

Chapter IV

Maternal intermittent hypoxia during pregnancy causes cardiorespiratory deficits and gut dysbiosis in adult male, but not female, offspring

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In preparation

Abstract

Sleep apnea during pregnancy can detrimentally impact neonatal health, but little is known regarding long-lasting consequences to the offspring. The *in utero* environment is well-known to affect the etiology of, and susceptibility to, many major public health problems, but prenatal triggers that increase susceptibility to ventilatory control disorders have been poorly investigated. We developed a model of sleep apnea in pregnancy by exposing rat dams to intermittent hypoxia, a hallmark of sleep apnea, during the latter half of pregnancy. Adult male offspring exposed to gestational intermittent hypoxia (GIH) exhibited an increased number of spontaneous apneas during presumptive sleep, elevated heart rate and increased mean arterial blood pressure. Furthermore, GIH male offspring exhibited altered gut microbiomes, with a decreased Firmicutes/Bacteroidetes ratio. By contrast, adult female offspring appeared largely unaffected. Collectively, these data indicate that intermittent hypoxia during pregnancy may increase risk for male offspring to develop sleep apnea, hypertension, and gut dysbiosis later in life.

Introduction

The *in utero* environment is well known to affect the etiology of and susceptibility to many major public health problems (Gozal et al., 2003; Santos and Dean, 2004; Dolinoy et al., 2007; Feinberg, 2007; Barouki et al., 2012; Balbus et al., 2013; Hanson and Gluckman, 2015; Khalyfa et al., 2017). For example, chronic or severe hypoxia during pregnancy is associated with an increased risk for neurological disorders in offspring (Amgalan et al., 2021), such as autism spectrum disorder (ASD), schizophrenia or intellectual disability (Modabbernia et al., 2016; Amgalan et al., 2021). Chronic/severe hypoxia may be experienced during a traumatic or disordered pregnancy, and physicians recognize the potential consequences to the child's health. Underappreciated is that many women experience recurrent episodes of brief hypoxia during pregnancy in the form of sleep apnea (SA). SA is characterized by recurrent cessation of breathing during sleep, causing pathological drops in blood oxygen levels (hypoxia) often hundreds of times each night. SA prevalence is dramatically increasing in pregnant women, almost doubling within the last decade (Spence et al., 2017; Liu et al., 2019). Current estimates suggest that 20% of all pregnant women (Pien et al., 2014), and >50% of women with high-risk pregnancies, develop SA by the third trimester (Johns et al., 2021). SA is associated with adverse pregnancy and perinatal outcomes (Bourjeily et al., 2013; Ding et al., 2014; Pamidi et al., 2014), but little is known about lasting effects of maternal SA on adult offspring physiology.

Evidence suggests that SDB is heritable (el Bayadi et al., 1990; Pillar and Lavie, 1995; Redline et al., 1997; Redline and Tishler, 2000) with nearly 40% of variance in AHI explained by familial factors. Familial aggregation of SDB is independent of obesity (Patel et al., 2008) and appears to be strongest when the mother has OSA (Au et al., 2021). While genetic abnormalities in upper airway anatomy may contribute in some cases, familial aggregation of SDB is apparent without overt craniofacial abnormalities and may be related to ventilatory control deficits, such as impaired neural control of the upper airway during sleep (Lavie and Rubin, 1984; Redline et al., 1997;

Redline and Tishler, 2000). Despite decades of research, the precise genetic factors that increase susceptibility for OSA remain poorly defined (Kent et al., 2010; Larkin et al., 2010; Patel et al., 2012; Cade et al., 2016). Strikingly, maternal obesity or diabetes -- both comorbidities of maternal SA -- strongly increase the risk of her offspring developing SA (Chen et al., 2019), suggesting that the heritability of SA may be influenced by the in utero environment.

Sleep apnea in humans has been associated with alterations in the microbial community that inhabits the gut (Valentini et al., 2020). The importance of a healthy and diverse gut microbiome in normal physiology is increasingly recognized, and gut dysbiosis is associated with a number of morbidities associated with sleep apnea, such as hypertension (Durgan, 2017), diabetes (Siljander et al., 2019), and obesity (Geng et al., 2022). In humans, sleep apnea is associated with reductions in microbial diversity, increases in Firmicutes/Bacteroidetes ratio, and reduced relative abundance of bacteria that produce short-chain fatty acids (SCFAs) (Valentini et al., 2020). SCFAs are a byproduct of bacterial fiber metabolism in the gut that have widespread effects throughout the body, including on immune and cardiovascular regulation (de Vos et al., 2022).

SA is frequently modeled in rodents by mimicking the nocturnal oxygen swings (intermittent hypoxia; IH) associated with the disorder (Chopra et al., 2016). Animal models indicate that the resulting chronic IH drives much of the morbidity associated with SA (Chopra et al., 2016), such as metabolic dysfunction (Carreras et al., 2012), cardiovascular dysfunction (Chu et al., 2015), gut dysbiosis (Moreno-Indias et al., 2015), and the risk for some cancers (Almendros and Gozal, 2018). To begin to understand the developmental consequences of maternal SA during pregnancy on offspring respiratory control and the gut microbiota, we developed a model of maternal SA in pregnancy by exposing rat dams to IH during the latter half of gestation (gestational intermittent hypoxia, GIH). We report that GIH increases spontaneous apnea

frequency during presumptive sleep and leads to gut dysbiosis, specifically in male offspring. GIH male offspring also exhibited increased heart rate and mean arterial pressure, which was driven by increased systolic blood pressure. Collectively, these data indicate for the first time that maternal IH, such as occurs in maternal SA, may increase risk for male offspring to develop gut dysbiosis and cardiorespiratory sequelae associated with SA.

Results

To determine if mimicking swings in oxygen associated with SA during pregnancy leads to cardiorespiratory deficits in adult offspring, pregnant dams were exposed to intermittent hypoxia or normoxia (respectively, gestational intermittent hypoxia, GIH; or gestational intermittent normoxia, GNX) during the last half of pregnancy. We chose this time period because most women who develop sleep apnea do so by the third trimester.(Pien et al., 2014) Offspring were raised to adulthood; separate groups of rats were randomly selected for breathing or cardiovascular measurements, and fecal samples were taken from both groups.

GIH increases spontaneous apnea frequency in adult male offspring

We first sought to investigate the frequency of apneas in adult GIH offspring . Breathing was measured in adult male and female GNX and GIH offspring during their subjective night using whole-body plethysmography by two investigators blinded to treatment. Presumptive sleep was scored using criteria outlined in Bastianini et al., 2017 (Bastianini et al., 2017). Adult male GIH offspring exhibited a significant increase in the total number of apneas during presumptive sleep (GIH males: 28.32 ± 1.531; GNX males: 22.41±1.517; **Figure 1A;** $p<0.02$). By contrast, no changes in total apneas were observed in female GIH offspring (GIH females: 24.37 ± 1.745; GNX females: 22.66 ± 1.226; **Figure 1A;** $p>0.05$**)**. Apneas were categorized as spontaneous (independent of a sigh), post-sigh (immediately after a sigh), or trailing post-sigh (within 10 missed breaths of a sigh, but with one or more breaths interposed - see methods for detailed description)

(**Figure 1B**). Male GIH offspring exhibited significant increases in spontaneous apneas (GIH males: 8.21 ± 0.0.65; GNX males 5.21 ± 0.48; **Figure 1C**, p=0.001), but not in post-sigh apneas (GIH males 10.03 ± 0.85; GNX males 10.67 ± 0.89; **Figure 1D**, p=0.84). A slight, but non-significant trend toward an increase in the number of trailing post-sigh apneas was also observed in male GIH offspring (GIH males 9.44 ± 0.71; GNX males 7.17 ± 0.89;**Figure 1E**, p=0.10). In all sigh categories, GIH male offspring exhibited slight decreases in the duration of the apneic episode (**Figures 1F-H**; p<0.05). Consistent with no change in total apneas (GIH females: 22.66±1.23; GNX females: 24.37±1.75; **Figure 1A**; p=0.67), female GIH offspring did not exhibit significant differences in the number or duration of spontaneous, post-sigh or trailing post-sigh apneas (not shown).

In 19/29 male GIH offspring, spontaneous apnea frequency was outside the upper 99% confidence interval for spontaneous apnea frequency in male GNX offspring (lower: 3.8, upper: 6.6 apneas/hr), suggesting that the penetrance of the increased apnea phenotype approached 66%. Spontaneous apnea frequency in affected GIH male offspring was 10.24 ± 0.53 apneas/hr, roughly double that observed in GNX male offspring (5.2 ± 0.5 apneas/hr; **Figure 2A**; p<0.0001). Affected GIH male offspring did not exhibit an increase in post-sigh apneas (GIH males: 11.1 ± 1.2; GNX males: 10.0 ± 0.9; **Figure 2B;** p=0.46), although a slight, but significant, increase in trailing post-sigh apneas was observed (GIH males: 9.8 ± 0.6; GNX males: 7.2 ± 0.9; **Figure 2C**; p=0.02). To determine the sleep cycle associated with increased spontaneous apneas, two blinded investigators scored the plethysmography traces for presumptive NREM/REM using criteria outlined in Bastianini et al., 2017. Increased spontaneous apneas in affected male GIH offspring were apparent in both presumptive NREM (GIH: 5.6 ± 0.9; GNX: 2.4 ± 0.4 apneas/hr presumptive NREM; **Figure 2D;** p<0.002) and REM sleep (GIH: 1.24 ± 0.15; GNX: 0.53 ± 0.08 apneas/min presumptive REM; **Figure 2E;** p<0.0003), roughly doubling in each state. There were no differences in the total amount of time marked as REM between GNX and GIH rats (GIH: 0.31

± 0.03; GNX: 0.37 ± 0.02) Post-sigh and trailing post-sigh apneas were relatively rare in REM, and thus were not included in this analysis.

No significant differences in T_V, f_R or V_E were observed between male GNX (T_V: 1.66 ± 0.06 ml, f_R: 90 ± 3 breaths/min; V_E: 147 ± 5 ml/min) and GIH (T_V: 1.59 ± 0.06 ml, f_R: 97 ± 3 breaths/min; or V_E: 156 ± 5 ml/min) offspring, or female GNX (T_V: 1.49 ± 0.06 ml; f_R: 85 ± 3 breaths/min; or V_E: 124 ± 4 ml/min) and GIH (T_V: 1.34 ± 0.03 ml, f_R: 85 ± 2 breaths/min; or V_E: 113 ± 3 ml/min) offspring (all $p>0.05$).

Adult GIH male offspring have hypertension

Since sleep apnea is strongly associated with hypertension, blood pressure and heart rate were measured in GIH male and female offspring, and GNX male and female offspring. Mean arterial pressure (MAP) was significantly increased in male GIH offspring, when compared to GNX counterparts (GIH: 112 ± 4, GNX: 96 ± 4 mmHg; $p=0.04$). Systolic blood pressure (BP) was significantly higher in male GIH offspring (GIH: 142 ± 8, GNX: 117 ± 5 mmHg; $p=0.04$), whereas no change was observed in mean diastolic blood pressure (GIH: 104 ± 6, GNX: 90 ± 5; $p=0.19$) or heart rate (GIH: 315 ± 10, GNX: 346 ± 11 beats/min; $p=0.08$). By contrast, no change in MAP (GIH: 120 ± 3, GNX: 122 ± 5 mmHg), systolic (GIH: 151 ± 4, GNX: 148 ± 6 mmHg) or diastolic (GIH: 104 ± 3, GNX: 110 ± 5 mmHg) BP, or heart rate (GIH: 356 ± 7, GNX: 388 ± 18 mmHg) were observed in female GIH offspring (all, $p>0.05$).

No change in adipose tissue weight or body weight

In subsets of rats, body weight was measured shortly after birth until early adulthood (P7-P77) and gonadal fat pad mass was assessed at 12 weeks of age. Contrary to previous reports (Song et al. 2021; Khalyfa et al. 2017; Weng et al. 2021), GIH did not result in significant differences in body weight in male or female offspring at any time point measured (Males: $p>0.05$, Females:

p>0.05; **Figure 3**). Fat pad ratio (avg. fat pad mass/total body mass) for male GNX and GIH (GNX: 0.0097 ± 0.0011; GIH: 0.01 ± 0.0012; p=0.85) and female GNX and GIH (GNX: 0.0030 ± 0.00055; GIH: 0.0026 ± 0.00095; p=0.50) offspring reveal no effect of GIH on gonadal fat pad mass.

Adult GIH male offspring have gut dysbiosis

To determine if gestational exposure to intermittent hypoxia influences the gut microbiome of adult offspring, we sequenced fecal samples from male and female offspring using 16s rRNA sequencing. Overall, we found that GIH skews the adult male offspring gut microbiome towards dysbiosis (**Figure 4**). GIH males displayed a reduction in alpha- and Beta-diversity, indicating less diverse bacterial taxa, compared to females and control males (**Figure 4A**). GIH exposure did not alter alpha- or Beta-diversity in female offspring. More specifically, we observed that GIH males have a decreased Firmicutes/Bacteroidetes ratio, with expansion of bacteria from the Bacteroidetes phyla and contraction of bacteria from the Firmicutes phyla (**Figure 4B**). Disruption of the Firmicute:Bacteroidete ratio is characteristic of gut dysbiosis (Stojanov et al., 2020). When we examined which species were present between the groups, we observed that GIH male offspring had decreased relative abundance of bacteria that produce the short-chain fatty acid butyrate, including *Roseburia* and *Ruminococcoceae* (**Figure 4B**).

Discussion

Here, we report that gestational intermittent hypoxia (GIH) has a long-lasting, sex-dependent impact on the health of the adult offspring. Specifically, male, but not female, GIH offspring exhibit gut dysbiosis, elevated blood pressure, and increased apneas during presumptive sleep. Strikingly, these three physiological factors–the gut microbiome, sleep-disordered breathing, and cardiovascular function–are closely intertwined and potentially mechanistically linked. The precise relationship between these three disordered systems is beyond the scope of the current study, but warrants further investigation. Taken together, our results suggest that male offspring

may be more vulnerable than female offspring to detrimental consequences of maternal sleep apnea in pregnancy.

The *in utero* environment can reprogram critical physiological systems, but this has been poorly evaluated in the context of respiratory motor control. Studies in animal models support the hypothesis that early *post*natal experiences can adversely impact the adult respiratory control system (Okubo and Mortola, 1990; Ling et al., 1996; Bavis et al., 2002, 2004; Fuller et al., 2002; Bisgard et al., 2003; Huang et al., 2004; Montandon et al., 2006, 2009; Reeves et al., 2006; Dumont and Kinkead, 2010; Nanduri et al., 2012; Kinkead et al., 2013, 2014; Mayer et al., 2014; Nanduri and Prabhakar, 2015; Hocker et al., 2019), but comparatively little is known regarding how the prenatal environment can shape adult respiratory control. Neuronal networks underlying breathing undergo a rapid period of development during the latter part of pregnancy to prepare the offspring for independent breathing upon birth (Greer et al., 2006), and as such may be particularly vulnerable to in utero insults. Indeed, we report that adult male, but not female, offspring born from dams exposed to maternal intermittent hypoxia during pregnancy have increased spontaneous apneas during presumptive sleep. No change in post-sigh apneas were observed, although modest increases in “trailing” apneas following a sigh were observed. Trailing post-sigh apneas were defined as an apnea that occurred within five breaths of a sigh, but with a normal breath interposed, and may indicate that GIH males have an extended period of vulnerability to respiratory instability following a sigh. Collectively, these data suggest that GIH may increase susceptibility to developing sleep apnea in adulthood.

Mechanisms that confer this increased risk are unknown, but SDB risk is 2-4 times greater in relatives of individuals with SDB (Patel et al., 2012), suggesting that SDB is heritable (el Bayadi et al., 1990; Pillar and Lavie, 1995; Redline et al., 1997; Redline and Tishler, 2000). Familial aggregation of SDB is independent of obesity and evident without overt craniofacial abnormalities

(Lavie and Rubin, 1984; Redline et al., 1997; Redline and Tishler, 2000). Specific gene mutations that impact heritability of SDB remain unknown (Kent et al., 2010; Larkin et al., 2010; Patel et al., 2012; Cade et al., 2016), but risk of SDB is higher in those whose mothers were obese, diabetic (Chen et al., 2019), or had their own SDB diagnosis (Au et al., 2021), thus epigenetic changes precipitated by an adverse *in utero* experience could contribute to the development of SDB later in life.

Our findings are supported by previous studies examining the impact of GIH on the health of adult offspring. For example, evidence in animal models suggests that GIH increases risk for obesity and metabolic dysfunction via epigenetic reprogramming of the peripheral inflammatory response (Khalyfa et al., 2017). GIH offspring have also been reported to have evidence of in utero growth restriction (Chen et al., 2018; Song et al., 2021), and high blood pressure as adults (Chen et al., 2018; Song et al., 2021). This model has also revealed that GIH offspring exhibit long-lasting behavioral and cognitive impairments accompanied by changes in the density of medial pre-frontal cortex dendritic spines (Vanderplow et al., 2022), which resemble deficits associated with autism spectrum disorder in humans. A common theme in these studies is that a more severe phenotype is apparent in males for reasons that are unclear. Although we did not observe changes in body weight or fat pad mass, we report that male offspring exhibited small, but significant increases in systolic blood pressure and mean arterial pressure, consistent with other recently published cardiovascular findings (Song et al., 2021).

We further found that GIH induces gut dysbiosis in adult male offspring, characterized by decreased alpha and beta diversity in the gut microbiome, a reduced Firmicute:Bacteroidete ratio, and decreased relative abundance of bacteria that produce the short-chain fatty acid (SCFA) butyrate. Multiple routes exist by which GIH could alter the maternal microbiome and induce a dysbiotic gut microbiome in her offspring. The precise etiology of adult GIH male gut dysbiosis

remains to be investigated, but one contributing factor could be the maternal experience during GIH exposure. The neonatal gut microbiome is derived from the maternal microbiome of the breast milk, vaginal canal, gut, skin, and placenta (Duranti et al., 2017; Socha-Banasiak et al., 2021), which are heavily influenced by the maternal experience during pregnancy (Bäckhed et al., 2015; Wang et al., 2020). Other models of prenatal insult, including maternal stress, result in gut dysbiosis (Golubeva et al., 2015; Jašarević et al., 2015; Zijlmans et al., 2015; Sun et al., 2021) and adverse physiological outcomes in the adult offspring, including an altered hypoxic ventilatory response, hypertension, and cognitive deficits (Golubeva et al., 2015). Sex-dependent differences in offspring gut dysbiosis have been noted following an adverse maternal experience during pregnancy despite cohousing of the litter with their mother (Jašarević et al., 2015; Gur et al., 2017; Rincel et al., 2019), highlighting that the gut microbiome is plastic and can be further influenced by the offspring's own physiology in a sex-dependent manner.

It is also possible that IH from increased spontaneous/trailing apneas could induce gut dysbiosis in the adult male GIH offspring (Moreno-Indias et al., 2015; Wu et al., 2016). Alterations in inspired oxygen, such as that experienced during an apnea, are mirrored in the lumen of the gut, damaging the intestinal wall, disrupting the balance of aerobic and anaerobic bacteria, and inducing gut dysbiosis (Moreno-Indias et al., 2015; Wu et al., 2016; Li et al., 2021). Humans with and animal models of sleep apnea have repeatedly reported gut dysbiosis (Moreno-Indias et al., 2015; Durgan et al., 2016; Valentini et al., 2020; Hu et al., 2021), including reduced species diversity and alterations in the Firmicute:Bacteroidete ratio. Collectively, our data raise the intriguing possibility that maternal sleep apnea during pregnancy may increase risk for her male offspring to develop sleep apnea themselves in adulthood, which may contribute to, or occur as a consequence of, dysbiosis of the gut microbiome.

While adult male GIH offspring suffer from a decreased Firmicute:Bacteroidete ratio, many studies of OSA/IH report an increase in the Firmicute:Bacteroidete ratio. We are unable to explain this disparity in the current study, as the gut microbiome exists in a complicated bidirectional relationship with many systems of the body, and OSA/IH is a widespread insult. Further, the Firmicute:Bacteroidetes ratio is widely regarded as a weak hallmark of any specific pathology, but rather an indicator of some disruption in the gut milieu (Magne et al., 2020). Inconsistencies in the exact profile of gut dysbiosis could be related to differences in housing conditions, study species, exposure paradigm, disease severity, or the presence of other comorbidities. Nevertheless, the dysbiotic gut of adult male GIH offspring exhibits reduced species diversity and a decrease in SCFA-producing bacteria consistent with other studies of OSA/IH (Valentini et al. 2020; Adnan et al. 2017).

A reduction in SCFAs is associated with increased inflammation due to the ability of SCFAs to impact immune function directly through receptor activation on immune cells (Maslowski et al., 2009) and indirectly through maintenance of the intestinal gut barrier (Usuda et al., 2021). While sleep apnea and its associated comorbidities can induce an inflammatory state (Boyd et al., 2004; Gozal and Kheirandish-Gozal, 2008; Kimoff et al., 2011; Unnikrishnan et al., 2015), inflammation is hypothesized to contribute to the development or worsening of sleep apnea (Patel et al., 2012) and gut dysbiosis independent of sleep apnea is associated with systemic inflammation (Malesza et al., 2021). Thus, gut dysbiosis and OSA likely exist in a vicious cycle that is complicated by the widespread effects of each pathology. Regardless of the exact relationship between GIH, sleep apnea, and gut dysbiosis, that GIH exposure results in sex-specific gut dysbiosis in the adult male offspring is a novel finding that may be tied to other pathological consequences of GIH (Mashaqi and Gozal, 2019).

We found that exposure to GIH alters cardiovascular function in male, but not female, adult offspring. Specifically, GIH male offspring have increased mean arterial and systolic blood pressure compared to GNX counterparts. In the human population (Peppard et al., 2000; Hou et al., 2018) the development of “sleep apnea” leads to high blood pressure. Gut dysbiosis has been demonstrated in humans and animals with hypertension comorbid with OSA (Yang et al., 2015; Li et al., 2017), and plays a pivotal role in the development of elevated blood pressure (Durgan et al., 2016; Adnan et al., 2017). As with OSA, a common thread in the dysbiotic gut in hypertension is reduced populations of SCFA-producing bacteria (Yang et al., 2015; Durgan et al., 2016; Adnan et al., 2017; Ganesh et al., 2018), and treatment with prebiotics, probiotics, or intracecal SCFAs rescue the hypertensive phenotype (Ganesh et al., 2018), potentially by activating SCFA receptors in the kidney (Pluznick et al., 2009) or vascular endothelium (Natarajan et al., 2016) that are involved in blood pressure regulation. Interestingly, *Roseburia* and *Rumonicoccaceae*, two butyrate-producing taxa that exhibit decreased abundance in adult male GIH offspring, are negatively correlated with systolic blood pressure (Durgan, 2017). Thus, restoring levels of SCFA-producing bacteria or delivering SCFAs may provide a target for future therapies of hypertension and OSA (Badran et al., 2020).

Alternatively, other pathophysiological consequences of sleep apnea may contribute to hypertension, such as elevations in sympathetic nervous system activity (Phillips and O’Driscoll, 2013), IH-induced endothelial dysfunction (Budhiraja et al., 2007), inflammation (Nadeem et al., 2013), or metabolic dysregulation (Seicean et al., 2008). Nevertheless, our findings of coincident hypertension, sleep apnea, and gut dysbiosis in adult male GIH offspring highlight the complicated web of these pathologies with the striking suggestion that they could be precipitated by perturbations in the prenatal environment. Whether gut dysbiosis is causative, coincident with, or independent of the other pathological consequences of GIH exposure provides exciting fodder for future investigations.

Conclusion

In the present study we show that gestational exposure to intermittent hypoxia, a model of maternal sleep apnea during pregnancy, leads to sex-specific detrimental outcomes in her adult male offspring. Adult male GIH offspring suffer from increased spontaneous apneas during presumptive sleep, and an increase in apneas trailing a sigh that may indicate a deficit in respiratory stability. Further, adult male offspring suffer from gut dysbiosis characterized by a decrease in species diversity, specifically in the bacterial populations that produce the short-chain fatty acids that regulate immune function. Finally, we report adult male GIH offspring have increased mean arterial pressure, driven by an increase in systolic pressure, which is a widely reported consequence of both sleep apnea and gut dysbiosis. Taken together, these studies demonstrate the far-reaching impacts of GIH both centrally and peripherally. This reinforces the idea of maternal sleep apnea as a consequential in utero insult and supports growing interest in the impact of sleep apnea in pregnancy on the health of the offspring. The exact mechanism by which GIH results in the reported pathologies remains to be investigated, but will provide critical insight to the long term consequences and anticipated healthcare burden of the increasing diagnosis of maternal sleep apnea during pregnancy.

Methods

Timed pregnant Sprague-Dawley rats (G9) were purchased from Charles River, fed breeders chow and water ad libitum, and gave birth in our facility. All rats were fed rat chow (dams were fed breeder chow) and water ad libitum, and housed under a standardized 12:12-h light-dark cycle. All procedures were completed in accordance with the National Institutes of Health (NIH) Guidelines for Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Wisconsin

Pregnant dams were exposed to gestational intermittent normoxia (GNX, controls) or gestational intermittent hypoxia (GIH) starting at G10. GIH exposure was defined as alternating

2-minute episodes of hypoxia (45 seconds down to 10.5%O_2) and normoxia (15 seconds up to 21%O_2) for 8 hours daily for 12 days. GNX exposure consisted of alternating episodes of normoxia, with the same time and gas flow parameters as the GIH condition. The day prior to birth (G21), dams were removed from the chamber to ensure offspring were never directly exposed to hypoxia. Within 3 days of birth, litters were culled to 8 pups (4 male, 4 female whenever possible). Pups were weaned at 21 days, and raised in our facility until time of study (8-16 weeks of age).

Breathing assessments

Breathing patterns were assessed in unanesthetized, unrestrained male and female GNX and GIH offspring (male GNX: 10.7 ± 0.6 wks, n=17; male GIH: 9.1 ± 0.3 wks, n=29; female GNX: 10.7 ± 0.4 wks, n=11; female GIH: 9.2 ± 0.3 wks, n=21) from several different litters (male GNX: 8; male GIH: 12; female GNX: 5; female GIH: 10 litters) using a whole-body flow through plethysmograph (Data Sciences International). A pressure calibration signal, the plethysmograph temperature, rat body temperature, ambient and chamber pressures, and rat body mass were used by the computer software to calculate breath-by-breath ventilatory variables using the equations described by Drorbaugh and Fenn (1955) as modified by Jacky (1978). Rats were placed in plethysmography chambers between 10-4pm to ensure an adequate duration of recording for robust apnea calculation; Dietgel was provided for nutritional support during the long recording period.

Two blinded investigators scored plethysmography traces for presumptive sleep based on simultaneous video and plethysmography recordings obtained in our own hands, in addition to criteria outlined in Bastianini et al., 2016. Using simultaneous EEG and plethysmography recordings, Bastianini and investigators found that vigilance state could be detected with ~90% accuracy based on baseline shifts in plethysmography recordings and the stability of breathing

frequency and amplitude. This technique enables vigilance state to be reliably inferred while avoiding invasive procedures that could impact natural breathing. In brief, breathing frequency, amplitude and baseline shifts were assessed by visual inspection. Wake was scored when baseline was irregular and obscured individual breaths for >2.5 sec, or when marked by a series of high amplitude breaths characteristic of sniffing. Restful breathing (presumptive NREM sleep) was scored when baseline was steady, and breathing frequency and amplitude were stable and regular for >30 sec. Presumptive REM sleep was scored when breathing frequency was irregular, breathing amplitude was low and irregular, and baseline was stable. Discrepancies between investigators were few (<1% of recording time), and were resolved between blinded investigators. High-amplitude excursions of the plethysmography signal with a waveform similar to that of surrounding breaths with an exaggerated exhalation were considered to be sighs, and were typically scored as presumptive NREM sleep unless accompanied by a baseline perturbation, in which case the epoch was scored as Wake. After presumptive sleep was scored, an in-house MatLab script was used to quantify the number of respiratory pauses that exceeded the apnea threshold (>2 missed breaths). These apneas were categorized as: 1) “post-sigh” – an apnea that immediately followed a sigh, 2) “trailing post-sigh” – any apneas occurring within a duration equivalent to 10 missed breaths (5 x apnea threshold) of a sigh, with one or more breaths interposed or 3) “spontaneous” – an apnea that was not associated with a sigh. Defining the apnea threshold and trailing post-sigh period based on each rat's apnea threshold accounted for rat-to-rat variation in baseline respiration.

Cardiovascular measurements

In separate animals, blood pressure and heart rate were measured in GIH male (n=10) and female (n=7) offspring, and GNX male (n=7) and female (n=8) offspring, using similar methods as Song et al., 2021.

Bodyweight and Fat Pad Measurements

Weekly changes in the mass of male GNX (n=14 from 4 litters), male GIH (n=20 from 6 litters) female GNX (n=18 from 4 litters) and female GIH (n=24 from 6 litters) offspring were recorded from 1 to 12 weeks of age. At 12 weeks of age, adult male and female GNX and GIH rats (GNX male n=5 from 5 litters, GIH male n=4 from 4 litters, GNX female n=7 from 7 litters, GIH female n=7 from 7 litters) were euthanized and the right and left gonadal fat pads were isolated and weighed. The weight of the right and left fat pad was averaged and expressed as a ratio of bodyweight (avg weight of fat pads/bodyweight).

Microbiome/Metagenomic analysis:

Fresh fecal samples from 12 week old GIH and GNX offspring (male and female) were collected in sterilized cages and immediately stored in microcentrifuge tubes. Fecal microbiota were analyzed through 16S rRNA sequencing by amplifying V3–V4 region of the 16S rRNA as described previously (PMID 31680682 and 27346372). Briefly, the R based platform Divisive Amplicon Denoising Algorithm 2 (DADA2) was used to trim, merge, and filter reads (Callahan et al. 2016). The data was mapped against the Greengenes reference database to assign taxa designations. α-diversity (Observed OTU number and Shannon index) was measured on unfiltered data, whereas β-diversity (Bray-Curtis and UniFrac distances) was measured on filtered data. PERMANOVA was used to test for an association among groups and the overall microbiota composition based on distance matrices (“adonis” function in R package “vegan”). Differential abundance analysis was performed using the Wilcoxon rank-sum test at phylum, family, genus and species levels. A false discovery rate (FDR) control based on the Benjamini-Hochberg procedure was used to correct for multiple testing. Pairwise correlation tests between microbial taxonomic composition at the species and genus levels with diet and glyphosate were performed using Spearman correlation with Benjamini-Hochberg FDR correction. The effect of each mediator (diet/glyphosate) was analyzed using the R package “mediation’’ (Tingley et al. ; Wang 2018; Wang 2019)) and the R package “iMediate” (Wang 2018; Wang 2019). Cage

effects were accounted for by incorporating mixed linear models utilizing R, where cages were the random effects or treatment was fixed. To assure quality of samples each DNA extraction, library preparation, and MiSeq/HiSeq analysis included positive and negative control samples.

For positive controls, fecal samples from 10-20 rats were pooled whereas multiple stocks of single-use Milli-Q ultrapure water were used as a negative control (stored at -70°C). During each batch of metagenomic sequencing described above, positive and negative reference samples were used as quality assurance for DNA extraction, library preparation, and sequencing. The normalization of OTU counts were conducted using existing methods implemented in R packages DESeq2 and edgeR and a method developed by Wang et al. The Random Forests (RF) method was used to predict bacteria associated with different groups/genotype based on the microbiota profile (genus-level relative abundance data) using default parameters of the R implementation of the algorithm (R package “randomForest”). PICRUSt2 was used to predict the abundance of functional categories, such as clusters of orthologous group (COG) categories and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways based on the 16S rDNA sequence data, and was compared among different groups.

Statistics

Total, post-sigh, trailing post-sigh and spontaneous apneas were quantified per hour of presumptive sleep for each animal. Tidal volume (V_T), respiratory frequency (f_R), minute volume ($V_E = V_T \times f_R$), total apneas/hr, and total apnea duration were analyzed using a Welch’s ANOVA test; individual comparisons between GNX-GIH males and GNX-GIH females were made with a Dunnet’s T3 post-hoc test (GraphPad Prism 9.0). The number and duration of spontaneous apneas, post-sigh apneas, and trailing post-sigh apneas per hour in male GIH offspring were compared to male GNX offspring using a Welch’s t-test. Statistical significance was determined to be $p<0.05$. Data are presented as mean ± SEM. Individual comparisons of fat pad ratio between

GNX-GIH males and GNX-GIH females were made using an unpaired t-test. Body mass over time was assessed using a two-way ANOVA with Bonferroni's post-hoc analyses for multiple comparisons.

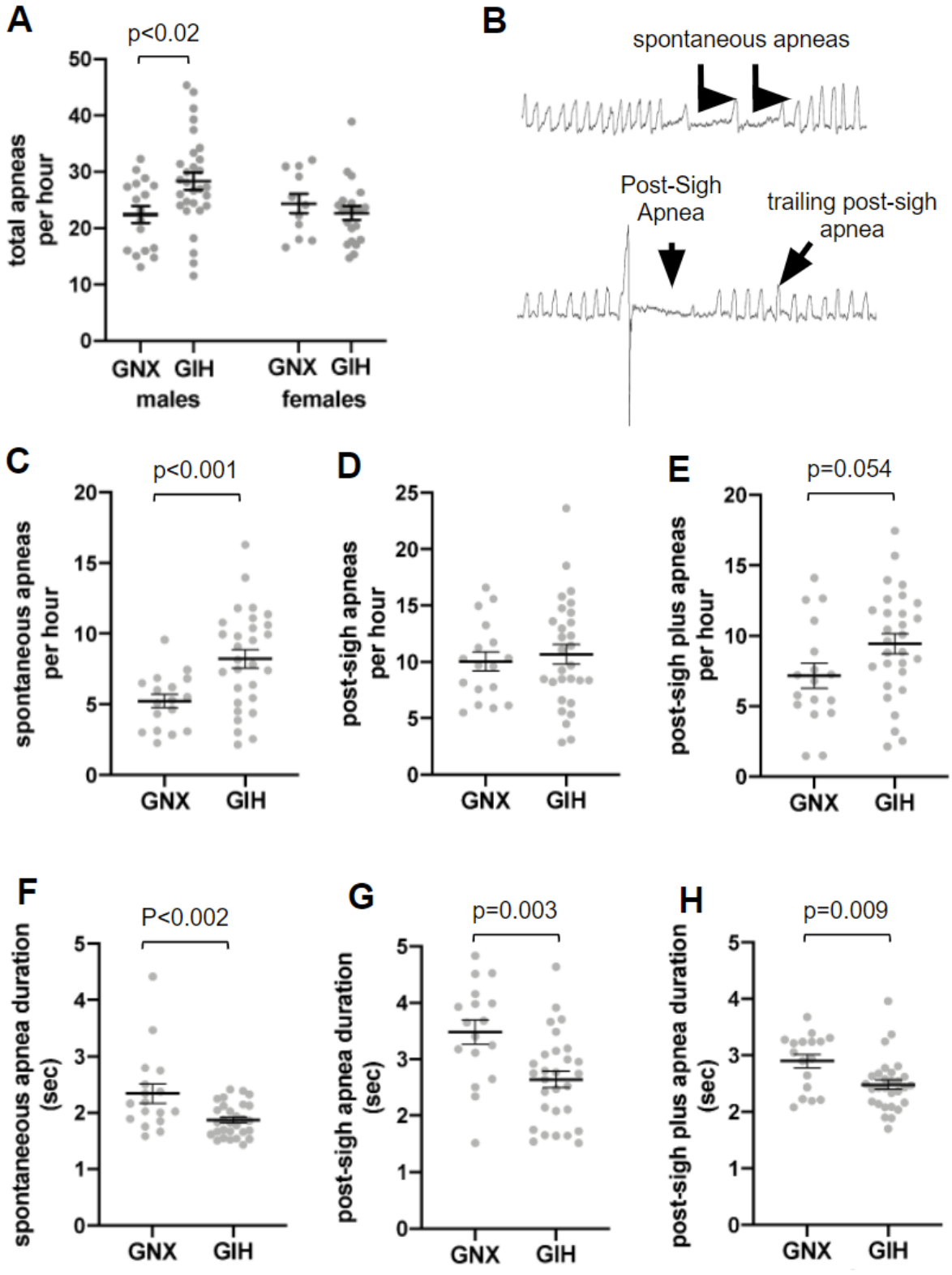

A
p<0.02
total apneas per hour
50
40
30
20
10
0
GNX GIH
males
GNX GIH
females
B
spontaneous apneas
Post-Sigh Apnea
trailing post-sigh apnea
C
p<0.001
spontaneous apneas per hour
20
15
10
5
0
GNX GIH
D
post-sigh apneas per hour
25
20
15
10
5
0
GNX GIH
E
p=0.054
post-sigh plus apneas per hour
20
15
10
5
0
GNX GIH
F
P<0.002
spontaneeous apnea duration (sec)
5
4
3
2
1
0
GNX GIH
G
p=0.003
post-sigh apnea duration (sec)
5
4
3
2
1
0
GNX GIH
H
p=0.009
post-sigh plus apnea duration (sec)
5
4
3
2
1
0
GNX GIH

Figure 1–Gestational intermittent hypoxia (GIH) increases spontaneous apnea frequency in adult male offspring. (**A)** Total apneas per hour in male and female offspring exposed to gestational intermittent hypoxia or normoxia (GNX). GIH male offspring (n=29) had significantly more apneas per hour than GNX controls (n=17) (GIH males: 28.32 ± 1.531; GNX males: 22.41±1.517; $p<0.02$). There was no difference observed between GIH (n=21) or GNX (n=11) female offspring (GIH females: 24.37 ± 1.745; GNX females: 22.66 ± 1.226; $p=0.67$). (**B)** Examples of spontaneous, post-sigh, and trailing post-sigh apneas observed in plethysmography recordings. (**C-E)** Frequency of spontaneous, post-sigh, and post-sigh plus apneas in GIH vs. GNX male offspring. GIH offspring exhibited significantly more spontaneous apneas (GIH males: 8.21 ± 0.0.65; GNX males 5.21 ± 0.48; $p=0.001$), no difference in the frequency of post-sigh apneas (GIH males 10.03 ± 0.85; GNX males 10.67 ± 0.89; $p<0.84$), and slightly more trailing post-sigh apneas (GIH males 9.44 ± 0.71; GNX males 7.17 ± 0.89; $p=0.10$) compared to GNX controls. **F-H)** Durations of spontaneous, post-sigh, and post-sigh plus apneas. Adult male GIH offspring exhibited shorter spontaneous apneas (GNX male: 2.342 ± 0.1732; GIH male: 1.870 ± 0.054; $p<0.002$), post-sigh apneas ($p=0.003$), and post-sigh plus apneas ($p=0.009$) than GNX controls.

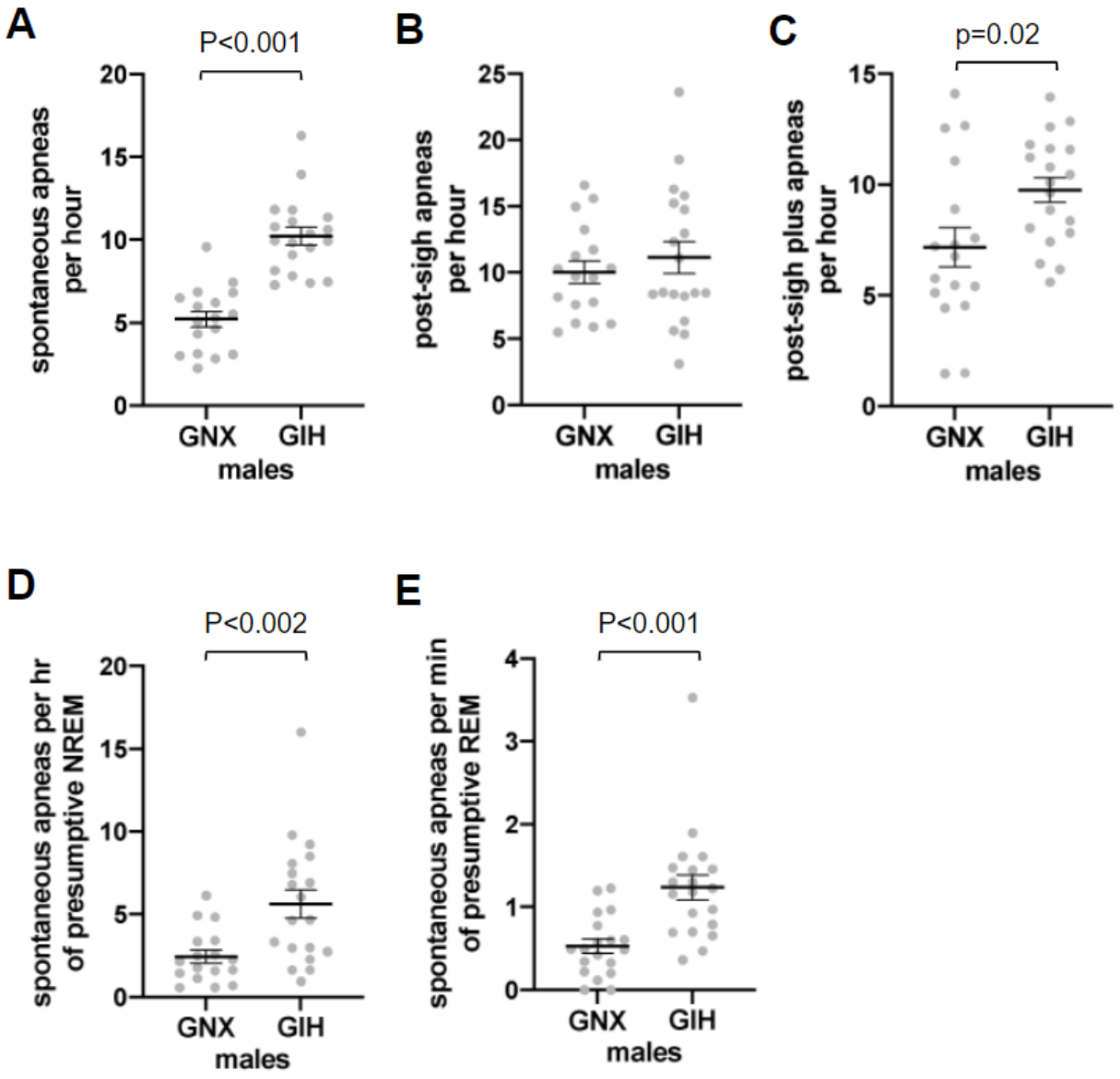


Figure 2–GIH increases spontaneous apnea frequency in affected adult male offspring. These comparisons focus on a subset of GIH male offspring displaying an increased apnea phenotype (n=19) compared to GNX controls (n=17). **(A-C)** Frequency of spontaneous, post-sigh, and post-sigh plus apneas in affected GIH male offspring. Affected GIH male offspring exhibited twice as many spontaneous apneas (0.24 ± 0.53 apneas/hr) as GNX controls (5.2 ± 0.5 apneas/hr) ($p<0.0001$), but there was no increase in the frequency of post sigh apneas (GIH males: 11.1±1.2 apneas/hr; GNX males: 10.0±0.9 apneas/hr; $p=0.46$). Affected GIH males

exhibited slightly more trailing post-sigh apneas than GNX controls (GIH males: 9.8±0.6 apneas/hr; GNX males: 7.2±0.9 apneas/hr, p=0.02). (**D)** Frequency of spontaneous apneas during presumptive NREM sleep. During presumptive NREM sleep, affected GIH male offspring exhibited more spontaneous apneas per hour than GNX controls (GIH: 5.6 ± 0.9; GNX: 2.4 ± 0.4 apneas/hr; $p<0.002$) (**E)** Frequency of spontaneous apneas during presumptive REM sleep. The number of spontaneous apneas during presumptive REM sleep was significantly greater in affected GIH male offspring than GNX controls (GIH: 1.24 ± 0.15; GNX: 0.53 ± 0.08 apneas/min; $p<0.0003$).

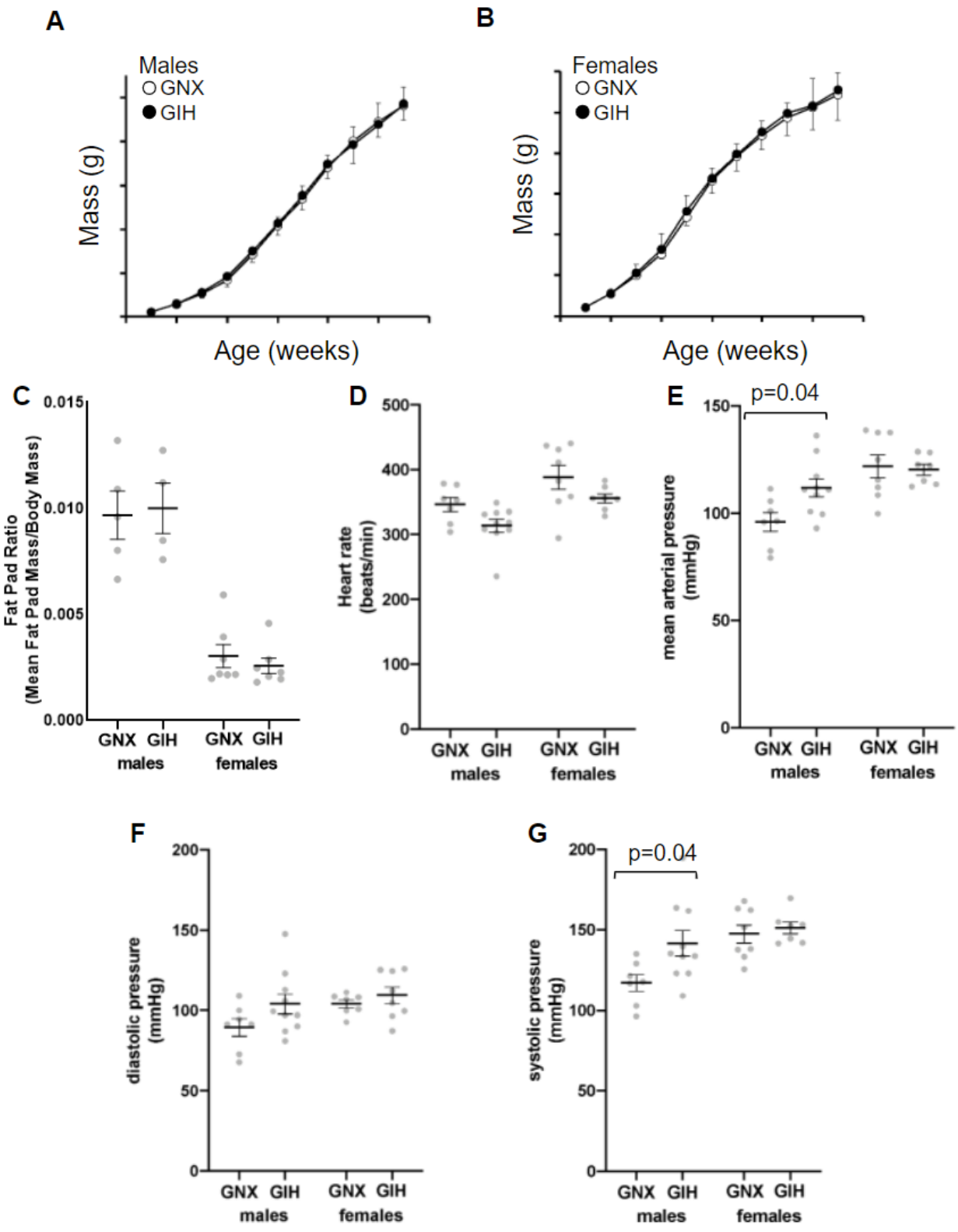
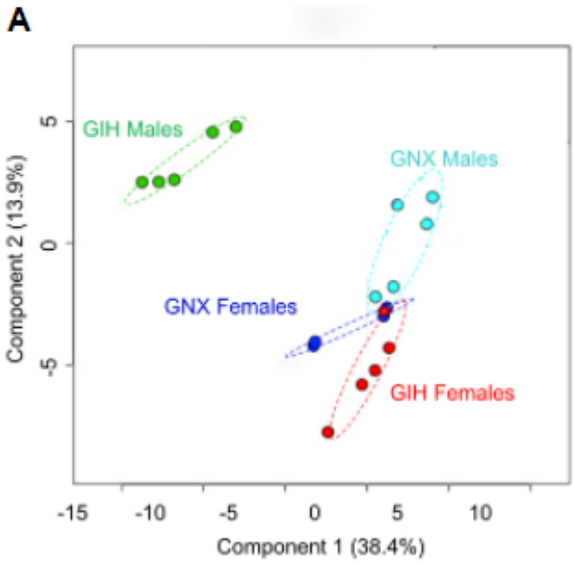

A
Males
GNX
GIH
Mass (g)
Age (weeks)
B
Females
GNX
GIH
Mass (g)
Age (weeks)
C
Fat Pad Ratio
(Mean Fat Pad Mass/Body Mass)
0.015
0.010
0.005
0.000
GNX GIH
males
GNX GIH
females
D
Heart rate
(beats/min)
500
400
300
200
100
0
GNX GIH
males
GNX GIH
females
E
p=0.04
mean arterial pressure
(mmHg)
150
100
50
0
GNX GIH
males
GNX GIH
females
F
diastolic pressure
(mmHg)
200
150
100
50
0
GNX GIH
males
GNX GIH
females
G
p=0.04
systolic pressure
(mmHg)
200
150
100
50
0
GNX GIH
males
GNX GIH
females

Figure 3 - Adult male GIH offspring exhibit cardiovascular deficits despite unchanged body mass

(A) Weekly changes in body mass of male GNX (n=14 from 4 litters) and GIH (n=20 from 6 litters) offspring reveal no effect of GIH on male body mass ($p>0.05$) **(B)** Weekly changes in body mass of female GNX (n=18 from 4 litters) and GIH (n=24 from 6 litters) offspring reveal no effect of GIH on female body mass ($p>0.05$) **(C)** Fat pad ratio (avg fat pad mass/body mass) for male GNX and GIH (GNX: n=5 from 5 litters, 0.0097 ± 0.0011; GIH: n=4 from 4 litters, 0.01 ± 0.0012; $p=0.85$) and female GNX and GIH (GNX: n=7 from 7 litters, 0.0030 ± 0.00055; GIH: n=7 from 7 litters, 0.0026 ± 0.00095; $p=0.50$) offspring reveal no effect of GIH on adult adiposity. **(D)** Heart rate does not differ between GNX or GIH adult male (GNX: 346 ± 11 beats/min, GIH: 315 ± 10 beats/min, $p=0.08$) or female (GNX: 388 ± 18 beats/min; GIH: 356 ± 7 beats/min; $p=0.24$) **(E)** Adult male GIH offspring suffer from increased mean arterial pressure (MAP) compared to their GNX counterparts (GIH: 112 ± 4, GNX: 96 ± 4 mmHg; $p=0.04$). No differences were observed between adult female GIH and GNX offspring MAP (GIH: 120 ± 3, GNX: 122 ± 5 mmHg, $p=0.96$) **(F)** Diastolic pressure does not differ between GNX and GIH males (GIH: 104 ± 6, GNX: 90 ± 5; $p=0.19$) or females (GIH: 104 ± 3, GNX: 110 ± 5 mmHg, $p=0.59$) **(G)** Adult male GIH offspring have increased systolic pressure relative to adult GNX males (GIH: 142 ± 8, GNX: 117 ± 5 mmHg; $p=0.04$). Female GNX and GIH do not exhibit differences in systolic blood pressure (GIH: 151 ± 4, GNX: 148 ± 6 mmHg; $p=0.83$).


A
GIH Males
GNX Males
GNX Females
GIH Females
Component 2 (13.9%)
Component 1 (38.4%)
5
0
-5
-15
-10
-5
0
5
10

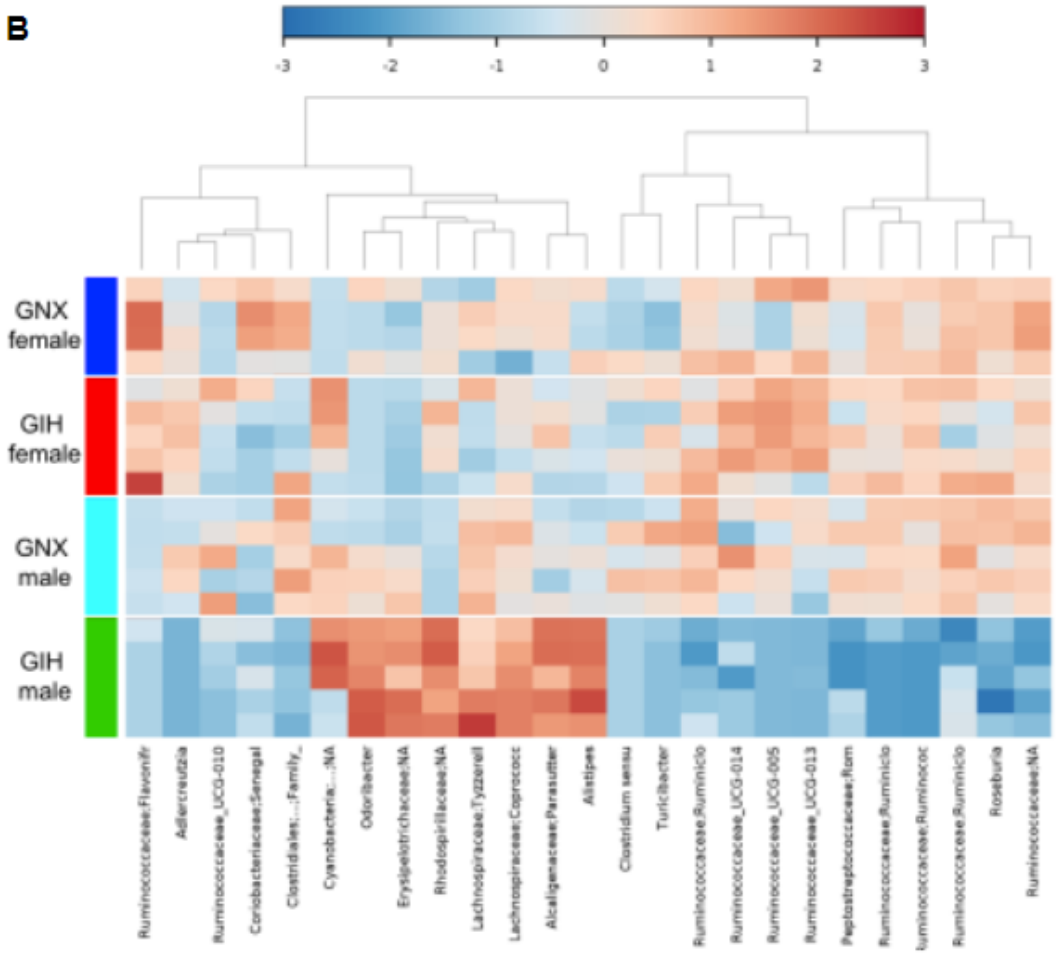

B
-3
-2
-1
0
1
2
3
GNX female
GIH female
GNX male
GIH male
Ruminococcaceae;Flavonifr
Adlercreutzia
Ruminococcaceae_UCG-010
Coriobacteriaceae;Senegal
Clostridiales;...Family_
Cyanobacteria;...;NA
Odoribacter
Erysipelotrichaceae;NA
Rhodospirillaceae;NA
Lachnospiraceae;Tyzzerel
Lachnospiraceae;Coprococc
Alcaligenaceae;Parasutter
Alistipes
Clostridium sensu
Turicibacter
Ruminococcaceae;Ruminicio
Ruminococcaceae_UCG-014
Ruminococcaceae_UCG-005
Ruminococcaceae_UCG-013
Peptostreptococcaceae;Rom
Ruminococcaceae;Ruminicio
Ruminococcaceae;Ruminicoc
Ruminococcaceae;Ruminicio
Roseburia
Ruminococcaceae;NA

Figure 4 - Microbial diversity in male GIH offspring differs from GNX controls and female GIH offspring.

Microbial DNA from 4-5 male and female GNX and GIH offspring fecal samples were extracted using the MoBio PowerSoil Kit. Paired end sequencing of the V3–V5 region of the 16s rRNA was performed. The raw 16S sequencing data were processed by IM-TORNADO to form operational taxonomic units (OTUs) at 97% similarity levels. A) Principal component clustering analysis shows that the GIH male biota are more similar to each other than any of the other treatment groups. Not only does bacterial composition differ between GIH and GNX males, it also differs from females of both groups. B) The heatmap shows the samples grouped by hierarchical clustering, indicating the bacterial families and genera that were most different. The red colors indicate greater expression while the blue indicates lower expression. There are several taxa that are more abundant in the GIH males than any of the other groups, and interestingly, there are several that are considerably lower. Of note, several of the taxa that are reduced are butyrate-producers.

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Chapter V

Recurrent hypoxia in a model of sleep apnea during pregnancy leads to microglia-dependent respiratory deficits and persistent neuroinflammation in adult male offspring

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ABSTRACT

Sleep apnea (SA) during pregnancy is dramatically increasing in prevalence and causes neonatal morbidity and mortality. Little is known regarding long-lasting consequences of maternal SA on adult offspring. SA is characterized by repeated cessations in breathing during sleep, resulting in intermittent hypoxia (IH). We show that gestational IH (GIH) in rats reprograms the male fetal neuroimmune system toward enhanced inflammation in a region- and sex-specific manner, which persists into adulthood. Male GIH offspring also had deficits in the neural control of breathing, specifically in the ability to mount compensatory responses to central apnea, an effect that was rescued by a localized anti-inflammatory or microglial depletion. Female GIH offspring appeared unaffected. These results indicate that SA during pregnancy sex- and region-dependently skews offspring microglia toward a pro-inflammatory phenotype, which leads to long-lasting deficits in the capacity to elicit important forms of respiratory neuroplasticity in response to breathing instability. These studies contribute to the growing body of recent evidence indicating that SA during pregnancy may lead to sex-specific neurological deficits in offspring that persist into adulthood.

INTRODUCTION

It is both scientifically thrilling and practically terrifying that a mother's experiences during pregnancy can contribute to disease development in her adult offspring (Bilbo et al., 2018). For example, prolonged or severe hypoxia during perinatal life is associated with an increased risk for neurological disorders in offspring (Amgalan et al., 2021), such as autism spectrum disorder (ASD), schizophrenia or intellectual disability (Dalman et al., 2001; Modabbernia et al., 2016); typically this form of hypoxia is associated with a traumatic or disordered pregnancy and physicians are well-aware of the risks to the child. Underappreciated is that many women commonly experience recurrent episodes of brief hypoxia during pregnancy in the form of sleep apnea (SA). SA is characterized by repeated pauses in breathing, and occurs in 10-30% of all pregnancies by the third trimester (Pien et al., 2014; Lockhart et al., 2015). Although SA in pregnancy is associated with adverse consequences to the neonate, including preterm birth and NICU admission (Ding et al., 2014), little is known regarding long-lasting effects of maternal SA on adult offspring. Recent evidence in animal models indicates that intermittent hypoxia (IH) associated with SA leads to life-long alterations in offspring physiology, with male offspring more severely affected. Adult male offspring born from rodent dams exposed to IH exhibit deficits in metabolism (Khalyfa et al., 2017; Cortese et al., 2021), cardiovascular function (Song et al., 2021) and have behavioral deficits characteristic of ASD in humans (Vanderplow et al., 2022). However, effects on other core physiological systems are unknown.

Sex-specific reprogramming of the neuroimmune response toward a persistently pro-inflammatory state is a common outcome of an adverse *in utero* environment and may

contribute to offspring cognitive deficits (Al-Haddad et al., 2019). Neuroinflammation in brain regions important in cognition undermines synaptic plasticity (Rizzo et al., 2018), a foundational piece of cognitive processing that enables the CNS to adapt to new experiences over the lifetime. Microglia play critical roles in creating and resolving neuroinflammation in the healthy and injured brain (Bilbo et al., 2018; Oh et al., 2020; Yegla et al., 2021). While the detrimental impact of an adverse perinatal environment on offspring cognitive function has been intensely investigated, potential effects of the perinatal environment on CNS regions important in breathing remain poorly understood.

Breathing is a complex neuromotor behavior that relies on a precisely coordinated pattern of muscle activation to hold the airway open and expand the thoracic cavity. Plasticity is a key element of a healthy respiratory control system since it adjusts muscle activation patterns to optimize breathing throughout the lifetime (Fuller and Mitchell, 2017). Neural circuits driving breathing are tuned for near constant activity from birth until death, and as such, are exquisitely sensitive to reductions in respiratory neural activity, even in the absence of a change in blood gases. Indeed, central hypopnea/apnea trigger a chemoreflex-independent, proportional enhancement in inspiratory motor output to muscles maintaining upper airway tone and expanding the thoracic cavity, a form of plasticity known as inactivity-induced inspiratory motor facilitation (iMF) (Braegelmann et al., 2017). Although respiratory neural activity is likely monitored in multiple neural circuits, local mechanisms operating within inspiratory motor neuron pools are key for iMF initiation (Streeter and Baker-Herman, 2014b).

We modeled SA during pregnancy by exposing rat dams to IH during their sleep phase in late gestation (gestational intermittent hypoxia; GIH). We report that GIH leads to long-lasting respiratory control and neuroimmune dysregulation in adult male, but not female, offspring. Adult male GIH offspring exhibit enhanced microglial inflammatory gene expression in spinal regions encompassing the phrenic motor pool, which interferes with the capacity to trigger iMF in response to recurrent central apnea. GIH-induced neuroinflammation was CNS-region specific, with no evidence for brainstem neuroinflammation. The capacity to elicit iMF could be rescued by a spinal anti-inflammatory or microglial depletion. Our results indicate that GIH sex- and region-specifically skews microglia toward a pro-inflammatory phenotype, impairing important forms of respiratory neuroplasticity elicited by breathing instability.

RESULTS

GIH impairs respiratory neuroplasticity

We first tested the hypothesis that the capacity to elicit respiratory neuroplasticity is impaired in adult offspring exposed to GIH compared to gestational intermittent normoxia (GNX)-exposed controls (Experimental Series 1). To induce respiratory plasticity, inspiratory neural activity was briefly silenced five times for ~1min each to mimic recurrent central apnea; rats continued to be ventilated during the central apnea so that no hypoxia or hypercapnia was experienced. Representative compressed phrenic neurograms show phrenic inspiratory motor output at baseline, during, and for 60 min following recurrent central apnea (**Fig. 1a**). Phrenic inspiratory activity was monitored for an equivalent duration in “time controls” exposed to the same surgical preparation, but with no central apneas. Recurrent central apneas triggered a compensatory

increase in phrenic inspiratory burst amplitude in both male (59.3±4.8% baseline, n=11, p=0.0001) and female (78±12.9% baseline, n=10, p<0.0001) GNX offspring relative to time controls (13.3±12.9% baseline, n=13), indicating iMF (**Fig. 1a, 1b**). Likewise, recurrent central apnea triggered robust iMF in female GIH offspring (63.6±11.7% baseline, n=9, p<0.0001) that was not statistically different from female GNX offspring (p=0.256; **Fig. 1b**). Strikingly, recurrent central apnea did not elicit iMF in male GIH offspring (3.6±6.8% baseline, n=9, p=0.42 compared to time controls; p<0.0001 compared to male GNX offspring). These data show that normal compensatory responses to reductions in respiratory neural activity are impaired by GIH in male, but not female, offspring.

GIH does not impact maternal care

Because other models of prenatal insult show changes in maternal care (Moore and Power, 1986), which can impact respiratory control in the offspring (Genest et al., 2007), we tested the hypothesis that GIH impacted maternal care. Several aspects of rodent maternal care were measured, including the time it took GNX and GIH mothers to approach their pups after they had been removed for 10 mins (GNX n=6, GIH n=6; **Suppl. Fig. 1a)**, retrieve the first pup (GNX n=11, GIH n =10; **Suppl. Fig. 1b)**, retrieve all pups (GNX n=11, GIH n=10; **Suppl. Fig. 1c)**, begin licking, grooming or sniffing the pups (GNX n=6, GIH n=6; **Suppl. Fig. 1d)** and begin crouching after all pups had been retrieved (GNX n=6, GIH n=5; **Suppl. Fig. 1e)**. We detected no differences in these measures of maternal care between GIH and GNX dams, suggesting that poor maternal care does not likely play a role in GIH-induced deficits in adult male offspring.

GIH increases spinal cord, but not brainstem, inflammation in male offspring

To test whether GIH induces neuroinflammation in CNS regions underlying breathing, we measured inflammatory gene expression in tissue homogenates from brainstem, where respiratory rhythm is generated, and cervical spinal cord segments C3-C6, where phrenic motor neurons reside. We focused on several general inflammatory markers that are increased in humans and rodent models with deficits in respiratory control, including interleukin-1 beta (IL-1β), s100a8 (a calcium binding protein), C-X-C motif chemokine ligand 10 (CXCL10), Janus kinase 1 (JAK1), and signal transducer and activator of transcription 1 (STAT1) (Jain et al., 2012; Anderson et al., 2014; Almatroodi et al., 2015). For male GIH offspring, no differences in brainstem inflammatory markers were detected **(Fig. 2a)** but increases in *Il1β* (GNX n=8, GIH n=7, p=0.04), *s100a8* (GNX n=7, GIH n=6, p=0.019), *Cxcl10* (GNX n=6, GIH n=6, p=0.078), *Jak1*, (GNX n=7, GIH n=5, p=0.046) and *Stat*1 (GNX n=6, GIH n=6, p=0.065) gene expression were observed in cervical spinal cord **(Fig. 2b)**. In contrast, *Il1β*, *s100a8*, and *Cxcl10* gene expression was reduced in adult female GIH offspring in both brainstem (*Il1β* GNX n=8, GIH n=7, p=0.035; *s100a8* GNX n=7, GIH n=6, p=0.11; *Cxcl10* GNX n=7, GIH n=7, p=0.02) **(Fig. 2c)** and cervical cord (*Il1β* GNX n=8, GIH n=7, p=0.019; *s100a8* GNX n=7, GIH n=8, p=0.17; *Cxcl10* GNX n=6, GIH n=7, p=0.04) **(Fig. 2d)**. These observations underscore critical sex differences in how offspring adapt to the maternal IH insult and indicate that spinal inflammation may play a role in GIH-induced neuroplasticity impairments.

Adult offspring brainstem and spinal microglial transcriptomes are differentially reprogrammed by GIH

Since microglia are important mediators of neuroinflammation, we next examined whether GIH skewed microglia toward a pro-inflammatory phenotype. RNA-sequencing was performed on microglia (CD11b+ cells) immunomagnetically isolated from brainstem and cervical spinal cord (C3-C6; n=5/treatment). Principal component analysis of brainstem and cervical spinal microglia transcriptomes revealed little variability between male GNX and GIH treatments in either region **(Fig. 3a),** although there were notable differences between brainstem and cervical spinal cord transcriptomes. While female microglia were sequenced as well **(Suppl. Fig 2**), the focus of our study relates to the deficits in male respiratory plasticity, thus our analyses focus on the male microglia RNA-sequencing. In isolated brainstem microglia from male GIH offspring, only 6 out of the 13,504 expressed genes were differentially expressed (4 up, 2 down) **(Fig. 3b; Table 1).** Similarly, of the 12,982 genes expressed in male GIH offspring cervical spinal microglia, only 29 were differentially expressed (6 up, 23 down) (FDR < 0.05; **Fig 3c; Table 1**). Despite the relatively low number of differentially expressed genes, all of the known differentially upregulated genes in the cervical spinal cord are putative targets of either NF-κB or STAT transcription factors (**Fig. 3d**). Notably, the IκB kinase epsilon isoform (Ikbkε) was 1 of the 6 upregulated transcripts in spinal microglia from male GIH offspring. Ikbkε targets the NF-κB inhibitory subunit (IκB) for degradation by the proteasome, resulting in activation of NF-κB-dependent inflammatory signaling, consistent with the enhanced neuroinflammatory gene expression data we observed in Figure 2.

We evaluated the FPKM values of all expressed genes within the male brainstem and cervical cord microglia RNA-Seq datasets. Interrogating for genes specifically associated with the Gene Ontology search terms “NF-κB signaling” (GO: 0038061) and “neuroinflammatory response” (GO: 0150076) we found the NF-κB target genes prostaglandin-endoperoxide synthase 2 (Ptgs2, or COX-2), Iκbkε, interleukin-1-receptor-associated-kinase-1 (Irak1), and leucine-rich repeat kinase 2 (Lrrk2) to be increased. In brainstem microglia, there were no detectable changes induced by GIH in the expression of any of these genes either by RNA-Seq FPKM **(Fig. 3e, left)** or qPCR analyses **(Fig. 3e, right)**. However, in cervical spinal microglia, GIH increased the expression of all four of these inflammatory genes both by RNA-Seq FPKM **(Fig. 3f, left)** and individual qPCR confirmation analyses **(Fig. 3f, right)**, indicating that NF-κB signaling pathways may be hyperactivated in male GIH cervical spinal microglia.

GIH-induced spinal neuroinflammation impairs iMF in adult male GIH offspring

Since iMF is a predominantly spinal form of neuroplasticity (Streeter and Baker-Herman, 2014b) and we observed increased neuroinflammation specifically in the spinal cord, we tested whether functional inhibition of local spinal inflammation, and NF-κB/STAT signaling in particular, could rescue iMF expression in adult male GIH offspring (Experimental Series 2). We intrathecally delivered vehicle (DMSO) or TPCA-1 (potent inhibitor of Iκ-kinases and STAT transcription factor activation) directly over the cervical spinal segments C3-C6, where phrenic motor neurons reside, prior to administering recurrent central apneas. Male GNX offspring treated with vehicle (55.8±9.9% baseline, n=10, p=0.02) or TPCA-1 (59.7±10.2% baseline, n=6, p=0.02) expressed iMF, whereas vehicle-treated adult male GIH offspring did not express iMF (32.5±7.6%, n=8, p=0.41)

relative to time controls (**Fig. 4a**, **b**). Although only a trend for a significant difference was observed in vehicle treated GIH and GNX rats (p=0.075), this was driven by one GIH rat that expressed iMF, and is a statistical outlier (Grubbs' Test p<0.05). With this rat removed, GIH vehicle rats were significantly different than GNX rats (p=0.02). Interestingly, local spinal TPCA-1 treatment in male GIH offspring restored the capacity to express iMF in response to recurrent central apnea (75.6±10.8%, n=7, p=0.001), which differed significantly from vehicle-treated male GIH offspring (p=0.004). These data indicate that GIH-induced spinal neuroinflammation abolishes the ability to elicit compensatory increases in phrenic inspiratory output in response to reductions in respiratory neural activity in adult male GIH offspring.

Aberrant microglial activities play a key role in impairing iMF in adult male GIH offspring

To determine whether aberrant microglial function, specifically, contributes to neuroinflammation-induced impairments in iMF, rats were treated with vehicle or the CSF1R inhibitor Pexidartinib (PLX3397; 80mg/kg daily for seven days, p.o.) to pharmacologically deplete microglia **(Fig. 5a-o)**. Microglial depletion was confirmed using immunohistochemistry. Compared to vehicle-treated control groups, PLX3397 significantly reduced Iba+ cell numbers by ~73% in the ventral horn (near phrenic motor neurons) of both GNX (Vehicle n=4, PLX n=6, p<0.0001) and GIH (Vehicle n=4, PLX n=5, p<0.0001) offspring **(Fig. 5b, d, m)**. PLX administration affected neither GFAP+ area fluorescence in GNX (Vehicle n=3, PLX n=5, p=0.424) or GIH offspring (Vehicle n=4, PLX n=3, p=0.682) **(Fig. 5f, h, n)** nor NeuN+ cell counts in GNX (Vehicle n=3, PLX n=6, p=0.754) or GIH offspring (Vehicle n=4, PLX n=5, p=0.895) **(Fig. 5j, l, o).** Consistent with other recent reports in the rat brain (Oh et al., 2020; Yegla et al., 2021), these results confirm that seven

days of PLX3397 treatment significantly decreased microglial numbers in the rat cervical spinal cord, without detectably impacting astrocyte activation status and neuron populations. To assess for evidence of astrogliosis in the brainstem, a key site involved in the neural control of breathing, we quantified GFAP+ area fluorescence in the brainstem and found no significant difference between PLX3397 and vehicle-treated rats (data not shown), which is consistent with other reports (Stokes et al., 2022). To further confirm spinal microglial cell depletion, RNA from offspring cervical spinal cord tissue was analyzed for the expression of other genes that are specific to microglia in the CNS **(Fig. 5p)**. Analysis of the integrin alpha M receptor CD11b, the fractalkine receptor CX3CR1 (C-X3-C motif chemokine receptor 1), and the $P2Y_{12}$ purinergic receptor genes by qRT-PCR confirmed significant reductions in their expression in both GNX and GIH offspring (n=5 each, $p<0.004$ vs. vehicle), indicating a significant loss of microglial cells following PLX treatment.

To test the hypothesis that depletion of inflammatory microglia rescues iMF in adult male GIH offspring (Experimental Series 3), we treated a separate cohort of animals with PLX3397. As expected, male GNX offspring treated with vehicle (55.8±10.4% baseline, n=7, p=0.033) or PLX (68.7±16.3% baseline, n=9, p=0.003) expressed significant iMF relative to time controls (19.9±6.7% baseline, n=8). Consistent with our earlier findings (**Fig. 1**), male GIH offspring treated with vehicle did not express iMF (15.9±6.1% baseline, n=9, p=0.796) (**Fig. 5q, r**). However, microglial depletion with PLX3397 rescued the capacity of adult male GIH offspring to elicit iMF (53.6±9.3% baseline, n=11, p=0.026 vs. time controls; p=0.011 vs. vehicle treated GIH offspring), indicating that microglial depletion in adulthood can restore the capacity to express iMF in

offspring that were exposed to GIH during fetal development. To determine whether microglia-dependent spinal neuroinflammation is also reversed by PLX treatment **(Fig. 5s)**, we analyzed the same inflammatory genes assessed in Fig 2. As before, we found that the inflammatory gene expression that was significantly enhanced in the GIH male cervical spinal cord homogenate (n=4-5; *Il1β* p=0.001; *s100a8* p=0.0006; *Cxcl10* p=0.002; *Jak 1* p=0.0007; *Stat1* p=0.0003) was significantly reduced by PLX treatment, especially in GIH males (*Il1β* GNX p=0.009, GIH p<0.001; *s100a8* GNX p=0.116, GIH p=<0.0001; *Cxcl10* GNX p=0.02, GIH p<0.0001; *Jak 1* GNX p=0.761, GIH p<0.0001; *Stat1* GNX p=0.377, GIH p<0.0001). Collectively, these data show that microglia are crucial mediators of GIH-induced impairment of iMF in adult males and support the idea that although the microglial reprogramming insult occurred *in utero* months earlier, the neuroplasticity deficit can be rescued in the adult male by depleting persistently inflammatory microglia.

Regulation of physiological variables

Supplementary Table 1 lists average age and body weight, and average Pa_{CO2}, Pa_{O2}, pH, mean arterial pressure (MAP) at baseline and 60 min after treatments. Small, but significant, differences in Pa_{CO2}, Pa_{O2}, pH, MAP, age, and bodyweight within or between some groups were noted in each experimental series, but these values were within normal physiological limits, not associated with iMF magnitude and were consistent with other reports using this anesthetized rat preparation (Dale-Nagle et al., 2011; Streeter and Baker-Herman, 2014a; Baertsch and Baker-Herman, 2015).

DISCUSSION

We demonstrate that gestational intermittent hypoxia (GIH), a hallmark of maternal SA during pregnancy, reprograms the male offspring neuroimmune system toward enhanced inflammation in CNS regions important in respiration, an effect that lasts into adulthood. Persistent neuroinflammation in male GIH offspring has lasting consequences on the ability to elicit compensatory neuroplasticity in response to a life-threatening event – reductions in respiratory neural activity. Several NF-κB pathway target genes are upregulated in adult male GIH microglia, but strikingly, only in the spinal cord and not in the brainstem. Depleting inflammatory microglia or directly inhibiting spinal NF-κB and STAT transcription factor activation rescued the capacity for adult male GIH offspring to exhibit respiratory plasticity. By contrast, adult female GIH offspring did not exhibit neuroinflammation or deficits in respiratory neuroplasticity; mechanisms that confer protection to females from the detrimental effects of maternal IH are not understood but provide an exciting direction for future study. Thus, we demonstrate for the first time a link between maternal IH, sex- and brain region-specific reprogramming of central immune processes, and detrimental effects on respiratory control in adult offspring. Further, our data indicate that microglia may be a source of cellular memory for *in utero* experiences, releasing neuromodulatory mediators that influence circuits regulating breathing.

Converging evidence suggests that many neurological disorders are the result of a complex interplay between genetics and early life experiences, particularly in the womb (Bilbo et al., 2018). Several prenatal factors have been implicated, such as maternal infection, stress, obesity, malnutrition, and environmental toxins. Although seemingly diverse, these factors share one

commonality: activation of the maternal immune system. Without question, IH associated with SA causes chronic inflammation in humans and animal models (McNicholas, 2009; Dempsey et al., 2010; Fung et al., 2012; Khalyfa et al., 2017), which is responsible for many of the morbidities associated with SA. The scientific community has recently begun to appreciate that maternal SA during pregnancy is detrimental to the health of the newborn (Brown et al., 2018; Johns et al., 2020); however, whether these detrimental effects extend into adulthood is unclear. Correlative evidence suggests that maternal SA during pregnancy may have life-long consequences on offspring neural function in humans. SA is most prevalent in women that are obese or of advanced age, and SA during pregnancy increases risk for gestational diabetes, hypertension, fetal growth restriction, premature birth, NICU admission and lower APGAR scores (Carnelio et al., 2017). These risk factors for, or complications of, SA during pregnancy increase susceptibility to the development of neural disorders in the offspring (Krakowiak et al., 2012; Carnelio et al., 2017). Despite this striking correlation, systematic investigations of a mechanistic link between maternal SA and aberrant neural outcomes in her offspring are lacking. Indeed, before such a link can be rigorously investigated in humans, animal models must necessarily provide the justification due to the expensive and time-consuming nature of epidemiological studies.

Given that neuroinflammation undermines plasticity in both the hippocampus (Rizzo et al., 2018) and the respiratory control system (Hocker et al., 2017), we investigated sex-specific differences in inflammatory gene expression in the brainstem and cervical spinal cord, CNS regions associated with the control of breathing, as a mechanism underlying GIH-induced respiratory control deficits. In male GIH offspring, we found enhanced neuroinflammatory gene expression

in the spinal cord, but not in the brainstem, which extends our previous observations of increased neuroinflammation in GIH neonates (Johnson et al., 2018). A tendency towards the opposite (decreased neuroinflammatory gene expression in brainstem and spinal cord) was observed in GIH females. Mechanisms underlying sex-specific effects of GIH are under investigation, but others report sexual dimorphisms in epigenetic gene alterations following prenatal stressors (Nätt et al., 2017; Lei et al., 2020), including late gestational sleep fragmentation (Khalyfa et al., 2015).

Epigenetic alterations of the neural immune system resulting from prenatal insults can have long-lasting neurological impacts on offspring (Bergdolt and Dunaevsky, 2019). Having observed spinal neuroinflammation in male GIH offspring, we performed RNA-seq on spinal microglia isolated from male GNX and GIH offspring. Although the mechanistic experiments described here focused on differentially upregulated genes in male GIH offspring spinal microglia related to inflammatory processes, there were also 23 differentially downregulated genes (Table 1). These downregulated genes included *Flt1*, which encodes the vascular endothelial growth factor receptor 1 protein, and *Epas1,* which encodes the hypoxia inducible factor 2a (HIF-2a) transcription factor. These observations suggest in addition to exaggerated inflammation in male GIH spinal microglia, aberrant function of hypoxia-responsive signaling pathways might also play a role. Additional studies will be required to probe the functional contributions of downregulated microglial genes in impaired respiratory neuroplasticity in adult male GIH offspring.

Although only six genes were upregulated in spinal microglia from adult male GIH offspring, of the four upregulated genes with known function (*Scarna2*, *Klkb1*, *Fcrl2* and *Ikbkε;* **Table 1**), all play a role in inflammation and/or are implicated in neural dysfunction (Chikaev et al., 2005; Khoddami and Cairns, 2013; Hayama et al., 2016; Yin et al., 2020). Recently, NF-κB was found to be upregulated in the nucleus accumbens of mouse offspring whose mothers were injected with poly I:C mid-gestation (Ketharanathan et al., 2021), setting a precedent for the dysregulation of offspring NF-κB in models of maternal immune activation. Analyses of upregulated genes from adult male GIH spinal microglia on the ENCODE database indicated that NF-κB and/or STAT transcription factors bind proximally, which guided our subsequent neurophysiological experiments. Local pharmacologic inhibition of NF-κB and STAT transcription factors in the spinal cord rescued expression of compensatory respiratory plasticity in adult male GIH offspring, indicating that region-specific inflammatory signaling plays a role in GIH-induced iMF impairment. Pharmacologic depletion of microglia also rescued iMF, further implicating altered spinal microglial inflammatory function as the underlying mechanism for impaired compensatory respiratory plasticity.

An important caveat to consider when using PLX drugs to reduce microglia number and reactivity is that PLX3397 inhibits CSF1R in all cells, not exclusively in microglia (Kumari et al., 2018; Han et al., 2020). Nevertheless, several lines of evidence indicate that CSF1R inhibition does not impact astrocyte (Qu et al., 2017) or neuronal function (Green et al., 2020), consistent with our findings that astrocyte and neuronal cell numbers were unaffected by PLX treatment, and that phrenic iMF was not impaired in PLX-treated adult male GNX offspring **(Fig. 5)**. While this latter finding

shows that phrenic iMF expression does not require microglia for normal expression of plasticity, inflammatory microglia seem sufficient to abolish phrenic iMF in the context of GIH-induced neuroinflammation.

We model SA in pregnancy by delivering IH to pregnant dams during their sleep phase; however, it is acknowledged that SA in humans induces concurrent pathologic conditions besides IH, such as sleep fragmentation, hypercapnia, excessive sympathetic activation, and drastic swings in intrathoracic pressure. Regardless, chronic IH alone replicates many of the core consequences of SA in humans (Chopra et al., 2016), allowing us to dissociate the other concomitant aspects of SA as causal, and simplifying the experimental pathologic insult. Thus, exposing pregnant animals to IH is a logical starting point for investigation into the long-lasting effects of SA on offspring, and mechanisms underlying deficits in respiratory control.

Although our studies are the first to show that GIH differentially reprograms adult male offspring spinal microglia to create deficits in the neural control of breathing in adulthood, other studies have interrogated the long-lasting consequences of maternal IH on adult offspring physiology. For example, late GIH exposure epigenetically reprograms adipocytes towards a pro-inflammatory phenotype, resulting in offspring metabolic dysfunction (Khalyfa et al., 2017; Cortese et al., 2021). Other studies have found that GIH offspring exhibit endothelial dysfunction (Badran et al., 2019), increased blood pressure (Chen et al., 2018; Song et al., 2021), altered gut microbiome (Cortese et al., 2021), and blunted hypoxic ventilatory responses (Gozal et al., 2003). Although we are beginning to recognize that GIH can have life-long effects on adult physiology,

comparatively little is known regarding adult offspring neural function. We recently reported that GIH has sexually dimorphic effects on offspring neural function, and permanently alters the male offspring brain in regions underlying social motivation and cognition, leading to a constellation of deficits that may have relevancy to autism spectrum disorder (Vanderplow et al., 2022). Our current study extends those findings to include CNS regions involved in breathing. GIH transforms developing microglia in the male offspring to a pro-inflammatory phenotype present in adulthood, which is linked to respiratory neural dysfunction that results in an inability to respond to reductions in respiratory neural activity with compensatory enhancements in phrenic inspiratory output (i.e., iMF). Strikingly, IH-induced enhancement of microglial pro-inflammatory activity is region specific, apparent only in the spinal cord, and not in the brainstem. These findings indicate that maternal SA may have a previously unrecognized, detrimental impact on male offspring neural function that may increase vulnerability to developing disorders of ventilatory control associated with breathing instability later in adulthood. Given the stark rise in prevalence of SA during pregnancy in recent years, future work examining additional consequences of GIH-induced microglial reprogramming on male offspring neural function are warranted.

MATERIALS AND METHODS

All animal experimental procedures were performed according to the NIH guidelines set forth in the Guide for the Care and Use of Laboratory Animals and were approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee. Sample sizes for each individual experiment is listed in the results section and figure legends.

Intermittent hypoxic exposures during pregnancy

Timed pregnant Sprague-Dawley rats (gestational age G9) were obtained from Charles River (RRID:RGD_734476; Wilmington, MA, USA) and housed in AAALAC-accredited facilities with 12 h:12 h light-dark conditions. Food and water were provided ad libitum. Beginning at G10, dams were exposed to intermittent hypoxia (GIH) which consisted of alternating 2-minute hypoxic (45 s down to 10.5% O2) and normoxic (15 s up to 21% O2) episodes for 8 h (9:00 am−5:00 pm) daily for 12 days. The apnea-hypopnea index (AHI) of 15 events/h generated by these parameters parallels the kinetics of moderate SA in children and adults (Lim et al., 2015; Brockmann et al., 2016). The control group (GNX) received alternating episodes of room air (normoxia) with the same time and gas flow parameters as GIH dams. Both groups were housed in standard microisolator cages with custom-made acrylic lids to deliver the gases. Rats were removed from the exposure system prior to their expected delivery date (G22), to prevent direct exposure of the pups to IH. Hereafter, offspring of GNX- and GIH-exposed dams are referred to as “GNX” and “GIH” rats, respectively. To control for potential differences in maternal care due to litter size, all litters were reduced to 8 pups (4 males and 4 females per litter if possible) by postnatal day 3.

***In vivo* electrophysiology preparations**

Adult rat offspring (8-16 weeks of age) exposed to GNX or GIH were induced with isoflurane (2.5-5%; balance O_2 and N_2), tracheotomized, mechanically ventilated (2-4 mL tidal volume; VentElite Small Animal Ventilator, Harvard Apparatus, Holliston, MA, USA), and bilaterally vagotomized to prevent mechanosensory feedback from ventilation. A tail vein catheter was placed for administration of fluids (1:5 sodium bicarbonate/Lactated Ringers Solution) and I.V. drugs.

Anesthesia was gradually converted from isoflurane to urethane (1.4-1.7 g/kg, I.V.), and depth of anesthesia was confirmed by monitoring pressor response. Rats were paralyzed with pancuronium bromide (1.0 mg/kg I.V.). Body temperature was maintained between 36-38ºC throughout the duration of the surgery and experimental protocol. Blood pressure was monitored, and blood samples were taken periodically from a femoral artery catheter to monitor blood-gas values using an ABL90 Flex (Radiometer, Brea, CA, USA). The phrenic nerve was cut distally, desheathed and inspiratory activity was measured using a bipolar or suction electrode. Raw signals were recorded and digitized with PowerLab data acquisition system (LabChart 8.0, ADInstruments, Colorado Springs, CO, USA), and compound action potentials were amplified (x10k), band-pass filtered (0.3-10 kHz) and integrated (time constant 50 ms). In a subset of rats, an intrathecal catheter was placed for drug delivery. A C2-C3 laminectomy was performed over the spinal midline and a small hole was cut in the dura. A silicone catheter (2 French; Access Technologies, Skokie, IL, USA) connected to a 30 µL Hamilton syringe containing TPCA-1 or vehicle was inserted into the intrathecal space and advanced caudally to lie on the dorsal surface of spinal segment C4.

Inactivity-induced inspiratory motor facilitation (iMF) protocol

To facilitate rapid induction of brief cessations in respiratory neural activity, rats were slightly hyperventilated and CO_2 was added to the inspired gas to maintain end-tidal CO_2 at ~45 mmHg. One hour following surgery, the CO_2 threshold for spontaneous breathing was determined (apneic threshold). Inspired CO_2 was lowered until phrenic activity ceased (apneic threshold), then raised slowly until phrenic activity resumed (recruitment threshold). Inspired CO_2 was then

maintained 2-3 mmHg above recruitment threshold to establish “baseline” respiratory neural activity. Following at least 20 minutes of stable baseline phrenic activity, two arterial blood samples were collected to establish baseline arterial PCO_2, PO_2, and pH values. A series of five intermittent central apneas (~1 min each) were induced by lowering inspired CO_2 below the apneic threshold until phrenic inspiratory output ceased, then inspired CO_2 was rapidly returned to baseline levels, at which point phrenic inspiratory activity resumed. Each central apnea was separated by 5 minutes. Importantly, rats remained mechanically ventilated during central apnea and did not experience hypoxia. Following the five central apneas, phrenic activity was recorded for 60 minutes under baseline conditions. Phrenic burst amplitude at 60 minutes post-apneas was compared to baseline phrenic burst amplitude to determine the magnitude of iMF (measured as percent change from baseline). To ensure blood-gas values were maintained at baseline levels following recurrent central apnea, arterial blood samples were drawn at 5-, 15-, 30- and 60-minute after the protocol. After the 60-minute blood draw, rats were euthanized with a urethane overdose.

TPCA-1 treatment

In Experimental Series 2, we delivered the Iκ-kinase inhibitor [5-(p-Fluorophenyl)-2-ureido]thiophene-3-carboxamide (TPCA-1; Sigma-Aldrich CAS 507475-17-4; SigmaAldrich, St. Louis, MO, USA) to examine the role of local inflammation in compensatory phrenic facilitation. TPCA-1 was dissolved in DMSO and diluted with artificial cerebral spinal fluid (in mM: 120 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 23 $NaHCO_3$, 10 glucose, bubbled with 95% O_2-5% CO_2). Vehicle or TPCA-

1 (1.4 µg in 10 µL) was delivered in 2 µL boluses over 2 min via an intrathecal catheter, 30-60 min prior to induced neural inactivity.

PLX3397 treatment

In Experimental Series 3, we pharmacologically depleted microglia cells in the CNS using PLX3397 (MedKoo Biosciences, Morrisville, NC, USA) to examine the role of microglia in iMF expression. This drug selectively kills microglia by inhibiting the tyrosine kinase of the CSF1 receptor, which is a receptor crucial to microglia survival. Each adult offspring rat received daily dosing of PLX3397 or vehicle for 7 consecutive days (80 mg/kg, P.O.). Drugs were formulated in DMSO, 1% PS80, and 2.5% hydroxycellulose.

Litters used in electrophysiological studies

Electrophysiological experiments included 1-2 offspring of each sex from a single litter. The distribution was as follows: Experimental Series 1: GNX male (11 rats from 9 litters), GIH male (9 rats from 8 litters), GNX female (10 rats from 7 litters), GIH female (9 rats from 9 litters), time control (13 rats from 9 litters); Experimental Series 2: GNX male vehicle (10 rats from 7 litters), GIH male vehicle (7 rats from 5 litters), GNX male TPCA (5 rats from 4 litters), GIH male TPCA (7 rats from 6 litters), time control (5 rats from 4 litters); Experimental Series 3: GNX male vehicle (7 rats from 7 litters), GIH male vehicle (9 rats from 8 litters), GNX male PLX (9 rats from 8 litters), GIH male PLX (11 rats from 8 litters).

Immunohistochemistry (IHC) and imaging

In adult offspring, the expression of astrocytes, microglia, and neurons were quantified using IHC following 7 days of PLX3397 treatment. Male GNX and GIH rats (1 rat from a litter) were transcardially perfused with 4% paraformaldehyde (PFA) in 1X phosphate buffered saline (pH 7.4). Cervical spinal cords were collected and post-fixed in 4% PFA for 24 h before being cryoprotected in 20% sucrose solution (1 day) followed by 30% sucrose solution (3 days). Coronal sections 40 µm thick were cut using a sliding microtome (SM200R, Leica Biosystems, Buffalo Grove, IL, USA) in the C3-C5 spinal regions. Spinal slices (n=2-4 from each rat) were incubated with antibodies to IBA1 (1:1000, anti-rabbit, 019-19741, Wako Chemicals, Richmond, VA, USA), GFAP (1: 250, anti-rabbit, ab5804, EMD MilliporeSigma, Burlington, MA, USA), and NeuN (1: 500, anti-mouse, MAV377, EMD MilliporeSigma, Burlington, MA, USA) to identify the expression of microglia, astrocytes, and neurons, respectively. Secondary antibodies were conjugated to Alexa Fluor fluorescent dyes (Invitrogen, Waltham, MA, USA). Images were obtained with a fluorescence microscope (BZX710 series microscope, Keyence, Itasca, IL, USA). Ventral horn images at 20x magnification were taken bilaterally for each slice. IBA+ and NeuN+ cells were hand counted by two blinded scorers using FIJI cell counter (ImageJ, public domain). GFAP+ cells were quantified by comparing percent area fluorescence via FIJI. The analysis threshold was set at default 30, 255.

Quantitative RT-PCR

Brainstem (medulla and caudal pons) and cervical spinal cord tissues (C3-C6) were isolated (n=5-8/treatment; 1 male and 1 female per litter) and sonicated in Tri-Reagent (Sigma, St. Louis, MO,

USA) and stored at −80°C. Total RNA was isolated with the addition of Glycoblue reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturers' protocols. Complementary DNA (cDNA) was synthesized from 1.0 µg of total RNA using MMLV reverse transcriptase and a cocktail of oligo dT and random primers (Promega, Madison, WI, USA). qPCR was performed using PowerSYBR green PCR master mix (Thermo Fisher Scientific, Warrington, UK) on an Applied Biosystems 7500 Fast system (Waltham, MA, USA). The ddCT method was employed to determine relative gene expression with respect to 18s ribosomal RNA in brainstem and cervical spinal cord tissue homogenates. The primer sequences used for qPCR are shown in Supplementary Table 2. Primers were designed to span introns wherever possible (NCBI Primer-BLAST) and were purchased from Integrated DNA Technologies (Coralville, IA, USA).

CD11b+ cell isolation and RNA sequencing

Adult offspring (n=5/treatment; 1 male and 1 female per litter) were euthanized and perfused with cold PBS to remove circulating immune cells from the vasculature of the CNS. Whole brainstems were dissected between the pontomedullary junction and the obex. Spinal cervical C2–C6 vertebrae were removed, and dorsal and ventral C3–C6 cervical spinal segments were extracted based on identification of the spinal roots. Tissues were dissociated into single cell suspensions using papain enzymatic digestion. CD11b+ cells were immunomagnetically isolated as previously described (Crain and Watters, 2009; Nikodemova and Watters, 2012; Crain et al., 2013; Crain and Watters, 2015). Isolated CD11b+ cells will be hereafter referred to as "microglia."

Total RNA was extracted from freshly isolated microglia with TriReagent according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO) as we have done before (Crain and Watters, 2009; Nikodemova and Watters, 2012; Crain et al., 2013; Crain and Watters, 2015). Total RNA was submitted to Novogene for library construction and paired-end (PE-150) sequencing with an Illumina NovaSeq.

Maternal care testing

To assess maternal competency of dams exposed to GIH relative to dams exposed to GNX, pup retrieval tests were performed on GIH and GNX litters on postnatal day 3 or 4. All pups were removed from the home cage and placed in under a heating lamp for 10 min before being returned to the home cage with 2 pups placed in the nest with the other pups scattered throughout the cage. Maternal behaviors were observed for 10 min, such as time (latency) to investigate, time to retrieve first pup, time to retrieve all pups, latency to lick, groom, or sniff pups, and time to crouch, burrow, or group pups. Dams that did not retrieve all pups within the 10-min testing period were given a maximal score of 600 s.

Statistical analysis

For all electrophysiology experiments, phrenic nerve burst amplitude was expressed as a percent change from baseline. Phrenic amplitude was measured just prior to blood samples taken at baseline, 15, 30, and 60 min after induced central apnea during the iMF protocol. Statistical differences between groups were determined using one-way ANOVA and Uncorrected Fischer's LSD *post hoc* test. Groups were considered significantly different when P values were <0.05.

Outliers were determined using Grubbs' Test with α=0.05. For RNA sequencing, index of the reference Rnor6.0 genome was built using Bowtie version 2.2.3. Reads were aligned using TopHat version 2.0.12. Gene counts were made using HTSeq version 0.6.1. Count files were imported to R and filtered such that only genes with a CPM 0.1 expressed in three samples were retained. Counts were normalized using the trimmed mean of M-values method and analyzed for differential expression using EdgeR (Robinson et al., 2010). Differentially expressed genes were identified as statistically significant if the false discovery rate (FDR) was 5%. Results were uploaded to the National Center for Biotechnology Information Gene Expression Omnibus with reference number **GSE142478** (https://www.ncbi.nlm). Transcriptomic Differences in Microglia 211 at ASPET Journals on July 1, 2021 jpet.aspetjournals.org Downloaded from nih.gov/gds). For all qRT-PCR analysis, experimental groups were compared back to their respective control, GNX.

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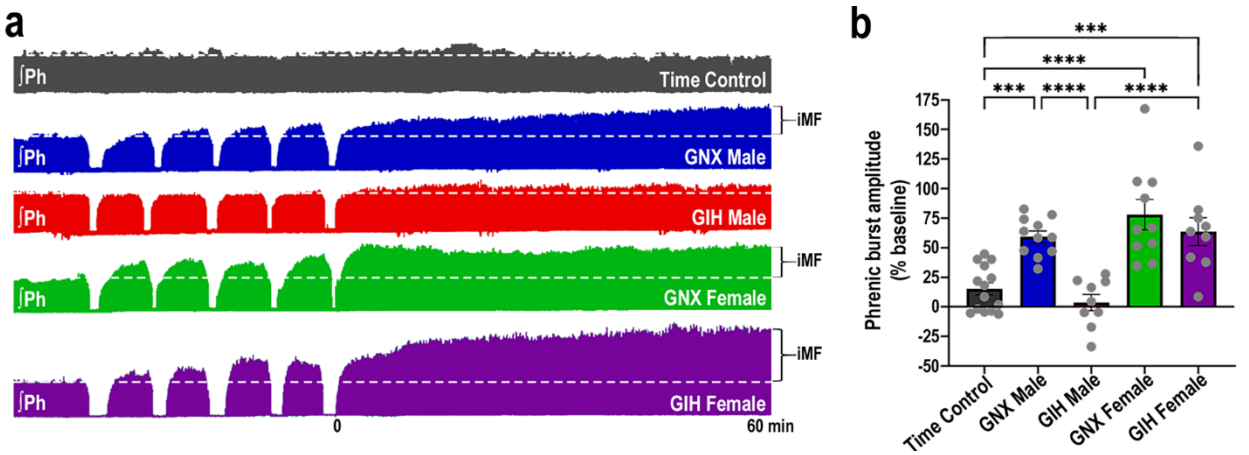


Figure 1: Gestational intermittent hypoxia (GIH) dysregulates compensatory respiratory neuroplasticity in adult male offspring.

(a) Representative compressed phrenic neurograms depicting phrenic burst amplitude before, during, and for 60 minutes following exposure to recurrent reductions in respiratory neural activity (5, ~1 min central apneas) or the equivalent duration in a rat not receiving central apnea (time control). Dotted white line represents baseline amplitude. **(b)** Average percent change (±SEM) in the amplitude of phrenic inspiratory output from baseline at 60 minutes following the fifth apnea. Treatment groups exposed to central apnea were compared to time controls that did not receive central apnea (n=13) to determine if respiratory neural activity deprivation triggered plasticity. In adult male and female rats exposed to gestational intermittent normoxia (GNX; males n=11, p=0.0001; females n=10, p=0.0001) and female GIH rats (n=9, p<0.0001), recurrent reductions in respiratory neural activity elicited a compensatory increase in phrenic inspiratory burst amplitude, indicating iMF. Conversely, recurrent central apnea did not elicit increased phrenic inspiratory burst amplitude in adult male GIH rats (n=9, p=0.42 relative to time controls; p<0.0001 relative to male GNX offspring), indicating that the ability to trigger compensatory enhancements in phrenic inspiratory output in response to respiratory neural activity deprivation (i.e., iMF) is impaired in adult male, but not female, offspring of dams exposed to intermittent hypoxia during gestation. Statistical analysis: one-way ANOVA ($F_{(4,48)}$ = 14.76, p<0.0001) followed by uncorrected Fischer's LSD *post hoc* test, ***p<0.001, ****p<0.0001).

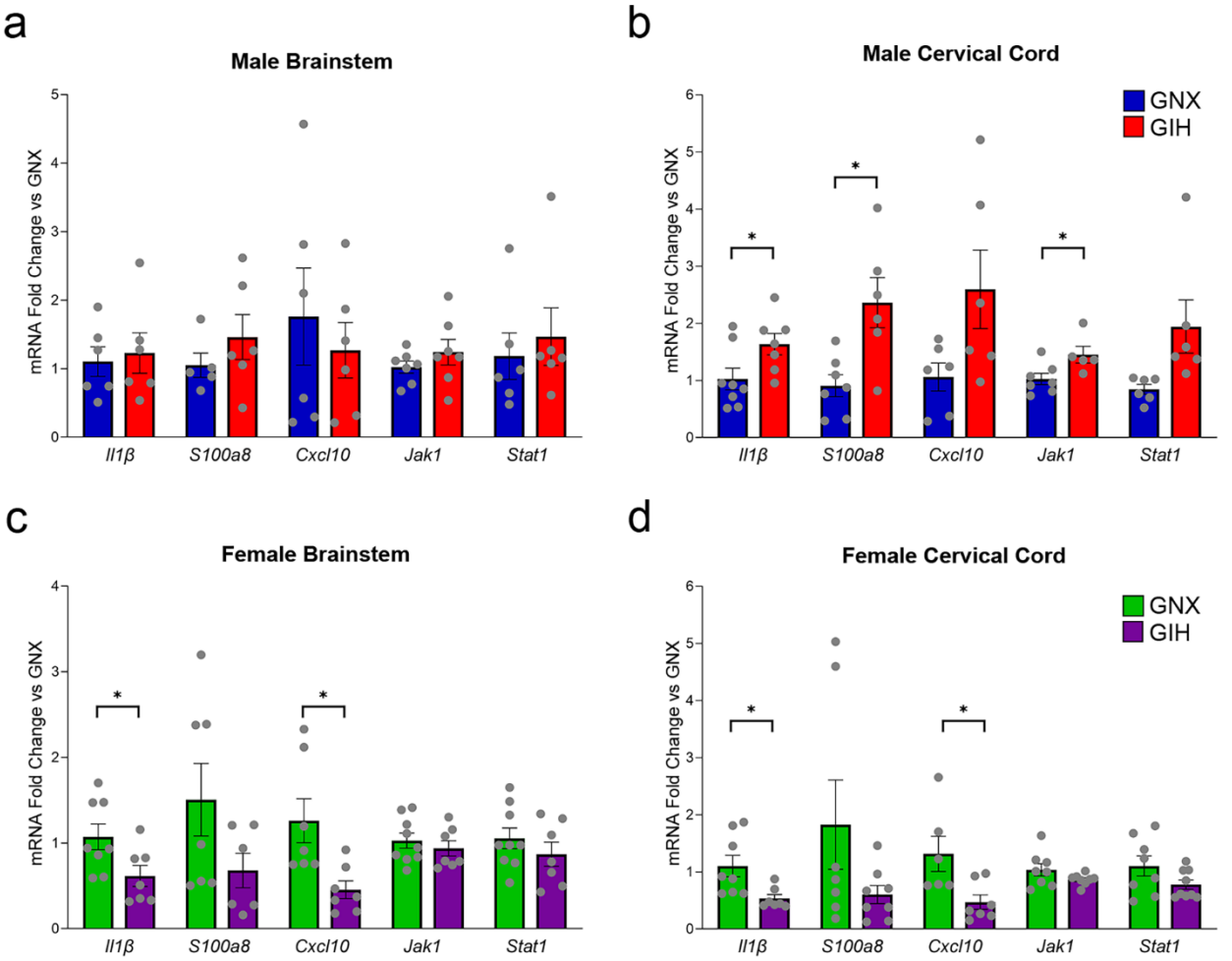


Figure 2: GIH males have increased inflammation in cervical spinal cord.
(a, b) Basal inflammatory gene expression was analyzed in adult male brainstem **(a)** and cervical spinal cord **(b)** tissue homogenates. Increases in *Il1β* (GNX,GIH n=8,7; t=2.285, df=12.94, F=1.170, p=0.04), *s100a8* (GNX,GIH n=7,6; t=3.029, df=6.885, F=4.482, p=0.019), *Cxcl10* (GNX,GIH n=6,6; t=2.101, df=6.249, F=7.876, p=0.078), *Jak1*, (GNX,GIH n=7,5; t=2.395, df=7.391, F=1.598, p=0.046) and *Stat*1 (GNX,GIH n=6,6; t=2.315, df=5.351, F=28.45, p=0.065) gene expression were seen in male spinal cord but not in brainstem. **(c, d)** Basal inflammatory gene expression was analyzed in adult female brainstem **(c)** and cervical spinal cord **(d)** tissue homogenates. In contrast to male offspring, decreases in *IL-1β, S100a8, Cxcl10* gene expression were seen in both female brainstem (*Il1β* GNX,GIH n=8,7; t=2.361, df=12.81, F=1.706, p=0.035; *s100a8* GNX,GIH n=7,6; t=1.763, df=8.503, F=5.149, p=0.11; *Cxcl10* GNX,GIH n=7,7; t=2.905, df=7.898, F=6.161, p=0.02) and cervical spinal cord (*Il1β* GNX,GIH n=8,7; t= 2.852, df=8.822, F=8.505, p=0.019; *s100a8* GNX,GIH n=7,8; t=1.537, df=6.5, F=21.02, p=0.17; *Cxcl10* GNX,GIH n=6,7; t=2.538, df=6.702, F=4.987, p=0.04). Collectively, these data demonstrate sex- and CNS region- specific changes in inflammatory gene expression induced by GIH exposure. *p<0.05 relative to GNX controls.

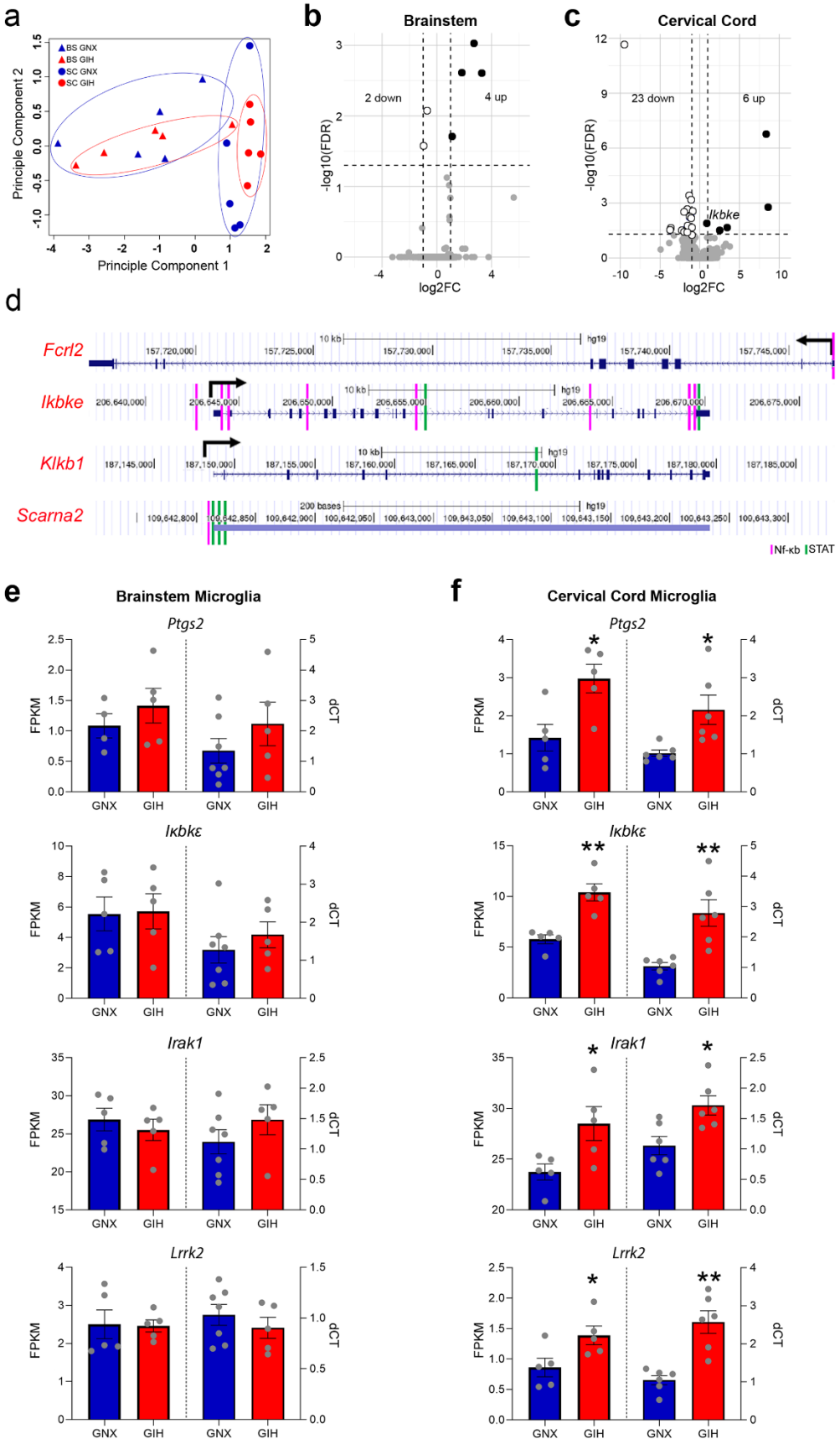

a
BS GNX
BS GIH
SC GNX
SC GIH
Principle Component 2
Principle Component 1
b
Brainstem
2 down
4 up
-log10(FDR)
log2FC
c
Cervical Cord
23 down
6 up
Ikbke
-log10(FDR)
log2FC
d
Fcrl2
Ikbke
Klkb1
Scarna2
Nf-kb
STAT
e
Brainstem Microglia
Ptgs2
Ikbkε
Irak1
Lrrk2
FPKM
dCT
GNX
GIH
f
Cervical Cord Microglia
Ptgs2
Ikbkε
Irak1
Lrrk2
FPKM
dCT
GNX
GIH

Figure 3: GIH differentially upregulates inflammatory gene transcripts in male spinal microglia **(a)** Principal component analysis of RNA-seq data from male brainstem and cervical spinal microglia (n = 5/treatment) show greater transcriptomic differences resulting from CNS region than by GIH treatment. **(b)** Volcano plot indicating differential gene expression in microglia isolated from the male GIH offspring brainstem. Few genes (6) were altered by GIH. Black dots represent significantly upregulated genes (FDR < 0.05); white dots represent significantly downregulated genes. FC, fold change. **(c)** Volcano plot of differential gene expression in male GIH offspring cervical spinal microglia. 29 genes were differentially expressed; black dots represent significantly upregulated genes (FDR < 0.05); white dots represent significantly downregulated genes. **(d)** Gene tracks from the ENCODE database of the 4 known upregulated genes in GIH male spinal microglia demonstrate binding sites for NF-κB (pink) and STAT (green) below each track, suggesting that differentially upregulated gene expression in GIH spinal microglia may be regulated by NF-κB and STAT transcription factor activity. **(e,f)** To ascertain evidence of enhanced basal inflammation in GIH offspring microglia, FPKM (fragments per kilobase of exon per million mapped fragments) analyses of RNA-seq data (left) and RT-qPCR (right) for the inflammatory genes Ptgs2, Ikbke, Irak1, and Lrrk2 were performed. **(e)** No differences in the expression of Ptgs2, Ikbke, Irak1, or Lrrk2 were observed in male brainstem microglia but **(f)** enhanced inflammatory gene expression for Ptgs2, Ikbke, Irak1 and Lrrk2 was observed in male GIH spinal microglia (Ptgs2 FPKM $t=3.029$, $df=7.96$, $F=1.152$, $p=0.016$ and dCT $t=2.888$, $df=5.489$, $F=20.40$, $p=0.031$; Ikbke FPKM $t=4.836$, $df=5.986$, $F=3.762$, $p=0.003$ and dCT $t=3.867$, $df=5.777$, $F=12.80$, $p=0.009$; Irak1 FPKM $t=2.581$, $df=5.690$, $F=4.512$, $p=0.044$ and dCT $t=3.04$, $df=9.985$, $F=1.08$, $p=0.013$; Lrrk2 FPKM $t=2.439$, $df=7.998$, $F=1.034$, $p=0.041$ and dCT $t=4.766$, $df=6.636$, $F=5.945$, $p=0.002$) . *$p<0.05$, **$p<0.01$ relative to GNX controls.

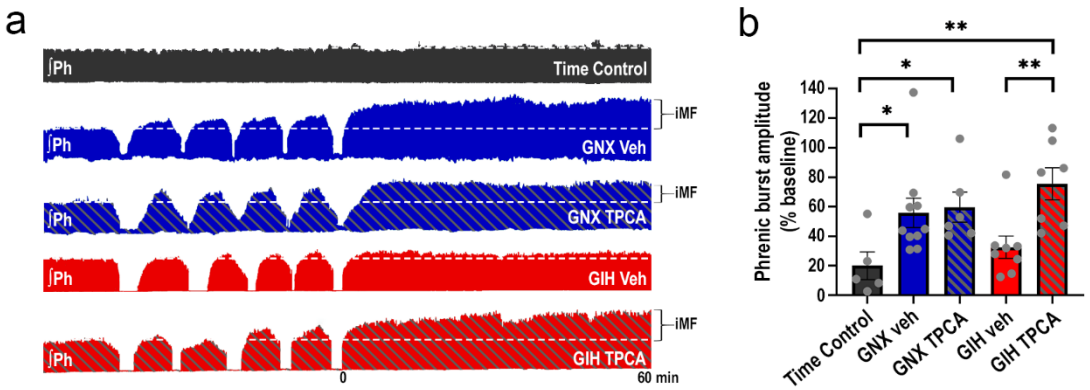


Figure 4: TPCA-1 administration to the phrenic motor pool restores iMF

(a) Representative compressed phrenic neurograms depicting phrenic burst amplitude before, during, and for 60 minutes following exposure to recurrent reductions in respiratory neural activity (5, ~1 min neural apneas) or the equivalent duration in a rat not receiving central apnea (time control). Dotted white line represents baseline amplitude. ~45 minutes prior to respiratory neural activity deprivation, rats received intrathecal injections of the Iκ-kinases/STAT inhibitor TPCA-1 or intrathecal vehicle in spinal regions encompassing the phrenic motor pool. **(b)** Average percent change (±SEM) in phrenic inspiratory burst amplitude from baseline at 60 minutes following the fifth central apnea. Treatment groups exposed to central apnea were compared to time controls that did not receive central apnea. GNX rats treated with vehicle or TPCA-1 retained their capacity to increase phrenic inspiratory output in response to recurrent reductions in respiratory neural activity (vehicle n=10, p=0.02; TPCA-1 n=6, p=0.02); male GIH rats treated with vehicle did not express iMF following five central apneas (n=8, p=0.41). However, local application of TPCA-1 to the phrenic motor pool of male GIH offspring rescued the capacity to trigger compensatory increases in phrenic inspiratory output following recurrent central apneas (n=7, p=0.001). These data demonstrate that GIH-induced spinal inflammation impairs the ability to elicit compensatory increases in phrenic inspiratory output in response to respiratory neural activity deprivation in adult male GIH offspring. Statistical analysis: one-way ANOVA ($F_{(4,29)}$ =4.090 , p=0.0095) followed by uncorrected Fischer's LSD *post hoc* test, *p<0.05, **p<0.01.

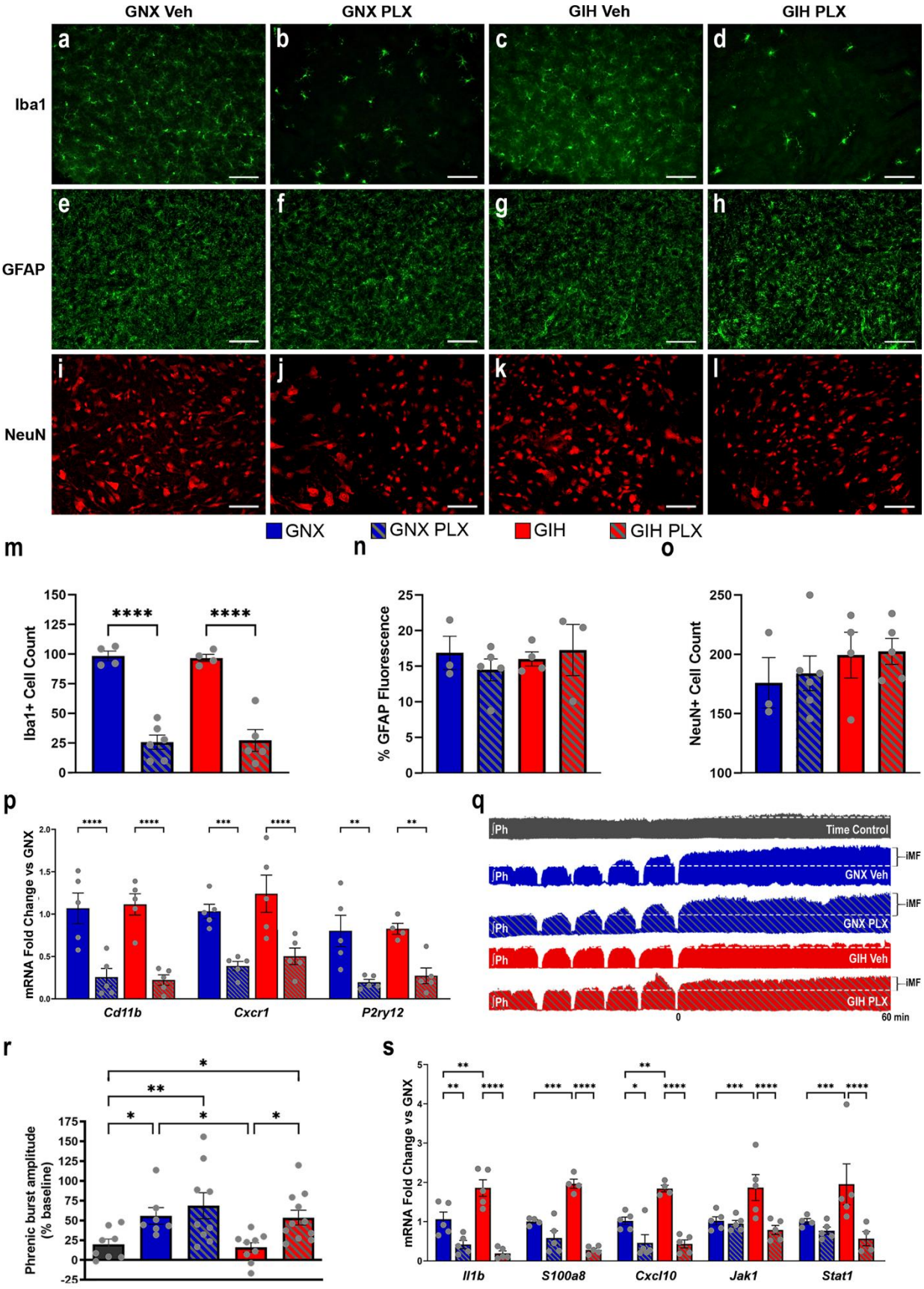

GNX Veh
GNX PLX
GIH Veh
GIH PLX
Iba1
GFAP
NeuN
a
b
c
d
e
f
g
h
i
j
k
l
GNX
GNX PLX
GIH
GIH PLX
m
n
o
Iba1+ Cell Count
% GFAP Fluorescence
NeuN+ Cell Count
****
p
q
mRNA Fold Change vs GNX
Cd11b
Cxcr1
P2ry12
∫Ph
Time Control
GNX Veh
GNX PLX
GIH Veh
GIH PLX
iMF
0
60 min
r
s
Phrenic burst amplitude (% baseline)
Il1b
S100a8
Cxcl10
Jak1
Stat1

Figure 5: PLX reduces Iba+ cells in cervical spinal cord and restores iMF
(a-l) Cervical spinal cord sections from PLX-treated GNX and GIH male rats were immunostained for Iba-1 (microglia) **(a-d)**, GFAP (astrocytes) **(e-h)**, and NeuN (neurons) **(i-l)**. Scale bar = 100um. **(m-o)** Cell count and percent-area fluorescence analyses indicated significant (~73%) depletion of Iba+ cells in both GNX ($p<0.0001$) and GIH ($p<0.0001$) offspring. No significant differences were detected in neurons (GNX p=0.754, GIH p=0.895) or astrocytes (GNX p=0.424, GIH p=0.682)**. (p)** Expression of the microglia-specific genes *Cd11b*, *Cx3cr1* and *P2ry12* was also significantly decreased by PLX treatment (n=5; two-way ANOVA F(3, 12)=9.758, p=0.0015; followed by uncorrected Fischer's LSD *post hoc* test). **(q)** Representative compressed phrenic neurograms depicting phrenic inspiratory output before, during, and for 60 minutes following exposure to recurrent reductions in respiratory neural activity (5, ~1 min neural apneas) or the equivalent duration in a rat not receiving central apnea (time control, n=8). Dotted white line represents baseline amplitude. **(r)** Average percent change (±SEM) in phrenic inspiratory burst amplitude from baseline at 60 minutes following the fifth central apnea. Male GNX rats treated with vehicle (n=7, p=0.033) and PLX3397 (n=9, p=0.003) showed robust responses to recurrent central apnea compared to time controls. Compared to time controls, PLX3397 treatment restored iMF (n=11, p=0.026), which was absent in vehicle-treated male GIH offspring (n=9, p=0.796). Statistical analysis: one-way ANOVA ($F_{(4,39)} = 4.88$, p=0.0027) followed by uncorrected Fischer's LSD *post hoc* test. **(s)** GIH-induced upregulation of *Il1β*, *S100a8*, *Cxcl10*, *Jak1*, and *Stat1* spinal gene expression was also significantly decreased by PLX treatment, suggesting a microglial source of neuroinflammation in the GIH spinal cord (n=4-5; two-way ANOVA F(4, 71)=11.46, $p<0.0001$; followed by uncorrected Fischer's LSD *post hoc* test). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

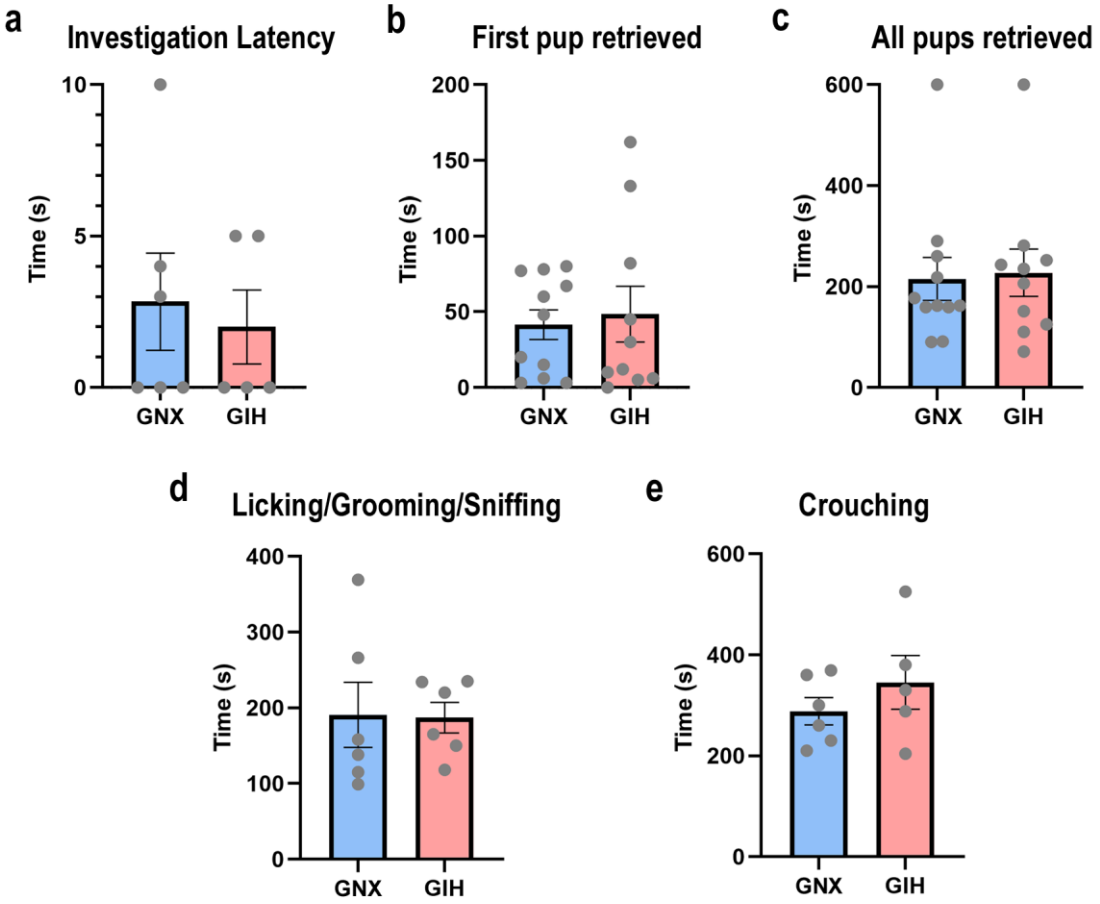
TABLES:

Brainstem Differentially Expressed Genes				
	Gene name	**Gene stable ID**	**FDR**	**log(Fold Change)**
Upregulated	AABR07000398.1	ENSRNOG00000047746	0.002	1.811
	AABR07015057.1	ENSRNOG00000060518	0.002	3.267
	AABR07015081.1	ENSRNOG00000047351	0.001	2.695
	F13a1	ENSRNOG00000015957	0.020	1.082
Downregulated	*Ccl4*	ENSRNOG00000011406	0.026	-0.960
	Ch25h	ENSRNOG00000019141	0.008	-0.709
Cervical Spinal Cord Differentially Expressed Genes				
	Gene name	**Gene stable ID**	**FDR**	**log(Fold Change)**
Upregulated	AABR07034362.2	ENSRNOG00000050586	0.022	3.390
	Fcrl2	ENSRNOG00000016164	0.030	2.500
	Ikbkε	ENSRNOG00000025100	0.013	0.868
	Klkb1	ENSRNOG00000014118	0.002	8.547
	LOC100910181	ENSRNOG00000051439	1.76E-07	8.332
	Scarna2	ENSRNOG00000051850	0.031	2.561
Downregulated	AABR07015081.1	ENSRNOG00000047351	0.030	-3.740
	Acox2	ENSRNOG00000007378	0.022	-3.635
	Bcam	ENSRNOG00000029399	0.003	-1.953
	Cxcl12	ENSRNOG00000013589	0.006	-1.333
	Cyyr1	ENSRNOG00000001544	0.002	-1.561
	Epas1	ENSRNOG00000021318	6.91E-4	-1.095
	Fcmr	ENSRNOG00000004441	0.047	-1.076
	Flt1	ENSRNOG00000000940	0.003	-0.860
	Igf2	ENSRNOG00000020369	0.030	-2.224
	Igfbp3	ENSRNOG00000061910	0.023	-1.599

	Plat	ENSRNOG00000019018	0.017	-1.497
	Plcl1	ENSRNOG00000032659	0.023	-1.649
	Pllp	ENSRNOG00000016558	0.038	-1.364
	Plxnd1	ENSRNOG00000025209	0.022	-1.009
	Podxl	ENSRNOG00000012495	0.040	-1.319
	RT1-Da	ENSRNOG00000032844	0.023	-1.202
	Sema3g	ENSRNOG00000018952	0.005	-1.322
	Slco1a4	ENSRNOG00000047493	0.004	-1.085
	Slco1c1	ENSRNOG00000009740	3.83E-4	-1.310
	Spock2	ENSRNOG00000061544	0.007	-1.045
	Stab1	ENSRNOG00000018434	6.91E-4	-1.034
	Ttr	ENSRNOG00000016275	2.21E-12	-9.480
	Wscd1	ENSRNOG00000007869	0.038	-1.850

Table 1: Differentially Expressed Genes

SUPPLEMENTARY FIGURES AND TABLES:



Supplementary Figure 1: Maternal care is uniform among GNX and GIH dams.
Maternal care was assessed in pregnant dams exposed to intermittent hypoxia or normoxia during gestation. No significant differences were observed among several measures used to assess maternal behavior after pup removal and replacement, including the time to investigate pups **(a)**, time to retrieve the first pup **(b)**, time to retrieve all pups into the nest **(c)**, time to lick/groom/sniff pups **(d)**, or time to crouch/nurse pups **(e)**. Data are displayed as mean ± SEM.

Supplementary Table 1

Experimental Series 1

	GNX Males		GIH Males		GNX Females		GIH Females		Time Controls	
	Baseline	60 min	Baseline	60 min	Baseline	60 min	Baseline	60 min	Baseline	60 min
paCO2 (mmHg)	*47.2 ± 0.1	*47.3 ± 0.9	*46.2 ± 1.1	*46.0 ± 1.0	51.7 ± 1.0	51.9 ± 0.9	49.8 ± 1.8	49.9 ± 1.9	51.2 ± 1.5	50.8 ± 1.5
paO2 (mmHg)	242.6 ± 12.3	236.2 ± 11.9	257.0 ± 11.5	245.2 ± 14.4	248.8 ± 14.7	201.4 ± 13.7*	231.3 ± 18.2	207.0 ± 13.0*	244.5 ± 9.8	224.8 ± 11.4*
pH	*7.45 ± 0.01	*7.34 ± 0.01	*7.35 ± 0.01	*7.35 ± 0.01	7.30 ± 0.01	7.29 ± 0.01	7.33 ± 0.01	7.33 ± 0.02	7.31 ± 0.01	7.31 ± 0.01
MAP (mmHg)	123 ± 7	*105 ± 8*	133 ± 12	116 ± 9	145 ± 12	134 ± 16	*101 ± 12	*91 ± 11	150 ± 8	137 ± 9*
Age (weeks)	*8.8 ± 0.2		10.5 ± 1.0		11.58 ± 0.7		11.3 ± 0.4		12.0 ± 0.4	
Body Weight (g)	395 ± 10		*458 ± 25		*300 ± 13		*284 ± 11		363 ± 30	

Experimental Series 2

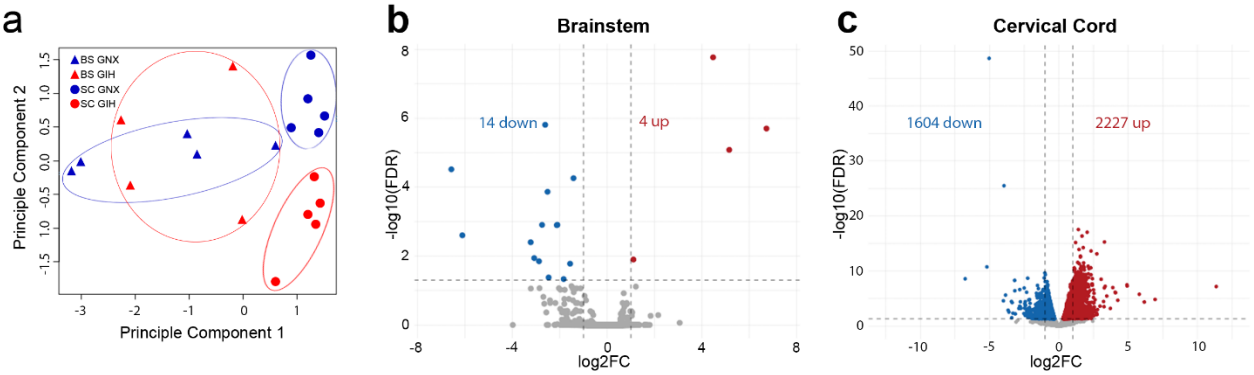
	GNX Male Veh		GNX Male TPCA		GIH Male Veh		GIH Male TPCA		Time Controls	
	Baseline	60 min	Baseline	60 min	Baseline	60 min	Baseline	60 min	Baseline	60 min
paCO2 (mmHg)	45.6 ± 1.1	45.5 ± 1.2	43.4 ± 5.6	43.3 ± 5.5	46.8 ± 0.9	45.8 ± 0.9 *	47.2 ± 1.5	47.6 ± 1.5	47.9 ± 2.4	47.7 ± 2.3
paO2 (mmHg)	253.4 ± 9.7	237.5 ± 10.6	261.0 ± 32.8	239.0 ± 31.9	253.3 ± 7.9	243.3 ± 10.9	284.6 ± 8.7	275.1 ± 13.4	250.0 ± 21.0	263.4 ± 13.4
pH	*7.36 ± 0.01	*7.35 ± 0.01	*7.37 ± 0.92	*7.38 ± 0.92	7.34 ± 0.01	*7.35 ± 0.01	7.33 ± 0.01	7.34 ± 0.01	7.32 ± 0.01	7.30 ± 0.02
MAP (mmHg)	131 ± 9	114 ± 8*	124 ± 17	103 ± 15*	128 ± 8	111 ± 10*	148 ± 6	138 ± 7	131 ± 10	111 ± 11*
Age (weeks)	10.6 ± 0.8		9.0 ± 1.3		10.6 ± 1.1		11.7 ± 1.3		9.7 ± 0.7	
Body Weight (g)	417 ± 23		380 ± 57		507 ± 31		462 ± 30		445 ± 38	

Experimental Series 3

	GNX Male Veh		GNX Male PLX		GIH Male Veh		GIH Male PLX		Time Controls	
	Baseline	60 min	Baseline	60 min	Baseline	60 min	Baseline	60 min	Baseline	60 min
paCO2 (mmHg)	45.6 ± 1.4	45.4 ± 1.2	46.4 ± 1.4	46.3 ± 1.3	45.9 ± 1.4	46.3 ± 1.5	*45.0 ± 1.0	45.3 ± 1.1	49.2 ± 1.6	48.6 ± 2.2
paO2 (mmHg)	256.3 ± 9.5	245.3 ± 17.5	*271.7 ± 12.0	235.2 ± 12.6*	232.7 ± 16.4	235.6 ± 16.0	*268.1 ± 9.9	*258.4 ± 8.0	231.9 ± 14.6	217.4 ± 11.3
pH	7.35 ± 0.01	7.36 ± 0.07	7.34 ± 0.01	7.35 ± 0.01	7.35 ± 0.01	7.35 ± 0.01	7.35 ± 0.01	7.36 ± 0.02	7.34 ± 0.01	7.34 ± 0.02
MAP (mmHg)	*131 ± 9	125 ± 6	*141 ± 12	*126 ± 12*	*131 ± 9	*125 ± 6	122 ± 8	*118 ± 8	89 ± 7	79 ± 8*
Age (weeks)	10.8 ± 1.0		*9.4 ± 0.6		10.7 ± 0.9		*9.0 ± 0.2		12.0 ± 0.2	
Body Weight (g)	*455 ± 35		*409 ± 17		*444 ± 31		*404 ± 15		302 ± 28	

Supplementary Table 1: Regulation of physiological variables

Values are expressed as mean ± SEM of arterial pCO_2, pO_2, pH, and mean arterial pressure (MAP) at baseline and 60 minutes following reduced respiratory neural activity.* indicates significant difference relative to equivalent time point in time controls within the experimental series. * indicates significant difference relative to baseline within the treatment.



Supplementary Figure 2: GIH differentially alters microglial transcriptome in female spinal microglia.

(a) Principal component analysis of RNA-seq data from male brainstem and cervical spinal microglia (n = 5/treatment) show greater transcriptomic differences resulting from CNS region than by GIH treatment. **(b)** Volcano plot indicating differential gene expression in microglia isolated from the female GIH offspring brainstem. Few genes (18) were altered by GIH. Red dots represent significantly upregulated genes (FDR < 0.05); blue dots represent significantly downregulated genes. FC, fold change. **(c)** Volcano plot of differential gene expression in female GIH offspring cervical spinal microglia. 3831 genes were differentially expressed; red dots represent significantly upregulated genes (FDR < 0.05); blue dots represent significantly downregulated genes.

Gene target	Forward Primer (5' → 3')	Reverse Primer (5' → 3')
18s	CGG GTG CTC TTA GCT GAG TGT CCC	CTC GGG CCT GCT TTG AAC AC
Ptgs2	CTC AGC CAT GCA GCA AAT CC	GGG TGG GCT TCA GCA GTA AT
Cxcl10	CCG CAT GTT GAG ATC ATT GCC	CTA GCC GCA CAC TGG GTA AA
Ikbkε	AGC CTG GCC AAG ATG TTT GA	TTC CTG CAT GTG GAA GAC CAG
Il1ϐ	CAG CTT TCG ACA GTG AGG AGA	TTG TCG AGA TGC TGC TGT GA
Irak1	CCT CCT CCA TCA AGC CAA GC	ACC ACC CTC TCC AAT CCT GA
Hmgb1	GGC GGC TGT TTT GTT GAC AT	ACC CAA AAT GGG AAG CA
Jak1	CAA GAA GAC GGA GGT GAA GC	GCA GAG AGG AGA GAT ACT GCA TTC
Lrrk2	AGG AAC CCA AGA ACA AAA AGA GAT	GTG GCG AGG ATG TCT GAT GT
S100a8	TGC CCT CAG TTT GTG CAG AAT AA	GTC TTT ATG AGC TGC CAC GC
Stat1	ACA AAG TCA TGG CTG CTG AGA	AAG TCT AGA AGG GTG GAC TTC AG
Tlr4	ATC TGA GCT TCA ACC CCC TG	TGT CTC AAT TTC ACA CCT GGA T

Supplementary Table 2: Primer sequences used for quantitative PCR

Chapter VI

Discussion

Summary

This dissertation builds on previous studies investigating the mechanisms underlying and impairing the compensatory response to reduced respiratory neural activity, called inactivity-induced inspiratory motor facilitation (iMF), which is critical to move toward a better understanding of the relevance of iMF in injury and disease. The overall purpose of this work was to investigate microglia-motor neuron interactions involved in iMF in the context of the healthy and inflamed CNS. Consistent with other forms of plasticity, the role of microglia in iMF expression is context-dependent. In the healthy CNS, microglia are required for expression of TNFα-dependent iMF induced by a prolonged neural apnea, but expression of retinoic-acid dependent iMF following recurrent neural apneas is cell autonomous (Chapter 2). On the other hand, we found that microglia impair expression of iMF following recurrent neural apneas in the context of neuroinflammation (Chapters 3 and 5). We expanded on this understanding by revealing sex-specific, long-lasting impairment of iMF induced by recurrent neural apneas in the adult male offspring of mothers exposed to gestational intermittent hypoxia (GIH), our animal model of maternal sleep apnea during pregnancy, which was due to reprogramming of microglia toward a pro-inflammatory phenotype (Chapter 5). Finally, we also describe that adult male GIH offspring may suffer from sleep apnea themselves, potentially indicating a role for an adverse in utero environment, microglial reprogramming, and impaired iMF in the heritability of sleep apnea (Chapter 4). The following discussion reflects on how these studies advance our understanding of microglia and neuroinflammation in respiratory neuroplasticity and diseases of impaired respiratory control.

Discussion

Throughout this thesis, we have probed the roles of neuroinflammation and microglia in activity-dependent plasticity of the respiratory control system, uncovering a context-dependent role for microglia in iMF. State-dependent effects of microglia and cytokine signaling, especially TNFα

and IL1β, which are primarily released by microglia (Cahoy et al., 2008; Zhang et al., 2014), is a consistent theme in various types of CNS plasticity.

Microglia, Cytokine Signaling, and Homeostatic Plasticity

In Chapter 2, we describe the differential requirement of microglia for expression of iMF. Specifically, iMF following a single, prolonged neural apnea, which is TNFα-dependent (Broytman et al. 2013), required microglia, while retinoic acid-dependent iMF induced by recurrent neural apneas did not. As the resident immune cells of the CNS, microglia are typically associated solely with the neuroimmune response, but they also play a critical role in synapse function in the absence of inflammatory stimuli. Microglia continually survey the CNS environment, making contact with synapses to “sample” their activity and make sure that all is well (Umpierre and Wu 2021). In the presence of hyper- or hypo-activity of a neuron, microglial contact with the affected synapse increases and adjustment of synaptic activity ensues (Eyo et al., 2014; Kato et al., 2016; Akiyoshi et al., 2018; Liu et al., 2019; Umpierre et al., 2020). Though hyper- and hypo-activity both stimulate microglial chemotaxis toward the affected synapse, they do so through distinct mechanisms. Hypoactivity-induced chemotaxis of microglia is mediated by the noradrenergic B2 receptor, in that reduced activation of the B2 receptor increases microglial chemotaxis and process extension (Stowell et al., 2019), and activation of the B2 receptor by NE inhibits it (Liu et al., 2019). On the other hand, neuronal hyperactivity leads to release of ATP, which is hydrolyzed to ADP and activates microglial P2Y12 receptors, stimulating microglial process extension toward the affected synapse (Haynes et al., 2006; Wu et al., 2007). Whether norepinephrine is involved in the microglial response to prolonged reductions in respiratory neural activity in the cervical spinal cord is unknown, but could be probed by future studies of microglia/motor neuron communication in iMF.

Once at the affected synapse, microglia appear to bidirectionally alter synaptic function, a reasonable assumption in the field based on data showing that activity of microglial processes at the aberrantly active synapse correlates with adjustment of synaptic activity (Umpierre et al., 2020). The mechanisms underpinning microglial regulation of synaptic activity are still under investigation (Wu et al., 2015), but TNFα signaling is well-documented in the response to reduced neural activity such as in iMF (Werneburg et al., 2017). In pyramidal neurons, the homeostatic increase in synaptic activity following prolonged activity deprivation, driven by trafficking of calcium-permeable AMPARs and internalization of inhibitory GABAARs, is TNFα-dependent (Stellwagen and Malenka, 2006). Accordingly, blocking TNFα in vivo prevents the homeostatic ocular dominance response to monocular sensory deprivation in the visual cortex (Kaneko et al., 2008; Ranson et al., 2012). Further, exogenous TNFα leads to a rapid increase in AMPAR expression (Stellwagen et al., 2005; He et al., 2012). Finally, blocking TNFα reduces hippocampal synaptic strength, indicating that TNFα signaling is necessary for baseline AMPAR expression in the hippocampus (Beattie et al., 2002). Previous studies from our group have established that TNFα signaling is required for expression of iMF following prolonged neural apnea, and that exogenous TNFα delivery results in phrenic motor facilitation (pMF; (Broytman et al., 2013). Based on the data described in Chapter 2, we hypothesize that soluble TNFα activates TNFR2 on phrenic motor neurons, leading to increased AMPAR expression and, therefore, phrenic motor output.

TNFα signaling can also reduce cell-surface AMPAR expression. In medium spiny neurons (MSNs) of the striatum, exogenous TNFα delivery results in endocytosis of surface AMPARs and a decrease in excitatory synaptic strength (Lewitus et al., 2014). The decrease in excitatory synaptic strength is considered adaptive in this context, and actually increases overall network excitability because MSNs are inhibitory. Differential activity of TNFα on receptor expression could be due to differences in brain region, context, or neuronal type. In Chapter 2 of this thesis,

we describe a differential effect of TNFα signaling in iMF, in that activation of TNFR1 actually constrains expression of iMF, which could be due to downregulation of AMPARs as seen in the striatum, or due to other context-, neuronal type-, or region-specific effects. Future studies should expand on these nuances to better understand TNFα-induced modulation of synaptic and network activity in homeostatic plasticity.

Historically, cytokine production under homeostatic conditions was hypothesized to be derived from astrocytes (Stellwagen and Malenka, 2006), but recent transcriptomic evidence indicates microglia produce TNFα almost exclusively (Cahoy et al., 2008; Zhang et al., 2014). There are limited investigations into specific non-neuronal cell types in homeostatic plasticity, but microglia are indicated as the source of TNFα in striatal plasticity induced by repeated cocaine administration (Lewitus et al., 2016). Similarly, we hypothesize that microglia are the source of TNFα release elicited by a prolonged neural apnea, which is consistent with their depletion impairing expression of TNFα-dependent iMF as described in Chapter 2.

There appears to be a delay of ~15 minutes between the onset of aberrant neuronal activity and synaptic contact by microglial processes (Umpierre et al., 2020). The time delay in the microglial response to hypoactivity may explain why iMF following a prolonged (~25-30 min) neural apnea involves microglia-dependent signaling, while iMF induced by intermittent (~1 min) neural apneas does not require intercellular communication between microglia and motor neurons.

Interestingly, exogenous delivery of IL-1β in the hippocampus or spinal cord also leads to increased excitatory signaling, like TNFα. While TNFα-induced increase in excitatory signaling is attributed to an increase in AMPARs and decreased GABAARs, IL-1β signaling increases AMPARs and NMDARs and decreases GABA-ergic and glycinergic transmission (Stellwagen et al., 2005; Kawasaki et al., 2008). Despite a similar effect of exogenous IL-1β delivery, IL-1β is

not known to be regulated by neural activity, and thus is not a focus in homeostatic plasticity (Stellwagen et al., 2005; Pribiag and Stellwagen, 2014).

Microglia and Cytokine Signaling in Other Forms of Plasticity

Microglia, TNFα, and IL-1β play critical roles in the induction of many other types of plasticity, including in the context of inflammatory stimuli. It is well-known that hippocampal LTP requires IL-1β signaling in the absence of inflammation (Rizzo et al., 2018). The plastic increase in intrinsic excitability of Purkinje cells in the cerebellum after an inflammatory stimulus requires TNFα released by activated microglia (Yamamoto et al., 2019). In the spinal cord, development of mechanical sensitization after inflammation, a form of spinal neuroplasticity, is dependent on microglia and activation of TNFα and IL-1-receptors (Maier et al., 1993; Cunha et al., 2008; Zhang et al., 2008; Boakye et al., 2021). In the carotid body, inflammation induced by chronic intermittent hypoxia (CIH) drives an increase in baseline frequency firing of the carotid sinus nerve, which can be replicated by administration of LPS or exogenous delivery of IL-1β . Following chronic sustained hypoxia exposure, treatment with non-specific microglia inhibitor minocycline reduces microglial activation (Tadmouri et al., 2014; MacFarlane et al., 2016) and cytokine expression (Hocker et al., 2017) in the nucleus tractus solitarius (NTS), and reduces the ventilatory acclimatization to hypoxia (Tadmouri et al., 2014), indicating that inflammation and/or microglial activation is required for the plastic response to chronic sustained hypoxia.

Increased expression of TNFα and IL-1β after microglial activation by inflammatory stimuli also impairs many forms of neuroplasticity. IL-1β may be required for LTP expression in the hippocampus, but high levels of IL-1β, either delivered exogenously or due to an inflammatory stimulus, impairs LTP expression (Katsuki et al., 1990; Bellinger et al., 1993; Murray and Lynch, 1998). Similarly, microglial activation and increased TNFα in neuroinflammation impairs multiple forms of plasticity (Liu et al., 2017b; Rizzo et al., 2018), and prolonged exposure to exogenous

TNFα actually leads to decreased AMPA-mediated excitatory signaling in hippocampal neurons (Furukawa and Mattson, 1998). Interestingly, the effect of exogenous TNFα delivery to the phrenic motor pool exhibits similar results, in that an intermediate dose of TNFα (25 ng) elicits pMF more effectively than higher doses. It is hypothesized that the “tipping point” at which cytokine signaling no longer increases excitatory transmission, and sometimes even reduces it, may serve to protect from excitotoxicity (Wu et al., 2015).

In Chapters 3 and 5, we describe inflammation-induced, microglia-dependent impairment of intermittent neural apnea-induced iMF by LPS or GIH exposure. There is extensive documentation of the effects of inflammation on another form of activity-independent phrenic motor plasticity induced by acute exposure to intermittent hypoxia (AIH) called phrenic long-term facilitation (pLTF) (Hocker et al., 2017). Inflammation induced by diverse systemic inflammatory stimuli impairs expression of some, but not all, forms of pLTF. Specifically, pLTF induced by moderate acute intermittent hypoxia (mAIH) is impaired by neuroinflammation resulting from lipopolysaccharide (Vinit et al., 2011; Huxtable et al., 2013), viral mimetic polyinosinic:polycytidylic acid (polyI:C; Hocker and Huxtable, 2019), and prolonged exposure to intermittent hypoxia (Huxtable et al., 2015, 2018), while severe AIH-induced pLTF seems to be inflammation-resistant (Agosto-Marlin et al., 2017; Hocker and Huxtable, 2019).

Inflammation-resistant pMF can be elicited by either sAIH exposure or exogenous delivery of adenosine, both of which activate adenosine 2A receptor and lead to pMF (Agosto-Marlin et al., 2017). Interestingly, adenosine, like TNFα, accumulates during the inflammatory response (Ribeiro et al., 2003). Additionally, preliminary evidence indicates that A2A receptor-dependent pMF, like TNFα-dependent iMF, is microglia-dependent (Tadjalli et al., 2021). The inflammation resistance of A2A-dependent pMF is hypothesized to act as a “backup” form of plasticity that can be elicited in the context of inflammation (Agosto-Marlin et al., 2017). In the studies

presented here, we did not investigate whether TNFα-dependent iMF induced by a prolonged neural apnea is inflammation resistant, but it would be interesting to test whether there is similar preservation of at least one form of activity-dependent plasticity in the context of inflammation, or whether accumulation of TNFα would lead to a ceiling or inhibitory effect as seen in other contexts (Furukawa and Mattson, 1998).

The specific inflammatory signaling molecules involved in impairment of iMF or pLTF by inflammation induced by peripheral inflammatory stimuli or GIH remain unknown, but they could be impaired by similar inflammatory signaling pathways. NFkB is a transcription factor activated during the immune response that mediates expression of a number of inflammatory cytokines (Liu et al., 2017a). The NSAID ketaprofen, which primarily inhibits COX1 and COX2 but can inhibit NFkB at high doses,(Cashman, 1996; Yin et al., 1998) rescued impairment of pLTF by inflammation induced by LPS or prolonged intermittent hypoxia exposure (Huxtable et al., 2013, 2015). The dose administered was not a conclusively “high dose”, but subsequent studies indicated that impairment of pLTF after IH was not mediated by COX-2, leaving a potential role for NFkB-dependent impairment of pLTF (Huxtable et al., 2018). In our GIH model, adult male offspring were unable to elicit iMF following recurrent reductions in respiratory neural activity (Chapter 5). RNA-sequencing data from adult male GIH microglia isolated from the cervical spinal cord, where phrenic motor neurons lie, indicated upregulation of signaling pathways related to NFkB and STAT. After inhibition of IκB kinase, which activates NFkB and STAT transcription factors, the capacity to elicit iMF was rescued. Thus, NFkB is not conclusively indicated in impairment of respiratory motor plasticity following various inflammatory stimuli, but converging evidence indicates it may play a role. Whether NFkB activation is similarly implicated in impairment of iMF following LPS administration is unknown but could be investigated in future studies.

While NFkB may represent a common feature of inflammation-induced impairment of respiratory motor plasticity, it upregulates the expression of a number of other pro-inflammatory molecules that could inhibit iMF or pLTF in the context of inflammation (Kawai and Akira, 2007). Indeed, activation of NFkB leads to increased expression of interleukins (IL-1β, IL-6, IL-18), TNFα, and COX-2 (Kawai and Akira, 2007). The role of specific cytokines in impairment of iMF by LPS or GIH has not been investigated, but studies of pLTF suggest a role for IL-1β. Inhibition of the IL-1 receptor after LPS rescues pLTF expression, but exogenous IL-1β application alone is not sufficient to impair pLTF (Hocker and Huxtable, 2018). The authors discussed that a lack of impairment of pLTF by IL-1β could be due to insufficient time between delivery and induction of pLTF, such that IL-1β must activate downstream signaling pathways that are required for impairment of pLTF. Another possibility is that a higher dose of IL-1β is required for outright impairment of pLTF, but a higher dose is not possible due to limitations in the method of delivery. Nevertheless, that inhibition of IL-1R rescues pLTF expression indicates that IL-1β may participate in inflammation-induced impairment of pLTF. Interestingly, exogenous IL-1β application at the highest deliverable dose induced phrenic motor facilitation (Hocker and Huxtable, 2018), which may be due to increased excitatory transmission as discussed earlier (Stellwagen et al., 2005). In the present studies, we did not investigate whether specific cytokines are responsible for impairment of iMF following GIH or LPS. However, adult male GIH offspring exhibit increased expression of IL-1β in the cervical spinal cord, which may contribute to their impaired iMF.

Another common pathway that could lead to impairment of both mAIH-induced pLTF and iMF is promotion of adenosine 2A receptor-dependent pMF. As described earlier, adenosine accumulates during the inflammatory response (Ribeiro et al., 2003), which could activate adenosine 2A receptors and elicit phrenic motor facilitation (pMF) by a similar pathway as that induced by severe AIH exposure (Agosto-Marlin et al., 2017), eventually acting through NMDAR

to increase phrenic motor output (Golder, 2009). NMDAR activation constrains iMF induced by prolonged (Streeter and Baker-Herman, 2014) and intermittent neural apneas (Fields et al., 2019). A2A-mediated pMF also impairs mAIH-induced pLTF, but the mechanism is not fully understood (Devinney et al., 2013). Preliminary evidence indicates that microglia are required for A2A-receptor mediated pMF (Tadjalli et al., 2021). Thus, it is an intriguing hypothesis that A2A-dependent pMF elicited by inflammation-induced adenosine could impair mAIH-induced pLTF and iMF via cross-talk inhibition. Inhibition of A2A receptors before attempted induction of mAIH-induced pLTF or intermittent apnea-induced iMF after inflammatory stimulus could reveal whether cross-talk inhibition by adenosine-dependent pMF is involved.

It is also possible that iMF and pLTF are impaired by distinct inflammatory signaling pathways. iMF induced by intermittent neural apneas is retinoic-acid dependent, and activated microglia produce cytochromes that increase catabolism of retinoic acid (Hellmann-Regen et al., 2013). Our working hypothesis is that reductions in intracellular calcium trigger activation of retinoic acid, which upregulates post-synaptic AMPAR translocation, resulting in an increase in phrenic motor output. However, retinoic acid is lipophilic, so it is also possible that it exerts its effect extracellularly (Chen et al., 2014), which could be impaired by cytochromes derived from activated microglia. Another possibility is that cytokine signaling induced by LPS increases calcium-permeable AMPAR expression on the post-synaptic membrane prior to attempted iMF induction (Stellwagen and Malenka, 2006). Increased calcium in the post-synaptic membrane could prevent disinhibition of RALDH (Wang et al., 2011), which would impact downstream retinoic acid signaling and prevent iMF expression.

Gestational Intermittent Hypoxia-Induced Deficits in Immune Signaling and Respiratory Control

That inflammation impairs iMF is significant given our hypothesis that iMF homeostatically protects and stabilizes respiratory output following reduced respiratory neural activity. This is especially relevant in the context of sleep apnea (SA), in which dozens of recurrent reductions in respiratory neural activity can occur each hour (Dempsey et al., 2010). Fields et al. (2019) demonstrated that co-induction of pLTF with iMF by a moderately hypoxic neural apnea, modeling that experienced in moderate to severe sleep apnea, occludes the expression of phrenic motor facilitation, which we hypothesize may contribute to the presumed lack of compensatory response to recurrent reductions in respiratory neural activity in SA. An additional possibility is that neuroinflammation, which is a comorbidity of SA, impairs compensatory responses to recurrent neural apneas, leading to a vicious cycle of hypoxia and inflammation.

In Chapters 4 and 5, we describe a slew of sex-specific deficits induced by GIH exposure, including increased neuroinflammation derived from aberrant microglia, impaired iMF, and increased spontaneous and trailing post-sigh apneas during presumptive sleep that suggests that adult male GIH offspring suffer from sleep apnea themselves. Adult respiratory control can be impacted by postnatal challenges like hyperoxia (Ling et al., 1996; Bavis et al., 2002, 2003; Fuller et al., 2002; Bisgard et al., 2003), hypoxia (Bavis et al., 2004; Reeves et al., 2006; Nanduri et al., 2012; Mayer et al., 2014; Hocker et al., 2019), stress (Dumont and Kinkead, 2010; Kinkead et al., 2013, 2014), nicotine (Huang et al., 2004), or caffeine (Montandon et al., 2006, 2009), and prenatal maternal stress (Golubeva et al., 2015). Our observation that prenatal exposure to IH leads to life-long respiratory deficits expands on this field and describes a novel role for in utero microglial reprogramming in impairing adult respiratory control, since depletion of microglia rescued expression of iMF in adult male GIH offspring. While we haven't tested the specific hypothesis that impaired iMF underlies the increase in apneas in adult male GIH

offspring, it could implicate epigenetic reprogramming of microglia in the heritability of sleep apnea.

Sleep apnea appears to be heritable in part (el Bayadi et al., 1990; Pillar and Lavie, 1995; Redline et al., 1997; Redline and Tishler, 2000), since risk of developing SA is 2-4X higher in individuals with a relative with SA (Patel et al., 2012). However, the precise mechanisms of heritability remain unresolved (Kent et al., 2010; Larkin et al., 2010; Patel et al., 2012; Cade et al., 2016). Children of mothers with OSA (Au et al., 2021) or its highly associated risk factors, including diabetes and obesity (Chen et al., 2019), have higher risk of developing SA themselves. Polymorphisms involved in immune function are also associated with sleep apnea (Larkin et al., 2010; Patel et al., 2012; Cade et al., 2016), and inflammation may underlie or worsen sleep apnea in humans (Patel et al., 2012). Collectively, these data indicate that differentially programmed immune function precipitated by an adverse in utero environment could contribute to heritability of sleep apnea. Reduction of inflammation by pharmacologic treatment or prescription of anti-inflammatory lifestyle interventions, like low-intensity exercise, could provide alternative or supplemental treatment options for individuals with sleep apnea. Even if future studies do not indicate a critical role for microglial reprogramming in the apnea phenotype exhibited by male GIH offspring, that GIH leads to immune reprogramming and deficits in respiratory control in adulthood advances our understanding of the long-term consequences that may be induced by maternal sleep apnea during pregnancy.

Maternal sleep apnea during pregnancy is increasing in parallel with the obesity epidemic, and increases the risk of a number of detrimental maternal and perinatal health outcomes, including gestational diabetes, fetal growth restriction, premature birth, NICU admission, and lower APGAR scores (Carnelio et al., 2017). However, the long-term consequences of maternal SA

during pregnancy remain underexplored. In animal models, GIH leads to adult metabolic (Khalyfa et al., 2017; Cortese et al., 2021) and endothelial dysfunction (Badran et al., 2019), high blood pressure (Song et al., 2021), altered hypoxic ventilatory responses (Gozal et al., 2003), cognitive impairments that resemble human autism spectrum disorder (Vanderplow et al., 2022), and alterations in the gut microbiome (Cortese et al., 2021). We expanded this onslaught of deficits to include increased neuroinflammation due to epigenetic reprogramming of microglia and impaired respiratory control suggestive of a sleep apnea phenotype in adult male offspring. Interestingly, alterations in the gut microbiome and high blood pressure, both of which we also report in Chapter 4, are both comorbidities of sleep apnea (Dempsey et al., 2010; Valentini et al., 2020).

The origin of gut dysbiosis in adult male GIH offspring remains unclear, but could be due to vertical transmission of a dysbiotic microbiome from the mother, since intermittent hypoxia exposure (Moreno-Indias et al., 2015) and maternal gestational stress (Jašarević et al., 2015) can alter the maternal microbiome. Alternatively, or in addition, the increase in apneas experienced by adult male GIH offspring exposes them to intermittent hypoxia, which could influence their gut microbiome at any point in life (Moreno-Indias et al., 2015). Regardless of the origin, adult male GIH offspring suffer from a decrease in butyrate-producing bacteria. Butyrate can potently influence immune function (Maslowski et al., 2009), and reduce microglial activation (Matt et al., 2018). Additionally, butyrate provides a major source of fuel for colonocytes (Canani et al., 2011). A deficit in butyrate-producing bacteria is associated with a “leaky” gut, in which the tight junction proteins that connect colonocytes and prevent translocation of bacterial products, like LPS, into the systemic circulation are broken down (Usuda et al., 2021). Thus, gut dysbiosis is very commonly associated with chronic systemic inflammation, and could exacerbate microglial dysfunction in adult male GIH offspring. Supplementation of butyrate or manipulations of the gut microbiome, such as fecal microbiome

transplant or probiotic supplementation, may provide relatively non-invasive treatment strategies to reduce neuroinflammation in adult male GIH offspring. Since the gut microbiome is extremely plastic, lifestyle interventions like increased fiber (Matt et al., 2018) or exercise (Mailing et al., 2019) can also increase populations of butyrate-producing bacteria, which may provide a new or supplemental option for treatment of sleep apnea in humans.

Conclusion

The work comprising this dissertation advances our understanding of the context-dependent effects of microglia in activity-dependent respiratory motor plasticity. We characterized a differential role for microglia in two different forms of iMF in the healthy CNS (Chapter 2). However, in the context of inflammation induced by LPS or GIH, we demonstrate that microglia have the capacity to impair expression of iMF induced by recurrent reductions in respiratory neural activity (Chapters 3 and 5). Finally, we characterize the widespread, long-lasting, sex-specific effects of gestational exposure to intermittent hypoxia on respiratory control, and the underlying cause of epiegenetic reprogramming of microglia toward a pro-inflammatory phenotype (Chapters 3 and 4). Collectively, these studies expand on previously defined pathways to iMF, and reveal potent inhibitory stimuli, our understanding of which may inform the harnessing of iMF for treatment of disorders characterized by respiratory instability.

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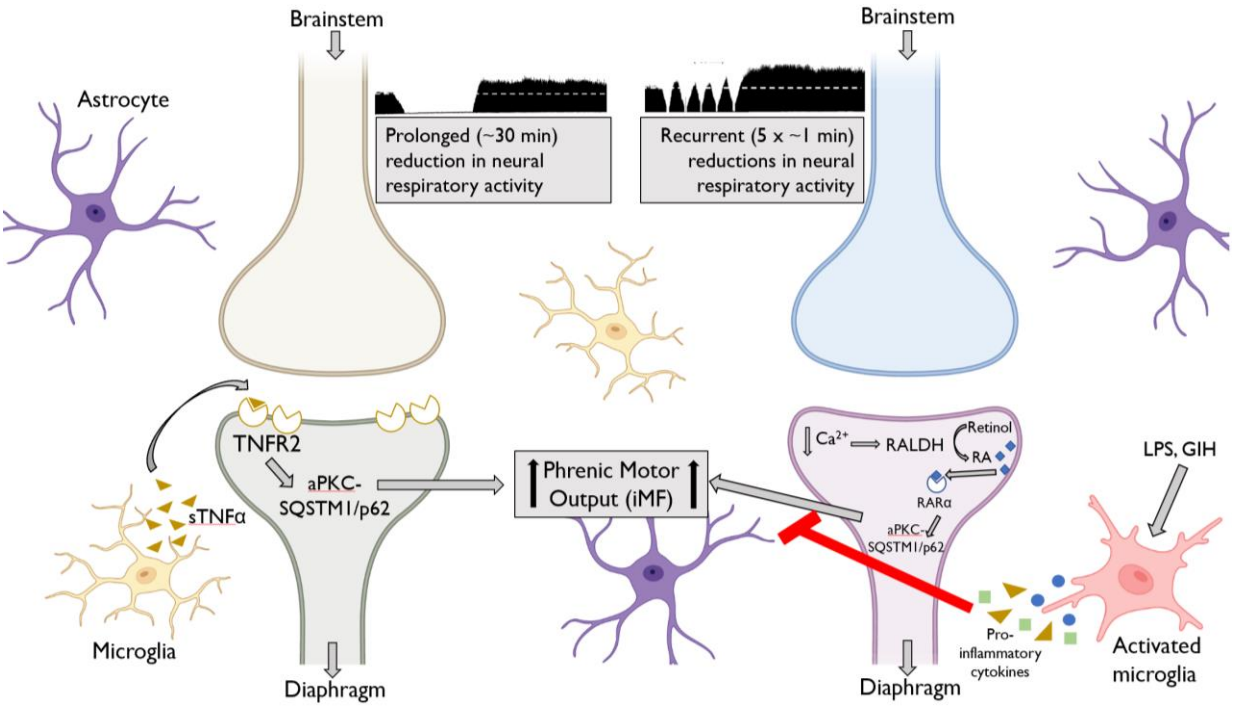

Figure 1: Working model of iMF
Prolonged reductions in neural respiratory activity prompts microglia to release soluble TNF-alpha, which activates TNFR2 on phrenic motor neurons, activating atypical PKC and leading to iMF expression. Recurrent reductions in neural respiratory activity reduces intracellular calcium, which disinhibits retinaldehyde dehydrogenase (RALDH), which converts retinol to retinoic acid (RA). RA activates retinoic acid receptor-alpha (RARa), which leads to activation of atypical PKC and an increase in phrenic motor output. Inflammatory stimuli, like lipopolysaccharide (LPS) or gestational exposure to intermittent hypoxia (GIH), activates microglia, which impairs expression of iMF following recurrent neural apneas.