Cellular mechanisms underlying how early life stressors disrupt respiratory control

by

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DISSERTATION ABSTRACT

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Title: Cellular mechanisms underlying how early life stressors disrupt respiratory control

Sufficient breathing is essential to maintaining homeostatic blood gases, yet stressors early in life undermine neural circuits controlling breathing in neonates, leading to potentially life-threatening respiratory insufficiency. Further, since respiratory control circuitry continues to develop postnatally, many early life stressors have lasting negative consequences for adult breathing and can increase risk for developing adult ventilatory control disorders, such as sleep apnea. For instance, neonatal inflammation acutely disrupted neonatal central respiratory activity and caused lasting disruption in adult respiratory control by abolishing respiratory motor plasticity, which disrupts the ability of the neural networks controlling breathing to learn and adapt. Yet, identifying mechanisms mediating this loss of adult plasticity and the extent of impairments to the neural control of breathing beyond plasticity are still necessary to understand how neonatal inflammation contributes to adult ventilatory insufficiencies and the risk for developing ventilatory control disorders. Additionally, infants exposed to maternal opioids have diverse negative health consequences, including respiratory distress. However, many factors contribute to these negative health outcomes, confounding the ability to understand how maternal opioids directly impact the neonatal respiratory system. Thus, this dissertation focuses on (1) identifying lasting disruptions in adult breathing after neonatal inflammation, (2) cellular mechanisms contributing to these impairments in adults after neonatal inflammation and (3) mechanisms underlying how maternal opioids impairs neonatal respiratory circuits.

I will begin by providing an overview of respiratory control circuitry and development, introducing microglia and their role in neuroinflammatory signaling, and why it is important to understand how early life stressors disrupt respiratory control in Chapter I. Our laboratory previously identified that neonatal inflammation caused lasting abolishment of adult respiratory motor plasticity, where loss of plasticity has the potential to disrupt the ability of the neural networks controlling breathing to learn and adapt. Chapter II of this dissertation extends this work by identifying medullary microglia (primary immune cells in the brain) as a key cell type likely contributing to this loss of adult plasticity. Further, we determined neonatal inflammation sex-dependently impaired respiratory control beyond plasticity, increasing our understanding of how neonatal inflammation may contribute to adult ventilatory insufficiencies and the risk for developing ventilatory control disorders. We identified that neonatal inflammation caused lasting augmentation of adult male hypercapnic responses, consistent with males being at increased risk for sleep apnea. Chapter III of this dissertation focuses on understanding how a subsequent adult inflammatory challenge affects breathing in adults after neonatal inflammation to determine their risk for breathing disruptions during illness and disease. This research significantly advances our understanding of how impairments in microglial inflammatory responses may contribute to adult breathing vulnerability after neonatal inflammation.

Finally, Chapter IV of this dissertation investigated mechanisms underlying how neonatal breathing is impaired by maternal opioids, another early life stressor. We determined maternal opioids directly impaired neonatal respiratory control networks, likely contributing to neonatal breathing deficits after maternal opioids, which we identified previously. Together this dissertation significantly advances our understanding of cellular mechanisms underlying how two early life stressors, neonatal inflammation and maternal opioids, disrupt respiratory control. Such an

understanding is necessary to develop novel therapeutic strategies to support breathing at all ages.

This dissertation includes previously published, co-authored material.

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Chapter I

INTRODUCTION & BACKGROUND

This chapter includes previously published co-authored material (Beyeler et al., 2019). A.G. Huxtable contributed to writing and provided editorial input in this section.

Breathing begins at birth and is vital to maintaining life at all ages. However, breathing is highly vulnerable to early life stressors with a significant portion of neonatal respiratory insufficiency and mortality caused by early life stressors (NICHD, 2024; WHO, 2024). However, an incomplete understanding of how early life stressors increase risk of neonatal and adult respiratory insufficiency limits the ability to develop novel therapeutics to enhance breathing at all ages. Thus, the focus of this dissertation is to identify cellular mechanisms contributing to impaired respiratory control by two early life stressors, neonatal inflammation or maternal opioids. Chapter II & III investigates inflammatory mechanisms underlying adult respiratory control impairments after neonatal inflammation and Chapter IV examines how maternal opioids directly impair neonatal respiratory control networks, likely contributing to neonatal breathing deficits after maternal opioids. The first chapter of this dissertation reviews our current understanding of respiratory control, the role of microglia in neural circuit function and how neonatal inflammation and maternal opioids impact neural circuits.

BREATHING & RESPIRATORY CONTROL

Breathing is a rhythmic and robust motor behavior vital to maintaining blood gas homeostasis and pH (reviewed in Del Negro et al., 2018). In addition to maintaining homeostasis, breathing must coordinate with other body functions, such as speaking or eating. Breathing, therefore, must adapt to changes in the environment and metabolic demands (Nattie & Li, 2012). The respiratory

system is also plasticity allowing breathing to increase in response to prior experiences, which likely contributes to maintaining breathing during injury or disease (reviewed in Feldman et al., 2003; Fuller & Mitchell, 2017; Mitchell & Baker, 2022; Mitchell & Johnson, 2003; Seven & Mitchell, 2019).

Brain regions involved in respiratory control

Breathing is controlled centrally by neural networks in the pons, medulla and ventral cervical spinal cord (reviewed in Del Negro et al., 2018). These respiratory networks in the pons and medulla consist of heterogenous populations of excitatory and inhibitory neurons, which generate respiratory rhythm and integrate sensory feedback to regulate respiratory-related motoneurons innervating inspiratory and expiratory muscles (reviewed in Del Negro et al., 2018; Gonye & Bayliss, 2023; Krohn et al., 2023; Tenorio-Lopes & Kinkead, 2021). The generally agreed upon kernel of respiratory rhythm generation and pattern formation is a group of about 3,000 interneurons (with equal proportion of excitatory and inhibitory neurons) in the ventral lateral medulla, known as the preBötzinger Complex (preBötC; Feldman, 1981; Feldman et al., 2003; Feldman & Ellenberger, 1988; Hayes & Del Negro, 2007; McCrimmon et al., 1989). Decades of research investigating how preBötC neurons generate rhythm have determined inspiratory rhythmogenesis is an emergent property from preBötC network synchronization in a relatively small number of intrinsically bursting preBötC neurons and recurrent synaptic excitation in preBötC neurons (Ashhad et al., 2022; Ashhad & Feldman, 2020; Cohen & Feldman, 1977; Del Negro et al., 2018; Feldman, 2011; Feldman & Del Negro, 2006; Feldman & Ellenberger, 1988, p. 198; Feldman & Janczewski, 2006; Lindsay & Feldman, 1993; McCrimmon et al., 1986). Type-1 preBötC neurons have pre-inspiratory firing patterns, are intrinsically bursting, and a significant

proportion (13%) of their connections have recurrent excitatory synaptic connections. Thus, these type 1 preBötC neurons are hypothesized to be the kernel of rhythmogenesis (Borrus et al., 2024; Del Negro et al., 2002, 2005; Gray et al., 1999; Kallurkar et al., 2020; Pace et al., 2007b, 2007a; Rekling et al., 2000; Rekling & Feldman, 1998). While respiratory rhythm and motor pattern were thought to be inseparable, recent work (Kallurkar et al., 2020; Kam et al., 2013) showed preBötC microcircuits have subthreshold bursts that occur without generating motor output and the recurrent excitation within preBötC microcircuits represent a central respiratory oscillator (Kallurkar et al., 2020). While the exact mechanism underlying how subthreshold pre-inspiratory burstlets generate inspiratory bursts in the preBötC and respiratory motor neurons is still under investigation, two main hypotheses exist. (1) Burst formation may depend on either enough pre-inspiratory neurons being activated during a burstlet that triggers a cascade activating all excitatory neurons in the preBötC (Kallurkar et al., 2020) or (2) it may depend on synchronization of pre-inspiratory burstlets to trigger a cascade within excitatory neurons in the preBötC (Ashhad & Feldman, 2020). Thus, inspiration originates in the medulla from rhythmic preBötC activity and propagates to premotor pools (such as para-hypoglossal and rostral ventral respiratory group, rVRG) before inspiratory motoneurons (such as hypoglossal and phrenic motoneurons), ultimately leading to contraction of inspiratory muscles.

The post-inspiratory phase of breathing occurs immediately after inspiration and enhances breathing by lengthening diaphragm contractions and increasing alveolar gas exchange due to increase lung inflation time per breath (Sears et al., 1982; Tuck et al., 2001). Two respiratory-related regions have been identified as contributing to post-inspiration; (1) the post-inspiratory complex (PICO), located rostral and medial to the preBötC (Anderson et al., 2016), and (2) the Kölliker-Fuse (KF) nucleus in the pons (Dutschmann & Dick, 2012). Both post-inspiratory-related

regions innervate the preBötC, facilitating the transition from inspiration to expiration, likely through mutual inhibition (reviewed in Anderson & Ramirez, 2017; Dutschmann, 2015). While questions remain as to whether PICO is an independent respiratory oscillator (Hülsmann, 2021), PICO activity retains rhythmicity even when isolated from the PreBötC, and stimulation of PICO interneurons evokes a post-inspiratory burst, but never inspiratory bursts (Anderson et al., 2016; Baertsch et al., 2018; Ramirez et al., 2016; Ramirez & Baertsch, 2018). Further, PICO is hypothesized to contribute to protecting airways by glottal constriction during expulsive respiratory behaviors (such as coughing and sneezing) and non-respiratory behaviors (such as swallowing and vocalization; (Dhingra et al., 2021). Post-inspiratory activity from the KF increases resistance of the upper airways and lengthens the time between breaths during vocalizations or speech (Dutschmann & Herbert, 2006; Poon & Song, 2014). While some reports have postulated post-inspiration is an obligatory phase of the breathing cycle (Richter & Spyer, 2001; Smith et al., 2013; Smith et al., 2007), post-inspiratory muscle activity intermittently stops during sleep and under anesthesia, demonstrating post-inspiration is not essential for breathing or respiratory rhythmogenesis (Dutschmann et al., 2014). Thus, the post-inspiratory phase of breathing is generated centrally by PICO and KF and significantly contributes to enhancing breathing.

Expiration occurs immediately after the post-inspiration and is passive during quiet breathing at rest, while active expiration emerges during times of increased metabolic demand, when at altitude, and intermittently during rapid eye movement (REM) sleep (Janczewski & Feldman, 2006; Pagliardini et al., 2011, 2012; A. Pisanski & Pagliardini, 2019; Saini & Pagliardini, 2017). Immediately after birth, the lateral pFRG contributes to pre-inspiratory and inspiratory rhythm generation, before switching to contributing to active expiration in adulthood (Hocker and

Morrison et al., 2021; Mellen & Thoby-Brisson, 2012; Takeda et al., 2001; Thoby-Brisson et al., 2009). In juvenile rodents, expiratory rhythm is maintained without preBötC activity (Janczewski & Feldman, 2006), demonstrating the function of the lateral pFRG shifts throughout development and likely contributes to respiratory rhythm generation. During quiet at rest breathing, the lateral pFRG is inhibited by the preBötC during inspiration, preventing co-activation of inspiratory and expiratory muscles (Janczewski & Feldman, 2006; Mellen et al., 2003; Sears et al., 1982; Takeda et al., 2001). During time of increased metabolic demand (such as exercise), during REM sleep, increased CO_2 or decreased O_2, lateral pFRG neurons are disinhibited and are active during late-expiration and are tightly coupled with preBötC activity(Huckstepp et al., 2016; Huckstepp et al., 2015). Another medullary region that contributes to expiration is the Bötzinger complex (BötC), which is located caudal to the pFRG and rostral to the preBötC, and consists primarily of inhibitory interneurons active during expiration and projecting to the preBötC (Smith et al., 2009). During active expiration, neural activity from the lateral pFRG and the BötC propagates to premotor neurons in the caudal VRG before innervating motoneurons controlling expiratory muscles, such as in the abdominal wall (e.g. transversus abdominis muscle, internal oblique muscle, external oblique muscle, and rectus abdominis muscle) and in the rib cage (e.g., the internal intercostal muscles and the triangularis sterni muscle; Abdala & Dhar, 2010; Cohen & Feldman, 1978; Fortuna et al., 2008; Pisanski et al., 2018; Smith et al., 2009). In conclusion, the lateral pFRG and BötC contribute to active expiration, while the lateral pFRG likely contributes to generating respiratory rhythm at younger ages.

Respiratory networks maintain blood gas CO_2 and O_2 within homeostatic ranges to sustain life (reviewed Guyenet & Bayliss, 2015; Prabhakar & Peng, 2017). A key contributor to hypercapnic ventilatory responses is a respiratory region located near the ventral surface of the

medulla, known as the retrotrapezoid nucleus (RTN). This region is directly sensitive to pH and CO_2 tension *in vivo* and *in vitro* (Guyenet & Bayliss, 2015, 2022). Acid-base changes are sensed in the RTN via proton-activated G protein-coupled receptor (GPR4) and a proton-modulated potassium channel (TASK-2) on RTN neurons and P2Y receptors on astrocytes in the RTN (Gourine & Funk, 2017; Guyenet et al., 2016; Guyenet & Bayliss, 2015, 2022, 2022). Other pontine and medullary regions have been shown to contribute to chemosensitive *in vitro*, including preBötC, medullary raphe, nucleus tractus solitarius (NTS), and locus coeruleus (Guyenet & Bayliss, 2022; Guyenet & Mulkey, 2010; SheikhBahaei et al., 2024). In summary, increases in arterial CO_2 or decreases in pH activate RTN, medullary raphe, NTS, and locus coeruleus neurons causing stimulation of the preBötC to increase breathing (reviewed in Guyenet and Bayliss, 2022, 2015; Guyenet and Mulkey, 2010).

To maintain homeostatic levels of oxygen carbon dioxide and pH, the peripheral carotid body senses arterial partial pressure of O_2 (PaO_2) by closing voltage independent (TASK) and voltage-dependent K+ channels in carotid body glomus cells causing depolarization (Gonzalez et al., 1994; Iturriaga et al., 2021; Nurse, 2005; Pardal & López-Barneo, 2002). Depolarization of carotid body glomus cells leads to release of excitatory neurotransmitters, such as acetylcholine and adenosine triphosphate, from the carotid sinus nerve that projects to medullary respiratory nuclei, including the NTS, dorsal motor nucleus of the vagus, and the medullary raphe (reviewed in Pilowsky, 2014). Excitation of these central respiratory control regions leads to stimulation of the preBötC to increase breathing (reviewed in Feldman et al., 2003; Guyenet & Bayliss, 2022). The hypoxic ventilatory response consists of two phases: phase I and phase II. Phase I is the early phase of the hypoxic ventilatory response and is characterized by a steep increase in ventilation (increased frequency and tidal volume), mainly mediated by the carotid body (reviewed in

Pamenter and Powell, 2016). While phase II is the late phase of the hypoxic ventilatory response and is characterized by ventilatory decline, mainly mediated by central respiratory nuclei, such as the RTN and NTS (reviewed in Gourine and Funk, 2017; Prabhakar and Peng, 2017). Collectively, these respiratory neural circuits contribute to sustaining adequate breathing throughout life and maintain homeostasis in the face of respiratory challenges.

Respiratory Plasticity

Respiratory plasticity is defined as a change in future behavior or activity based on prior experience (Mitchell and Johnson, 2003). While the physiological role of plasticity is still under investigation, it likely compensates and stabilizes breathing during pathology, after injury, after pregnancy, across development as an individual ages, and during infection (Fuller and Mitchell, 2017). Respiratory plasticity has been observed as lasting increases in carotid sinus nerve activity, amplitude (motor plasticity) or frequency (frequency plasticity) in peripheral and respiratory-related nerves (reviewed in Feldman et al., 2003; Baker-Herman and Mitchell, 2008) and medullary respiratory centers (Pamenter and Powell, 2016).

One commonly studied model of adult respiratory motor plasticity is long-term facilitation (LTF), evoked by acute intermittent hypoxia (AIH, 3 × 5 minutes of hypoxia, 5–10.5% O_2). At least two working models describe the cellular mechanisms of LTF (reviewed in Perim and Mitchell, 2019). The "Q-pathway" is elicited by moderate AIH (mAIH, 3 × 5 minutes of hypoxia, PaO_2 = 35–45 mmHg; Baker-Herman et al., 2004). Moderate AIH activates medullary raphe nuclei, inducing release of serotonin in the vicinity of the phrenic motoneurons, which activates G_q protein-coupled serotonin (5-HT_2) receptors (Bach and Mitchell, 1996; Ling et al., 2001; Baker-Herman and Mitchell, 2002; Macfarlane et al., 2009). This activation of serotonin receptors results

in a cascade requiring spinal ERK-MAP kinase activity (Hoffman et al., 2012), new BDNF protein synthesis (Baker-Herman and Mitchell, 2002; Baker-Herman et al., 2004), activation of TrkB receptors (Dale et al., 2017), and increased activity of protein kinase C-θ (Devinney et al., 2015). Alternatively, the "S-pathway" is evoked by severe acute intermittent hypoxia (sAIH, 3 × 5 minutes of hypoxia, PaO_2 = 25–35 mmHg; Nichols et al., 2012; Seven et al., 2018). Severe AIH activates G_S protein-coupled metabotropic receptors, such as adenosine 2A (A_{2A}) and serotonin ($5\text{-}HT_7$) receptors (Golder et al., 2008; Hoffman and Mitchell, 2011). The mechanisms underlying S-pathway plasticity include increased cyclic adenosine monophosphate (cAMP) signaling (Fields and Mitchell, 2017), activation of exchange protein activated by cAMP (Fields, Springborn and Mitchell, 2015), Akt and mammalian target of rapamycin (Dougherty et al., 2015). In contrast to the Q-pathway, the mechanisms underlying S-pathway-evoked plasticity require new synthesis of TrkB, but not BDNF (Golder et al., 2008; Seven et al., 2018). These two pathways, however, do not occur independently. Co-activation of Q- and S-pathway-evoked plasticity results in cross-talk inhibition, where the S-pathway inhibits the Q-pathway-evoked plasticity through PKA (Fields and Mitchell, 2017; Perim et al., 2018, 2019), limiting or even abolishing expression of respiratory motor plasticity (reviewed in Devinney et al., 2016; Perim and Mitchell, 2019). Understanding the mechanisms underlying respiratory motor plasticity is important as AIH is being utilized therapeutically as a treatment to enhance motor recovery following spinal cord injury (Christiansen et al., 2018). However, enhanced motor recovery is not observed in all spinal cord injury patients (Christiansen et al., 2018), indicating impaired respiratory motor plasticity may limit its use as a treatment in these patients. This inhibition of plasticity may be a result of cross-talk inhibition or inhibition by other signaling mechanisms, such as inflammation. Enhancing our understanding of how inflammation influences plasticity has the potential to improve therapeutic efficacy of using

plasticity in patients with spinal cord injury or diseases impacting respiratory control, such as amyotrophic lateral sclerosis or obstructive sleep apnea.

Development of respiratory control circuits

Essential components of respiratory networks begin developing *in utero*. Neurogenesis of phrenic motoneurons occurs within the ventricular zone of the neural tube, peaking at E11 (Altman & Bayer, 1984). These phrenic motor neurons are linked by gap junctions and migrate to the ventral cervical spinal cord along radial glia arriving at the ventral horn of the cervical spinal cord by E15(Ren and Greer, 2003) and immediately begin innervating the diaphragm, a major inspiratory muscle, via neuromuscular junctions (reviewed in Mantilla and Sieck, 2008). The peak preBötC neurogenesis occurs at E12.5 – E13.5 in rats, whereby these neurons migrate from the ventricular zone to their final location in the ventrolateral medulla (Pagliardini et al., 2003). Once preBötC neurons reach the ventrolateral medulla, they quickly establish key connections with the rostral ventral respiratory group at E17 (Pagliardini et al., 2003). Thus, critical components of the respiratory circuitry are intact at E17, when fetal breathing movements commence (reviewed in Greer, 2012; Greer and Martin-Caraballo, 2017). Fetal breathing movements facilitate circuit maturation by augmenting initially weak signals from the preBötC (Kobayashi et al., 2001), increasing membrane potential of phrenic motoneurons (Ren and Greer, 2003), and facilitating lung proliferation and differentiation (Hooper and Harding, 1990; Bissonnette et al., 1991; Liu et al., 1992; Kitterman, 1996; Liu and Post, 2000; Hooper and Wallace, 2006). Precise timing of each of these events is critical for the respiratory control network to maintain blood gases immediately at birth without external assistance.

Early in life breathing is unstable and chemosensitivity is blunted, contributing to increased apneas and increased vulnerability in newborn infants (Sterni et al., 1999; Fairchild et al., 2016). Rodents have proven to be a useful model to study respiratory dysfunctions early in life, such as apnea of prematurity, since the newborn rodent (P0–4) respiratory networks are comparable to the respiratory system at the third trimester in humans (Di Fiore et al., 2013). The first few days of life (P0-4) in rodents represents a critical window due to shifts in oscillator dominance and expression of neurotransmitter receptors, resulting in overall decreased excitation in the respiratory control network. Inspiratory rhythm generation is initially maintained by both the pFRG and the preBötC *in utero* and in newborn rodents (P0–4), with the pFRG being the initial dominant oscillator (Onimaru and Dutschmann, 2012). During the first week of life, the preBötC shifts to become the dominant oscillator and the pFRG plays a larger role in active expiration and, possibly chemoreception (Barnes et al., 2007; Huckstepp et al., 2015). Further research is needed to understand the development of PICO rhythm generating networks or its role in respiratory network maturation early in life.

At the cellular level, the expression of glutamate and NMDA receptors are moderately decreased in P3–4 rats in regions important for respiratory control (preBötC, NTS, and Amb, XII, and DMV) compared to the first week of life, resulting in an overall decrease in excitability (Liu and Wong-Riley, 2002, 2005). Additionally, expression of $GABA_A$, $GABA_B$, and glycine receptors transiently plateaus in P3–4 rats and transiently decreases from P10-12, before continuing to increase throughout neonatal development, further contributing to overall decreased excitability (Liu et al., 2006). Receptors for other neuromodulators, such as thyrotropin-releasing hormone, serotonin (5-HT_{1A}, 5-HT_{1B}, and 5-HT_{2A}) are also low during the first few days of life in rodents (P0–4) before peaking at P5 to P11 (Liu and Wong-Riley, 2008, 2010a, 2010b).

Collectively, these fluctuations in receptor expression decreases overall excitability of respiratory control regions, potentially contributing to the increased apnea frequency and increased variability in breathing frequency (Wong-Riley et al., 2013).

In addition to the developmental processes occurring in rhythm generating nuclei, changes in chemoreception occur early in life, contributing to increased apnea frequency in neonates (Prabhakar et al., 2007). The insensitivity of the newborn carotid body to O_2 contributes to blunting hypoxia responses (Sterni et al., 1999; Bairam et al., 2001; Prabhakar et al., 2007; MacFarlane et al., 2013) and limits the ability of newborns to compensate and stabilize breathing during a hypoxic challenge (Julien et al., 2008). Thus, newborn developmental processes in respiratory rhythm generating nuclei and chemoreceptive regions contribute to increased frequency of apneas in preterm infants and newborns, potentially leaving the newborn respiratory control network more vulnerable to stressors.

ROLE OF MICROGLIA IN NEURAL CIRCUIT FUNCTION & BREATHING

Microglia are key immune cells in the CNS and contribute significantly to early life neural circuit development, including development of respiratory circuits (Cabirol et al., 2022). Microglia and mononuclear phagocytes originate from early myeloid progenitor cells in the embryonic yolk sac (Colonna & Butovsky, 2017; Harry, 2013; Rosin et al., 2021; reviewed in Bilbo & Schwarz 2012). Microglia progenitor cells enter the developing CNS at E9.5 in mice and immediately begin proliferating and migrating throughout the entire parenchyma (Ginhoux et al., 2010; Gomez Perdiguero et al., 2015; Kierdorf et al., 2013; Low & Ginhoux, 2018; Palis & Yoder, 2001; Schulz et al., 2012; Schwarz et al., 2012; Thion et al., 2018; Utz et al., 2020). Distribution of microglia throughout all brain tissue contributes to microglia differentiation and maturation (Ginhoux et al.,

2010; Thion et al., 2018; reviewed in Bilbo & Schwarz 2012). The microglial migration speed and proliferation rates vary significantly based on brain region and sex (Dadwal & Heneka, 2023; Schwarz et al., 2012). Identification of specific chemokines (including CCL2, CCL3, CCL6, CCL7, CCL12 and chemokine ligand 6) upregulated at birth in rodent hippocampus and cortex suggests developmental expression of chemokines likely contributes to regional migration of immature microglia throughout the parenchyma (Schwarz et al., 2012; reviewed in Bilbo & Schwarz, 2012). After the initial microglial infiltration there is minimal contributions of circulating monocytes to microglial populations and instead maintain their population through proliferation (Goldmann et al., 2013; Hashimoto et al., 2013; Tay et al., 2017). In addition to microglia, CNS-associated macrophages enter the brain at E12.5 and colonize CNS interfaces, such as the perivascular space, the choroid plexus, and meninges (Ginhoux et al., 2010; Goldmann et al., 2016; Schulz et al., 2012; Utz et al., 2020). Unlike microglia, these macrophages are partially replaced by bone marrow-derived circulating monocytes in adulthood (Goldmann et al., 2016; Utz et al., 2020).

Microglia rapidly proliferate and increase in density from weeks 1-3 in rodents (Nikodemova et al., 2015). This is transient, however, as by weeks 5-6 microglia reduce in number and maintain a stable number of microglia into adulthood (Nikodemova et al., 2015). Microglia significantly contribute to neural development by expressing inflammatory signals to contribute to remodeling dendritic spines and elimination of synapses (Bobotis et al., 2023; Hong et al., 2016; Schafer & Stevens, 2015; Tremblay, 2011) and contribute to neuron migration and axon guidance (Harry, 2013; Kettenmann et al., 2011). Adult microglia density and microglial activation in response to inflammatory challenges are highly influenced by CNS region (reviewed in Dadwal & Heneka, 2023; Furube et al., 2018; St-Pierre et al., 2022), highlighting the importance of

understanding regional differences in microglia and how these differences contribute to neural circuit dysfunction. Within respiratory circuits, the genetic ablation of microglia during *in utero* mouse development disrupts embryonic respiratory activity and likely contributes to neonatal death in this model, since these embryos are unable to sustain breathing activity (Cabirol et al., 2022). Thus, microglia are likely essential for development of respiratory control, and increased understanding of how stressors early in life (such as neonatal inflammation) disrupt microglia in respiratory control regions, thereby disrupting development of respiratory control circuits, is needed to develop novel treatments to support breathing in neonates and adults after early life stressors.

Microglia significantly contribute to development of neural circuits and sexual dimorphisms in the brain, which likely contribute to sex differences in response to early life stressors. Microglia directly contribute to sex differences in neural circuit development (reviewed in Lenz & McCarthy, 2015). At E12 (in mice) activation of sex hormone receptors expressed on microglia leads to the refinement and sculpting of neural circuits, leading to sexual dimorphisms in sex-related behaviors in healthy adults (Lenz et al., 2011, 2013). Further, ample evidence supports the hypothesis that disruptions in development of microglia during early life likely contribute to sex differences in multiple CNS pathologies, such as early onset cognitive decline (Bilbo, 2010; Bilbo et al., 2012; Ince et al., 2023; Leuner, 2024), mood and social disorders (Cao et al., 2021; Gracia-Rubio et al., 2016; Kentner et al., 2010; Saavedra et al., 2021; Sequeira & Bolton, 2023; Wang et al., 2016; Wang et al., 2017), neurodevelopmental disorders (reviewed in Hanamsagar & Bilbo, 2016), and neuropsychiatric disorders (reviewed in Hollander et al., 2020). Thus, investigating how early life stressors, such as neonatal inflammation, disrupt microglia in respiratory control regions is needed to understand how early life stressors contribute to neonatal

and adult ventilatory insufficiency and provide the framework for development of novel treatments to support breathing at all ages.

Microglia activation

Within respiratory control regions, moderate systemic inflammation (3 mg/kg, LPS, i.p.) blunts short-term hypoxic phrenic responses in cats (Fernández et al., 2008), piglets (McDeigan et al., 2003), and adult rats (Vinit et al., 2011; Huxtable et al. 2013), demonstrating inflammation impairs central respiratory neural circuits. Even low levels of inflammation disrupt respiratory circuits (0.1 mg/kg LPS, i.p.) in adults abolished respiratory motor plasticity (Huxtable et al., 2011, 2013; Hocker and Huxtable, 2018). These impairments in respiratory control are associated with increased microglial inflammatory gene expression (Huxtable et al., 2011, 2013; Hocker and Huxtable, 2018), suggesting inflammatory signaling likely contributes to acute disruptions in respiratory circuits after inflammatory challenges. Microglia are a key cell type mediating neuroinflammation and likely underlie inflammation-induced impairments in respiratory control; however, few studies have focused on the specific contribution of microglia to respiratory effects from inflammation. Throughout the CNS microglia become activated and contribute to the resolution of neuroinflammation after inflammatory challenges, including repair of neural tissue after injury. Activated microglia are characterized as microglia with ameboid shape, increased proliferation, and/or increased inflammatory signaling (reviewed in Badoer, 2010; Borst et al., 2021) and microglia activation contributes to modulating neural circuits related to sickness behaviors after adult systemic inflammation in mice (LPS-induced; Béchade et al., 2021; Torii et al., 2022). While microglial inflammatory signaling likely contributes to the respiratory effects of

inflammation, this cell type is not well studied within respiratory control. Thus, this research aims to advance our understanding of microglia activation within respiratory control regions.

Further, region and sex differences in microglia activation contribute to acute inflammatory responses after systemic inflammation. For instance, while systemic inflammation activates adult microglia throughout the brain, regional differences in microglia caused significant increases in number of Iba-1 positive cells and density of microglia in the substantia nigra and entorhinal cortex compared to other regions, including the cerebellum, striatum, and hippocampus (Brandi et al., 2022). Additionally, systemic inflammation-induced microglial activation contributes to transient decreases in dopaminergic dendrites specifically in the substantia nigra, yet future research is required to identify the physiological consequences of these regional differences in microglial activation (Brandi et al., 2022). Growing evidence highlights the importance of advancing our understanding of heterogeneity in microglial activation to determine how microglia contribute to development of neurodegenerative disorders, such as Parkinson's disease (Badanjak et al., 2021; Ho, 2019; Isik et al., 2023), Alzheimer's disease (Sarlus & Heneka, 2017; Singh, 2022; St-Pierre et al., 2022), and Amyotrophic Lateral Sclerosis (Du et al., 2017; Geloso et al., 2017). Regional differences in microglial activation within respiratory control regions is less well established, recent evidence shows healthy adult medullary microglial transcriptomes are distinct in cervical spinal microglia (Ewald et al., 2020), suggesting microglia likely have region specific responses to inflammation within respiratory control regions.

Microglia are sexually dimorphic with neonatal males have increased microglial number and activated morphology in the preoptic area (Lenz et al., 2013), hippocampus, parietal cortex, and amygdala compared to females (Schwarz et al., 2012). However, the vast majority of studies

investigating microglial inflammatory responses to systemic inflammation have been performed exclusively in males (reviewed in Hoogland et al., 2015), restricting our understanding of female microglia. This has further hampered our understanding of how activation of female microglia impacts neural circuits function and limits development of therapeutics optimized to treat neuroinflammation in females. The importance of sex differences in microglia within the respiratory control network has been highlighted by Ewald et al., 2020, who showed that within these respiratory control regions, male microglial transcriptomes are distinct from female microglia, suggesting distinct inflammatory mechanisms in male and female microglia likely contribute to disruptions in respiratory after inflammation. Thus, our studies here will significantly advance our understanding of regional and sex differences in microglia within respiratory control regions.

Microglia priming

Similar to innate immune memory in peripheral immune cells, microglia can have long lasting reprogramming in response to inflammatory challenges, termed microglial priming (reviewed in Hoeijmakers et al., 2016; Leuner, 2024; Lima et al., 2022; Perry & Holmes, 2014). Reprograming of microglia occurs through epigenetic dysregulation in microglia (Crews et al., 2024; Martins-Ferreira et al., 2021; Zhang et al., 2022). A key aspect of microglia priming is the initial inflammatory challenge occurring during a critical period of development, such as early life, leading to lasting exaggeration of microglial inflammatory responses during future inflammatory challenges (Hoeijmakers et al., 2016). One common example is that neonatal inflammation primes adult hippocampal microglia for exaggerated inflammatory responses contributing to age-related cognitive decline (Bilbo, 2010; Williamson et al., 2011) and pathology of Alzheimer's disease

(reviewed in Dziabis & Bilbo, 2022; Hanamsagar & Bilbo, 2016). Microglia priming also leads to CNS pathologies, including Parkinsons disease, Alzheimer's disease and Amyotrophic Lateral Sclerosis (reviewed in Anwar & Rivest, 2020; Dziabis & Bilbo, 2022; Hoeijmakers et al., 2016; Leuner, 2024; Lima et al., 2022; Perry & Holmes, 2014). While ample evidence shows microglia priming disrupts neural circuit function and increases the risk for developing neurological diseases, little is known about microglia priming in respiratory control regions or how microglia priming contributes to breathing vulnerability during illness and disease.

Within respiratory control circuits, gestational intermittent hypoxia (an early life stressor modeling maternal sleep disordered breathing) sex-dependently blunted neonatal microglial inflammatory responses. Although LPS-induced systemic inflammation decreases neonatal respiratory neural activity in controls, gestational intermittent hypoxia attenuated LPS-induced decreases in respiratory neural activity (Johnson et al., 2018). These data suggest this early life stressor reprograms microglia within respiratory control regions, potentially having protective effects on neonatal respiratory circuits. While we are beginning to understand how other early life stressors impact microglia inflammatory responses, whether neonatal inflammation primes microglia in cortical regions and increases adult risk for ventilatory insufficiency remains unclear. Thus, this dissertation investigating whether neonatal inflammation primes microglia in respiratory control regions to a subsequent adult inflammatory challenge will provide novel insights into how neonatal inflammation contributes to adult vulnerability for ventilatory insufficiency.

EARLY LIFE STRESS DISRUPTS RESPIRATORY CONTROL

Many early life stressors acutely disrupt neonatal respiratory control increasing neonatal breathing vulnerability. Gestational intermittent hypoxia (Gozal et al., 2003; Johnson et al., 2017), gestational ethanol exposure (Dubois & Pierrefiche, 2020), perinatal inflammation (Camacho-Hernández et al., 2022; Morrison et al., 2019), perinatal nicotine exposure (Cholanian et al., 2017; Ferng & Fregosi, 2015; Luo et al., 2004), perinatal anti-depressant drug exposure (Biancardi et al., 2022), and maternal opioids (Hocker and Morrison et al. 2019) decrease neonatal respiratory activity and/or neonatal breathing, demonstrating early life stressors increase neonatal risk of mortality and highlighting the importance of advancing our understanding of cellular mechanisms underlying these neonatal respiratory control disruptions to facilitate development of novel therapeutics to support neonatal breathing. Thus, this dissertation will advance our understanding of neonatal ventilatory insufficiency by studying how maternal opioids (an increasingly common early life stressor) disrupt neonatal respiratory circuits.

Some of these early life stressors also have enduring consequences on the adult respiratory system (Biancardi et al., 2022; Dubois & Pierrefiche, 2020; Genest et al., 2004; Hocker et al., 2019; Kinkead et al., 2009; Soliz et al., 2016), likely contributing to adult ventilatory insufficiency. While much research has identified how neonatal inflammation acutely disrupts neonatal respiratory control (Camacho-Hernández et al., 2022; Hofstetter et al., 2007; Hofstetter & Herlenius, 2005; Morrison et al., 2020), much less is known about how neonatal inflammation cause lasting disruptions in adult respiratory control. Our laboratory recently identified that neonatal inflammation causes lasting abolishment of adult respiratory motor plasticity in males and females (Hocker et al., 2019), which limits the adult respiratory system from learning and adapting over time, and likely restricts respiratory compensation during illness, injury or disease

in adults after neonatal inflammation. Lasting inflammatory signaling likely contributes to abolishment of plasticity, as an acute anti-inflammatory treatment (ketoprofen) restores plasticity in adults (Hocker et al., 2019). However, inflammatory gene expression in homogenate samples from the medulla or cervical spinal cord were unchanged in adults after neonatal inflammation. Thus, the inflammatory mechanisms underlying how neonatal inflammation abolishes plasticity remains unresolved and whether neonatal inflammation impairs respiratory control beyond abolishing plasticity remains unclear, limiting our ability to understand the lasting consequences of neonatal inflammation on adult ventilatory insufficiency.

SPECIFIC AIMS

This dissertation advances our understanding of the cellular mechanisms underlying disrupted respiratory control after early life stressors, neonatal inflammation and maternal opioids. The first aim will focus on the lasting effects of neonatal inflammation on adult microglia in respiratory control regions, increasing our understanding of an underappreciated cell type in respiratory control and providing mechanistic insights that may help in developing clinical strategies to promote plasticity after neonatal inflammation. Additionally, these studies will determine lasting effects of neonatal inflammation on adult breathing, furthering our understanding of how neonatal inflammation contributes to adult risks for ventilatory control disorders. Next, I investigated the impact of neonatal inflammation on microglial inflammatory responses and breathing during adult inflammation, providing novel insights into how neonatal inflammation may effect risks for adult breathing vulnerability during infection, illness or disease. These studies expand our understanding of how sex impacts the lasting impacts of neonatal inflammation on adult breathing, and emphasizes the need to further understand the effects of

inflammation on female respiratory control. Lastly, I examined how maternal opioids directly impaired neonatal respiratory control networks, determining central components of neonatal breathing deficits after maternal opioids, foundational to development of novel therapeutics to support breathing in these infants. Overall, this research provides unique insights into how early life stressors undermine neonatal and adult respiratory control, significantly advancing our understanding of how neonatal inflammation and maternal opioids contribute to respiratory insufficiency and provide key cellular insights needed to develop novel treatments to improve breathing at all ages.

CHAPTER II

LASTING SEX-DEPENDENT ACTIVATION OF ADULT MEDULLARY MICROGLIA AND SEX SPECIFIC EFFECTS ON SIGHS AND CHEMOREFLEXES AFTER NEONATAL INFLAMMATION

This chapter includes material being prepared for submission for publication in the *eLife Sciences Journal*, co-authored with Deanna L. M. Plunkett, Jyoti J. Watters, Adrianne G. Huxtable. I led these studies. I designed, collected and analyzed data for microglia experiments, analyzed the data for the breathing experiments, and wrote the manuscript. Deanna L. M. Plunkett contributed to collecting data for microglia frequency experiments. Jyoti Watters contributed to designing the experiments and provided guidance for this study. Dr. Adrianne Huxtable designed, contributed to writing, provided guidance and editorial assistance.

Introduction

Neonatal inflammation has lasting negative consequences for adult central nervous system (CNS) health. Many of these negative consequences are mediated, at least in part, by adult microglia, key resident immune cells in the CNS (reviewed in Bilbo et al., 2012; Bilbo & Schwarz, 2012; Schwarz & Bilbo, 2012; Williamson et al., 2011). Neonatal inflammation primes adult microglia for exaggerated adult inflammatory responses by augmenting microglia proliferation, changing microglia morphology and augmenting inflammatory signaling after subsequent adult inflammatory challenges (reviewed in Bilbo et al., 2012; Bilbo & Schwarz, 2012). These primed microglia contribute to impaired hippocampal-dependent learning in adults after neonatal inflammation (Bilbo et al., 2005) and an increased risk for age-related cognitive decline (Bilbo, 2010; Bland, Beckley, Young, et al., 2010). Furthermore, lasting activation of adult microglia is

also associated with long-term sex-specific effects of neonatal inflammation on adult male and female behaviors (Bilbo, 2010; Bland, Beckley, Watkins, et al., 2010; Bland, Beckley, Young, et al., 2010; Cao et al., 2021; Hanamsagar & Bilbo, 2017), such as long-term increases in adult male depressive-like behaviors (Kentner et al., 2010) and decreased reproductive behaviors in adult females (Sylvia & Demas, 2017). Thus, microglia likely play a key role in the lasting sex-dependent disruptions to adult behavior after neonatal inflammation; yet, their role in respiratory control regions after neonatal inflammation remain largely unknown.

We recently demonstrated that the lasting negative consequences of neonatal inflammation extend to the neural control of breathing by abolishing adult respiratory motor plasticity (Hocker et al., 2019). Such a loss of plasticity may prevent the adult respiratory system from learning and adapting over time, limiting the ability for respiratory compensation during illness, injury or disease in adults after neonatal inflammation. Abolished adult respiratory motor plasticity after neonatal inflammation is due, in whole or in part, to lasting adult inflammation since respiratory motor plasticity was restored with an adult anti-inflammatory treatment (Hocker et al., 2019). Surprisingly, neonatal inflammation did not increase adult inflammatory gene expression in homogenates from respiratory control regions (medulla or cervical spinal cord) nor was adult microglia density or morphology qualitatively changed within these regions (Hocker et al., 2019). Microglia, however, comprise only a small portion of cells within homogenates (reviewed in Shabab et al., 2017). Thus, specific or isolated changes to adult microglia after neonatal inflammation were likely diluted within homogenate samples, highlighting a need for the specific, quantitative assessment of adult microglia in respiratory control regions after neonatal inflammation. Therefore, the present study was designed to specifically determine the lasting effects of neonatal inflammation on adult microglia within key respiratory control regions, the

medulla and ventral cervical spinal cord, thereby advancing our understanding of the inflammatory mechanisms underlying the lasting impact of neonatal inflammation on adult respiratory control.

While neonatal inflammation abolishes adult respiratory motor plasticity (Hocker et al., 2019), the broader impact of neonatal inflammation on adult neural control of breathing was not assessed. Since acute, adult inflammation abolishes respiratory motor plasticity (Hocker & Huxtable, 2018, 2019; Huxtable et al., 2011, 2013, 2015), increases eupneic breathing and blunts chemoreflexes (Huxtable et al., 2011), we hypothesized that lasting adult inflammation after neonatal inflammation would also impair adult eupneic breathing or chemoreflexes. Our previous work (Morrison et al., 2020) and others (Lorea-Hernández et al., 2016; MacFarlane et al., 2016) demonstrated a role for neonatal microglia in impairing respiratory control, but whether these effects last into adulthood has not been studied and is essential for developing a better understanding of adult ventilatory insufficiencies. Thus, we hypothesized that neonatal inflammation would activate adult microglia in respiratory control regions and disrupt adult male and female breathing behaviors.

Collectively, our findings here highlight sex- and region-specific roles for adult microglia after neonatal inflammation in impairing respiratory control. Neonatal inflammation increased the number of medullary microglia and TNFα gene expression in adult males, while neonatal inflammation only increased medullary microglial IL-6 gene expression and induced no changes in medullary microglia number in adult females. Further, increases in microglia number and inflammatory gene expression were specific to medullary and not ventral cervical spinal microglia, suggesting region-specific activation of medullary microglia likely contributes to abolishment of adult male and female respiratory motor plasticity after neonatal inflammation. In addition to abolished respiratory motor plasticity, neonatal inflammation also sex-specifically augmented

male HCVR and decreased female sighs during eupnea. Thus, despite loss of plasticity in both sexes, adult male and female microglia are differentially activated by neonatal inflammation and adult male and female breathing behaviors are distinctly impacted by neonatal inflammation. In conclusion, neonatal inflammation causes lasting sex- and region-dependent activation of adult microglia, likely contributing to lasting inflammation in adults after neonatal inflammation and sex-specific changes to adult breathing behaviors.

Methods

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Oregon and conformed to the policies of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Timed pregnant Sprague-Dawley dams (E17) were purchased in pairs from a commercial vendor (Envigo, colony 206, 202 or 231) and monitored daily until giving birth. Rats were housed under standard conditions (12:12h light/dark cycle) with food and water *ad libitum*.

Neonatal treatments

Details for neonatal treatments have been described previously (Hocker et al., 2019). In brief, postnatal day 4 (P4) neonates were stratified between a pair of dams to control for potential differences in environment or maternal care. Dams fostered an equal number of neonates. Neonatal inflammation was induced with lipopolysaccharide (LPS). Before P4 neonates were injected with LPS (1 mg/kg, i.p., 10 mg/mL stock solution, 0111:B4, Sigma-Aldrich) or an equivalent volume by weight of sterile saline (i.p.) as a vehicle control. The LPS stock solution was sonicated and diluted in sterile saline. Neonates were weaned at P21 and aged to adulthood (males >300g and females >250g). Separate cohorts of animals were used for microglia (12 dams total) and breathing assessments (6 dams total), with microglia assessed in adults after neonatal inflammation (males n=18; females n=16) and adults after neonatal saline (males n=18; females n=18), while breathing behaviors were measured in adults after neonatal inflammation (males n=9; females n=7) and adults after neonatal saline (males n=9; females n=7).

Adult microglia frequency and isolation

To investigate the impact of neonatal inflammation on microglia density (microglia frequency) and inflammatory gene expression, adult medullas (between the pontomedullary junction and C1), ventral cervical spinal cords (C3-C6), and cortical tissues were extracted and dissociated, as previously described (Crain et al., 2009; Huxtable et al., 2013b; Nikodemova & Watters, 2012). In brief, anesthetized adult rats were transcardially perfused with ice cold phosphate buffered saline (PBS: 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4) before adult medullas, ventral cervical spinal cords, and cortices were extracted. Tissues were mechanically dissociated with a razor blade, enzymatically dissociated with papain to break down the extracellular matrix (MP Biomedicals, 1:100), and incubated with DNAse (Worthington, 1:25 at 37ºC for 16 min) to digest DNA from lysed cells. Tissue was filtered with cell strainers (40µm) and myelin removed with centrifugation in percoll (23%, GE Healthcare in PBS, 1050xg at 4ºC for 15 min) to generate single-cell homogenates.

Microglia frequency

Single cell homogenates were incubated (4°C for 10 min) with antibodies for CD11b conjugated-PE (Miltenyi, 1:25) or CD11b conjugated-PE-Vio770 (Miltenyi Biotec, 130-123-288, 1:25) and CD45 conjugated-APC (R&D systems, FAB114A, 1:25) in 0.5% BSA, 2mM EDTA, PBS and incubated with DAPI to identify dead cells. Microglia density was assessed as a frequency (%$CD11b^{high}CD45^{low}$/homogenates) and measured in single-cell homogenates with flow cytometry.

Fluorescently labeled single-cell homogenates are identified using flow cytometry (10 min, Beckman-Coulter, Gallios©). Raw data were analyzed with Kaluza (version 2.1, Beckman-Coulter) by plotting side scatter height versus side scatter area and gating to encompass only single

cells and exclude non-single cell events, as described previously (Maciorowski et al., 2017; McKinnon, 2018; Nikodemova et al., 2015; Nikodemova & Watters, 2012). To identify only live cells, density plots of DAPI florescent intensity versus forward scatter area were gated to capture DAPI negative cells and exclude dead cells from further analyses. To identify microglia ($CD11b^{high}CD45^{low}$ cells) within single-cell homogenates, density plots of CD11b intensity versus CD45 intensity were gated with a Cartesian grid, as previously described (Crain et al., 2009; Huxtable et al., 2013b; Nikodemova & Watters, 2012). Microglia frequency was defined as the percentage of $CD11b^{high}CD45b^{low}$ cells within homogenates.

Microglia isolations

To assess adult microglia inflammatory gene expression, adult microglia were immunomagnetically isolated, as described previously (Crain et al., 2009; Huxtable et al., 2013; Nikodemova and Watters, 2012). In brief, single cell homogenates were dissociated, as described above. Microglia were immunomagnetically isolated by incubating homogenates with CD11b antibodies conjugated to a magnetic bead (microbead-conjugated CD11b, Miltenyi Biotec, 130-105-634, 1:25 dilution, 4°C for 10 min, 0.5% BSA, 2mM EDTA, PBS). Microglia were immunomagnetically separated with MS columns (Miltenyi Biotec) and stored in Tri-reagent (Sigma, MO, USA) at -80ºC for qRT-PCR.

Adult microglia inflammatory gene expression after neonatal inflammation was evaluated with qRT-PCR, as previously described (Crain et al., 2013; Hocker et al., 2019; Morrison et al., 2019; Nikodemova et al., 2016). In brief, total RNA was extracted using Tri-reagent (Sigma, MO, USA) with Glycoblue (Invitrogen, Carlsbad, CA, USA). Synthesis of cDNA was performed using MMLV reverse transcriptase (BioRad). Quantitative PCR was performed with Power SYBR

Green Solution (Applied Biosystems, Foster City, CA, USA) using a BIO-RAD CFX96© Real-time PCR qPCR Detection System. Inflammatory genes were analyzed in medullary and ventral cervical spinal microglia using the following primers:

IL-6	forward 5’	GTG GCT AAG GAC CAA GAC CA,
	reverse 5’	GGT TTG CCG AGT AGA CCT CA
IL-1β	forward 5’	CTG CAG ATG CAA TGG AAA GA,
	reverse 5’	TTG CTT CCA AGG CAG ACT TT
COX-2	forward 5’	TGT TCC AAC CCA TGT CAA AA,
	reverse 5’	CGT AGA ATC CAG TCC GGG TA
TNFα	forward 5’	TCC ATG GCC CAG ACC CTC ACA C
	reverse 5’	TCC GCT TGG TGG TTT GCT ACG
iNOS	forward 5’	AGG GAG TGT TGT TCC AGG TG,
	reverse 5’	TCT GCA GGA TGT CTT GAA CG
IL-18	forward 5’	TGG AGA CTT GGA ATC AGA CC,
	reverse 5’	GGC AAG CTA GAA AGT GTC CT
IFNα	forward 5’	CCT CAG CCT CTT CAC ATC AA,
	reverse 5’	TGT GGC TCA GGA CTC ATT TC
18s	forward 5’	CGG GTG CTC TTA GCT GAG TGT CCC G,
	reverse 5’	CTC GGG CCT GCT TTG AAC AC

Gene expression was normalized to 18s and relative gene expression levels were determined by the ΔΔCt method (Livak & Schmittgen, 2001), as in our previous publications (Hocker et al., 2019a; Hocker & Huxtable, 2019; Huxtable et al., 2013c, 2015a; Huxtable et al., 2018; Huxtable, Peterson, et al., 2018; Morrison et al., 2020). Comparisons were not made between genes since the relative expression between genes does not significantly advance our understanding of their role in impairing adult ventilatory control. Primer efficiency was assessed using standard curves, as previously reported (Crain et al., 2009).

Whole-body plethysmography experiments

Ventilation was measured in freely moving, unanesthetized adult males and females using 2L custom built whole-body plethysmography chambers, similar to those described previously (Hodges et al., 2002, 2013; Kaplan et al., 2016; Mouradian et al., 2012). Rectal temperatures were assessed pre- and post-plethysmography. Prior to each experiment, the chambers were calibrated by pipetting 1mL of air into the chamber at least 20 times. The plethysmography chamber temperatures and humidity levels were measured throughout experiments (Vernier© and Logger lite© software). Mass flow controllers (Alicat Scientific©, Tucson, AZ) controlled the flow rate and composition of oxygen, nitrogen, and carbon dioxide into each chamber at 4L/min. Breathing was measured as changes in pressure using a spirometry pod (ADInstruments®, Colorado Springs, CO) and analyzed with LabChart software (PowerLab System, ADInstruments®, LabChart v8.1). The plethysmography recordings occurred between 11:00 am and 9:00 pm. To assess hypercapnic ventilatory response (HCVR), inflow gas was switched to 5% CO_2 (21% O_2, balance N_2, 10 min). After a 20-minute recovery period in normoxia (21% O_2, balance N_2), the hypoxic ventilatory response (HVR) was assessed during inflow gas of 10% oxygen (balanced N_2, 10 min), followed by normoxia (21% O_2, balance N_2, 15 min).

Plethysmography analyses

Ventilation was assessed as described previously for neonates (Hocker et al., 2021). In brief, frequency (breaths/minute) and tidal volume (V_T, mL/100g) were analyzed from segments of continuous breathing (>30 sec/segment) without sniffing, sighs or post-sigh apneas in LabChart (v8, AD Instruments®). Baseline breathing was determined by averaging 10 minutes of eupneic breathing during normoxia (30 min, 21% O_2, balance N_2) prior to chemoreflex challenges. Body temperatures were estimated during eupnea, hypercapnia, and hypoxia by generating a trend line

from the pre- and post-rectal temperatures. Chamber temperatures and humidity were extrapolated throughout the experiment by correlating the time of each breath with concurrent chamber temperature and humidity at that time. Tidal volumes were calculated using average chamber pressure calibrations (1mL), body temperatures, chamber temperatures, humidity and barometric pressures (Drorbaugh & Fenn, 1955). Minute ventilation (V_E, mL/min/kg) was calculated as the product of frequency and V_T. The custom-built plethysmograph is most effective for assessing respiratory frequency and is not optimized for absolute quantification of tidal volume. Thus, tidal volume and minute ventilation are reported as percent changes from baseline within each treatment during HCVR and HVR. The peak HCVR (the last 2 minutes) and phase I HVR (peak hypoxic response, minutes 4-6 in hypoxia) were assessed as the rolling averages in 2 min bins for frequency, V_T, and V_E (Hodges et al., 2002, 2013; Kaplan et al., 2016; Mouradian Jr et al., 2012). Phase II HVR (hypoxic ventilatory decline) was assessed as the average frequency, V_T, and V_E during last 2 min of the hypoxic stimulus (Hodges et al., 2002, 2013; Kaplan et al., 2016; Mouradian Jr et al., 2012).

Statistical analysis

GraphPad Prism (version 10.2) was used for statistical analyses. A three-way ANOVA was used to assess the effect of neonatal treatment (saline, LPS), CNS region (medulla, cervical spinal cord, cortex), and sex (male, female) on microglia frequency. Within each gene, a two-way ANOVA was used to assess the impact of neonatal treatment (saline, LPS) and sex (male, female) on microglia inflammatory gene expression. Eupneic breathing frequency, tidal volume and minute ventilation were assessed with two-way ANOVAs. To determine whether ventilation increased in

response to respiratory challenges (hypercapnia or hypoxia), three-way RM ANOVAs were used to assess the impact of treatment (saline, LPS), sex (male, female) and time (0-10 mins during hypercapnia or hypoxia) from baseline (pre-hypercapnia or hypoxia challenge). To determine whether neonatal and adult treatments impacted ventilation throughout hypercapnia or hypoxia, neonatal treatment and sex were collapsed into one factor and assessed with a two-way ANOVA at each time point during the response. Bonferroni *post hoc* tests were used to limit type I errors. For all tests, $p<0.05$ was considered significant and all data are expressed as mean±SD.

Results

Neonatal inflammation region- and sex-dependently increased adult microglia.

We previously demonstrated lasting adult inflammatory signal(s) contribute to abolishment of respiratory motor plasticity (Hocker et al., 2019), yet the source and identity of the lasting inflammatory signal(s) has not been established. Although we previously identified neonatal inflammation did not change adult microglial density or morphology in the preBötzinger Complex (preBötC) or around phrenic motor neurons in the spinal cord (Hocker et al., 2019), our previous qualitative immunohistochemistry analyses were restricted to small respiratory regions and respiratory circuitry is distributed throughout the medulla and cervical spinal cord (Del Negro et al., 2018). Thus, there is a need for specific and quantitative assessment of adult microglia after neonatal inflammation throughout respiratory control regions. Here, we used flow cytometry to assess the impact of neonatal inflammation on adult microglia number in the medulla and ventral cervical spinal cord. Microglia number is measured as adult microglia frequency and defined as the percentage of microglial cells within homogenate samples of dissociated cells (%$CD11b^{high}CD45^{low}$/homogenates; Nikodemova & Watters, 2012) isolated from adult respiratory control regions (medulla, location of respiratory rhythm and pattern generating regions; or ventral cervical spinal cord, location of the phrenic motoneurons) and the forebrain (cortex, as a positive control). Adults were treated as neonates with either neonatal inflammation (1mg/kg lipopolysaccharide LPS, i.p., P4) or neonatal saline (vehicle control), as described previously (Hocker et al., 2019). Neonatal treatment (LPS or saline, $p<0.0001$), region (medulla, ventral cervical spinal cord, or cortex, $p<0.0001$) and the interactions between neonatal treatment-region ($p<0.0001$) and region-sex ($p=0.047$) had significant main effects on adult microglia frequency.

However, neither sex (p=0.38), the interactions between neonatal treatment-sex (p=0.48), nor neonatal treatment-sex-region (p=0.82) had significant main effects on adult microglia frequency.

In assessing pairwise differences, neonatal inflammation region- and sex-dependently increased the number of adult microglia, as supported by the main effects. Within the medulla, microglia frequency increased in adult males after neonatal inflammation (23±3% microglia) compared to adult males after neonatal saline (12±2% microglia, p=0.0027, **Fig. 1**), demonstrating neonatal inflammation increased adult male medullary microglia number. In contrast, medullary microglia frequency was similar in adult females after neonatal inflammation (17±3% microglia) and adult females after neonatal saline (11±3% microglia, p=0.12), demonstrating female medullary microglia number was unaffected by neonatal inflammation. Between sexes, medullary microglia frequency increased in adult males after neonatal inflammation compared to adult females after neonatal saline (p=0.002), providing further support that neonatal inflammation sex-dependently increased medullary microglia number. However, medullary microglia frequency was similar between adult males and females after neonatal inflammation (p=0.3), adult males and females after neonatal saline (p=0.9), and adult females after neonatal inflammation and adult males after neonatal saline (p=0.15). Thus, these data provide the first quantitative evidence that neonatal inflammation causes lasting activation of adult male medullary microglia.

In the ventral cervical spinal cord, there were no pairwise differences in microglia frequency (adult males after neonatal inflammation=13±5% microglia, adult males after neonatal saline=11±3% microglia; adult females after neonatal inflammation=16±6% microglia, adult females after neonatal saline=13±5% microglia, p>0.9). Thus, the effects of neonatal inflammation on adult microglia number are region specific, with no significant changes in the ventral cervical spinal cord.

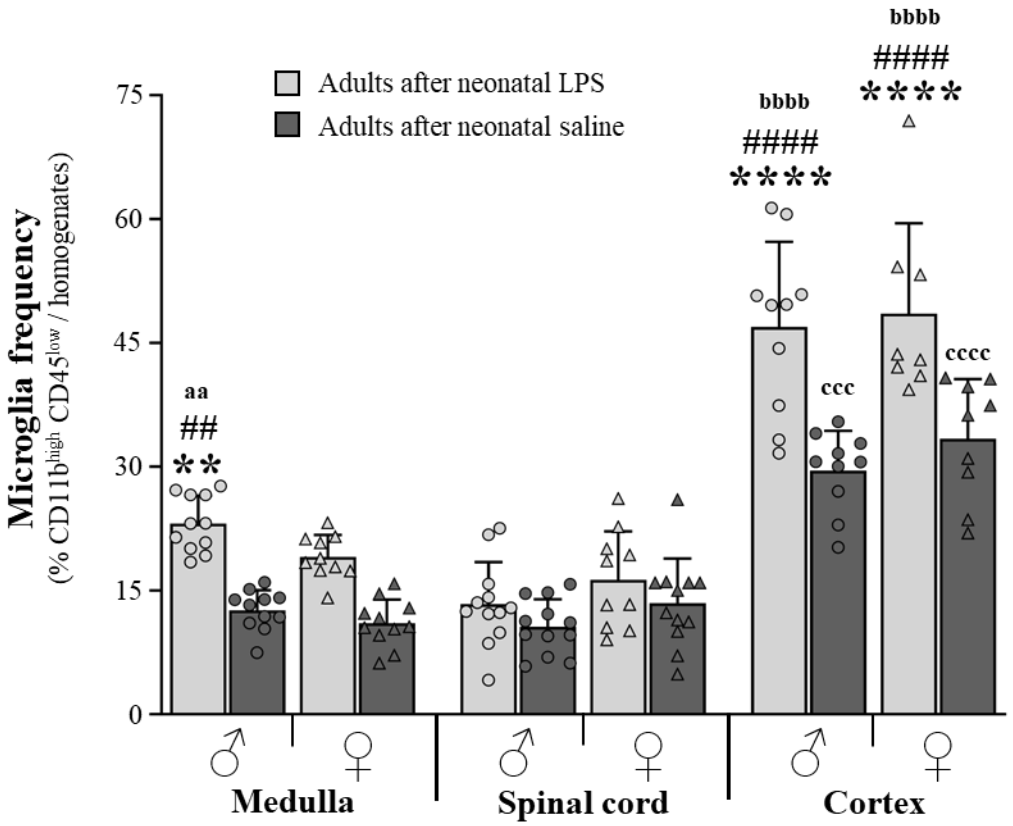


Figure 1. Neonatal inflammation region- and sex-dependently increased adult microglia. In the medulla, microglia frequency (%CD11b^{high}CD45^{low}/homogenates) increased in adult males (circles, ♂) after neonatal inflammation (light gray bars) from adult males and females after neonatal saline (dark gray bars), while microglia frequency was unchanged in adult females (triangles, ♀). Adult male medullary microglia frequency after neonatal inflammation was also increased compared to all spinal microglia, except adult females after neonatal inflammation. In the spinal cord, adult microglia frequency was unaffected by neonatal inflammation. In the cortex, microglia frequency increased in all adults after neonatal inflammation compared to all adults after neonatal saline, regardless of sex. Cortical microglia frequency in adults after neonatal inflammation also increased compared to all medullary and spinal microglia. In adults after neonatal saline, cortical microglia frequency increased compared to adult medullary and spinal microglia, except adult male medullary microglia after neonatal inflammation. (**p<0.001, ****p<0.00001 different from adults after neonatal saline within region and sex; ## p<0.001, #### p<0.00001 different from adults after neonatal saline of the opposite sex; aa p<0.01 different from all adult male spinal microglia and adult female spinal microglia after neonatal saline; bbbb p<0.00001 different from all adult medullary and spinal microglia; ccc p<0.0001, cccc p<0.00001 different from adult medullary microglia after neonatal saline, adult female medullary microglia after neonatal inflammation and all adult spinal microglia; Three-way ANOVA; Bonferroni post-hoc test.)

Microglia frequencies in the cortex were assessed as a positive control since neonatal inflammation is known to increase adult cortical microglia density (Bilbo, 2010; Bilbo et al., 2005; Bilbo & Schwarz, 2012; Bland, Beckley, Watkins, et al., 2010; Bland, Beckley, Young, et al.,

2010; Cao et al., 2021; Mallard et al., 2019; Williamson et al., 2011). As expected, microglia frequency in the cortex increased in adult males after neonatal inflammation (47±10% microglia) compared to adult males after neonatal saline (30±5% microglia, $p<0.0001$) and similarly increased in adult females after neonatal inflammation (49±11% microglia) compared to adult females after neonatal saline (33±7% microglia, $p<0.0001$). Between sexes, cortical microglia frequency was similar between adult males and females after neonatal inflammation ($p=0.9$) and adult males and females after neonatal saline ($p=0.9$). As expected, cortical microglia frequency increased in adult males after neonatal inflammation compared to adult females after neonatal saline ($p<0.0001$) and increased in adult females after neonatal inflammation compared to adult males after neonatal saline ($p<0.0001$). Thus, unlike the medulla or ventral cervical spinal cord, neonatal inflammation increased adult cortical microglia number in both sexes.

When comparing between regions, adult microglia frequency within respiratory control regions (medullary and ventral cervical spinal cord) was similar in healthy adults (adults after neonatal saline); however, neonatal inflammation caused lasting regional differences in adult microglia number in respiratory control regions. Microglia frequency increased in adult males after neonatal inflammation in the medulla compared to adult male ventral cervical spinal microglia from both treatment groups (adult males after neonatal inflammation $p=0.006$; adult males after neonatal saline $p=0.001$) and spinal microglia in adult females after neonatal saline ($p=0.005$), but was similar to spinal microglia in adult females after neonatal inflammation ($p=0.075$). In the cortex, neonatal inflammation increased adult male cortical microglia frequency compared to all medullary microglia (adult males after neonatal inflammation $p=0.0003$; adult males after neonatal saline $p<0.0001$; adult females after neonatal inflammation $p=0.0005$; adult females after neonatal saline $p=0.0002$) and ventral cervical spinal microglia (adult males after neonatal inflammation

p=0.0003; adult males after neonatal saline p=0.0002; adult females after neonatal inflammation p<0.0001; adult females after neonatal saline p<0.0001). In adult males after neonatal saline, cortical microglia frequency increased compared to medullary microglia in adults after neonatal saline (male p=0.002, females p=0.014), medullary microglia in adult females after neonatal inflammation (p=0.034), and all ventral cervical spinal microglia (adult males after neonatal inflammation p=0.0018; adult males after neonatal saline p=0.0016; adult females after neonatal inflammation p=0.0015; adult females after neonatal saline p=0.0031), but were similar to adult male medullary microglia after neonatal inflammation (p=0.09). Similarly, cortical microglia frequency in adult females after neonatal saline increased compared to medullary microglia in adults after neonatal saline (males p=0.0012; females p=0.0022), medullary microglia in adult females after neonatal inflammation (p=0.0043), and all ventral cervical spinal microglia (adult males after neonatal inflammation p=0.0032) adult males after neonatal saline p=0.008; adult females after neonatal inflammation p=0.0023; adult females after neonatal saline p=0.0033), but was similar to medullary microglia in adult males after neonatal inflammation (p=0.07). Thus, neonatal inflammation increases microglia number region-dependently, whereby cortical microglia are more abundant than microglia within the respiratory control network, but neonatal inflammation only increased adult male microglia number within the medulla.

Neonatal inflammation sex-dependently increased medullary microglial inflammatory gene expression.

To identify potential microglial inflammatory mechanisms contributing to lasting inflammatory signaling in adults after neonatal inflammation, the long-term impact of neonatal inflammation on adult microglial inflammatory gene expression was assessed by

immunomagnetically isolating medullary and ventral cervical spinal microglia in adult males and females after neonatal inflammation or neonatal saline. In adult medullary microglia**,** neonatal treatment ($p=0.056$) trended toward significantly affecting microglial TNFα expression, and there was a significant main effect of sex ($p=0.048$) and the interaction between neonatal treatment-sex ($p=0.045$). When assessing pairwise differences, medullary TNFα gene expression increased in adult males after neonatal inflammation compared to adult males after neonatal saline ($p=0.048$, **Fig. 2A**), but was unchanged in adult females after neonatal inflammation compared to adult females after neonatal saline ($p=0.8$), demonstrating neonatal inflammation increased adult male, but not female, medullary microglial TNFα gene expression. Between sexes, medullary microglial TNFα gene expression was similar between adult males and females after neonatal saline ($p=0.87$), but was increased in adult males after neonatal inflammation compared to adult females after neonatal inflammation ($p=0.03$). Medullary microglial TNFα gene expression increased in adult males after neonatal inflammation compared to adult females after neonatal saline ($p=0.046$), but was similar in adult females after neonatal inflammation compared to adult males after neonatal saline ($p=0.9$), demonstrating neonatal inflammation sex-dependently increased adult male, but not female, medullary microglial TNFα gene expression. Collectively, these data provide further support for sex-specific activation of adult male medullary microglia after neonatal inflammation.

Neonatal treatment ($p=0.0002$), sex ($p=0.025$) and the interaction between neonatal treatment-sex ($p<0.0001$) had significant main effects on adult medullary microglial IL-6 gene expression. While medullary microglial IL-6 gene expression was unchanged in adult males after neonatal inflammation compared to adult males after neonatal saline ($p=0.9$), IL-6 gene expression increased in adult females after neonatal inflammation compared to adult females after neonatal saline ($p<0.0001$), demonstrating neonatal inflammation increased adult female, but not male,

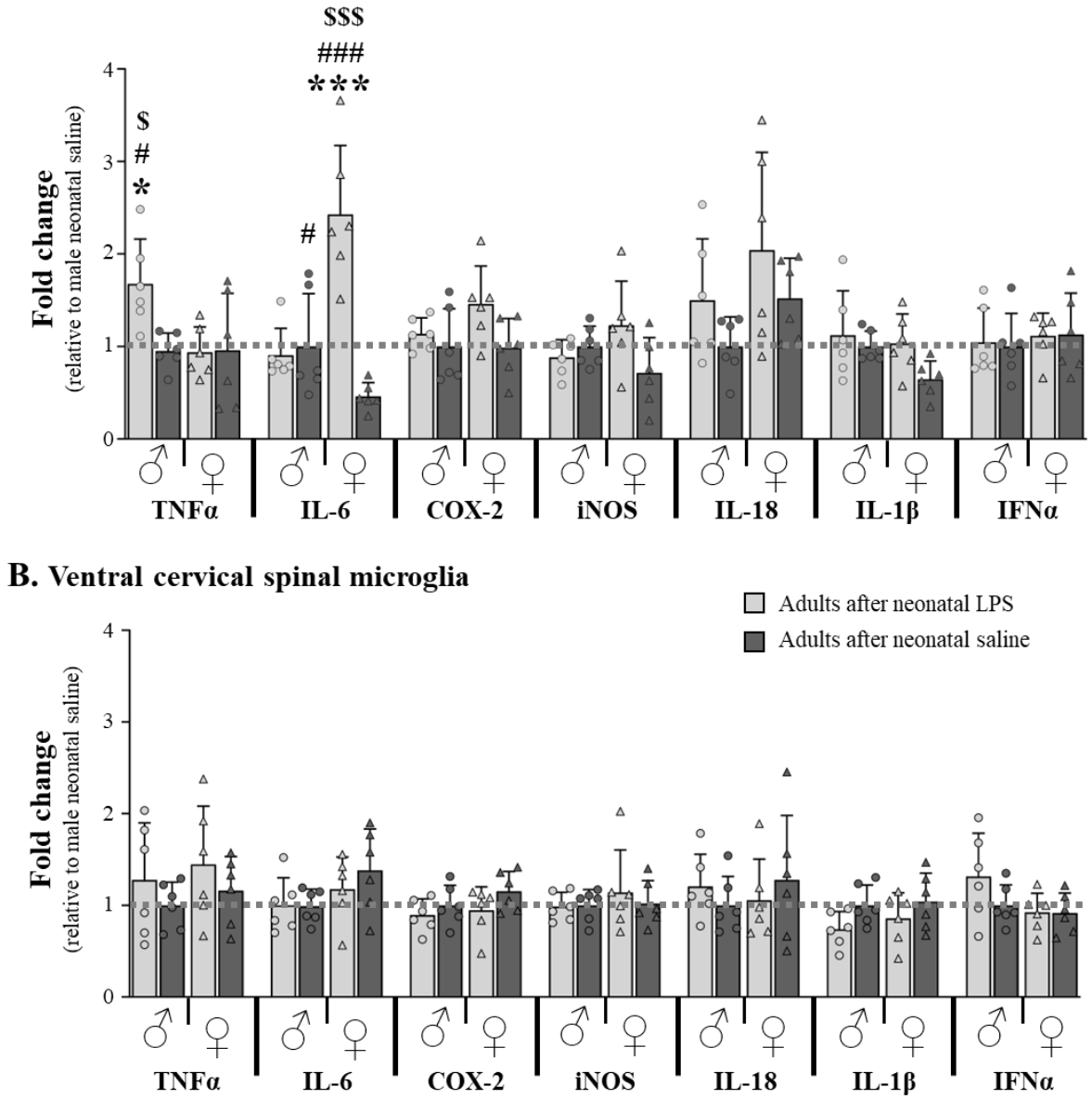


Figure 2. Neonatal inflammation region- and sex-dependently increased microglia inflammatory gene expression. (**A.**) In medullary microglia, TNFα gene expression was increased in adult males (circles, ♂) after neonatal inflammation (light gray bars) from neonatal saline (dark gray bars) and females after neonatal inflammation, but was unchanged between treatment groups in adult females (triangles, ♀). Microglial IL-6 gene expression in adult males was unchanged between treatment groups, but increased in adult males after neonatal saline compared to adult females after neonatal saline. Adult female microglial IL-6 gene expression after neonatal inflammation was increased from all other groups. Adult male and female microglial COX-2, iNOS, IL-18, IL-1β, IFNα medullary gene expressions were unchanged after neonatal inflammation. In ventral cervical spinal microglia (**B.**), all genes (TNFα, IL-6, COX-2, iNOS, IL-18, IL-1β, IFNα) were unchanged in adults after neonatal inflammation or between sexes. (* $p<0.05$, *** $p<0.0001$ different from adults after neonatal saline within a gene and sex; # $p<0.05$, ### $p<0.0001$ different from adults after neonatal saline of the opposite sex within a gene; $ $p<0.05$, $$$ $p<0.0001$ different from adults after neonatal inflammation of the opposite sex within a gene; Two-way ANOVAs, Bonferroni post-hoc test.)

medullary microglial IL-6 gene expression. Between sexes, medullary microglial IL-6 gene

expression increased in adult females after neonatal inflammation compared to adult males after neonatal inflammation ($p=0.0002$), demonstrating neonatal inflammation sex-dependently increased medullary microglial IL-6 gene expression. Medullary microglial IL-6 gene expression was higher in adult males after neonatal saline compared to adult females after neonatal saline ($p=0.049$), supporting sex differences in healthy adult medullary microglial IL-6 gene expression. Medullary microglial IL-6 gene expression was, however, similar between adult males after neonatal inflammation and adult females after neonatal saline ($p=0.69$), but increased in adult females after neonatal inflammation compared to adult males after neonatal saline ($p=0.0004$). Thus, neonatal inflammation increased medullary microglial TNFα gene expression in males and IL-6 gene expression in females, supporting sex-dependent increases in adult medullary microglial inflammatory gene expression after neonatal inflammation.

In contrast to TNFα and IL-6 gene expression, neonatal treatment ($p=0.041$) had a significant effect on medullary microglial COX-2 gene expression, but there was no main effect of sex ($p=0.28$) nor the interaction between neonatal treatment-sex ($p=0.24$) and there were no pairwise differences ($p>0.16$). Neither neonatal treatment ($p=0.17$) nor sex ($p=0.83$) significantly influenced adult medullary microglial iNOS expression, but the interaction between neonatal treatment-sex ($p=0.033$) had a significant a main effect on iNOS expression. However, there were no pairwise differences in adult medullary microglial iNOS expression ($p>0.9$). Medullary microglial IL-18, IL-1β and IFNα gene expression were not significantly affected by neonatal treatment (IL-18 $p=0.081$, IL-1β $p=0.063$; IFNα $p=0.92$), sex (IL-18 $p=0.07$, IL-1β $p=0.1$; IFNα $p=0.51$) nor the interaction between neonatal treatment and sex (IL-18 $p=0.1$, IL-1β $p=0.32$; IFNα $p=0.85$) and there were no pairwise differences ($p>0.9$). Thus, neonatal inflammation increased

specific medullary microglial inflammatory genes, but did not induce generalized inflammation associated with common inflammatory genes.

In contrast to medullary microglia, neonatal inflammation did not significantly change adult ventral cervical spinal microglial gene expression in either sex. Neonatal treatment, sex, nor the interaction between neonatal treatment-sex significantly impacted ventral cervical spinal microglial gene expression in any genes assessed ($p>0.9$). Accordingly, there were no pairwise differences in any ventral cervical spinal microglial inflammatory genes ($p>0.9$; **Fig. 2B**). Overall, neonatal inflammation sex-dependently activates adult medullary microglia, but not microglia in the ventral cervical spinal cord. Since increased inflammatory gene expression in microglia is associated with activated microglia, medullary microglia are likely a source of lasting adult inflammation in respiratory control regions after neonatal inflammation.

Neonatal inflammation did not impair adult eupneic breathing, but decreased adult female sighs during eupnea.

To investigate the long-term effects of neonatal inflammation on adult breathing behaviors beyond respiratory motor plasticity, eupneic breathing (breathing at rest) and chemoreflexes were measured with plethysmography in freely moving, unanesthetized adult males and females after neonatal inflammation or neonatal saline. Neither neonatal treatment ($p=0.80$), sex ($p=0.57$), nor the interaction between treatment-sex ($p=0.97$) had significant main effects on adult eupneic breathing frequency (breaths/min) and there were no pairwise differences (adult males after neonatal inflammation=91±6 breaths/min, adult males after neonatal saline=92±11 breaths/min; adult females after neonatal inflammation=89±13 breaths/min, adult females after neonatal

saline=90±10 breaths/min, p>0.9, **Fig. 3B**). Thus, contrary to our hypothesis, neonatal inflammation did not impair adult eupneic breathing frequency in either sex.

In contrast to adult eupneic breathing frequency, sex (p<0.0001) had a significant main effect on adult eupneic tidal volume (V_T, mL/100g). However, neither neonatal treatment (p=0.18) nor the interaction between neonatal treatment-sex (p=0.65) had a significant main effect on adult eupneic V_T. Adult eupneic V_T was unchanged by neonatal inflammation in adult males (adult males after neonatal inflammation=0.57±0.07 mL/100g, adult males after neonatal saline=0.65±0.05 mL/100g p=0.99, **Fig. 3C**) or adult females (adult females after neonatal inflammation=0.80±0.19 mL/100g, adult females after neonatal saline=0.84±0.13 mL/100g, p=0.99). However, eupneic V_T in females after neonatal inflammation was higher than adult males after neonatal inflammation (p=0.0035), but was not significantly different from adult males after neonatal saline (p=0.11). Additionally, eupneic V_T was higher in females after neonatal saline compared to males, regardless of neonatal treatment (adult males after neonatal inflammation p=0.001; adult males after neonatal saline p=0.032), demonstrating sex differences in adult eupneic V_T. Despite no impairments in adult eupneic V_T, adult females had higher eupneic V_T than males.

Due to sex differences in adult eupneic V_T, adult eupneic minute ventilation (V_E, mL/100g/min) was significantly affected by sex (p<0.0001) and neonatal treatment (p=0.029), but was not affected by the interaction between treatment-sex (p=0.59). Contrary to our hypothesis, adult eupneic V_E was unchanged by neonatal inflammation in adult males (adult males after neonatal inflammation=50±4 mL/100g/min; adult males after neonatal saline=59±9 mL/100g/min, p=0.24) or females (adult females after neonatal inflammation=68±9 mL/100g/min, adult females after neonatal saline=73±12 mL/100g/min, p=0.9, **Fig. 3D**). Between sexes, eupneic V_E increased

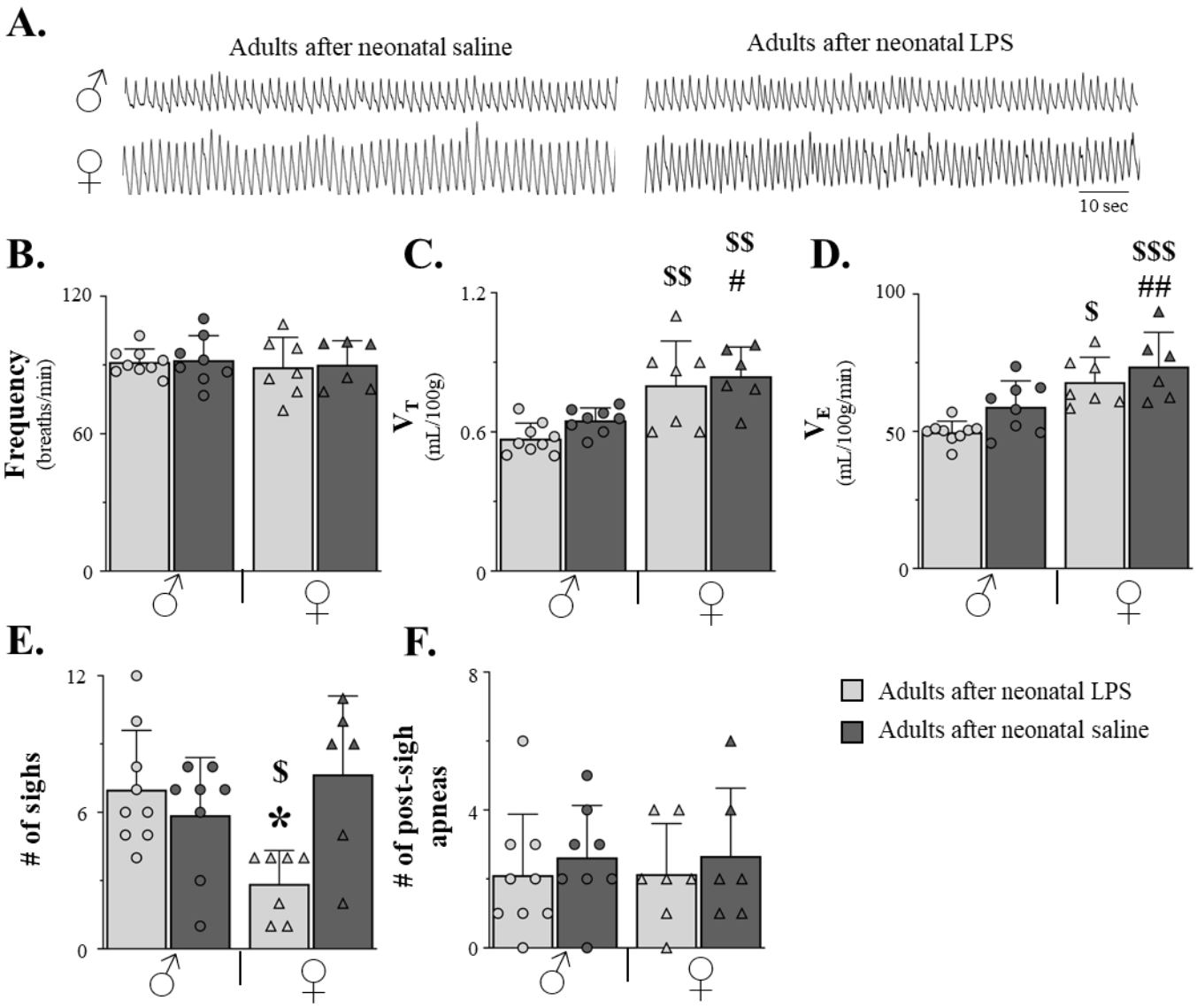


Figure 3. Adult eupneic breathing was not impaired by neonatal inflammation, but neonatal inflammation decreased adult female sighs during eupnea. Representative plethysmography traces (**A.**) showed adult eupnea was unaffected by neonatal inflammation in either sex, yet adult female eupneic breathing was increased compared to males, regardless of treatment. Adult male (circle, ♂) and female (triangle, ♀) eupneic frequencies (breaths/min; **B.**) and tidal volumes (V_T, mL/100g; **C.**) were unchanged within a sex by neonatal inflammation (light gray bars). However, V_T in adult females after neonatal inflammation increased compared to adult males after neonatal inflammation, while V_T in adult females after neonatal saline (dark gray bars) increased compared to males, regardless of treatment. Adult male minute ventilation (V_E, mL/100g/min; **D.**) was unaffected by neonatal inflammation. Due to higher female V_T, V_E in adult females after neonatal inflammation increased compared to adult males after neonatal inflammation, while V_E in adult females after neonatal saline (dark gray bars) increased compared to males, regardless of treatment. The number of sighs (**E.**) in adult males were unchanged by neonatal inflammation, but decreased in adult females after neonatal inflammation compared to adult females after neonatal saline and adult males after neonatal inflammation. Adult post-sigh apnea number (**F.**) was unchanged by neonatal inflammation in either sex. (* $p<0.05$ different from adults after neonatal saline within sex; # $p<0.05$, ## $p<0.001$ different from adults after neonatal saline of the opposite sex; $ $p<0.05$, $$ $p<0.001$ $$$ $p<0.0001$ different from adults after neonatal inflammation of the opposite sex; Two-way ANOVAs; Bonferroni post-hoc test.)

in adult females after neonatal inflammation compared to adult males after neonatal inflammation ($p=0.021$), but was similar to adult males after neonatal saline ($p=0.36$). However, eupneic V_E

increased in adult females after neonatal saline compared to adult males, regardless of treatment (adult males after neonatal inflammation p=0.0001; adult males after neonatal saline p=0.0029), further supporting sex differences in eupneic V_E in healthy adults. In summary, adult females have increased eupneic breathing, driven by increases in V_T, compared to adult males. However, contrary to our hypothesis, adult eupneic breathing was not impaired by neonatal inflammation in either sex.

Multiple respiratory-related behaviors occur during eupnea, including spontaneous apneas, sighs, and post-sigh apneas, whereby changes in the incidence of these behaviors are indicators of respiratory health or disease (Brown & Bates, 2000; Fleming et al., 1984; Hoch et al., 1998). Thus, we measured spontaneous apneas, sighs, and post-sigh apneas in adults after neonatal inflammation or neonatal saline. Spontaneous apneas are defined as cessation of breathing for more than 2.5 breaths absent of a sigh (Fleming et al., 1984; Hoch et al., 1998) and rarely occur in healthy adult eupneic breathing, since increases in spontaneous apneas threaten homeostatic maintenance of blood gases (reviewed in Ishikawa & Oks, 2021). Neonatal inflammation did not affect spontaneous apneas, as spontaneous apneas were not observed in any treatment group or sex during eupnea.

Although sighs and post-sigh apneas are important breathing behaviors supporting adult lung health, little is known about how neonatal inflammation impacts these adult breathing behaviors. Sighs contribute to healthy lung function by protecting against atelectasis, the gradual collapse of small alveoli (reviewed in Ramirez et al., 2022; Severs et al., 2022; Yackle et al., 2017), and are identified as a breath with more than twice the average tidal volume (Li et al., 2016; Ramirez, 2014; Ramirez et al., 2022; Severs et al., 2022). Post-sigh apneas are a cessation of breathing for more than 2.5 breaths immediately after a sigh (reviewed in Dempsey, 2019;

Dempsey et al., 2010) and likely represent a refractory period after a sigh (Alsahafi et al., 2015; Baertsch et al., 2018; Guerrier et al., 2015; Kottick et al., 2017; Ramirez et al., 2022; Zavala-Tecuapetla et al., 2014). Adult eupneic sighs were significantly affected by the interaction between neonatal treatment-sex ($p=0.0043$), but not neonatal treatment ($p=0.063$) nor sex ($p=0.23$) alone. The number of sighs during eupnea were unchanged in adult males after neonatal inflammation (7±3 sighs) compared to adult males after neonatal saline (6±3 sighs, $p=0.9$, **Fig. 3E**). While in adult females, the incidence of sighs during eupnea decreased after neonatal inflammation (3±1 sighs) compared to adult females after neonatal saline (8±3 sighs, $p=0.014$), demonstrating neonatal inflammation decreased sighs in adult females during eupnea. Between sexes, sighs during eupnea were decreased in adult females after neonatal inflammation compared to males after neonatal inflammation ($p=0.021$), but similar between adult males and females after neonatal saline ($p=0.9$). Between treatment group and sex, sighs during eupnea were similar between adult males after neonatal inflammation and adult females after neonatal saline ($p=0.99$), and between adult females after neonatal inflammation and adult males after neonatal saline ($p=0.19$). In contrast to sighs, neither neonatal treatment ($p=0.41$), sex ($p=0.95$), nor the interaction between neonatal treatment and sex ($p=0.98$) had a significant main effect on the number of post-sigh apneas during eupnea and there were no pairwise differences (adult males after neonatal inflammation=2±2 post-sigh apneas, adult males after neonatal saline=3±2 post-sigh apneas, adult females after neonatal inflammation=2±1 post-sigh apneas, adult females after neonatal saline=3±2 post-sigh apneas; $p>0.9$, **Fig. 3F**). Thus, neonatal inflammation decreased adult female, but not male, sighs during eupnea, demonstrating neonatal inflammation has sex-specific impairments on sigh behavior.

Neonatal inflammation augmented hypercapnic ventilatory responses in adult males, but not females.

The consequences of neonatal inflammation on adult hypercapnic chemoreflexes were assessed in adult males and females after neonatal inflammation or neonatal saline. Time ($p<0.0001$), neonatal treatment ($p=0.0028$), and the interactions between neonatal treatment-sex ($p=0.0006$), and neonatal treatment-sex-time ($p=0.040$) had main effects on V_E throughout the adult hypercapnic ventilatory response (HCVR). However, neither sex ($p=0.14$) nor the interactions between sex-time ($p=0.26$) and neonatal treatment-time ($p=0.17$) had main effects on adult V_E during HCVR. Adult V_E increased from baseline at each timepoint during HCVR in all treatment groups ($p<0.0004$, **Fig. 4A & B**), demonstrating all adults had stereotypical increases in ventilation during hypercapnia (reviewed in Nattie & Li, 2012).

Comparing breathing between neonatal treatment-sex over time during HCVR showed time ($p=0.0001$), neonatal treatment-sex ($p=0.0001$) and the interaction between neonatal treatment-sex-time ($p=0.020$) had main effects on adult V_E during HCVR. Accordingly, pairwise differences support V_E during HCVR increased at all timepoints in adult males after neonatal inflammation compared to adult males after neonatal saline (0-2mins, $p=0.018$; 2-4mins, $p=0.0073$; 4-6mins, $p=0.0016$; 6-8mins, $p=0.0086$; 8-10mins, $p=0.0064$; **Fig. 4A**). However, V_E during hypercapnia was unchanged at any time point in females after neonatal inflammation compared to females after neonatal saline ($p=0.99$ at all timepoints). Between sexes, V_E during hypercapnia was increased from 4-8mins in adult males after neonatal inflammation and adult females after neonatal inflammation, but was similar at all other timepoints (0-2mins, $p=0.38$; 2-4mins, $p=0.65$; 4-6mins, $p=0.014$; 6-8mins, $p=0.042$; 8-10mins, $p=0.13$). V_E during hypercapnia was also similar between adult males and females after neonatal saline at all timepoints (0-2mins,

p=0.99; 2-4mins, p=0.38; 4-6mins, p=0.99; 6-8mins, p=0.99; 8-10mins, p=0.30). Between treatments and sex, V_E during hypercapnia was similar between males after neonatal inflammation and females after neonatal saline at all timepoints, despite a trend for augmented V_E from 0-2mins (0-2mins, p=0.074; 2-4mins, p=0.99; 4-6mins, p=0.46; 6-8mins, p=0.20; 8-10mins, p=0.23). Similarly, V_E during hypercapnia was similar between females after neonatal inflammation and adult males after neonatal saline at all timepoints (0-2mins, p=0.99; 2-4mins, p=0.35; 4-6mins, p=0.99; 6-8mins, p=0.99; 8-10mins, p=0.99). Thus, HCVR was not impaired in adults after neonatal inflammation in either sex, but neonatal inflammation augmented adult male HCVR.

Focusing on the peak hypercapnic response, sex had a significant main effect on adult breathing during the last 2 minutes of the HCVR (p=0.012, gray box in **Fig. 4A**). However, neither neonatal treatment (p=0.13) nor the interaction between neonatal treatment-sex (p=0.76) had a significant main effect on adult breathing frequency during the peak HCVR. Breathing frequencies during peak HCVR were similar in adult males after neonatal inflammation (46±14% change from baseline) compared to adult males after neonatal saline (54±18% change from baseline, p=0.9, **Fig. 4C**) and between adult females after neonatal inflammation (27±15% change from baseline) and adult females after neonatal saline (39±22% change from baseline, p=0.9). When comparing between sexes, breathing frequencies during peak HCVR were similar between adult males and females after neonatal inflammation (p=0.21) and between adult males and females after neonatal saline (p=0.68). Between neonatal treatments and sex, breathing frequencies during peak HCVR were also similar between adult males after neonatal inflammation and adult females after neonatal saline (p=0.9), but lower in adult females after neonatal inflammation compared to adult males after saline (p=0.032), suggesting the combination of neonatal inflammation and sex may have effects on adult female breathing frequency during HCVR.

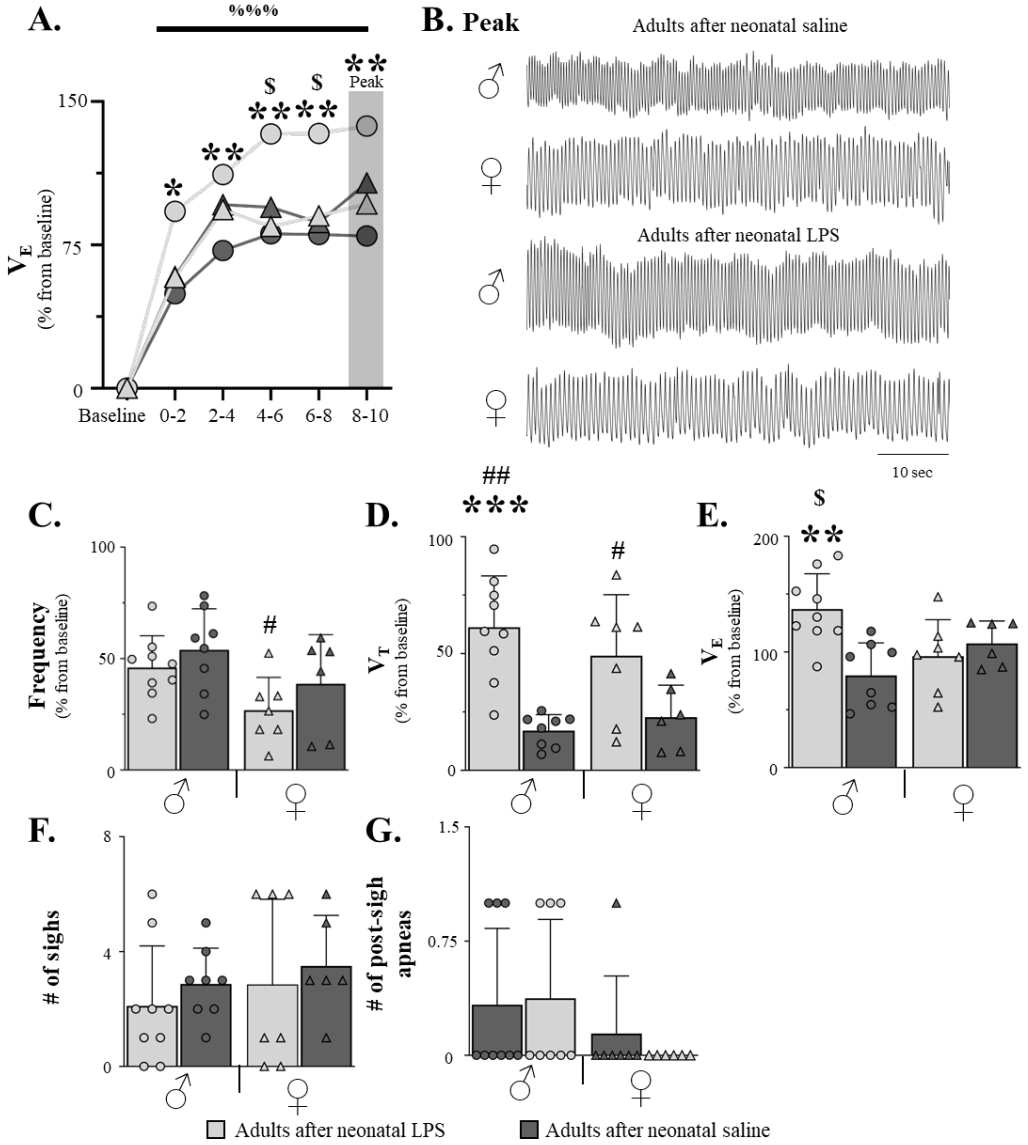


Figure 4. Neonatal inflammation augmented adult male, but not female, hypercapnic ventilatory responses. Average adult minute ventilation (V_E) over time (**A.**) increased at all timepoints in all treatment groups. V_E was augmented in adult males (circles, ♂) after neonatal inflammation (light gray line) compared to adult males after neonatal saline (dark gray line) at all timepoints and compared to females (triangles, ♀) after neonatal inflammation from 4-8 mins of (**A.**). Representative plethysmography traces (**B.**) showed breathing increased during the peak hypoxic ventilatory response (HCVR; gray box in **A**.) in males after neonatal inflammation compared to adults after neonatal saline. Adult breathing frequencies (**C.**) during the peak HCVR in adult males were unchanged by neonatal inflammation, but decreased in females after neonatal inflammation compared to adult males after neonatal saline. Tidal volumes (V_T, **D.**) during HCVR increased in adult males after neonatal inflammation from all adults after neonatal saline and increased in adult females after neonatal inflammation compared to adult males after neonatal saline. V_E (**E.**) during HCVR increased in adult males after neonatal inflammation from adult males after neonatal saline and females after neonatal inflammation, while female V_E during HCVR was unaffected by neonatal inflammation. Sighs (**F.**) and post-sigh apneas (**G.**) during HCVR were unchanged by neonatal inflammation in either sex. (%%% $p<0.0001$ different from baseline, * $p<0.05$ ** $p<0.001$ *** $p<0.0001$ different from adults after neonatal saline within sex, $ $p<0.05$ different from adults after neonatal inflammation of the opposite sex; # $p<0.05$, ## $p<0.001$ different from adults after neonatal saline of the opposite sex; Three-way and Two-way RM ANOVAs; Bonferroni post-hoc test.)

In contrast to adult breathing frequencies during peak HCVR, neonatal treatment ($p=0.0001$) had a significant main effect on adult V_T during peak HCVR. However, neither sex

(p=0.65) nor the interaction between neonatal treatment-sex (p=0.21) had a significant main effect on adult V_T during peak HCVR. In contrast to adult breathing frequencies, V_T during peak HCVR was increased in adult males after neonatal inflammation (61±22% change from baseline) compared to adult males after neonatal saline (16±7% change from baseline, p=0.0003, **Fig. 4D**), demonstrating neonatal inflammation augmented adult male V_T during peak HCVR. While in adult females, V_T during peak HCVR was similar in adult females after neonatal inflammation (49±26% change from baseline) to adult females after neonatal saline (23±14% change from baseline, p=0.11). When comparing between sexes, adult V_T during peak HCVR was similar between adult males and females after neonatal inflammation (p=0.99) and between adult males and females after neonatal saline (p=0.99). Between treatment groups and sex, V_T during peak HCVR was increased in adult males after neonatal inflammation compared to adult females after neonatal saline (p=0.0038), and increased in adult females after neonatal inflammation compared to adult males after neonatal saline (p=0.017). Thus, neonatal inflammation augmented adult male V_T during peak HCVR.

In assessing adult V_E during peak HCVR, neonatal treatment (p=0.035) and the interaction between neonatal treatment-sex (p=0.003) had significant main effects on V_E during peak HCVR. However, sex (p=0.54) did not have a main effect on adult V_E during peak HCVR. For pairwise differences, V_E during peak HCVR increased in adult males after neonatal inflammation (137±30% change from baseline) compared to adult males after neonatal saline (79±28% change from baseline, p=0.0017, **Fig. 4E**), demonstrating neonatal inflammation augmented adult male peak HCVR. However, V_E during peak HCVR was similar in adult females after neonatal inflammation (96±31% change from baseline) to adult females after neonatal saline (107±19% change from baseline, p=0.99), demonstrating female V_E during peak HCVR was unaffected by

neonatal inflammation. Between sexes, V_E during peak HCVR was increased in adult males after neonatal inflammation compared to adult females after neonatal inflammation (p=0.049), and similar between adult males and females after neonatal saline (p=0.48). Between treatment and sex, V_E during peak HCVR was similar between adult males after neonatal inflammation compared to adult females after neonatal saline (p=0.34), and similar in females after neonatal inflammation and adult males after neonatal saline (p=0.99). In conclusion, neonatal inflammation increased adult male, but not female, V_E during peak HCVR (primarily driven by an increase in V_T), demonstrating lasting sex-specific changes to adult HCVR after neonatal inflammation.

The number of spontaneous apneas, sighs, and post-sigh apneas during HCVR were unaffected by neonatal treatment or sex. Spontaneous apneas were not observed in any treatment group or sex during HCVR. Neonatal treatment (p=0.39), sex (p=0.37), nor the interaction between neonatal treatment-sex (p=0.94) had main effects on sighs during HCVR, and there were no pairwise differences (adult males after neonatal inflammation=2±2 sighs, adult males after neonatal saline=3±1 sighs, adult females after neonatal inflammation=3±3 sighs, adult females after neonatal saline=4±2 sighs, p>0.9, **Fig. 4F**). Neonatal treatment (p=0.75), sex (p=0.084), nor the interaction between neonatal treatment-sex (p=0.56) had main effects on post-sigh apneas during HCVR, and there were no pairwise differences (adult males after neonatal inflammation=0.3±0.5 post-sigh apneas, adult males after neonatal saline=0.4±0.5 post-sigh apneas, adult females after neonatal inflammation=0.1±0.4 post-sigh apneas, adult females after neonatal saline=0±0 post-sigh apneas, p>0.9, **Fig. 4G**). Thus, neonatal inflammation did not impair sighs or post-sigh apneas during the peak HCVR.

Neonatal inflammation caused sex differences in adult hypoxic ventilatory responses.

Adult hypoxic ventilatory responses (HVRs) were assessed in adult males and females after neonatal inflammation or neonatal saline to determine the lasting impact of neonatal inflammation on respiratory responses to hypoxia. The hypoxic ventilatory response is characterized by an initial increase in breathing during HVR phase I before breathing declines toward baseline during HVR phase II (Pamenter & Powell, 2016). Time ($p<0.0001$), neonatal inflammation ($p=0.042$), sex ($p<0.0041$) and the interactions between neonatal treatment-sex ($p=0.015$), neonatal treatment-time ($p=0.048$), and sex-time ($p<0.046$) had main effects on V_E during hypoxia. While the interaction between neonatal treatment-time-sex ($p=0.13$) did not have a main effect on V_E during hypoxia. All adults increased breathing during hypoxia after 4 mins, with the exception of 8-10mins in females after neonatal saline, supporting that all adults responded to hypoxia (adult males after neonatal inflammation: 0-2mins, $p=0.0002$, 2-4mins, $p=0.0001$, 4-6mins, $p=0.0001$, 6-8mins, $p=0.0001$, 8-10mins, $p=0.0001$; adult males after neonatal saline: 0-2mins, $p=0.99$, $p=0.0001$ for all other timepoints; adult females after neonatal inflammation: 0-2mins, $p=0.99$, 2-4mins, $p=0.19$, 4-6mins, $p=0.0003$, 6-8mins, $p=0.0008$, 8-10mins, $p=0.010$; adult females after neonatal saline: 0-2mins, $p=0.99$, 2-4mins, $p=0.0007$, 4-6mins, $p=0.0009$, 6-8mins, $p=0.0061$, 8-10mins, $p=0.064$, **Fig. 5A**).

Comparing between neonatal treatment-sex over time during HVR showed time ($p=0.0001$), neonatal treatment-sex ($p=0.0006$), and the interaction between neonatal treatment-sex-time ($p=0.0065$) had main effects on adult V_E during HVR. Pairwise differences show V_E during hypoxia increased from 6-8mins in adult males after neonatal inflammation compared to adult males after neonatal saline and trended toward increased from 0-2mins (0-2mins $p=0.067$, 2-4mins $p=0.22$, 4-6mins $p=0.45$, 6-8mins $p=0.031$, 8-10mins $p=0.17$). V_E during hypoxia was similar between adult females after neonatal inflammation and adult females after neonatal saline

(0-2mins, p=0.99; 2-4mins, p=0.79; 4-6mins, p=0.99; 6-8mins, p=0.99; 8-10mins, p=0.99), suggesting neonatal inflammation causes sex differences in adult HVR. Between sex, V_E increased in adult males after neonatal inflammation compared to adult females after neonatal inflammation (0-2mins, p=0.059; 2-4mins, p=0.048; 4-6mins, p=0.090; 6-8mins, p=0.035; 8-10mins, p=0.023), suggesting neonatal inflammation causes lasting sex differences in HVR. In contrast, V_E during hypoxia was similar between adult males and females after neonatal saline (p=0.99 at all timepoints). Between treatment groups and sex, V_E during hypoxia increased in adult males after neonatal inflammation compared to adult females after neonatal saline (0-2mins, p=0.18; 2-4mins, p=0.077; 4-6mins, p=0.074; 6-8mins, p=0.068; 8-10mins, p=0.0074), further supporting sex differences in HVR after neonatal inflammation. V_E during hypoxia was similar between adult females after neonatal inflammation and adult males after neonatal saline (0-2mins, p=0.99; 2-4mins, p=0.92; 4-6mins, p=0.99; 6-8mins, p=0.99; 8-10mins, p=0.99, **Fig. 5A & B**). Thus, neonatal inflammation likely cause sex differences in adult ventilation during HVR.

Breathing frequency and tidal volume during HVR phase I were unchanged after neonatal inflammation. Neither neonatal treatment (p=0.44), sex (p=0.28), nor the interaction between neonatal treatment-sex (p=0.88) had a main effect on adult breathing frequency and there were no pairwise differences (adult males after neonatal inflammation=44±23% change from baseline, adult males after neonatal saline=37±20% change from baseline; adult females after neonatal inflammation=34±22% change from baseline, adult females after neonatal saline=29±16% change from baseline, p>0.99, **Fig. 5C**). For adult V_T during HVR phase I, neither neonatal treatment (p=0.13), sex (p=0.41), nor the interaction between neonatal treatment-sex (p=0.38) had significant main effects and there were no pairwise differences (adult males after neonatal inflammation=33±16% change from baseline, adult males after neonatal saline=16±16% change

from baseline; adult females after neonatal inflammation=21±18% change from baseline, adult females after neonatal saline=17±23% change from baseline, p>0.43, **Fig. 5D**). Thus, neonatal inflammation did not impair frequency or tidal volume during HVR phase I.

Similar to breathing frequency and V_T, adult V_E during HVR phase I was not affected by neonatal inflammation. Sex (p=0.034) had a main effect on adult V_E during HVR phase I. However, neither neonatal treatment (p=0.14) nor the interaction between neonatal treatment-sex (p=0.19) had significant main effects on adult V_E during HVR phase I. Pairwise differences showed adult V_E during HVR phase I was unchanged by neonatal inflammation in adult males (adult males after neonatal inflammation=86±29% change from baseline, adult males after neonatal saline=59±31% change from baseline, p=0.12) or in females (adult females after neonatal inflammation=52±22% change from baseline, adult females after neonatal saline=50±20% change from baseline, p=0.99, **Fig. 5E**). Between sexes, V_E during HVR phase I was similar between adult males and females after neonatal inflammation (p=0.085) or after neonatal saline (p=0.99), despite a trend for increased V_E during HVR phase I in males. Between treatment groups and sex, V_E during HVR phase I was similar between adult males after neonatal inflammation compared to adult females after neonatal saline (p=0.086). In contrast, V_E during HVR phase I was similar between adult females after neonatal inflammation and males after neonatal saline (p=0.99). Thus, adult breathing during HVR phase I was unchanged by neonatal inflammation.

After the initial increase in breathing during HVR phase I, breathing during hypoxia declines toward baseline during HVR phase II (Pamenter & Powell, 2016). Sex (p=0.015) had a main effect on adult breathing frequency during HVR phase II. However, neither neonatal treatment (p=0.83) nor the interaction between neonatal treatment-sex (p=0.89) had main effects on adult breathing frequency during HVR phase II. Further, there were no pairwise differences

(adult males after neonatal inflammation=47±20% change from baseline, adult males after neonatal saline=46±9% change from baseline; adult females after neonatal inflammation=32±19% change from baseline, adult females after neonatal saline=30±15% change from baseline, p>0.35, **Fig. 5F**). For adult V_T during HVR phase II, neither neonatal treatment (p=0.082), sex (p=0.49) nor the interaction between neonatal treatment-sex (p=0.22) had significant main effects on adult V_T during HVR phase II and there were no pairwise differences between groups (adult males after neonatal inflammation=24±20% change from baseline, adult males after neonatal saline=4±14% change from baseline; adult females after neonatal inflammation=11±13% change from baseline, adult females after neonatal saline=8±23% change from baseline, p>0.16, **Fig. 5G**). Thus, neonatal inflammation did not impair frequency or V_T during HVR phase II.

Adult V_E during HVR phase II was significantly affected by neonatal treatment (p=0.046) and sex (p=0.0062), but not the interaction between neonatal treatment-sex (p=0.12). Pairwise differences showed V_E during HVR phase II similar in adult males after neonatal inflammation (83±27% change from baseline) compared to adult males after neonatal saline (49±30% change from baseline, p=0.057) during HVR phase II. In contrast, V_E during HVR phase II was similar between adult females after neonatal inflammation (41±21% change from baseline) and adult females after neonatal saline (36±17% change from baseline, p=0.99, **Fig. 5H**). Between sexes, V_E during HVR phase II increased in adult males after neonatal inflammation compared to adult females after neonatal inflammation (p=0.015), but was similar between adult males and females after neonatal saline (p=0.99). Between treatment groups and sex, V_E during HVR phase II increased in males after neonatal inflammation compared to females after neonatal saline (p=0.0087), but was similar in adult females after neonatal inflammation compared to adult males

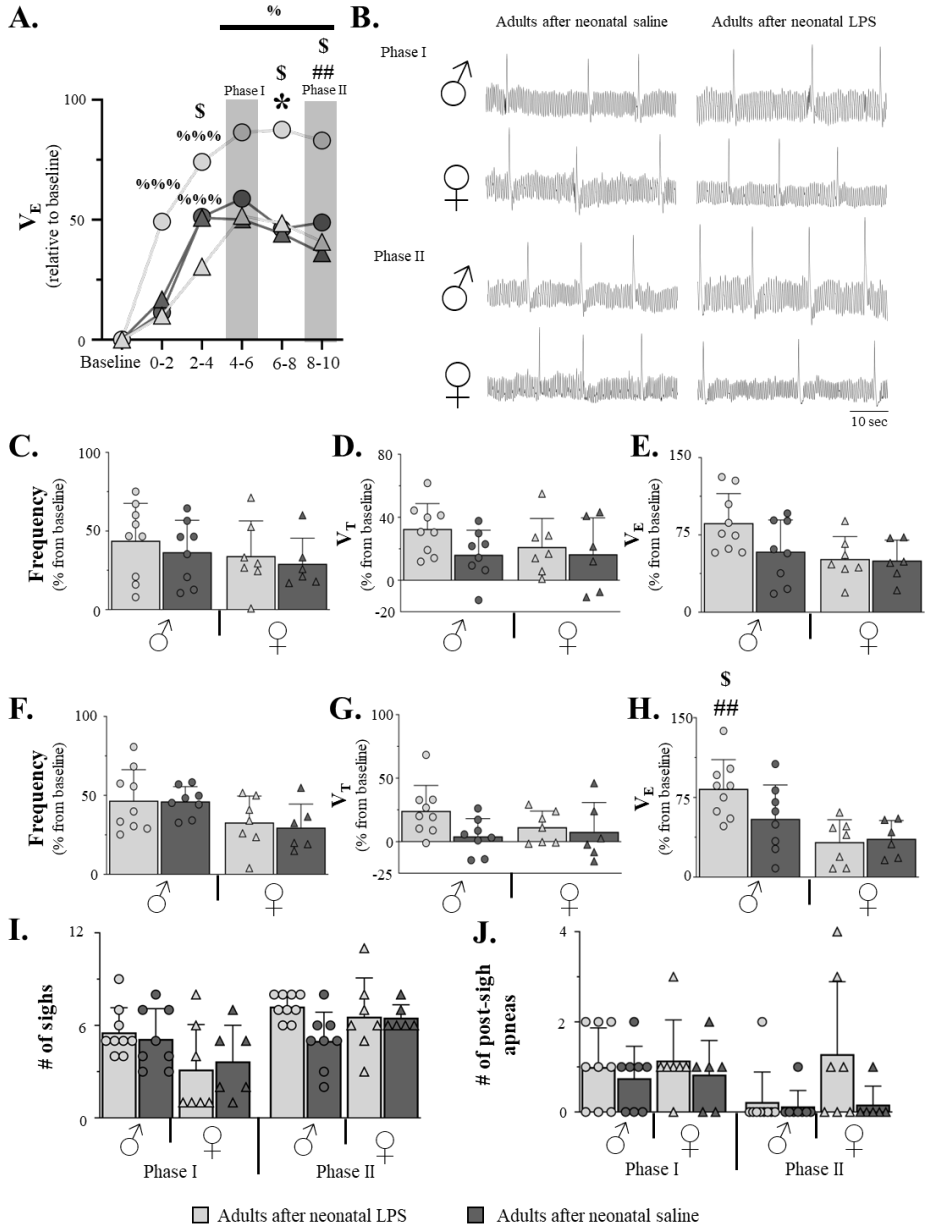


Figure 5. Neonatal inflammation augmented adult male hypoxic ventilatory responses compared to adult females. Average adult minute ventilation (V_E) over time (**A.**) increased from baseline at all timepoints in adult males (circles, ♂) after neonatal inflammation (light gray line), increased after 2 mins of hypoxia in males after neonatal saline (dark gray line), increased after 4 mins of hypoxia in females (triangles, ♀) after neonatal inflammation, and increased from 2-8 mins of hypoxia in females after neonatal saline. HVR phase I and phase II is highlighted in gray (**A.**). Plethysmography traces from HVR phase I show breathing was similar in all adults and during phase II (**B.**) males after neonatal inflammation have increased breathing than females, regardless of treatment. During HVR phase I, adult breathing frequencies (**C.**), V_T (**D.**) and V_E (**E.**) were unchanged by neonatal inflammation in either sex. During HVR phase II, adult breathing frequencies (**F.**), V_T (**G.**), and were unaffected by neonatal inflammation in either sex. However, during HVR phase II V_E (**H.**) increased in adult males after neonatal inflammation compared to all females, regardless of neonatal treatment. During HVR phase I and II, the number of sighs (**I.**) and post-sigh apneas (**J.**) were unchanged by neonatal inflammation in either sex. (% $p<0.05$, %%% $p<0.0001$ different from baseline; \$ $p<0.05$ different from adults after neonatal inflammation of the opposite sex; * $p<0.05$ different from adults after neonatal saline within sex; # $p<0.05$ ## $p<0.01$ different from adults after neonatal saline of the opposite sex; Three-way and Two-way RM ANOVAs, Bonferroni post-hoc test.)

after neonatal saline (p=0.99). Thus, neonatal inflammation may cause sex differences in HVR phase II.

Spontaneous apneas and sighs during hypoxia were not affected by neonatal treatment. Spontaneous apneas were not observed in any treatment group or sex during HVR. For sighs during HVR phase I, sex ($p=0.025$) had a main effect on the number of sighs during HVR phase I; however, neonatal treatment ($p=0.95$) nor the interaction between neonatal treatment-sex ($p=0.56$) had main effects on sighs during HVR phase I and there were no pairwise differences (adult males after neonatal inflammation=6±2 sighs, adult males after neonatal saline=5±2 sighs; adult females after neonatal inflammation=3±3 sighs, adult females after neonatal saline=4±2 sighs, $p>0.9$, **Fig. 5J**). During HVR phase II, neither neonatal inflammation ($p=0.071$), sex ($p=0.49$), nor the interaction between neonatal treatment-sex ($p=0.089$) had main effects on the number of sighs during HVR phase II. Pairwise differences showed sighs during HVR phase II were similar in males after neonatal inflammation (7±1 sighs) compared to males after neonatal saline (5±2 sighs, $p=0.061$), but were trending toward being increased in males after neonatal inflammation. There were no other pairwise differences between treatment groups or sex (adult females after neonatal inflammation=7±3 sighs, adult females after neonatal saline=7±1 sighs, $p>0.46$). Thus, neonatal inflammation may effect male sighs during HVR phase II, without changing sighs in females.

Post-sigh apneas were unaffected during HVR phase I or II. In HVR phase I, post-sigh apneas during HVR were not significantly affected by neonatal treatment ($p=0.36$), sex ($p=0.71$) nor the interaction between neonatal treatment-sex ($p=0.92$) and there were no pairwise differences (adult males after neonatal inflammation=1±1 post-sigh apneas, adult males after neonatal saline=1±1 post-sigh apneas; adult females after neonatal inflammation=1±1 post-sigh apneas, adult females after neonatal saline=1±1 post-sigh apneas, $p>0.9$, **Fig. 5I**). Similarly, neither neonatal treatment ($p=0.076$), sex ($p=0.12$) nor the interaction between neonatal treatment-sex ($p=0.11$) had significant main effects on the number of post-sigh apneas during HVR phase II.

Further, there were no pairwise differences between treatment groups and sex (adult males after neonatal inflammation=0.22±0.67 post-sigh apneas, adult males after neonatal saline=0.13±0.35 post-sigh apneas, adult females after neonatal inflammation=1.2±1.6 post-sigh apneas, adult females after neonatal saline=0.17±0.41 post-sigh apneas, $p>0.11$). In conclusion, neonatal inflammation did not affect sighs or apneas during HVR in either sex.

Discussion

Inflammation during critical periods of early life development has lasting negative consequences for the adult nervous system, including the respiratory control network (Hocker et al., 2019). We previously determined lasting adult inflammation contributes to abolishment of respiratory motor plasticity after neonatal inflammation (Hocker et al., 2019), yet the lasting mechanisms underlying abolished plasticity and the long-term effects of neonatal inflammation on adult breathing beyond respiratory motor plasticity were unknown. Here, we identified medullary microglia as a source of lasting adult inflammation after neonatal inflammation. Lasting sex-specific changes in adult medullary microglia number (increased in males, but not females) and microglial inflammatory gene expression (increased TNFα expression in males and increased IL-6 expression in females) suggest different inflammatory mechanisms underlie adult male versus female impairments after neonatal inflammation, including respiratory motor plasticity and breathing. Despite abolished respiratory motor plasticity in both sexes, neonatal inflammation did not change adult male eupneic breathing, but augmented hypercapnic ventilatory responses (HCVR) and had sex specific effects on adult male hypoxic ventilatory responses (HVR) where by males after neonatal inflammation had augmented HVR compared to females. In adult females, neonatal inflammation decreased sighs during eupnea, but did not disrupt chemoreflexes. Enhancements in adult male chemoreflexes is consistent with an increased male risk for developing adult ventilatory control disorders, such as sleep apnea (Dempsey, 2019; Dempsey et al., 2010; Trombetta et al., 2013). Collectively, neonatal inflammation sex-dependently activated adult medullary microglia, likely contributing to lasting adult inflammation in respiratory control regions, sex-specific augmentation of male chemoreflexes, and decreases in female sigh behaviors during eupnea.

Our previous study determined that respiratory motor plasticity was abolished after neonatal inflammation (Hocker et al., 2019), but did not identify the respiratory control regions associated with the lasting adult impairments. In our current study, neonatal inflammation specifically activated adult medullary microglia, without changing ventral cervical spinal microglia. While the cervical spinal cord is the presumptive site of respiratory motor plasticity (Agosto-Marlin & Mitchell, 2017; Dale et al., 2017; Dale-Nagle et al., 2010; Devinney et al., 2015; Hoffman et al., 2012), the lack of inflamed spinal microglia further supports our previous work demonstrating that phrenic motor neurons themselves are still capable of phrenic motor facilitation after neonatal inflammation (Hocker et al., 2019). The results from our study suggest the restoration of adult respiratory motor plasticity by the non-steroidal anti-inflammatory drug (ketoprofen; Hocker et al., 2019) is likely caused by reducing inflammation in activated adult medullary microglia. Such a hypothesis is supported by other work demonstrating medullary respiratory regions are involved in respiratory motor plasticity (reviewed in Feldman et al., 2003; Mitchell & Baker, 2022; Mitchell & Johnson, 2003; Perim & Mitchell, 2019). Specifically, serotonergic neurons in the medulla are activated by acute intermittent hypoxia (AIH), whereby serotonin is released in and around phrenic motor neurons to elicit plasticity (Agosto-Marlin & Mitchell, 2017; Dale et al., 2017; Dale-Nagle et al., 2010; Devinney & Mitchell, 2018; Hoffman & Mitchell, 2011; Holtman et al., 1986; Kinkead et al., 1998, 2001). Thus, we hypothesize that activated medullary microglia may impair medullary serotonergic neurons, reduce serotonin during AIH, and contribute to impaired plasticity. In support of this, neonatal inflammation evoked by sustained hypoxia, rather than neonatal LPS as done here, decreased neonatal serotonergic signaling in the nucleus tractus solitaries (NTS) and dorsal medial vagus nerve (DMVN; MacFarlane et al., 2016; Mayer et al., 2013). Thus, our research identified medullary microglial activation likely contributes to

inflammatory signaling underlying abolishment of respiratory motor plasticity and highlights a need for future investigations to determine the effects of neonatal inflammation on adult medullary serotonin signaling.

The lasting activation of adult medullary microglia does not preclude the involvement of other cell types to impaired adult respiratory control. Astrocytes are activated by microglia during neuroinflammatory challenges (Kwon & Koh, 2020; Sarlus & Heneka, 2017; Singh, 2022; Singh et al., 2011) and astrocytes are critical to breathing and chemoreflexes (Erlichman et al., 2010; Fitzgerald & Rocher, 2021; Fukushi et al., 2021; Gourine & Funk, 2017; Guyenet & Stornetta, 2022; Huckstepp et al., 2018; Hülsmann et al., 2000; Huxtable et al., 2010; Marina et al., 2016; Mouradian et al., 2021). Further, our previous work suggested a potential role for astrocytes in abolished respiratory motor plasticity (Hocker et al., 2019). Additional experiments investigating the interplay between the activated microglia shown here and effects on medullary or spinal astrocytes in adults after neonatal inflammation are needed.

Sex-dependent activation of adult medullary microglia after neonatal inflammation (increased number and TNFα gene expression in males, and increased IL-6 gene expression in females) provides necessary insights into inflammatory mechanisms underlying abolishment of respiratory motor plasticity. The increased adult male medullary microglia may be due to either increased migration of microglia (Chausse et al., 2021; Harry, 2013; Kielian, 2004) or increased microglia proliferation (Gao et al., 2013; Garden & Möller, 2006; McLarnon, 2017). Further, increased microglial TNFα gene expression begins to triangulate on precise lasting inflammatory contributors in adult males after neonatal inflammation. TNFα gene expression also increased in neonatal medullary and spinal cord homogenates immediately after neonatal inflammation and remained elevated 24 hrs later (Morrison et al., 2020). Here, we show for the first time that this

increase is maintained into adulthood in male medullary microglia, supporting that the initial stimulus transforms male medullary microglia into a lasting inflammatory state. Spinal TNFα is required for inactivity induced respiratory motor plasticity in adult males (Baertsch & Baker, 2017; Baertsch & Baker-Herman, 2015; Broytman et al., 2013); however, this form of plasticity has not been assessed after neonatal inflammation. However, similar to cross-talk pathways for plasticity after AIH, whether TNFα facilitates or abolishes plasticity may be a question of dose and/or location (Navarrete-Opazo & Mitchell, 2014; Perim et al., 2018; Perim & Mitchell, 2019). Too much TNFα might abolish respiratory motor plasticity, while some is necessary for other forms of plasticity. Alternatively, increased medullary TNFα gene expression after neonatal inflammation induces medullary inflammation, which may impair serotonergic neurons critical for plasticity.

Despite abolished plasticity in both sexes, adult female medullary microglia did not change in number nor TNFα gene expression after neonatal inflammation. Yet, IL-6 gene expression increased in the female medulla. While increases in spinal IL-6 gene expression were associated with abolishing respiratory motor plasticity after prolonged intermittent hypoxia (Huxtable et al., 2015), these data were from homogenates, not isolated microglia, and medullary IL-6 gene expression was not evaluated (Huxtable et al., 2015). Thus, similar to TNFα expression in males, investigations into IL-6 signaling in respiratory control lag behind, including in adults after neonatal inflammation. While little is known about the role of IL-6 expression in abolishing plasticity, elevated IL-6 is predictive of severe respiratory distress during COVID (Broman et al., 2021; Ragab et al., 2020), and increased IL-6 gene expression is associated with respiratory distress after viral infections (Bociąga-Jasik et al., 2011; Chiaretti et al., 2013). Thus, our data demonstrating females have increased medullary microglial IL-6 expression after neonatal inflammation and our previous data showing females have abolished respiratory motor plasticity

after neonatal inflammation together suggest adult females after neonatal inflammation may have impaired adult inflammatory responses, which likely negatively impact respiratory control during infection and illness. Additionally, increases in IL-6 are associated with inducing preterm labor (Romero et al., 2012), fetal inflammatory response syndrome (Xiong & Wintermark, 2020), neonatal sepsis (Qiu et al., 2018), and increased risk of neurodevelopmental disorders (Nist & Pickler, 2019), suggesting a need for longitudinal studies and emphasizing the importance of increasing our understanding of how IL-6 expression impacts respiratory insufficiency at all ages, particularly in females after neonatal inflammation. Importantly, sex-specific activation of adult medullary microglia suggest distinct inflammatory mechanisms in adult males and females after neonatal inflammation may underlie impaired adult respiratory control, including abolishment of respiratory motor plasticity.

Our previous work was the first to demonstrate loss of adult respiratory motor plasticity after neonatal inflammation, but we did not investigate impairments in adult breathing. To understand the extent of respiratory impairments after neonatal inflammation, we assessed adult breathing after neonatal inflammation in both males and females. Similar to the isolated microglia data, neonatal inflammation sex-specifically impaired adult breathing behaviors. In adult males, neonatal inflammation did not impair eupneic breathing, consistent with our previous research showing neonatal inflammation did not impair baseline phrenic motor activity (Hocker et al., 2019). However, neonatal inflammation augmented adult male chemoreflexes. Enhanced HCVR, as seen in males after neonatal inflammation, is known to be a contributing factor to the pathology of sleep apnea by increasing loop gain (Caravita et al., 2022; Hudgel et al., 1998; Rosenzweig et al., 2015; Trombetta et al., 2013; Verbraecken et al., 1998). Thus, augmented HCVR may be indicative of adult males after neonatal inflammation being at increased risk for developing sleep

apnea (reviewed in Behrents et al., 2019). Further, augmented tidal volume combined with increased inflammation from medullary microglia during HCVR in adult males suggests investigations into medullary regions involved in modulating respiratory amplitude, such as the RTN, medullary raphe, and NTS (reviewed in Nattie & Li, 2012) will likely lend significant insight into whether there is generalized medullary inflammation or whether it is confined to specific medullary respiratory regions.

We determined neonatal inflammation did not change adult HVR within sex, consistent with our results showing phrenic motor activity during hypoxia was not changed in adults after neonatal inflammation (Hocker et al., 2019); however, comparisons between sexes identified augmented HVR in adult males after neonatal inflammation compared to females, regardless of neonatal treatment. Our research does not rule out the possible that enhanced chemoreflexes, as seen in adult males after neonatal inflammation, have protective effects on adult male respiratory control. However, maternal neonatal separation, another early life stressor, sex specifically augments adult male HVR (Genest et al., 2004, 2007; Kinkead et al., 2005) leading to these adult males being at increased risk for developing sleep apnea (Kinkead et al., 2009), providing evidence that enhanced HVR can have maladaptive effects on adult male respiratory control. Further, increases in serum TNFα contribute to the pathology of sleep apnea (Yi et al., 2022), highlighting the need to understand how lasting increases in medullary microglial TNFα gene expression contributes to lasting augmentation of HVR in adult males after neonatal inflammation compared to females.

In females, neonatal inflammation did not impair minute ventilation during eupnea; however, neonatal inflammation significantly decreased adult female sighs during eupnea. Consistent with other studies (reviewed in Gargaglioni et al., 2019; Lozo et al., 2017), we showed adult female eupneic breathing was increased compared to males regardless of neonatal treatment, which

manifested as increased tidal volume. Females are thought to maintain higher tidal volumes than males to compensate for sex differences in lung and airway anatomy (reviewed in Gargaglioni et al., 2019; Lozo et al., 2017). However, our data showing baseline sex differences in IL-6 gene expression in medullary microglia (increased IL-6 gene expression in adult males after neonatal saline compared to adult females after neonatal saline) suggest medullary IL-6 signaling may contribute to sex differences in breathing in healthy adults. Sighs during eupnea are generated in the preBötC, mediated by neuronal-glia coupling involving purinergic and calcium signaling (Borrus et al., 2020, 2024; Severs et al., 2023). Since our previous research identified lasting disruption to purinergic signaling contributes to abolishment of respiratory motor plasticity in adults after neonatal inflammation (Hocker et al., 2019), we postulate that neonatal inflammation may disrupt medullary purinergic signaling in females to decrease sighs and contribute to disruption of respiratory motor plasticity.

Overall, this study significantly advances our understanding of cellular and molecular mechanisms impairing adult respiratory control after neonatal inflammation. We identified medullary microglia as a source of lasting inflammation, likely contributing to abolished adult respiratory motor plasticity. Our research is the first to identify sex-specific changes in adult sigh behaviors and chemoreflexes after neonatal inflammation, supporting that the deficits in adults after neonatal inflammation are not confined to adult respiratory motor plasticity. Further, our results are consistent with other models of early life stress, such as neonatal maternal separation (Baldy et al., 2018) and sustained hypoxia (MacFarlane et al., 2016), which likely contribute to adult respiratory deficits, like sleep apnea. Collectively, we hypothesize that early life stressors alter the developmental trajectory of the respiratory control network, in part, by causing lasting activation of microglia in respiratory control regions to increase risk of developing respiratory

control disorders in adulthood (reviewed in Bilbo et al., 2012; Bilbo & Schwarz, 2009; Schwarz & Bilbo, 2012; Williamson et al., 2011). Together, our novel findings provide a framework for future studies to triangulate on the role of adult medullary microglia in understanding how neonatal inflammation contributes to adult risk for ventilatory insufficiencies and ventilatory control disorders.

CHAPTER III

NEONATAL AND ADULT INFLAMMATION AUGMENTED MICROGLIAL INFLAMMATORY RESPONSES, WITHOUT IMPAIRING BREATHING.

This chapter includes material being prepared for submission for publication in the *Frontiers in Physiology*, co-authored with Robyn Naidoo, Deanna L. M. Plunkett, Jyoti J. Watters, Adrianne G. Huxtable. I led these studies. I designed, collected and analyzed data for microglia experiments, analyzed the data for the breathing experiments, and wrote the manuscript. Robyn Naidoo designed and collected the data for the breathing experiments. Deanna L. M. Plunkett contributed to collecting data and analyzing the microglia frequency experiments. Jyoti Watters contributed to designing the experiments and provided guidance for this study. Dr. Adrianne Huxtable designed, contributed to writing, provided guidance and editorial assistance.

Introduction

Certain adults are more vulnerable to respiratory instability during illness and disease (Capelastegui, España, Bilbao, et al., 2008; Capelastegui, España, Quintana, et al., 2008), yet the underlying factors contributing to this respiratory instability are unclear. Neonatal inflammation is one potential factor that may increase adult vulnerability to respiratory instability during illness and disease. Adults after neonatal inflammation are at increased risk for cognitive disruptions (Bilbo, 2010; Bland, Beckley, Young, et al., 2010) and thermoregulation impairments during subsequent adult inflammation (Ellestad et al., 2009; reviewed in Kentner & Pittman, 2010), demonstrating neonatal inflammation can cause lasting impairments to adult behaviors in response to subsequent inflammatory challenges. Disruptions in cognitive behaviors after neonatal inflammation are mediated, at least in part, by primed microglia with heightened and prolonged microglial inflammatory responses after subthreshold adult inflammation (Bilbo & Schwarz, 2009;

Bland, Beckley, Young, et al., 2010). Thus, primed microglia after neonatal inflammation contribute to disrupting adult behaviors during otherwise harmless adult inflammatory challenges, yet the impact of microglia priming in respiratory control regions has not been investigated.

We recently demonstrated neonatal inflammation (lipopolysaccharide, LPS 1mg/kg, i.p., P4) abolish adult male and female respiratory motor plasticity (Hocker et al., 2019) and cause lasting augmentation of male HCVR, demonstrating neonatal inflammation causes lasting sex-specific disruptions in adult respiratory control (Beyeler et al., *in preparation*). Since augmented chemoreflexes contribute to pathology of sleep apnea (Fung, 2014; Iturriaga et al., 2021; Trombetta et al., 2013), neonatal inflammation may contribute to a heightened male risk for developing sleep apnea. While a single bout of neonatal inflammation leads to lasting disruptions in adult respiratory control (Hocker et al., 2019), it is unclear whether these adults are more vulnerable to subsequent, subthreshold inflammatory insults.

These impairments in breathing in adults after neonatal inflammation are likely mediated, in part, by lasting inflammation in adult medullary microglia (Beyeler et al., *in preparation*). Neonatal inflammation increased male medullary microglia number and TNFα gene expression and female IL-6 gene expression in microglia, demonstrating neonatal inflammation leads to lasting increases in microglia originating pro-inflammatory signaling in respiratory control regions. Determining whether these inflamed microglia disrupt how microglia respond to future inflammatory challenges is needed to understand how neonatal inflammation contributes to adult risk for breathing disruptions during illness or disease. Thus, the goal of this study is to advance to determine in microglia are primed to even low levels of adult inflammatory stimuli and whether such microglia changes contribute to impaired breathing.

Adults often encounter diverse inflammatory stimuli and non-respiratory microglia are primed for exaggerated proinflammatory IL-1β gene expression after neonatal and adult LPS (lipopolysaccharide, component of bacterial cell wall; reviewed in Bilbo et al., 2012; Bilbo & Schwarz, 2012), demonstrating neonatal inflammation increases susceptibility for cognitive disfunction during subsequent adult homotypic inflammation (same as neonatal stimulus). However, viral infections are very common (Obasi et al., 2014) and induce distinct inflammatory profiles through TLR3 activation (Reimer et al., 2008). Yet, the effect of neonatal inflammation on microglial primed to heterotypic inflammation (different than neonatal inflammation) is not well understood. Furthermore, how polyIC-induced inflammation (polyinosinic:polycytidylic acid, a viral mimetic) impacts eupneic breathing and chemoreflexes has not been investigated, limiting our understanding of how viral inflammation impact respiratory control. Thus, we investigated how microglial inflammatory responses and breathing after neonatal and adult subthreshold homotypic (neonatal LPS + adult LPS) or heterotypic inflammation (neonatal LPS + adult polyIC). Since neonatal inflammation sex-dependently activated adult medullary microglia and disrupted adult breathing (Beyeler et al., *in preparation*), we hypothesize that the combination of neonatal and adult treatments will sex-dependently prime adult microglia in respiratory control regions and contribute to impaired adult breathing.

Methods

All experiments were approved by the Institutional Animal Care and Use Committees at the University of Oregon and conformed to the policies of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Female Sprague Dawley rats (Envigo Colony 206, 202 or 231) were housed under standard conditions (12:12 hr light/dark cycle) with food and water *ad libitum* and monitored daily until giving birth.

Neonatal treatments

Litters from a pair of dams were stratified and balanced, each fostering similar numbers of males and females, to control for between litter effects. On postnatal day 4 (P4), the stratified neonates from one dam were injected with lipopolysaccharide (LPS, 1 mg/kg, i.p.), while the stratified neonates from the second dam were injected with sterile saline (i.p.) as the vehicle control, as described previously (Hocker et al. 2019 and Beyeler et al. *in preparation*). This LPS dose was selected based on our previous research demonstrating this dose of LPS at P4 caused acute disruption to neonatal respiratory activity (Morrison et al., 2020), lasting abolishment of adult respiratory motor plasticity (Hocker et al., 2019), and lasting activation of adult medullary microglia (Beyeler et al. *in preparation*). Neonates were weaned at P21 and weighed weekly.

Adult treatments

After aging to adulthood (males ≥300g and females ≥250g), adults were subsequently treated with subthreshold LPS, polyIC or sterile saline before adult microglia and breathing assessments. An LPS (0.5 - 1000 µg/kg, i.p.) and polyIC dose responses (100 – 1000 µg/kg, i.p.) were conducted in healthy adult rats. These preliminary experiments identified 25 µg/kg LPS and 479 µg/kg polyIC as the half-maximal effective concentrations (EC_{50}) to increase microglia number using the Hill equation. To remain on the linear portion of the dose response curves, 24

μg/kg LPS was selected as the adult subthreshold homotypic inflammatory challenge and 478 μg/kg polyIC was selected as the adult subthreshold heterotypic inflammatory challenge.

Experimental design

In our first set of experiments (11 dams total), adult microglia frequency was assessed with flow cytometry in six adult treatment groups 24hrs after adult treatments: neonatal saline + adult saline, neonatal LPS + adult saline, neonatal saline + adult LPS, neonatal LPS + adult LPS, neonatal saline + adult polyIC and neonatal LPS + adult polyIC. Since respiratory motor plasticity is abolished 3hrs after systemic LPS and continues to be abolished for at least 24hrs (Huxtable et al. 2013), in a second cohort of animals (8 dams total), adult breathing was assessed both 3hrs and 24hrs after adult treatment in the following groups: neonatal saline + adult saline, neonatal LPS + adult saline, neonatal saline + adult LPS and neonatal LPS + adult LPS. In contrast, adult inflammatory responses increase 24hrs after systemic polyIC (Hocker & Huxtable, 2019). Thus, in a third set of experiments (10 dams total), we assessed breathing 24hrs after adult treatment in the following groups: neonatal saline + adult saline, neonatal LPS + adult saline, neonatal saline + adult polyIC and neonatal LPS + adult polyIC. The impact of neonatal and adult inflammation on adult microglial inflammatory gene expression was determined by isolating adult microglia after measuring breathing in the second and third set of experiments.

Microglia assessments

To investigate the impact of neonatal and adult subthreshold inflammation on microglia priming, adult medullas (between the pontomedullary junction and C1), ventral cervical spinal cords (C3-C6) and prefrontal cortex tissues were extracted and dissociated, as previously described (Crain et al., 2009; Huxtable et al., 2013b; Nikodemova & Watters, 2012; Beyeler et al. in preparation). In brief, adult rats were transcardially perfused with ice cold phosphate buffered

saline (PBS: 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4) before extracting medullas, ventral cervical spinal cords, and cortices. Tissues were mechanically dissociated with a razor blade, enzymatically dissociated with papain to break down the extracellular matrix (MP Biomedicals, 1:100), and incubated with DNAse (Worthington, 1:25 at 37ºC for 16 min) to digest DNA from lysed cells. Tissue was filtered with cell strainers (40µm) and myelin removed with centrifugation in percoll (23%, GE Healthcare in PBS, 1050xg at 4ºC for 15 min) to generate single-cell homogenates.

Microglia frequency

Microglia abundance (microglia frequency, %$CD11b^{high}CD45^{low}$/homogenates) was determined as described in (Crain et al., 2009; Huxtable et al., 2013; Nikodemova & Watters, 2012; Beyeler et al. *in preparation*). In brief, microglia were identified in single cell homogenates with fluorophore conjugated antibodies, CD11b (Miltenyi Biotec, 1:100 dilution) and CD45 (Miltenyi Biotec, 1:100 dilution) using flow cytometry (Beckman Coulter, Gallios©). Microglia were identified by plotting live, single cells and counting microglia as the percent $CD11b^{high}/CD45^{low}$ cells within single cell homogenates.

Microglia isolations

To assess microglia inflammatory gene expression (IL-6, IL-1β, IL-18, TNFα, iNOS, COX-2, and IFNα), adult microglia were isolated from single cell homogenates by incubating cells with CD11b magnetic bead conjugated antibodies (Miltenyi Biotec, 1:50 dilution) and immunomagnetically separated, as described previously (Nikodemova and Watters, 2012). RNA was extracted from adult microglia using Tri-reagent (Sigma, MO, USA) with Glycoblue (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from RNA using MMLV reverse transcriptase (BioRad) and quantified with SYBR green (BioRad). Primer efficiency was assessed

with standard curves (Crain et al., 2009). Ct values were normalized to 18s before using the ΔΔCt method to measure gene expression (Livak and Schmittgen, 2001; Nikodemova and Watters, 2012). Data are expressed as fold change relative to male neonatal saline + adult saline inflammatory gene expression.

IL-6	forward 5’	GTG GCT AAG GAC CAA GAC CA,
	reverse 5’	GGT TTG CCG AGT AGA CCT CA
IL-1β	forward 5’	CTG CAG ATG CAA TGG AAA GA,
	reverse 5’	TTG CTT CCA AGG CAG ACT TT
COX-2	forward 5’	TGT TCC AAC CCA TGT CAA AA,
	reverse 5’	CGT AGA ATC CAG TCC GGG TA
TNFα	forward 5’	TCC ATG GCC CAG ACC CTC ACA C
	reverse 5’	TCC GCT TGG TGG TTT GCT ACG
iNOS	forward 5’	AGG GAG TGT TGT TCC AGG TG,
	reverse 5’	TCT GCA GGA TGT CTT GAA CG
IL-18	forward 5’	TGG AGA CTT GGA ATC AGA CC,
	reverse 5’	GGC AAG CTA GAA AGT GTC CT
IFNα	forward 5’	CCT CAG CCT CTT CAC ATC AA,
	reverse 5’	TGT GGC TCA GGA CTC ATT TC
18s	forward 5’	CGG GTG CTC TTA GCT GAG TGT CCC G,
	reverse 5’	CTC GGG CCT GCT TTG AAC AC

Whole-body plethysmography experiments

Ventilation was measured in freely moving, unanesthetized adult males and females using 2L custom built whole-body plethysmography chambers, similar to those described previously (Hodges et al., 2002, 2013; Kaplan et al., 2016; Mouradian et al., 2012). Rectal temperatures were assessed pre- and post-plethysmography. Prior to each experiment, the chambers were calibrated by pipetting 1mL of air into the chamber at least 20 times. The plethysmography chamber

temperatures and humidity levels were measured throughout experiments (Vernier© and Logger lite© software). Mass flow controllers (Alicat Scientific©, Tucson, AZ) controlled the flow rate and composition of oxygen, nitrogen, and carbon dioxide into each chamber at 4L/min. Breathing was measured as changes in pressure using a spirometry pod (ADInstruments®, Colorado Springs, CO) and analyzed with LabChart software (PowerLab System, ADInstruments®, LabChart v8.1). The plethysmography recordings occurred between 7:00 am and 4:00 pm. To assess hypercapnic ventilatory response (HCVR), inflow gas was switched to 5% CO_2 (21% O_2, balance N_2, 10 min). After a 20-minute recovery period in normoxia (21% O_2, balance N_2), the hypoxic ventilatory response (HVR) was assessed during inflow gas of 10% oxygen (balanced N_2, 10 min), followed by normoxia (21% O_2, balance N_2, 15 min).

Plethysmography analyses

Ventilation was assessed as described previously for neonates (Hocker et al., 2021). In brief, frequency (breaths/minute) and tidal volume (V_T, mL/100g) were analyzed from segments of continuous breathing (>30 sec/segment) without sniffing, sighs or post-sigh apneas in LabChart (v8, AD Instruments®). Baseline breathing was determined by averaging 10 minutes of eupneic breathing during normoxia (30 min, 21% O_2, balance N_2) prior to chemoreflex challenges. Body temperatures were estimated during eupnea, hypercapnia, and hypoxia by generating a trend line from the pre- and post-rectal temperatures. Chamber temperatures and humidity were extrapolated throughout the experiment by correlating the time of each breath with concurrent chamber temperature and humidity at that time. Tidal volumes were calculated using average chamber pressure calibrations (0.5mL), body temperatures, chamber temperatures, humidity and barometric pressures (Drorbaugh & Fenn, 1955). Minute ventilation (V_E, mL/min/kg) was calculated as the

product of frequency and V_T. The custom-built plethysmograph is most effective for assessing respiratory frequency and is not optimized for absolute quantification of tidal volume. Thus, tidal volume and minute ventilation are reported as percent changes from baseline within each treatment during HCVR and HVR. The peak HCVR (the last 2 minutes) and phase I HVR (peak hypoxic response, minutes 4-6 in hypoxia) were assessed as the rolling averages in 2 min bins for frequency (Hodges et al., 2002, 2013; Kaplan et al., 2016; Mouradian Jr et al., 2012). Phase II HVR (hypoxic ventilatory decline) was assessed as the average V_T during last 2 min of the hypoxic stimulus (Hodges et al., 2002, 2013; Kaplan et al., 2016; Mouradian Jr et al., 2012).

Statistics

GraphPad Prism (version 9.3) was used for statistical analyses. Two-way ANOVAs were used to assess the effect of neonatal treatment and adult treatment on microglia frequency. We did not compare microglia frequency between regions, since we previously determined microglia frequency is significantly different between respiratory control regions and non-respiratory control regions (Beyeler. et. al*., in preparation*). Within each gene, a Two-way ANOVA was used to assess the impact of neonatal and adult treatment on microglia inflammatory gene expression. Comparisons were not made between genes since the relative expression between genes does not significantly advance our understanding of their role in impairing adult ventilatory control. Two-way ANOVAs were used to assess the effects of neonatal and adult treatment on breathing during eupnea and chemoreflexes. To determine whether ventilation increased in response to respiratory challenges (hypercapnia or hypoxia), three-way RM ANOVAs were used to assess the impact of neonatal treatment (saline, LPS), adult treatment (saline, LPS, polyIC) and time (0-10 mins during hypercapnia or hypoxia) from baseline (pre-hypercapnia or hypoxia challenge). To determine whether neonatal and adult treatments impacted ventilation throughout hypercapnia or hypoxia,

neonatal and adult treatment were collapsed into one factor and assessed with a two-way ANOVA at each time point during the response. Bonferroni post hoc tests were used to limit type I errors. For all tests, $p<0.05$ was considered significant and all data are expressed as mean±SD.

Results

Neonatal and adult inflammation stimulus-specifically increased microglia in respiratory control regions.

We previously demonstrated neonatal inflammation led to lasting activation of adult microglia (Beyeler et al*., in preparation*), suggesting neonatal inflammation likely has functional consequences for how microglia respond to future inflammatory challenges. In non-respiratory control regions, neonatal inflammation primes adult microglia, in part, by increasing microglia density in response to subsequent adult inflammation (Bland et al., 2010 and reviewed in Bilbo and Schwarz, 2012; Mallard et al., 2019). Thus, we investigated the impact of neonatal inflammation on adult microglia priming in respiratory control regions. Flow cytometry was used to measure adult microglia frequency, defined as the percentage of microglial cells within homogenate samples of dissociated cells (%$CD11b^{high}CD45^{low}$/homogenates) isolated from adult respiratory control regions (medulla, location of respiratory rhythm and pattern generating regions; or ventral cervical spinal cord, location of the phrenic motoneurons) and the forebrain (prefrontal cortex, as a positive control). Neonates were treated with either neonatal inflammation (lipopolysaccharide, LPS, 1mg/kg, i.p., P4) or neonatal saline (vehicle control), as described previously (Hocker et al., 2019; Beyeler et al*., in preparation*), and adults were treated with subsequent adult subthreshold inflammation (LPS, 24µg/kg, i.p. or polyIC 478µg/kg, i.p.) or saline (vehicle control).

Neonatal inflammation primed adult male medullary microglia to increase in response to subthreshold adult inflammation. In males, neonatal treatment ($p=0.0001$), adult treatment ($p=0.0043$), and the interaction between neonatal-adult treatment ($p=0.0097$) had significant main

effects on microglia frequency (% $CD11b^{high}CD45b^{low}$ cells / homogenates). Pairwise differences showed medullary microglia frequency increased in males after neonatal LPS + adult saline (18.1±2.4% microglia) compared to adult males after neonatal saline, regardless of adult treatment (neonatal saline + adult saline=10.1±1.8% microglia, p=0.0006, neonatal saline + adult LPS=10.3±1.9% microglia, p=0.0008, neonatal saline + adult polyIC=10.7±1.5% microglia, p=0.0024, **Fig. 1A**), demonstrating neonatal inflammation alone cause a lasting increase in adult male medullary microglia, consistent with previous studies (Beyeler et al*., in preparation*). Neonatal LPS + adult LPS (25.4±5% microglia) also increased male medullary microglia frequency compared to males after neonatal LPS + adult saline ($p<0.0019$), and compared to all males after neonatal saline (neonatal saline + adult saline, p=0.0001, neonatal saline + adult LPS, p=0.0001, neonatal saline + adult polyIC, p=0.0001), demonstrating neonatal and adult homotypic inflammation primes male medullary microglia. Further, neonatal LPS + adult polyIC (25.1±6.6% microglia) increased adult male medullary microglia frequency compared to males after neonatal LPS + adult saline ($p<0.0027$), and compared to all males after neonatal saline (neonatal saline + adult saline, p=0.0001, neonatal saline + adult LPS, p=0.0001, neonatal saline + adult polyIC, p=0.0001), demonstrating neonatal and adult heterotypic inflammation primes male medullary microglia. Additionally, medullary microglia were similar between males after neonatal LPS + adult LPS and neonatal LPS + adult polyIC (p=0.99). Thus, adult male medullary microglia increased after neonatal inflammation, regardless of adult treatment. Within males after neonatal saline, there were no pairwise differences in spinal microglia frequency between adult treatment groups (p=0.99 for all comparisons), demonstrating the adult inflammatory challenges used here were subthreshold to increasing adult microglia number in otherwise healthy adults. In summary,

neonatal inflammation primed microglia to increase in response to adult subthreshold adult bacterial and viral inflammation.

In the spinal cord, neonatal treatment ($p=0.0003$), adult treatment ($p=0.0036$) and the interaction between neonatal-adult treatment ($p=0.0001$) had main effects on adult male spinal microglia number. Pairwise differences showed spinal microglia frequency in males after neonatal LPS + adult saline (9.4±2.5% microglia) were similar to all spinal microglia after neonatal saline, regardless of adult treatment (neonatal saline + adult saline=9.6±2% microglia, $p=0.99$, neonatal saline + adult LPS=8.9±1.2% microglia, $p=0.99$, neonatal saline + adult polyIC=9.9±2% microglia, $p=0.99$), showing adult male ventral cervical spinal microglia were unchanged by neonatal inflammation alone. Neonatal LPS + adult LPS (16.9±3.4% microglia) increased adult male spinal microglia compared males after neonatal LPS + adult saline ($p=0.0001$), compared to all adult males after neonatal saline (neonatal saline + adult saline, $p=0.0001$, neonatal saline + adult LPS, $p=0.0001$, neonatal saline + adult polyIC, $p=0.0001$), and compared to males after neonatal LPS + adult polyIC (12.1±4.7% microglia, $p=0.013$), demonstrating neonatal inflammation primed adult male spinal microglia to adult homotypic adult inflammation. In contrast, neonatal LPS + adult polyIC ($p=0.92$) did not change male spinal microglia frequency compared to neonatal LPS + adult saline ($p=0.84$), and all male spinal microglia after neonatal saline (neonatal saline + adult saline, $p=0.99$, neonatal saline + adult LPS, $p=0.40$, neonatal saline + adult polyIC, $p=0.99$), demonstrating male spinal microglia were unchanged by neonatal and adult heterotypic inflammation. Within adults after neonatal saline, there were no pairwise differences in spinal microglia frequency between adult treatment groups ($p=0.99$ for all comparisons), demonstrating adult inflammatory challenges were subthreshold to increasing adult

ventral cervical spinal microglia in healthy adults. These data demonstrate adult male spinal microglia are stimulus-specifically primed by neonatal inflammation.

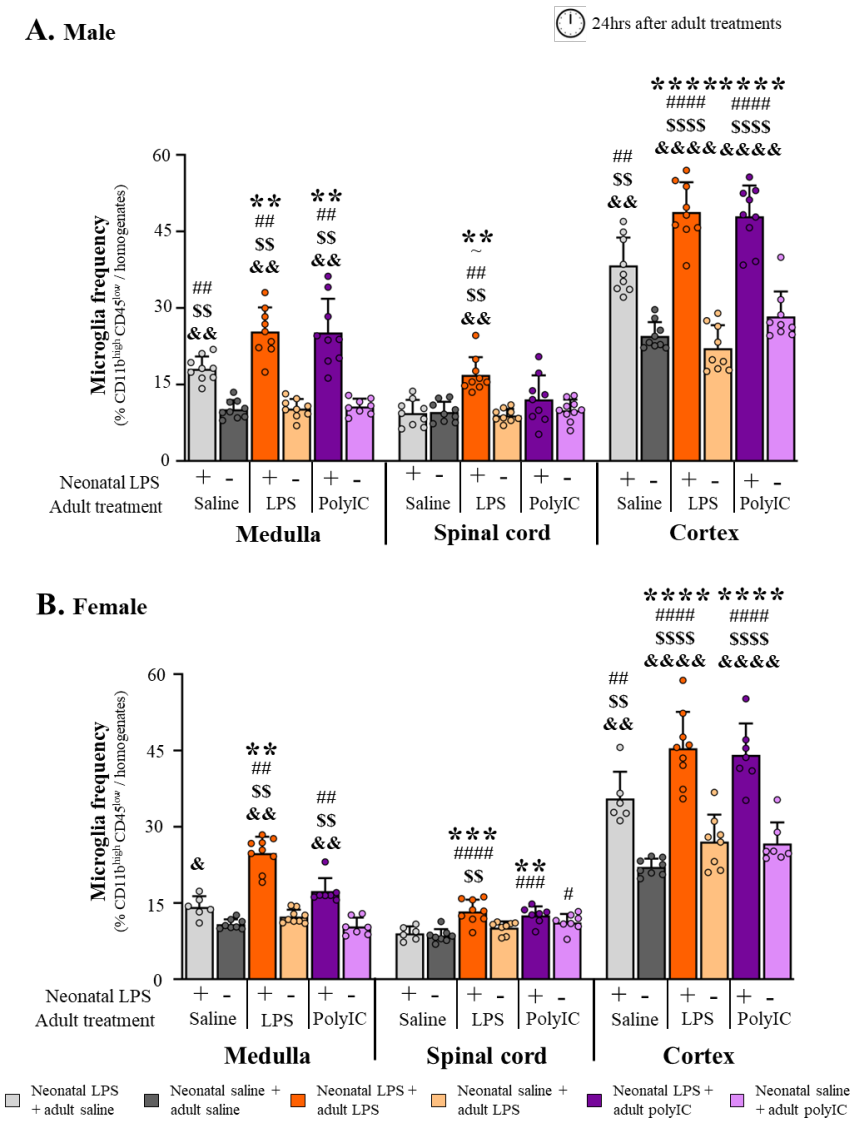


Figure 1. Adult microglia stimulus-specifically increased after the combination of neonatal and adult inflammation. In males (**A.**), medullary microglia frequency increased in all males after neonatal LPS + adult saline compared to all males after neonatal saline, regardless of adult treatment, and increased in adult males after neonatal LPS + adult LPS and neonatal LPS + adult polyIC compared to all other treatment groups. Spinal microglia frequency increased in males after neonatal LPS + adult LPS compared to all other groups. Cortical microglia frequency increased in males after neonatal LPS + adult saline compared to all females after neonatal saline, regardless of adult treatment, and increased in adult males after neonatal LPS + adult LPS and neonatal LPS + adult polyIC compared to all other treatment groups. In females (**B.**), medullary microglia frequency increased in females after neonatal LPS + adult LPS compared to all females after neonatal saline, regardless of adult treatment. Adult female spinal microglia were increased after neonatal LPS + adult LPS compared to neonatal LPS + adult saline, neonatal saline + adult saline, neonatal saline + adult LPS. Further, spinal microglia increased in females after neonatal LPS + adult polyIC compared to neonatal LPS + adult saline and neonatal saline + adult saline, and increased in females after neonatal saline + adult polyIC compared to neonatal saline + adult saline. Cortical microglia frequency increased in all females after neonatal LPS + adult saline from all female microglia after neonatal saline, regardless of adult treatment, and increased in females after neonatal LPS + adult LPS and neonatal LPS + adult polyIC compared to all other groups. (** $p<0.001$, **** $p<0.00001$ different from neonatal LPS + adult saline; ## $p<0.001$, #### $p<0.00001$ different from neonatal saline + adult saline; $$ $p<0.001$, $$$$ $p<0.00001$ different from neonatal saline + adult LPS; && $p<0.001$, &&&& $p<0.00001$ from neonatal saline + adult polyIC; Two-way ANOVAs, Bonferroni post-hoc.)

Similar to respiratory control regions, neonatal treatment ($p=0.0001$), adult treatment ($p=0.0009$) and the interaction between neonatal-adult treatment ($p=0.0015$) had main effects on adult male cortical microglia number. Pairwise differences showed cortical microglia increased in males after neonatal LPS + adult saline (38.3±5.4% microglia) compared to all males after neonatal saline (neonatal saline + adult saline=24.5±2.7% microglia, $p=0.0001$, neonatal saline + adult LPS=22.1±4.5% microglia, $p=0.0001$ and neonatal saline + adult polyIC=28.3±4.8% microglia, $p=0.0016$), demonstrating neonatal inflammation alone cause a lasting increase in adult male cortex microglia, consistent with previous studies (Bilbo, 2010; Beyeler et al., *in preparation*). Neonatal LPS + adult LPS (48.8±5.8% microglia) increased male cortical microglia compared to males after neonatal LPS + adult saline ($p=0.0008$), compared to all males after neonatal saline (neonatal saline + adult saline, $p=0.0001$, and compared to neonatal saline + adult LPS, $p=0.0001$ and neonatal saline + adult polyIC, $p=0.0001$), demonstrating neonatal and adult homotypic inflammation primes cortical microglia. Similarly, neonatal LPS + adult polyIC (47.9±6% microglia) increased male cortical microglia frequency compared to males after neonatal LPS + adult saline ($p=0.0027$), compared to all males after neonatal saline (neonatal saline + adult saline, $p=0.0001$, neonatal saline + adult LPS, $p=0.0001$ and neonatal saline + adult polyIC, $p=0.0001$), demonstrating neonatal and adult heterotypic inflammation primes male cortical microglia. Cortical microglia were similar in males after neonatal LPS + adult LPS to neonatal LPS + adult polyIC ($p=0.9$), providing further evidence that neonatal inflammation primes cortical microglia, regardless of adult treatment. Within adults after neonatal saline, there were no pairwise differences in cortical microglia frequency between adult treatment groups ($p>0.17$), showing adult inflammatory challenges were subthreshold to increasing adult male cortical microglia in

healthy adults. In summary, neonatal inflammation primed adult cortical microglia, regardless of adult treatment.

Neonatal inflammation stimulus-specifically primed adult female medullary microglia to increase in response to adult inflammation. In females, neonatal treatment ($p=0.0001$), adult treatment ($p=0.0001$) and the interaction between neonatal-adult treatment ($p=0.0034$) had significant main effects on female microglia frequency. When assessing pairwise differences, medullary microglia frequency was similar in adult female after neonatal LPS + adult saline (14±2.1% microglia) to females after neonatal saline + adult saline (11±1% microglia, $p=0.08$) and neonatal saline + adult LPS (12±1.3% microglia, $p=0.99$), but increased from neonatal saline + adult polyIC (10±1.8% microglia, $p=0.039$, **Fig. 1B**), showing adult female medullary microglia were not affected by neonatal inflammation alone. Neonatal LPS + adult LPS (25±3.2% microglia) increased female medullary microglia compared to females after neonatal LPS + adult saline ($p=0.0001$), all females after neonatal saline (neonatal saline + adult saline, $p=0.0001$, neonatal saline + adult LPS, $p=0.0001$, neonatal saline + adult polyIC, $p=0.0001$) and compared to females after neonatal LPS + adult polyIC ($p=0.0001$), demonstrating female cortical microglia were primed after neonatal and adult homotypic inflammation. In contrast, neonatal LPS + adult polyIC (17±2.6% microglia) were similar between female medullary microglia and females after neonatal LPS + adult saline ($p=0.19$), demonstrating neonatal inflammation did not prime female medullary microglia to heterotypic inflammation. However, neonatal LPS + adult polyIC increased female medullary microglia compared to all females after neonatal saline (neonatal saline + adult saline, $p=0.0001$; neonatal saline + adult polyIC, $p=0.0001$; neonatal saline + adult LPS, $p=0.0006$), demonstrating the combination of neonatal and adult heterotypic inflammation increased microglia. Within adults after neonatal saline, there were no pairwise differences in spinal

microglia frequency between adult treatment groups (p=0.99 for all comparisons), showing the adult inflammatory challenges used here were subthreshold to increasing adult microglia number in otherwise healthy adults. In summary, neonatal and adult homotypic inflammation primed adult female medullary microglia, without priming female medullary microglia to heterotypic inflammation.

In contrast to adult medullary microglia, neonatal treatment (p=0.0001) and adult treatment (p=0.0015), but not the interaction between neonatal-adult treatment (p=0.085), had main effects on adult female spinal microglia frequency. Pairwise differences showed spinal microglia in females after neonatal LPS + adult saline (9±1.3% microglia) were similar to females after neonatal saline + adult saline=8.5±1.3% microglia (p=0.99), and females after neonatal saline + adult LPS=10±1.2% microglia (p=0.33), demonstrating female spinal microglia were not affected by neonatal inflammation alone. Neonatal LPS + adult LPS (13±2.3% microglia) increased adult female spinal microglia compared to females after neonatal LPS + adult saline (p=0.0003), compared to females after neonatal saline + adult saline (p=0.0001) and compared to females after neonatal saline + adult LPS (p=0.0049), demonstrating neonatal and adult homotypic inflammation primes female spinal microglia. However, spinal microglia were similar in females after neonatal LPS + adult LPS to females after neonatal LPS + adult polyIC (p=0.99) and neonatal saline + adult polyIC (p=0.17), demonstrating neonatal inflammation has stimulus specific effects on adult female microglia. In contrast, neonatal LPS + adult polyIC (13±1.7% microglia) increased adult female spinal microglia compared to females after neonatal LPS + adult saline (p=0.0071) and neonatal saline + adult saline (p=0.0005), but was unchanged from females after neonatal saline + adult polyIC (p=0.99). Further, neonatal saline + adult polyIC increased adult female spinal microglia frequency compared to females after neonatal saline + adult saline (p=0.045),

suggesting female spinal microglia may have increased responses to viral challenges. Within females after neonatal saline, there were no other pairwise differences in spinal microglia frequency in females after neonatal saline (between neonatal saline + adult saline and neonatal saline + adult LPS, p=0.92; between neonatal saline + adult LPS and neonatal saline + adult polyIC, p=0.99), suggesting adult LPS treatments were subthreshold to increasing adult female spinal microglia number. In conclusion, neonatal and adult homotypic inflammation primes adult spinal microglia, while neonatal and adult heterotypic inflammation did not prime female spinal microglia number.

Similar to female respiratory control regions, neonatal treatment (p=0.0001) and adult treatment (p=0.0009), but not the interaction between neonatal-adult treatments (p=0.43) had main effects on adult female cortical microglia. Pairwise differences showed cortical microglia increased in females after neonatal LPS + adult saline (36±5.3% microglia) compared to all microglia after neonatal saline (neonatal saline + adult saline=22±1.7% microglia, p=0.0005; neonatal saline + adult LPS=27±5.3% microglia, p=0.0034; neonatal saline + adult polyIC=27±4.1% microglia, p=0.049), demonstrating neonatal inflammation alone causes a lasting increase in adult female cortical microglia, consistent with previous studies (Bilbo, 2010; Beyeler et al.*, in preparation*). Neonatal LPS + adult LPS (45±7.2% microglia) increased adult female cortical microglia frequency compared to females neonatal LPS + adult saline (p=0.016) and compared to all females after neonatal saline (neonatal saline + adult saline, p=0.0001, neonatal saline + adult LPS, p=0.0001, neonatal saline + adult polyIC, p=0.0001), demonstrating neonatal and adult homotypic inflammation primes female cortical microglia. Similarly, neonatal LPS + adult polyIC (44±6.2% microglia, p=0.9) increased female cortical microglia frequency compared to female after neonatal LPS + adult saline (p=0.090), and all females after neonatal

saline (neonatal saline + adult saline, p=0.0001, neonatal saline + adult LPS, p=0.0001, neonatal saline + adult polyIC, p=0.0001), demonstrating neonatal and adult heterotypic inflammation primes female cortical microglia. Thus, neonatal inflammation primed adult female microglia, regardless of adult treatment.

Adult male and female medullary microglia gene expression was stimulus-specifically primed after neonatal and adult inflammation.

To identify inflammatory genes involved in adult microglia priming after neonatal inflammation, inflammatory gene expression was assessed 24hrs after adult treatments in immunomagnetically isolated adult medullary and spinal microglia in all treatment groups and sexes. Neonatal treatment (p=0.0001) had main effects on male medullary microglial TNFα gene expression, while neither adult treatment (p=0.056) nor the interaction between neonatal-adult treatments (p=0.084) had main effects on male medullary microglial TNFα gene expression. Pairwise differences showed medullary microglial TNFα gene expression increased in males after neonatal LPS + adult saline (3.9±1.5 fold change) compared to males after neonatal saline (neonatal saline + adult saline=1±0 fold change, p=0.0087; neonatal saline + adult LPS=1.1±0.2 fold change, p=0.013; neonatal saline + adult polyIC=1.2±0.2 fold change, p=0.19), demonstrating neonatal inflammation caused lasting increases in adult male medullary TNFα gene expression (**Fig. 2A**). Neonatal LPS + adult LPS (6.4±2.3 fold change) increased male medullary microglial TNFα gene expression compared to all treatment groups (neonatal LPS + adult saline, p=0.026; neonatal saline + adult saline, p=0.0001; neonatal saline + adult LPS, p=0.0001; neonatal saline + adult polyIC, p=0.0001), except neonatal LPS + adult polyIC (5.2±2.2 fold change, p=0.99), demonstrating neonatal inflammation primes adult male TNFα gene expression. Medullary

microglial TNFα gene expression in males after neonatal LPS + adult polyIC were similar to males after neonatal LPS + adult saline (p=0.99), but increased from males after neonatal saline (neonatal saline + adult saline p=0.0001; neonatal saline + adult LPS p=0.0001; neonatal saline + adult polyIC p=0.0001), demonstrating neonatal and adult heterotypic inflammation did not prime male medullary TNFα gene expression. Within adults after neonatal saline, there were no differences between adult treatment groups (p=0.99 for all comparisons), demonstrating the adult inflammatory challenges used here were subthreshold to increasing adult microglial TNFα gene expression in otherwise healthy adults. In conclusion, neonatal and adult homotypic inflammation primed adult male medullary microglial TNFα gene expression, but was not primed to neonatal and adult heterotypic inflammation.

Similar to TNFα gene expression, neonatal treatment (p=0.0001), adult treatment (p=0.0001) and the interaction between neonatal-adult treatments (p=0.0001) had main effects on adult male medullary IL-6 gene expression. Pairwise differences showed medullary microglial IL-6 gene expression was similar between males after neonatal LPS + adult saline (1.2±0.3 fold change) and all males after neonatal saline (neonatal saline + adult saline=1±0 fold change, p=0.99; neonatal saline + adult LPS=1.0±0.2 fold change, p=0.99; neonatal saline + adult polyIC=1.1±0.2 fold change, p=0.99), demonstrating neonatal inflammation alone did not change adult male medullary IL-6 gene expression. However, neonatal LPS + adult LPS (3.8±0.8 fold change) increased adult male medullary IL-6 gene expression compared to all other groups (neonatal LPS + adult saline p=0.0001, neonatal saline + adult saline, p=0.0001; neonatal saline + adult LPS, p=0.0001; neonatal LPS + adult polyIC=1.9±1.0 fold change, p=0.0001; neonatal saline + adult polyIC, p=0.0001), demonstrating neonatal and adult homotypic inflammation primed medullary

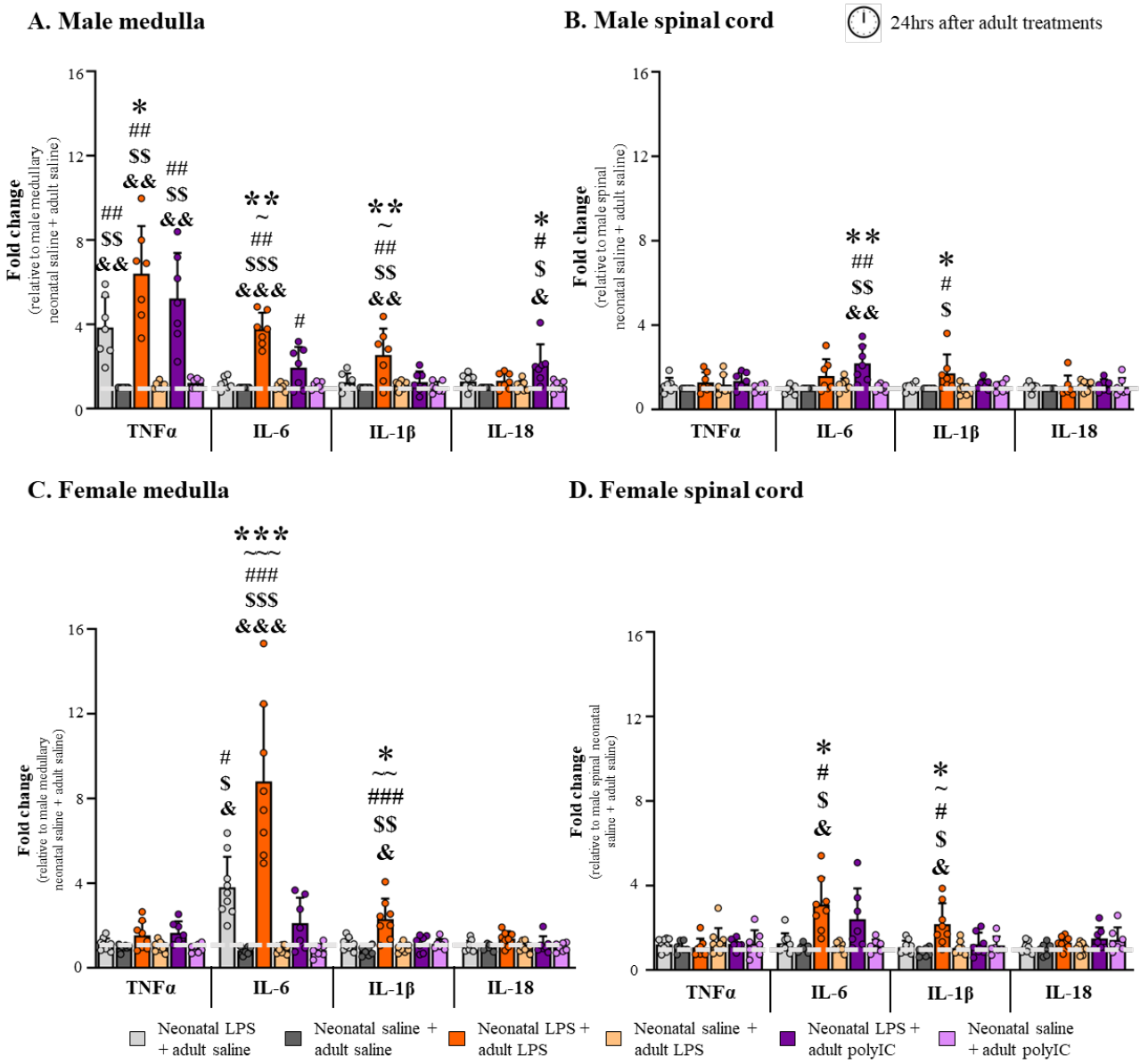


Figure 2. Adult microglial inflammatory gene expression region- and stimulus-specifically increased after neonatal and adult inflammation. Adult male medullary (**A.**) microglial TNFα gene expression increased in all males after neonatal LPS, regardless of adult treatment, compared to all males after neonatal saline and increased in adult males after neonatal LPS + adult LPS compared to males after neonatal LPS + adult saline. Medullary microglial IL-6 and IL-1β gene expression increased after neonatal LPS + adult LPS compared to all other groups. Medullary microglial IL-18 gene expression increased in adult males after neonatal LPS + adult polyIC compared to all males after neonatal saline and neonatal LPS + adult saline. Adult male spinal (**B.**) TNFα and IL-18 gene expression was unaffected by any treatment. Spinal IL-6 gene expression increased in males after neonatal LPS + adult polyIC compared to all males after neonatal saline and neonatal LPS + adult saline. Spinal IL-1β gene expression increased in males after neonatal LPS + adult LPS compared to males after neonatal LPS + adult saline, neonatal saline + adult saline, and neonatal saline + adult LPS. Adult female medullary (**C.**) microglial TNFα and IL-18 gene expression was unchanged in any treatment. Medullary microglial IL-6 gene expression increased in females after neonatal LPS + adult saline compared to all females after neonatal saline, regardless of treatment, whereas medullary IL-6 gene expression increased in females after neonatal LPS + adult LPS compared all other groups. Medullary microglial IL-1β gene expression increased in females after neonatal LPS + adult LPS from all other treatment groups. Adult female spinal (**D.**) TNFα and IL-18 gene expression was unchanged in any treatment group. Spinal IL-6 gene expression increased in females after neonatal LPS + adult LPS compared to all females except neonatal LPS + adult polyIC, while spinal IL-1β gene expression increased in females after neonatal LPS + adult LPS compared to all other treatment groups. (* $p<0.05$, ** $p<0.001$ different from neonatal LPS + adult saline; # $p<0.05$, ## $p<0.001$, ### $p<0.0001$ different from neonatal saline + adult saline; $ $p<0.05$, $$ $p<0.001$, $$$ $p<0.0001$ different from neonatal saline + adult LPS; ~ $p<0.05$, ~~$p<0.001$ different from neonatal LPS + adult polyIC; & $p<0.05$, && $p<0.001$, &&& $p<0.0001$ from neonatal saline + adult polyIC; One-way ANOVAs, Bonferroni post-hoc.).

microglial IL-6 gene expression. In contrast, neonatal LPS + adult polyIC increased male medullary IL-6 gene expression compared to males after neonatal saline + adult saline ($p=0.034$),

but was unchanged from all other groups (neonatal LPS + adult saline, p=0.21; neonatal saline + adult LPS, p=0.051; neonatal saline + adult polyIC, p=0.069), demonstrating neonatal and adult heterotypic inflammation has effects on male medullary microglial IL-6 gene expression, without priming these microglia. Within adult males after neonatal saline, medullary IL-6 gene expression was unchanged by any adult treatments (p=0.99 for all comparisons), demonstrating the adult dose of inflammation was subthreshold to increase adult male medullary IL-6 gene expression in otherwise healthy adults. In conclusion, neonatal and adult homotypic inflammation primed adult male medullary microglial IL-6 gene expression: however, neonatal and adult heterotypic inflammation did not prime adult medullary microglia.

Similar to TNFα and IL-6 gene expression, neonatal treatment (p=0.0013), adult treatment (p=0.0042) and the interaction between neonatal-adult treatments (p=0.016) had main effects on adult male medullary microglial IL-1β gene expression. Pairwise differences showed medullary microglial IL-1β gene expression was similar between males after neonatal LPS + adult saline (1.3±0.4 fold change) and all males after neonatal saline (neonatal saline + adult saline=1.0±0.0 fold change, p=0.99, neonatal saline + adult LPS=1.1±0.2 fold change, p=0.99; neonatal saline + adult polyIC=1.0±0.3 fold change, p=0.99), demonstrating neonatal inflammation alone did not change IL-1β gene expression. In contrast, neonatal LPS + adult LPS (2.5±1.3 fold change) increased males medullary IL-6 gene expression compared to all other treatment groups (neonatal LPS + adult saline, p=0.0037, neonatal saline + adult saline, p=0.0003; neonatal saline + adult LPS, p=0.0010; neonatal LPS + adult polyIC=1.3±0.5 fold change, p=0.0038; neonatal saline + adult polyIC p=0.0004), demonstrating neonatal and adult homotypic inflammation primes microglia. Since medullary microglial IL-1β gene expression was unchanged in males after neonatal LPS + adult polyIC compared to males after neonatal LPS + adult saline (p=0.99) and

compared to all males after neonatal saline (p=0.99 for all comparisons), neonatal inflammation did not prime medullary microglial IL-1β gene expression to adult heterotypic inflammation. Thus, adult male medullary IL-1β gene expression is stimulus specifically primed to neonatal and adult homotypic inflammation.

Neonatal treatment (p=0.0010) and adult treatment (p=0.036) had main effects on adult male medullary microglial IL-18 gene expression, while the interaction between neonatal-adult treatments (p=0.063) did not have a main effect on adult male medullary microglial IL-18 gene expression. Pairwise differences showed medullary microglial IL-18 gene expression was similar in males after neonatal LPS + adult saline (1.3±0.4 fold change) to all males after neonatal saline (neonatal saline + adult saline=1.0±0.0 fold change, p=0.99; neonatal saline + adult LPS=1.1±0.3 fold change, p=0.99; neonatal saline + adult polyIC=1.1±0.2 fold change, p=0.99), demonstrating neonatal inflammation alone did not change male medullary IL-18 gene expression. Neonatal LPS + adult LPS (1.3±0.4 fold change) did not change male medullary IL-18 gene expression compared males after neonatal LPS + adult saline (p=0.99) and compared to all males after neonatal saline (p=0.99 for all males after neonatal saline groups), demonstrating neonatal and adult homotypic inflammation did not prime male medullary IL-18 gene expression. In contrast, neonatal LPS + adult polyIC (2.1±1.0 fold change) increased male medullary microglial IL-18 gene expression compared to males after neonatal LPS + adult saline (p=0.041) and compared to all neonatal saline groups (neonatal saline + adult saline, p=0.0017; neonatal saline + adult LPS, p=0.0036; neonatal saline + adult polyIC, p=0.0038), demonstrating neonatal and adult heterotypic inflammation primed adult male medullary microglial IL-18 gene expression. Medullary microglial IL-18 gene expression was similar in males after neonatal LPS + adult polyIC and neonatal LPS + adult LPS (p=0.061), suggesting neonatal inflammation stimulus specifically primed male medullary IL-18

gene expression. In summary, neonatal and adult heterotypic inflammation primed male medullary IL-18 gene expression, but was unchanged after neonatal and adult homotypic inflammation.

Adult male medullary microglial COX-2, iNOS and IFNα gene expression were not significantly affected by any combination of neonatal and adult treatments. Neonatal treatment ($p=0.049$) had a main effect on male medullary COX-2 gene expression. However, neither adult treatment ($p=0.059$) nor the interaction between neonatal and adult treatment ($p=0.23$) had main effects on male medullary COX-2 gene expression and there were no pairwise differences between treatment groups ($p>0.15$, **Table. 1**). In contrast, neither neonatal treatment ($p=0.63$), adult treatment ($p=0.27$) nor the interaction between neonatal-adult treatments ($p=0.27$) had main effects on male medullary iNOS expression and there were no pairwise differences between groups ($p>0.70$, **Table. 1**). Additionally, neither neonatal treatment ($p=0.33$), adult treatment ($p=0.46$) nor the interaction between neonatal-adult treatments ($p=0.15$) had main effects on male medullary IFNα expression and there were no pairwise differences between groups ($p>0.93$, **Table. 1**). Thus, neonatal inflammation likely primes specific microglial inflammatory genes, without inducing a generalized inflammatory response.

Table 1. COX-2, iNOS and IFNa gene expression.

			COX-2 fold change		**iNOS fold change**		**IFNα fold change**	
Sex	**Region**	**Treatment groups**	mean	SD	mean	SD	mean	SD
Male	medulla	Neonatal LPS + adult saline	0.96	0.19	0.97	0.32	1.31	0.51
		Neonatal saline + adult saline	1.00	0.00	1.00	0.00	1.00	0.00
		Neonatal LPS + adult LPS	1.54	0.54	1.29	0.44	1.31	0.46
		Neonatal saline + adult LPS	1.11	0.41	1.04	0.29	1.11	0.25
		Neonatal LPS + adult polyIC	1.11	0.30	1.00	0.26	0.95	0.32
		Neonatal saline + adult polyIC	1.10	0.30	1.09	0.30	1.14	0.29
		Neonatal LPS + adult saline	0.96	0.19	1.37	0.40	1.08	0.30

	spinal cord	Neonatal saline + adult saline	1.00	0.00	1.00	0.00	1.00	0.00
		Neonatal LPS + adult LPS	1.54	0.54	1.05	0.25	1.13	0.31
		Neonatal saline + adult LPS	1.11	0.41	1.08	0.25	1.05	0.20
		Neonatal LPS + adult polyIC	1.33	0.56	1.24	0.35	1.09	0.20
		Neonatal saline + adult polyIC	1.02	0.16	1.15	0.44	1.05	0.20
Female	medulla	Neonatal LPS + adult saline	0.97	0.27	1.06	0.26	1.10	0.23
		Neonatal saline + adult saline	0.92	0.19	1.09	0.12	0.95	0.13
		Neonatal LPS + adult LPS	1.15	0.32	1.23	0.31	1.37	0.49
		Neonatal saline + adult LPS	0.98	0.23	1.03	0.23	0.96	0.22
		Neonatal LPS + adult polyIC	1.15	0.32	1.13	0.30	1.28	0.38
		Neonatal saline + adult polyIC	1.02	0.24	1.04	0.22	1.28	0.28
	spinal cord	Neonatal LPS + adult saline	0.99	0.28	0.80	0.22	1.06	0.25
		Neonatal saline + adult saline	1.05	0.13	0.98	0.25	1.10	0.28
		Neonatal LPS + adult LPS	0.88	0.26	1.11	0.19	1.05	0.35
		Neonatal saline + adult LPS	1.11	0.38	1.04	0.24	1.12	0.22
		Neonatal LPS + adult polyIC	0.92	0.24	1.06	0.31	1.33	0.69
		Neonatal saline + adult polyIC	1.07	0.32	1.13	0.24	1.12	0.28

Interestingly, different inflammatory genes were primed in adult male spinal microglia by the combination of neonatal and adult treatments. Neither neonatal treatment ($p=0.16$), adult treatment ($p=0.13$) nor neonatal-adult treatment interaction ($p=0.58$) had main effects on male spinal TNFα gene expression and there were no pairwise differences between treatment groups (neonatal saline + adult saline 1.0±0.0 fold change; neonatal LPS + adult saline 1.2±0.4 fold change; neonatal saline + adult LPS 1.2±0.5 fold change; neonatal LPS + adult LPS 1.3±0.5 fold change; neonatal saline + adult polyIC 1.0±0.2 fold change; neonatal LPS + adult polyIC 1.4±0.4 fold change; $p>0.70$ **Fig. 2B**), demonstrating spinal microglial TNFα gene expression is not primed to either homotypic or heterotypic inflammation, despite priming in medullary microglial gene expression.

In contrast, neonatal treatment ($p=0.0051$), adult treatment ($p=0.021$) and the interaction between neonatal-adult treatments ($p=0.029$) had main effects on male spinal IL-6 gene expression. Pairwise differences showed spinal IL-6 gene expression increased in males after

neonatal LPS + adult polyIC (2.2±0.9 fold change) compared to males after neonatal LPS + adult saline ($p=0.0048$) and all males after neonatal saline (neonatal saline + adult saline=1.0±0.0 fold change, $p=0.0055$, neonatal LPS + adult saline=0.9±0.2 fold change, $p=0.020$, neonatal saline + adult LPS=1.2±0.3 fold change $p=0.0074$), but was similar to neonatal LPS + adult LPS (1.6±0.8 fold change, $p=0.58$). There were no other pairwise differences between groups ($p>0.99$). Thus, neonatal and adult heterotypic inflammation primed male spinal IL-6 gene expression, but neonatal and adult homotypic inflammation did not prime male spinal IL-6 gene expression.

Similar to male spinal IL-6 gene expression, neonatal treatment ($p=0.015$) and the interaction between neonatal-adult treatments ($p=0.039$) had main effects on male spinal IL-1β gene expression. However, adult treatment ($p=0.14$) did not have a main effect on male spinal IL-1β gene expression. Pairwise differences showed spinal IL-1β gene expression increased in males after neonatal LPS + adult LPS (1.7±0.9 fold change) compared to neonatal LPS + adult saline (1.0±0.2 fold change, $p=0.049$), neonatal saline + adult saline (1±0 fold change, $p=0.035$), and neonatal saline + adult LPS (0.94±0.3 fold change, $p=0.015$), with no other differences between treatment groups (neonatal LPS + adult polyIC=1.2±0.2 fold change, $p>0.11$). Thus, neonatal and adult homotypic inflammation primed male spinal IL-1b gene expression, but neonatal and adult heterotypic inflammation did not prime spinal IL-1b gene expression. Similar to male spinal TNFα gene expression, neonatal treatment ($p=0.70$), adult treatment ($p=0.64$) nor the interaction between neonatal-adult treatments ($p=0.96$) had main effects on male spinal IL-18 gene expression and there were no pairwise differences (neonatal saline + adult saline 1.0±0.0 fold change; neonatal LPS + adult saline 1.2±0.4 fold change; neonatal saline + adult LPS 1.2±0.5 fold change; neonatal LPS + adult LPS 1.3±0.5 fold change; neonatal saline + adult polyIC 1.0±0.2 fold change; neonatal LPS + adult polyIC 1.4±0.4 fold change, $p>0.99$). In summary, the combination of neonatal and

adult treatments had stimulus specific effects on adult spinal IL-6 and IL-1β gene expression, without impacting TNFα or IL-18 gene expression.

Adult male spinal microglial COX-2, iNOS and IFNα gene expression were not affected by any combination of neonatal and adult treatments. Neither neonatal treatment ($p=0.11$), adult treatment ($p=0.47$), nor the interaction between neonatal-adult treatments ($p=0.68$) had a main effect on male spinal COX-2 gene expression and there were no pairwise differences between treatment groups ($p>0.99$, **Table. 1**). Similarly, neither neonatal treatment ($p=0.36$), adult treatment ($p=0.75$) nor the interaction between neonatal-adult treatments ($p=0.57$) had main effects on male spinal iNOS expression and there were no pairwise differences between groups ($p>0.99$, **Table. 1**). Additionally, neither neonatal treatment ($p=0.15$), adult treatment ($p=0.86$) nor the interaction between neonatal-adult treatments ($p=0.90$) had main effects on male spinal IFNα expression and there were no pairwise differences between groups ($p>0.93$, **Table. 1**). Thus, neonatal inflammation likely primes specific spinal microglial inflammatory genes, without inducing a generalized inflammatory response.

When focusing on female microglial inflammatory gene expression, neonatal treatment ($p=0.0026$) had main effects on adult female medullary microglial TNFα gene expression. However, neither adult treatment ($p=0.17$) nor neonatal-adult treatments ($p=0.39$) had main effects on microglial TNFα gene expression and there were no pairwise differences between treatment groups (neonatal saline + adult saline 1.0±0.5 fold change; neonatal LPS + adult saline 1.1±0.3 fold change; neonatal saline + adult LPS 1.0±0.2 fold change; neonatal LPS + adult LPS 1.5±0.7 fold change; neonatal saline + adult polyIC 0.9±0.2 fold change; neonatal LPS + adult polyIC 1.7±0.5 fold change; $p>0.102$; **Fig. 2C**). In contrast, neonatal treatment ($p=0.0001$), adult treatment ($p=0.0001$) and the interaction between neonatal-adult treatments ($p=0.0001$) had main effects on

adult female medullary microglial IL-6 gene expression. Medullary microglial IL-6 gene expression was increased in females after neonatal LPS + adult saline (3.9±1.5 fold change) compared to all females after neonatal saline (neonatal saline + adult saline=1±0 fold change, p=0.032, neonatal saline + adult LPS=1.1±0.2 fold change, p=0.033, neonatal saline + adult polyIC=1.2±0.2 fold change, p=0.032). Further, neonatal LPS + adult LPS (3.8±0.8 fold change) increased female medullary microglial IL-6 gene expression compared to all other treatment groups ($p<0.0002$), demonstrating neonatal and adult homotypic inflammation primes female medullary microglial IL-6 gene expression. In contrast, neonatal LPS + adult polyIC (2.1±1.2 fold change) did not change adult female medullary IL-6 gene expression from any treatment group (p=0.99 for all comparisons), demonstrating neonatal and adult heterotypic inflammation did not prime adult male medullary IL-6 gene expression.

Interleukin gene expression was affected by the combination of neonatal and adult treatments. While IL-1β, but not IL-18, gene expression was stimulus specifically primed by the combination of neonatal and adult treatments. Neonatal treatment (p=0.0047), adult treatment (p=0.018), and the interaction between neonatal-adult treatments (p=0.0048) had main effects on adult female medullary microglial IL-1β gene expression. Pairwise differences showed neonatal LPS + adult LPS (2.5±1.3 fold change) increased female medullary microglial IL-1β gene expression compared to all other treatment groups (neonatal LPS + adult saline=1.3±0.4 fold change, p=0.017; neonatal saline + adult saline=1.0±0.0 fold change, p=0.0004; neonatal saline + adult LPS=1.1±0.2 fold change, p=0.0016; neonatal LPS + adult polyIC=1.3±0.5 fold change, p=0.0048; neonatal saline + adult polyIC=1.0±0.3 fold change, p=0.022). Since neonatal LPS + adult polyIC was unchanged from neonatal LPS + adult saline (p=0.99) and all neonatal saline groups (p=0.99 for all comparisons), demonstrating adult female medullary microglial IL-1β gene

expression was stimulus specifically primed to neonatal and adult homotypic inflammation. In contrast, adult treatment ($p=0.044$) had a main effect on female medullary microglial IL-18 gene expression. However, neither neonatal treatment ($p=0.15$) nor the interaction between neonatal-adult treatments ($p=0.38$) had main effects on female medullary microglial IL-18 gene expression and there were no pairwise differences (neonatal saline + adult saline 1.0±0.0 fold change; neonatal LPS + adult saline 1.2±0.4 fold change; neonatal saline + adult LPS 1.2±0.5 fold change; neonatal LPS + adult LPS 1.3±0.5 fold change; neonatal saline + adult polyIC 1.0±0.2 fold change; neonatal LPS + adult polyIC 1.4±0.4 fold change, $p>0.13$). Thus, IL-1β gene expression was stimulus specifically primed by the combination of neonatal and adult treatments, while IL-18 was unchanged by the combination of neonatal and adult treatments.

Adult female medullary microglial COX-2, iNOS and IFNα gene expression were not affected by any combination of neonatal and adult treatments. Neither neonatal treatment ($p=0.12$), adult treatment ($p=0.15$), nor the interaction between neonatal-adult treatments ($p=0.55$) had a main effect on female medullary COX-2 gene expression and there were no pairwise differences between treatment groups ($p>0.19$, **Table. 1**). Similarly, neither neonatal treatment ($p=0.54$), adult treatment ($p=0.84$), nor the interaction between neonatal-adult treatments ($p=0.74$) had main effects on female medullary iNOS expression and there were no pairwise differences between groups ($p>0.99$, **Table. 1**). Additionally, neither neonatal treatment ($p=0.12$), adult treatment ($p=0.083$), nor the interaction between neonatal-adult treatments ($p=0.46$) had main effects on female medullary IFNα expression and there were no pairwise differences between groups ($p>0.52$, **Table. 1**). Thus, neonatal inflammation likely primes specific female medullary microglial inflammatory genes, without inducing a generalized inflammatory response.

Similar to female medullary microglia, there were no changes in female spinal TNFα gene expression, while neonatal and adult homotypic inflammation primed spinal microglial IL-6 gene expression. For female spinal TNFα gene expression, neither neonatal treatment ($p=0.60$), adult treatment ($p=0.70$), nor the interaction between neonatal-adult treatments ($p=0.47$) had main effects on adult female spinal microglial TNFα gene expression and there are no pairwise differences (neonatal saline + adult saline 1.0±0.5 fold change; neonatal LPS + adult saline 1.1±0.3 fold change; neonatal saline + adult LPS 1.0±0.2 fold change; neonatal LPS + adult LPS 1.5±0.7 fold change; neonatal saline + adult polyIC 0.9±0.2 fold change; neonatal LPS + adult polyIC 1.7±0.5 fold change, $p>0.99$; **Fig. 2C**). In contrast, female spinal IL-6 gene expression was significantly affected by neonatal treatment ($p=0.0003$) and adult treatment ($p=0.038$). However, the interaction between neonatal-adult treatments ($p=0.057$) did not have a main effect on female spinal IL-6 gene expression. Pairwise differences showed neonatal LPS + adult LPS (2.2±0.9 fold change) increased female spinal microglial IL-6 gene expression compared to females after neonatal LPS + adult saline (2.2±0.9 fold change, $p=0.019$), and compared to all females after neonatal saline groups (neonatal saline + adult saline 1.0±0.5 fold change, $p=0.0063$; neonatal saline + adult LPS 1.0±0.2 fold change, $p=0.0078$; neonatal saline + adult polyIC, 0.9±0.2 fold change, $p=0.013$), demonstrating neonatal and adult homotypic inflammation primed adult female spinal microglial IL-6 gene expression. In contrast, neonatal LPS + adult polyIC (2.2±0.9 fold change) did not change female spinal microglial IL-6 gene expression compared to all other groups ($p>0.12$), demonstrating neonatal and adult heterotypic inflammation did not prime adult female spinal microglial IL-6 gene expression.

Neonatal and adult homotypic inflammation primed female spinal medullary microglial IL-1β gene expression, while no changes in IL-18 gene expression. Female spinal IL-1β gene

expression was significantly affected by neonatal treatment (p=0.027) and adult treatment (p=0.026). However, the interaction between neonatal and adult treatment (p=0.074) did not have a main effect on female spinal IL-1β gene expression. Pairwise differences showed neonatal LPS + adult LPS (2.1±0.9 fold change) increased female spinal microglial IL-1β gene expression compared to females after neonatal LPS + adult saline (1.1±0.3 fold change, p=0.041), neonatal saline + adult saline (1.0±0.0 fold change, p=0.0066), and neonatal saline + adult LPS (1.0±0.3 fold change, p=0.042), but was similar to neonatal LPS + adult polyIC (1.2±0.9 fold change, p=0.064) and neonatal saline + adult polyIC (1.1±0.4 fold change, p=0.080), demonstrating neonatal and adult homotypic inflammation primes female spinal microglial IL-1β gene expression. There were no other differences between treatment groups (p=0.99, for all other comparisons), demonstrating neonatal and adult heterotypic inflammation did not prime female spinal microglial IL-1β gene expression. In contrast, adult treatment (p=0.040) had a main effect on spinal microglial IL-18 gene expression. However, neither neonatal treatment (p=0.42) nor the interaction between neonatal-adult treatment (p=0.89) had main effects on female spinal microglial IL-18 gene expression and there were no pairwise differences between treatment groups (neonatal saline + adult saline 1.0±0.0 fold change; neonatal LPS + adult saline 1.2±0.4 fold change; neonatal saline + adult LPS 1.2±0.5 fold change; neonatal LPS + adult LPS 1.3±0.5 fold change; neonatal saline + adult polyIC 1.0±0.2 fold change; neonatal LPS + adult polyIC 1.4±0.4 fold change, p>0.63). In summary, neonatal and adult homotypic inflammation primed female spinal microglial IL-1β gene expression, without effecting female spinal IL-18 gene expression.

Adult female spinal microglial COX-2, iNOS and IFNα gene expression were not affected by any combination of neonatal and adult treatments. Neither neonatal treatment (p=0.37), adult treatment (p=0.84), nor the interaction between neonatal-adult treatments (p=0.66) had a main

effect on female spinal COX-2 gene expression and there were no pairwise differences between treatment groups ($p>0.99$, **Table. 1**). Similarly, neither neonatal treatment ($p=0.16$), adult treatment ($p=0.24$), nor the interaction between neonatal-adult treatments ($p=0.44$) had main effects on female spinal iNOS expression and there were no pairwise differences between groups ($p>0.60$, **Table. 1**). Additionally, neither neonatal treatment ($p=0.46$), adult treatment ($p=0.58$), nor the interactions between neonatal-adult treatments ($p=0.62$) had main effects on female spinal IFNα expression and there were no pairwise differences between groups ($p>0.99$, **Table. 1**). Thus, neonatal inflammation likely primes specific female spinal microglial inflammatory genes, without inducing a generalized inflammatory response.

Male and female breathing was unaffected by any combination of neonatal and adult treatments, 3hrs after adult treatments.

Since neuroinflammatory responses and acute disruptions in respiratory control peak 3hrs after systemic LPS (Huxtable et al. 2013), in our first set of plethysmography experiments focused on adult breathing during the peak inflammatory response, 3hrs after adult treatments. Adult male eupneic breathing was similar between all treatment groups during the peak inflammatory response (**Fig. 3A**). Adult male eupneic breathing frequency (breaths/min) were not significantly affected by neonatal treatment ($p=0.53$), adult treatment ($p=0.067$), nor the interaction between neonatal-adult treatments ($p=0.85$). In assessing pairwise differences, adult male eupneic breathing frequency was unchanged by any combination of neonatal and adult treatments ($p>0.48$, **Fig. 3B**). Similarly, adult male eupneic tidal volume (V_T, mL/100g) was not significantly affected by neonatal treatment ($p=0.24$), adult treatment ($p=0.26$), nor the interaction between neonatal-adult treatments ($p=0.62$), and there were no pairwise differences between any treatment groups ($p>0.70$, **Fig. 3C**). Consistent with eupneic breathing frequency and tidal volume, adult male

eupneic minute ventilation (V_E, mL/100g/min) was not significantly affected by neonatal treatment (p=0.080), adult treatment (p=0.76), nor the interaction between neonatal-adult treatments (p=0.42) and there were no pairwise differences between any treatment groups (p>0.59, **Fig. 3D**). In summary, adult male breathing frequency, tidal volume and minute ventilation during eupnea were unchanged during the peak inflammatory response, 3 hours after adult treatments.

Similar to male breathing, adult female eupneic breathing was unaffected in any group during the peak inflammatory response (**Fig. 3E**). Neonatal treatment (p=0.20), adult treatment (p=0.78), nor the neonatal-adult treatment interaction (p=0.28) significantly affected female eupneic breathing frequency and there were no pairwise differences between treatments (p>0.99, **Fig. 3F**). Female eupneic V_T was not affected by neonatal treatment (p=0.36), adult treatment (p=0.9), nor interaction between neonatal-adult treatment (p=0.44) and there were no pairwise differences in any group (p>0.99, **Fig. 3G**). Female eupneic V_E was not affected by neonatal treatment (p=0.13), adult treatment (p=0.89), nor interaction between neonatal-adult treatment (p=0.61) and there were no pairwise differences in any treatment group (p>0.99, **Fig. 3H**). In summary, adult male and female eupneic breathing was maintained in all treatment groups during the peak inflammatory response, demonstrating no combination of neonatal and adult subthreshold treatments impaired male or female breathing during eupnea.

Eupnea (breathing at rest) includes multiple respiratory-related behaviors, including spontaneous apneas, sighs, and post-sigh apneas, and differences in the abundance of these respiratory behaviors are important indicators of respiratory health or pathology (Brown & Bates, 2000; Fleming et al., 1984; Hoch et al., 1998). Thus, we measured spontaneous apneas, sighs, and post-sigh apneas in all treatment groups. Adult male spontaneous apneas during eupnea were not significantly affected by either neonatal treatment (p=0.68), adult treatment (p=0.48), nor the

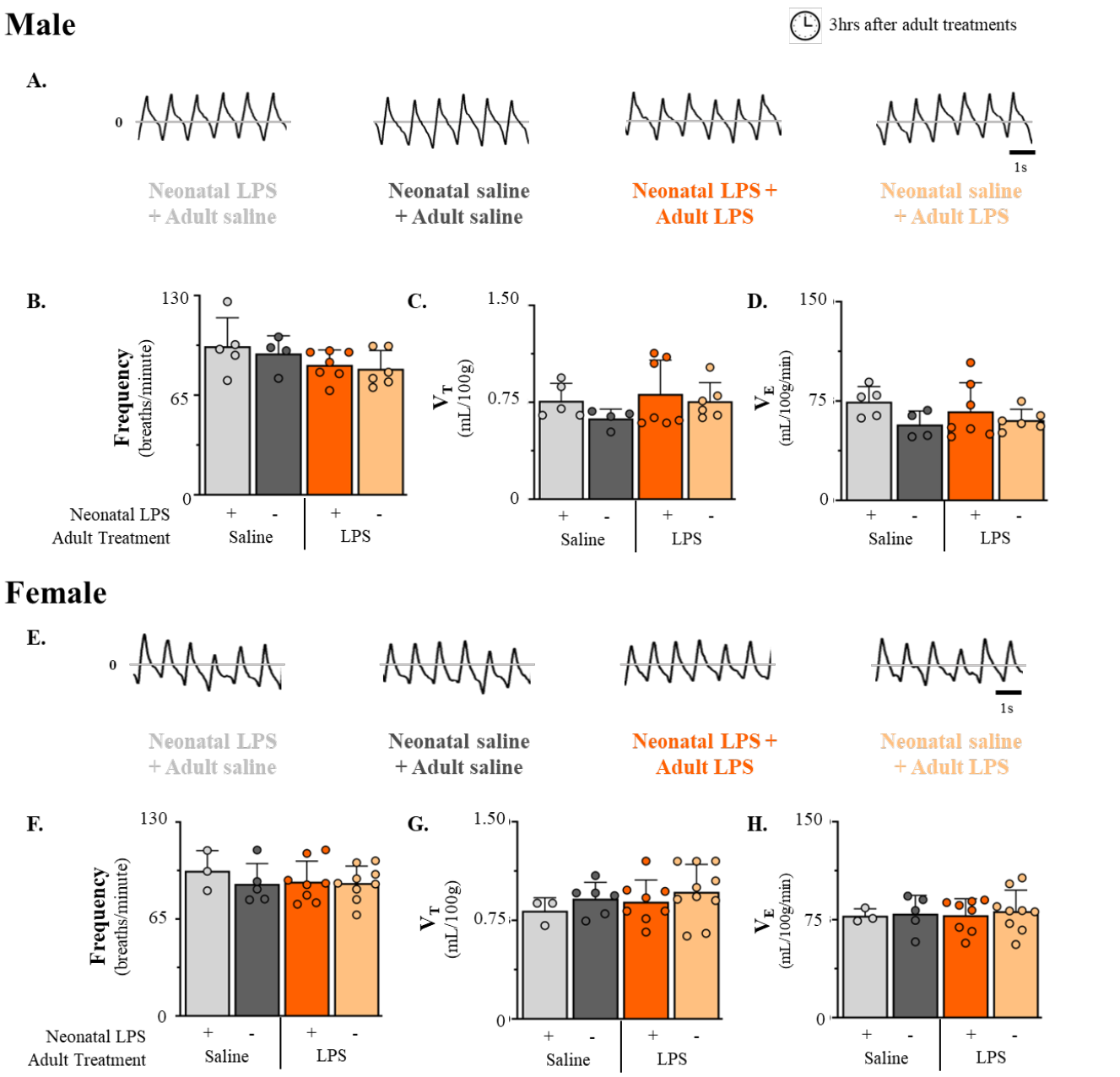


Figure 3. Male and female eupneic breathing was unchanged by any combination of neonatal and adult treatment. Plethysmography traces show breathing 3 hours after adult treatments and demonstrate adult male breathing (**A.**). Adult male eupneic breathing frequency (breath/min, **B.**), tidal volume (V_T, mL/100g, **C.**), and minute ventilation (V_E, mL/100g/min, **D.**) were unchanged by any combination of neonatal and adult treatment. Similarly, Plethysmography traces (**E.**) show adult female breathing was unchanged by any combination of neonatal and adult treatment, with no changes in adult female breathing frequency (**F.**), tidal volume (**G.**) and minute ventilation (**H.**). (Two-way ANOVAs).

interaction between neonatal-adult treatment (p=0.99) and there were no pairwise differences between groups p>0.99, **Table 2**). Adult male sighs during eupnea were not significantly affected by either neonatal treatment (p=0.27), adult treatment (p=0.91), nor the interaction between neonatal-adult treatments (p=0.27) and there were no pairwise differences between treatment

groups (p>0.94, **Table 2**). Similar to sighs, post-sigh apneas during eupnea in males were not significantly affected by neonatal treatment (p=0.79), adult treatment (p=0.65), or the interaction between neonatal-adult treatment (p=0.49) and there were no pairwise differences (p>0.99, **Table 2**). Thus, neonatal and adult inflammation did not affect male spontaneous apneas, sighs or post-sigh apneas during the peak inflammatory response, 3 hours after adult treatments.

We previously showed neonatal inflammation causes a lasting decrease in adult female sighs during eupnea (Beyeler et al., *in preparation*); thus, the effects of neonatal and adult inflammation on spontaneous apneas, sighs and post-sigh apneas during eupnea were assessed in adult females. Spontaneous apneas were not significantly affected by either neonatal treatment (p=0.96), adult treatment (p=0.96), nor the interaction between neonatal-adult treatment (p=0.82) and there were no pairwise differences between groups p>0.99, **Table 2**). Consistent with our previous findings (Beyeler et al*., in preparation*), neonatal treatment (p=0.0014) had a significant main effect on adult female sighs during eupnea. However, neither adult treatment (p=0.090), nor the interaction between neonatal-adult treatments (p=0.69) had a main effect on female sighs during eupnea. Accordingly, pairwise differences showed sighs during eupnea were decreased in females after neonatal LPS + adult saline compared to neonatal saline + adult LPS (p=0.011) and decreased in females after neonatal LPS + adult LPS compared to neonatal saline + adult LPS (p=0.0084), with no other differences between treatment groups (p>0.38). Thus, neonatal inflammation decreases adult female sighs during eupnea, which remained decreased after subsequent adult inflammatory challenges. In contrast to neonatal inflammation's impact on adult female sighs during eupnea, post-sigh apneas were unchanged by any treatment in females. Neonatal treatment (p=0.91), adult treatment (p=0.42), nor the interaction between neonatal-adult treatments (p=0.91) did not significantly affect post-sigh apneas during eupnea and there were no

pairwise differences (p>0.9). In summary, neonatal inflammation decreased adult female sighs, which remained decreased during subsequent adult inflammation, without affecting spontaneous apneas or post-sigh apneas.

Table 2. Spontaneous apneas, sighs and post-sigh apneas during eupnea, 3hrs after adult treatments.

		Number of spontaneous apneas		Number of sighs		Number of post-sigh apneas	
	Treatment groups	mean	SD	mean	SD	mean	SD
Male	Neonatal LPS + adult saline	0.40	0.55	1.40	1.10	0.60	0.55
	Neonatal saline + adult saline	0.50	0.60	2.50	1.30	0.75	0.96
	Neonatal LPS + adult LPS	0.57	0.54	2.00	1.20	1.00	0.82
	Neonatal saline + adult LPS	0.67	0.52	2.00	0.89	0.67	0.82
Female	Neonatal LPS + adult saline	0.33	0.58	0$^{\$}$	0$^{\$}$	1.00	1.00
	Neonatal saline + adult saline	0.40	0.55	1.20	0.84	1.00	1.00
	Neonatal LPS + adult LPS	0.38	0.52	0.50$^{\$}$	0.54$^{\$}$	0.75	0.71
	Neonatal saline + adult LPS	0.33	0.50	2.00	1.10	0.67	0.71

$ different from neonatal saline + adult LPS, Two-way ANOVAs, Bonferroni post hoc.

Sex-dependent augmentation of hypercapnic ventilatory responses after neonatal LPS + adult saline, 3 hours after adult treatments.

Neonatal inflammation causes a lasting augmentation in male hypercapnic ventilatory responses (HCVR; Beyeler et al*., in preparation*), suggesting males may be more susceptible to disruptions in hypercapnic responses during future inflammatory challenges. Thus, we measured HCVR in all treatment groups and in both sexes, 3 hours after adult treatments. Time ($p<0.0001$), adult treatment ($p=0.018$), and the interaction between adult treatment-time ($p=0.013$) had main effects on V_E during male HCVR. However, neither neonatal treatment ($p=0.28$) nor the interactions between neonatal-adult treatments ($p=0.16$), neonatal treatment-time ($p=0.25$), and between neonatal-adult treatment-time ($p=0.35$) had main effects on male V_E during HCVR. Male V_E increased from baseline at each timepoint during HCVR in all treatment groups ($p<0.0045$),

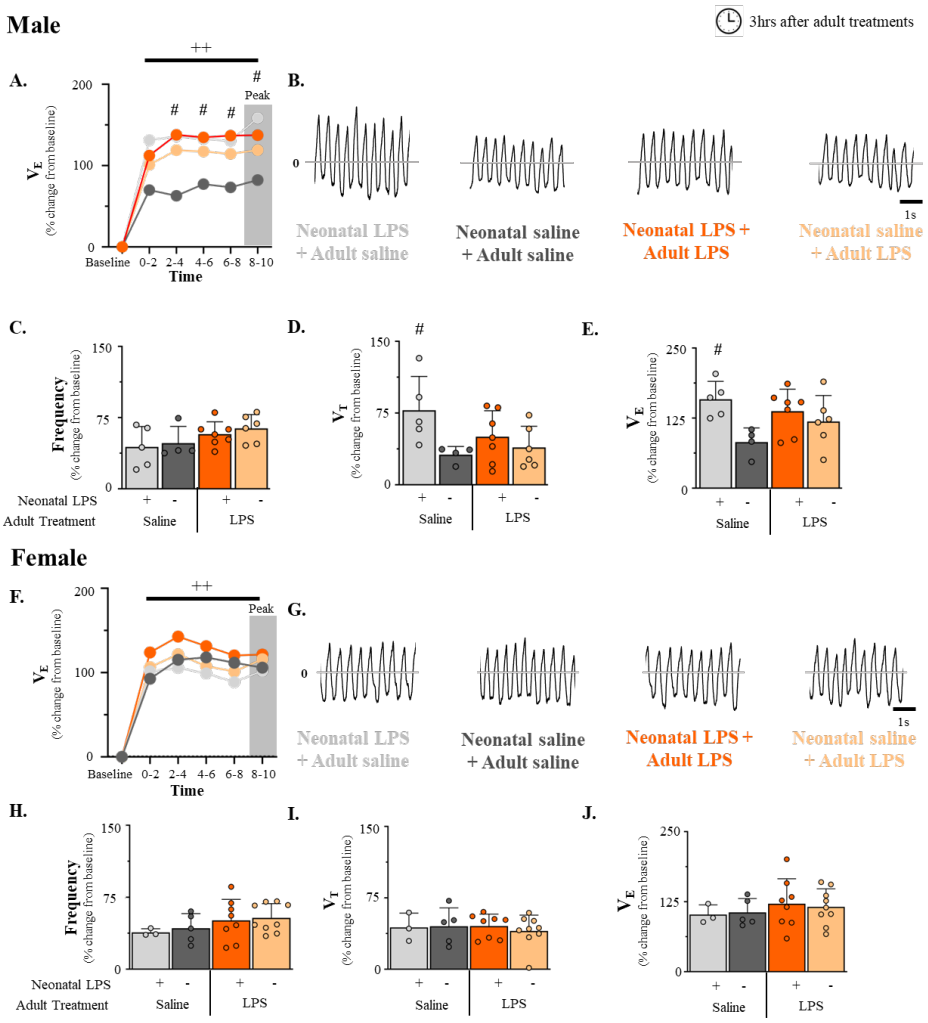


Figure 4. Neonatal LPS + adult saline augmented male, but not female, hypercapnic ventilatory responses. Minute ventilation during hypercapnia increased from baseline at all timepoints and in all treatment groups 3 hours after adult treatments (**A.**). Minute ventilation (V_E) increased in males after neonatal LPS + adult saline compared to neonatal saline + adult saline after 2mins of hypercapnia, and increased in males after neonatal LPS + adult LPS compared to males after neonatal saline + adult saline from 2-4mins and 6-8mins of hypercapnia. Plethysmography traces during the peak hypercapnic ventilatory response (HCVR) show breathing was augmented in adult males after neonatal LPS + adult saline from males after neonatal saline + adult saline (**B.**). Adult male breathing frequency (breath/min, **C.**) during peak HCVR unchanged by any combination of neonatal and adult treatments. Tidal volume (V_T, mL/100g, **D.**) during peak HCVR increased in males after neonatal LPS + adult saline compared to males after neonatal saline + adult saline. V_E (mL/100g/min, **E.**) during peak HCVR increased in males after neonatal LPS + adult saline compared to males after neonatal saline + adult saline. In adult females, minute ventilation increased from baseline at all timepoints during HCVR (**F.**) and was unchanged by any combination of neonatal and adult treatments (**G.-J.**). (++ $p>0.001$ different from baseline; # $p<0.05$ different from neonatal saline + adult saline; Three- and Two-way RM ANOVAs, Bonferroni post-hoc.)

except for 2-4mins in males after neonatal saline + adult saline ($p=0.054$, **Fig. 4A & B**), suggesting all adults increased ventilation during hypercapnia (reviewed in Nattie & Li, 2012).

Comparing neonatal-adult treatment within a timepoint, time ($p=0.0001$) had a main effect on male V_E during HCVR. However, treatment ($p=0.081$) and the interaction between treatment-time ($p=0.064$) did not have a main effect on male V_E during HCVR. Pairwise comparisons within

timepoints showed V_E during HCVR increased after 2mins in males after neonatal LPS + adult saline compared to neonatal saline + adult saline (0-2mins p=0.19, 2-4mins p=0.040, 4-6mins p=0.016, 6-8mins p=0.036, 8-10mins p=0.031), demonstrating neonatal inflammation augments male breathing during HCVR. Further, neonatal LPS + adult LPS increased V_E during HCVR from 2-4 and 6-8mins compared to neonatal saline + adult saline (0-2mins p=0.45, 2-4mins p=0.049, 4-6mins p=0.054, 6-8mins p=0.041, 8-10mins p=0.12). However, there were no other between treatment differences in V_E during HCVR (p>0.33), demonstrating the combination of neonatal and adult homotypic inflammation did not further augment or impair adult male hypercapnic responses.

Focusing on the peak HCVR (last 2 minutes of HCVR, gray box in **Fig. 4A**), male breathing frequency during peak HCVR was not significantly affected by neonatal treatment (p=0.49), adult treatment (p=0.058), or the interaction between neonatal-adult treatments (p=0.89) and there were no pairwise differences between treatment groups (p>0.39, **Fig. 4C**), demonstrating the combination of neonatal and adult homotypic treatments. In contrast, neonatal treatment (p=0.0058) had a main effect on male tidal volume during peak HCVR; however, neither adult treatment (p=0.31) nor the interaction between neonatal-adult treatments (p=0.14) had main effects on V_T during peak HCVR. Neonatal LPS + adult saline increased male V_T during peak HCVR compared to males after neonatal saline + adult saline (p=0.041), with no other differences between treatment groups (p>0.061, **Fig. 4D**). Thus, adult males after neonatal inflammation have lasting augmentation of V_T during HCVR; however, V_T returns to control levels in males after neonatal and adult homotypic inflammation.

Driven by the effects of neonatal inflammation on V_T during HCVR, neonatal treatment (p=0.01) had a main effect on V_E during peak HCVR. However, neither adult treatment (p=0.64)

nor the interaction between neonatal-adult treatment (p=0.096) had main effects on V_E during peak HCVR. In assessing pairwise differences, neonatal LPS + adult saline increased male V_E during peak HCVR compared to neonatal saline + adult saline (p=0.046), but there were no other between treatment differences (p>0.19). Thus, neonatal inflammation alone induces a lasting augmentation of adult male HCVR; however, breathing returns to control levels after subsequent adult homotypic inflammation.

In adult females, no combination of neonatal and adult treatments affected breathing during HCVR, 3 hours after adult treatments. During hypercapnia, time (p=0.0001) had a main effect on female V_E. Female V_E increased from baseline at each timepoint during HCVR in all treatment groups (p<0.0001, **Fig. 4F & G**), demonstrating all females had stereotypical increases in ventilation during hypercapnia (reviewed in Nattie & Li, 2012). However, neither neonatal treatment (p=0.28), adult treatment (p=0.77), nor the interactions between neonatal-adult treatments (p=0.34), neonatal treatment-time (p=0.72), adult treatment-time (p=0.91), nor neonatal-adult treatment-time (p=0.51) had main effects on female V_E during hypercapnia. Comparing neonatal-adult treatment and time showed, time (p=0.0001) had a main effect on adult female V_E during HCVR. However, neither treatment (p=0.47), nor the interaction between treatment-time (p=0.86) had main effects on adult female V_E during HCVR. Pairwise comparisons showed female V_E was unchanged by any combination of neonatal and adult treatment at any timepoint during hypercapnia (p>0.29, **Fig. 4F**).

Focusing on peak HCVR, female breathing frequency was not significantly affected by neonatal treatment (p=0.65), adult treatment (p=0.13), nor the interaction between neonatal- adult treatment (p=0.91) and there were no pairwise differences between any treatment groups (p>0.55, **Fig. 4H**). Female V_T during peak HCVR was not significantly affected by neonatal treatment

(p=0.77), adult treatment (p=0.80), or the interaction between neonatal-adult treatments (p=0.65) and there were no pairwise differences (p>0.99, **Fig. 4I**). Similar to breathing frequency and tidal volume, female V_E during peak HCVR was not significantly affected by neonatal treatment (p=0.95), adult treatment (p=0.35) or the interaction between neonatal-adult treatments (p=0.76) and there were no pairwise differences between treatment groups (p>0.99, **Fig. 4J**). Thus, female breathing during HCVR was unaffected by any combination of neonatal and adult treatments.

The combination of neonatal and adult treatments did not affect spontaneous apneas, sighs or post-sigh apneas during HCVR. There were no spontaneous and post-sigh apneas during HCVR in any animals (**Table 3**). Male sighs during HCVR were not significantly affected by neonatal treatment (p=0.73), adult treatment (p=0.73), or the interaction between neonatal-adult treatments (p=0.73) and there were no pairwise differences between treatment groups (p>0.99, **Table 3**). Female sighs during HCVR were not significantly affected by neonatal treatment (p=0.53), adult treatment (p=0.99), or the interaction between neonatal-adult treatments (p=0.81) and there were no pairwise differences between treatment groups (p>0.99). Thus, spontaneous apneas, sighs or post-sigh apneas during HCVR were not affected in either sex by any combination of neonatal and adult treatment.

Table 3. Spontaneous apneas, sighs and post-sigh apneas during hypercapnia, 3hrs after adult treatments.

		Number of spontaneous apneas		Number of sighs		Number of post-sigh apneas	
	Treatment groups	mean	SD	mean	SD	mean	SD
Male	Neonatal LPS + adult saline	0.00	0.00	3.60	1.67	0.00	0.00
	Neonatal saline + adult saline	0.00	0.00	4.00	1.41	0.00	0.00
	Neonatal LPS + adult LPS	0.00	0.00	4.00	1.15	0.00	0.00
	Neonatal saline + adult LPS	0.00	0.00	4.00	1.10	0.00	0.00
Female	Neonatal LPS + adult saline	0.00	0.00	4.00	1.00	0.00	0.00

	Neonatal saline + adult saline	0.00	0.00	3.80	1.30	0.00	0.00
	Neonatal LPS + adult LPS	0.00	0.00	4.13	1.13	0.00	0.00
	Neonatal saline + adult LPS	0.00	0.00	3.67	1.22	0.00	0.00

Adult male hypoxic ventilatory responses were not affected by any combination of neonatal and adult treatments.

We previously demonstrated neonatal inflammation increased respiratory responses to hypoxia in adult males compared to females (Beyeler et al., *in preparation*); however, whether adults after neonatal inflammation are more susceptible to disruptions in hypoxic ventilatory responses during subsequent adult inflammatory challenges remains unclear. Thus, hypoxic ventilatory responses (HVRs) were assessed in adult males and females in all treatment groups. Time ($p<0.0001$) had a main effect on V_E during hypoxia. However, neither neonatal treatment ($p=0.15$), adult treatment ($p=0.41$), nor the interactions between neonatal-adult treatment ($p=0.74$), neonatal treatment-time ($p=0.12$), adult treatment-time ($p=0.73$), nor neonatal-adult treatment-time ($p=0.77$) had main effects on male V_E during HVR. Neonatal LPS + adult saline did not increase V_E from baseline during hypoxia at any timepoint (0-2mins, $p=0.20$; 2-4mins, $p=0.38$; 4-6mins, $p=0.32$; 6-8mins, $p=0.17$; 8-10mins, $p=0.13$, **Fig. 5A**). Neonatal saline + adult saline increased V_E from baseline between 2-6 mins of hypoxia (0-2mins, $p=0.86$; 2-4mins, $p=0.035$; 4-6mins, $p=0.041$; 6-8mins, $p=0.064$; 8-10mins, $p=0.13$). In contrast, neonatal LPS + adult LPS increased V_E from baseline at all timepoints (0-2mins, $p=0.041$; 2-4mins, $p=0.0035$; 4-6mins, $p=0.0038$; 6-8mins, $p=0.0064$; 8-10mins, $p=0.014$). Neonatal saline + adult LPS increased V_E from baseline after 4 mins (0-2mins, $p=0.18$; 2-4mins, $p=0.13$; 4-6mins, $p=0.0061$; 6-8mins, $p=0.025$; 8-10mins, $p=0.029$). In summary, while males after neonatal LPS + adult saline and neonatal saline

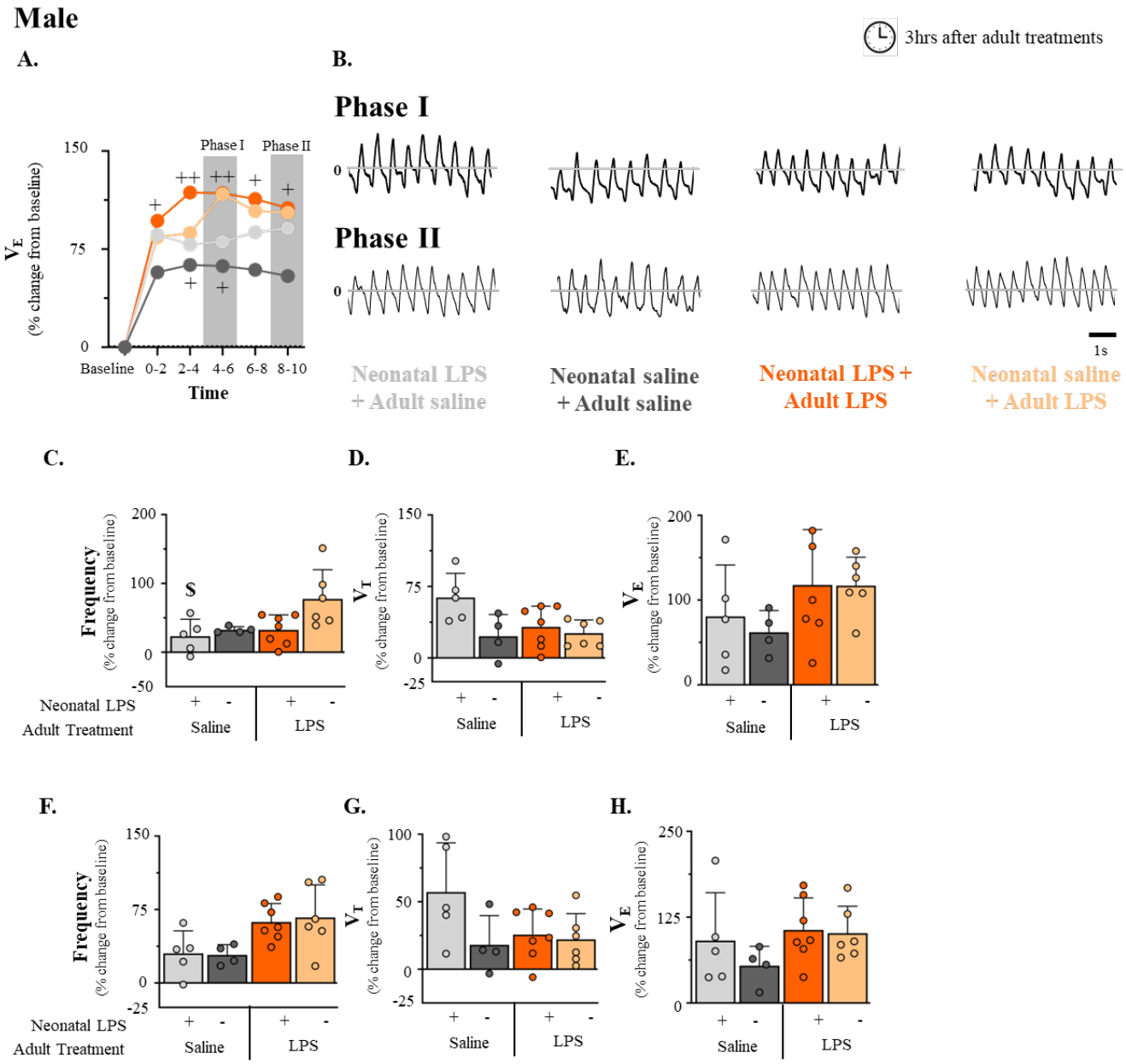


Figure 5. Augmented tidal volume during hypoxic ventilatory responses in males after neonatal LPS + adult saline. Minute ventilation during the hypoxic ventilatory response (HVR) increased from baseline from 2-6mins in males after neonatal saline + adult saline, at all timepoints in males after neonatal LPS + adult LPS, and after 4 mins in males after neonatal saline + adult LPS, but was unchanged from baseline in males after neonatal LPS + adult saline (**A.**). Plethysmography traces show breathing was unchanged by any combination of neonatal and adult treatment (**B.**). Adult male breathing frequency (breath/min, **C.**) during HVR phase I decreased in adult males after neonatal LPS + adult saline compared to males after neonatal saline + adult LPS. Tidal volume (V_T, **D.**) and minute ventilation (V_E, **E.**) during HVR phase I was unchanged by any combination of neonatal and adult treatment. Similarly, during HVR phase II breathing frequency (**F.**), tidal volume (**G.**), and minute ventilation (H.) was unchanged by any combination of neonatal and adult treatments. (+ p<0.05 different from baseline; $ p<0.05 different from neonatal saline + adult LPS; Three- and Two-way RM ANOVAs, Bonferroni post-hoc.)

+ adult saline did not increase breathing in response to hypoxia, males after neonatal LPS + adult LPS and neonatal saline + adult LPS increased breathing in response to hypoxia (**Fig. 5B**). Time (p=0.0001) had a main effect on adult male V_E during HVR. However, neither treatment (p=0.42)

nor the interaction between treatment-time (p=0.52) had main effects on adult male V_E during HVR and there were no between treatment differences within a timepoint (p>0.12). Thus, no combination of neonatal and adult homotypic inflammation affected adult male breathing during hypoxia, 3 hours after adult treatments.

HVR is characterized as an initial increase in breathing, termed Phase I, followed by a slight decrease in breathing while remaining above baseline, termed Phase II (Mitchell et al., 2001; Pamenter & Powell, 2016). Focusing on HVR phase I (left gray box in **Fig. 5A**), male breathing frequency during HVR phase I was significantly affected by neonatal treatment (p=0.040) and adult treatment (p=0.041), but not the interaction between neonatal-adult treatments (p=0.17). Pairwise differences show breathing frequency decreased in males after neonatal LPS + adult saline compared to males after neonatal saline + adult LPS (p=0.032), with no other differences between treatment groups (p>0.062, **Fig. 5C**), suggesting neonatal inflammation alone may have lasting effects on adult male breathing frequency. Similar to male breathing frequency, male V_T during HVR phase I was significantly affected by neonatal treatment (p=0.0058). However, neither adult treatment (p=0.40) nor the interaction between neonatal-adult treatment (p=0.14) had main effects on male V_T during HVR phase I and there were no significant differences between treatment groups (p>0.052, **Fig. 5D**). Thus, no combination of neonatal and adult homotypic inflammation affected male V_T during HVR phase I. In assessing V_E during HVR phase I, neither neonatal treatment (p=0.67), adult treatment (p=0.056), nor the interaction between neonatal and adult treatment (p=0.70) had main effects on male V_E during hypoxia HVR phase I and there were no significant pairwise differences between treatment groups (p>0.61, **Fig. 5E**). Thus, no combination of neonatal and adult treatments affected adult male breathing during HVR phase I, despite effects on frequency.

HVR phase II was not significantly changed by any combination of neonatal and adult treatments. Breathing frequency during HVR phase II was significantly affected by adult treatment ($p=0.0031$). However, neither neonatal treatment ($p=0.88$) or the interaction between neonatal-adult treatments ($p=0.77$) had main effects on male breathing frequency during HVR phase II and there were no pairwise differences between treatment groups ($p>0.12$, **Fig. 5F**). For V_T during HVR phase II, neither neonatal treatment ($p=0.060$), adult treatment ($p=0.21$) or the interaction between neonatal-adult treatments ($p=0.11$) had main effects on V_T during HVR phase II. Further, there were no pairwise differences between treatment groups ($p>0.17$, **Fig. 5G**). Consistent with frequency and tidal volume during HVR phase II, neither neonatal treatment ($p=0.14$), adult treatment ($p=0.25$) nor the interaction between neonatal-adult treatments ($p=0.54$) had main effects on male V_E during HVR phase II and there were no pairwise differences ($p=0.63$, **Fig. 5H**). Thus, male breathing during HVR phase II was not changed by any combination of neonatal and adult treatments.

Female hypoxic ventilatory responses were not affected by any combination of neonatal and adult treatments.

We previously showed neonatal inflammation did not disrupt HVR in adult females (Beyeler et. al*., in preparation*), yet whether females after neonatal inflammation are susceptible to disrupted hypoxia responses during subsequent inflammatory challenges remains unknown. Thus, we measured adult female HVR responses in all treatment groups. Time ($p=0.0001$) had a main effect on female V_E during hypoxia. However, neither neonatal treatment ($p=0.11$), adult treatment ($p=0.75$), nor the interactions between neonatal-adult treatments ($p=0.90$), adult treatment-time ($p=0.94$), neonatal treatment-time ($p=0.27$), nor neonatal-adult treatment-time

(p=0.90, **Fig. 6A & B**) had significant effects on adult female HVR. Neonatal LPS + adult saline did not increase female V_E from baseline at any timepoint during hypoxia (p=0.99 for all timepoints). Neonatal saline + adult saline did not increase female V_E from baseline at any timepoint during hypoxia (0-2mins, p=0.29; 2-4mins, p=0.49; 4-6mins, p=0.14; 6-8mins, p=0.22; 8-10mins, p=0.17). Neonatal LPS + adult LPS increased female V_E from baseline during hypoxia after 2 mins in hypoxia (0-2mins, p=0.062; 2-4mins, p=0.027; 4-6mins, p=0.016; 6-8mins, p=0.026; 8-10mins, p=0.016). Neonatal saline + adult LPS increased female V_E from baseline during hypoxia from 4-8 mins in hypoxia (0-2mins p=0.37, 2-4mins p=0.21, 4-6mins p=0.014, 6-8mins p=0.0085, 8-10mins p=0.060, **Fig. 6A**). In summary, females after neonatal LPS + adult saline and neonatal saline + adult saline did not respond to hypoxia, while females after neonatal LPS + adult LPS and neonatal saline + adult LPS responded to hypoxia, 3hrs after adult treatments. Comparing neonatal-adult treatment within a timepoint, time (p=0.0001) had a main effect on adult female V_E during HVR. However, neither treatment (p=0.40) nor the interaction between treatment-time (p=0.79) had main effects on adult female V_E during HVR and there were no between treatment differences within a timepoint (p>0.31). Thus, no combination of neonatal and adult homotypic inflammation impaired adult female breathing during hypoxia, 3 hours after adult treatments.

Focusing on female HVR phase I, neither neonatal treatment (p=0.62), adult treatment (p=0.054), nor the interaction between neonatal-adult treatment (p=0.53). Pairwise comparisons showed no differences in female breathing frequency during HVR phase I between any treatment groups (p>0.28, **Fig. 6C**). For female V_T during HVR phase I, neither neonatal treatment (p=0.16), adult treatment (p=0.54), nor the interaction between neonatal-adult treatments (p=0.86) had main effects on female V_T during HVR phase I and there were no pairwise differences between treatment

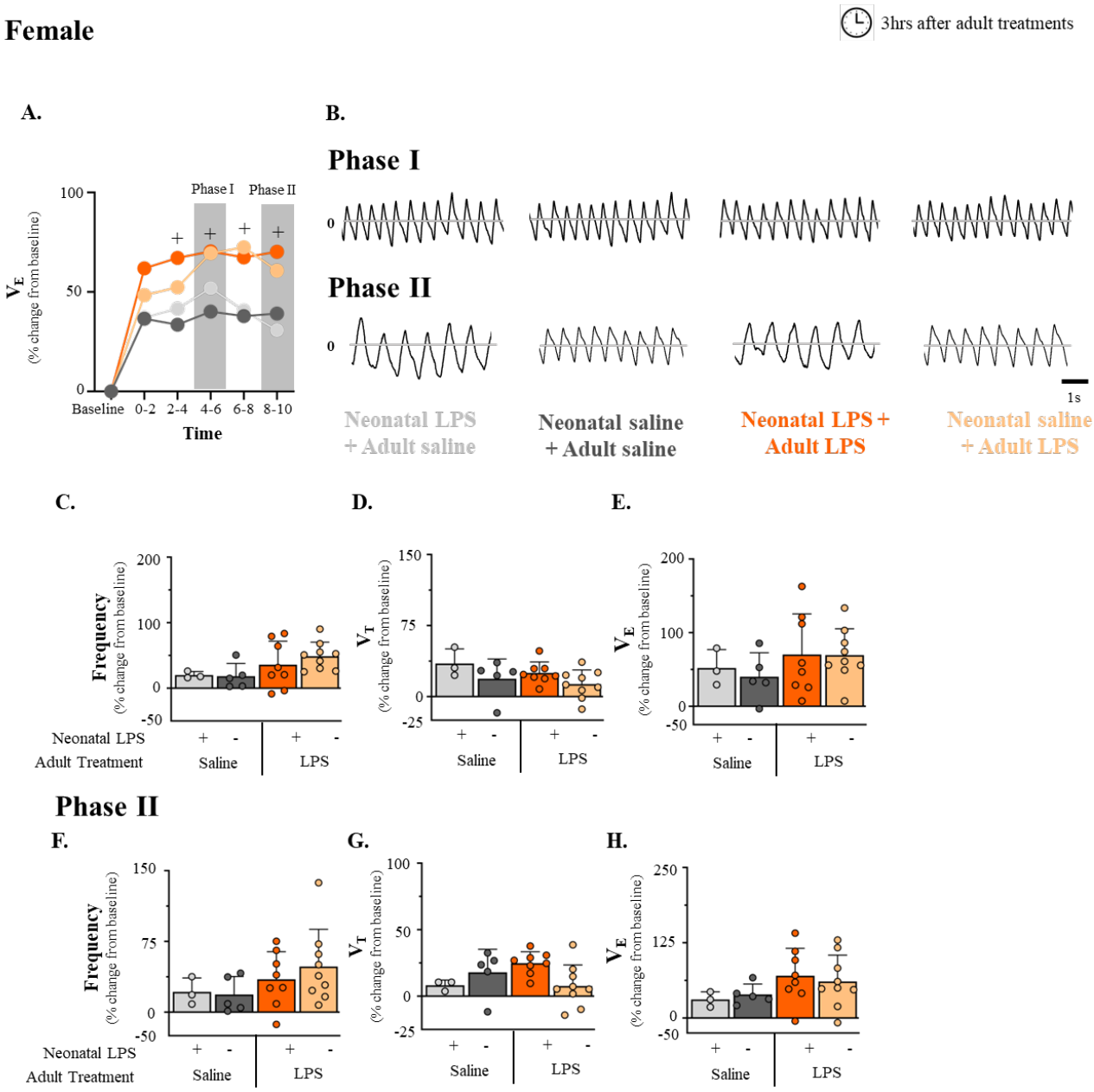


Figure 6. Female hypoxic ventilatory responses were not affected by any combination of neonatal and adult treatments. Minute ventilation during the hypoxic ventilatory response (HVR) increased from baseline from 4-8mins in males after neonatal saline + adult LPS and after 2mins in males after neonatal LPS + adult LPS, but was unchanged from baseline in males after neonatal LPS + adult saline and neonatal saline + adult saline at any timepoint (**A.**). Plethysmography traces (**B.**) show breathing adult females was similar in all treatment groups during HVR phase I and II. Breathing frequency (**C.**), tidal volume (**D.**) and minute ventilation (**E.**) during HVR phase I were unchanged by any combination of neonatal and adult treatment. Similarly, during HVR phase II breathing frequency (**F.**), tidal volume (**G.**) and minute ventilation were similar in all treatment groups. (+ $p<0.05$ different from baseline, Three- and Two-way RM ANOVAs, Bonferroni post-hoc.)

groups ($p>0.92$, **Fig. 6D**). For female V_E during HVR phase I, neither neonatal treatment ($p=0.74$), adult treatment ($p=0.21$), nor the interaction between neonatal and adult treatment ($p=0.77$) had

main effects on female V_E during HVR phase I and there were no pairwise differences between treatment groups ($p>0.99$, **Fig. 6E**). In summary, female breathing during HVR phase I was not changed by any combination of neonatal and adult treatments, 3 hours after adult treatments.

Similar to effects on HVR phase I, breathing during HVR phase II in females was not disrupted by any combination of neonatal and adult treatments. Breathing frequency during HVR phase II was not significantly affected by neonatal treatment ($p=0.70$), adult treatment ($p=0.13$), nor the interaction between neonatal-adult treatments ($p=0.55$) and there were no pairwise differences ($p>0.34$, **Fig. 6F**). In contrast, V_T during HVR phase II was significantly affected by the interaction between neonatal-adult treatments ($p=0.032$). However, neither neonatal treatment ($p=0.53$) nor adult treatment ($p=0.59$) had main effects on female V_T during HVR phase I, with no pairwise differences between groups ($p>0.091$, **Fig. 6G**). For female V_E during HVR, neither neonatal treatment ($p=0.97$), adult treatment ($p=0.086$), nor the interaction between neonatal-adult treatments ($p=0.60$) had a main effect on V_E during HVR phase II and there were no pairwise differences between groups ($p>0.87$, **Fig. 6H**). Thus, female breathing during HVR was not changed by any combination of neonatal and adult treatments.

Spontaneous apneas, sighs and post-sigh apneas during HVR phase I or II were not affected by any combination of neonatal and adult treatment. There were no spontaneous apneas during HVR in any animal (**Table 4.**). For male sighs during HVR, neither neonatal treatment (phase I $p=0.49$, phase II $p=0.73$), adult treatment (phase I $p=0.34$, phase II $p=0.56$), nor the interaction between neonatal-adult treatments (phase I $p=0.62$, phase II $p=0.73$) had main effects on male sighs during HVR and there were no pairwise differences between treatment groups (phase I $p>0.61$, phase II $p>0.99$, **Table 4.**). Similarly, post-sigh apneas during HVR phase I or II were not significantly affected by neonatal treatment (phase I $p=0.11$, phase II $p=0.51$), adult treatment

(phase I p=0.82, phase II p=0.93), or the interaction between neonatal-adult treatments (phase I p=0. 058, phase II p=0.51) and there were no pairwise differences between treatment groups (phase I p>0.15, phase II p>0.99, **Table 4.**). Thus, the combination of neonatal and adult treatments did not affect apneas or sighs during HVR.

Female spontaneous apneas, sighs and post-sigh apneas during HVR phase I or II were not affected by any combination of neonatal and adult treatment. There were no spontaneous apneas during HVR in any animal (**Table 4.**). During HVR phase I and II, neither neonatal treatment (phase I p=0.52, phase II p=0.31), adult treatment (phase I p=0.27, phase II p=0.42) nor the interaction between neonatal and adult treatment (phase I p=0.87, phase II p=0.76) had main effects on female sighs and there were no pairwise differences between treatment groups (phase I p>0.99, phase II p>0.99, **Table 4.**). Similarly, female post-sigh apneas during HVR phase I or II were not significantly affected by neonatal treatment (phase I p=0.78, phase II p=0.065), adult treatment (phase I p=0.93, phase II p=0.48) or the interaction between neonatal and adult treatment (phase I p=0.78, phase II p=0.43) and there were no pairwise differences between treatment groups (phase I p>0.99, phase II p>0.35, **Table 4.**). Thus, the combination of neonatal and adult treatments did not affect female apneas or sighs during HVR.

Table 4. Spontaneous apneas, sighs and post-sigh apneas during hypoxia, 3hrs after adult treatments.

		Number of spontaneous apneas		Number of sighs		Number of post-sigh apneas	
	Treatment groups	mean	SD	mean	SD	mean	SD
Phase I	Neonatal LPS + adult saline	0.00	0.00	10.00	2.24	4.80	0.84
	Neonatal saline + adult saline	0.00	0.00	10.25	1.71	7.00	0.82
	Neonatal LPS + adult LPS	0.00	0.00	10.57	2.82	5.86	1.35
	Neonatal saline + adult LPS	0.00	0.00	12.00	3.35	5.67	1.86
Phase II	Neonatal LPS + adult saline	0.00	0.00	11.00	2.74	4.17	1.17
	Neonatal saline + adult saline	0.00	0.00	11.00	2.94	4.17	1.47

	Neonatal LPS + adult LPS	0.00	0.00	11.29	2.87	4.57	1.40
	Neonatal saline + adult LPS	0.00	0.00	12.17	2.79	3.86	1.35
Phase I	Neonatal LPS + adult saline	0.00	0.00	11.33	3.06	6.33	1.15
	Neonatal saline + adult saline	0.00	0.00	11.80	2.59	6.00	1.58
	Neonatal LPS + adult LPS	0.00	0.00	10.11	1.76	6.11	1.27
	Neonatal saline + adult LPS	0.00	0.00	10.89	2.03	6.11	1.36
Phase II	Neonatal LPS + adult saline	0.00	0.00	12.00	3.61	6.00	1.00
	Neonatal saline + adult saline	0.00	0.00	12.80	2.77	4.20	1.30
	Neonatal LPS + adult LPS	0.00	0.00	10.75	2.31	5.13	1.36
	Neonatal saline + adult LPS	0.00	0.00	12.22	2.17	4.25	1.28

Sex-specific effects of neonatal and adult treatments on eupneic breathing, 24hrs after adult treatments.

Adults responses to inflammation are highly stimulus specific (Elisia et al., 2017), yet little is known about how neonatal inflammation may impact adult respiratory control during a subsequent heterotypic (different from neonatal stimulus) inflammatory challenge. Thus, we expanded our adult treatment groups to also include adult polyIC treatments. Since respiratory motor plasticity was disrupted and increased inflammatory gene expression increased 24hrs after acute, adult polyIC (Hocker & Huxtable, 2019), we only measured breathing in all treatment groups 24hrs after adult treatments, as our previous work would indicate no impact at an earlier time point.

Adult male eupneic breathing was unchanged by any combination of neonatal and adult treatment, 24 hours after adult treatments (**Fig. 7A**). Neither neonatal treatment ($p=0.98$), adult treatment ($p=0.17$) nor the interactions neonatal-adult treatments ($p=0.98$) had main effects on adult male eupneic breathing frequency and there were no pairwise differences ($p>0.99$, **Fig. 7B**). However, neonatal treatment ($p=0.0078$) had main effects on adult male eupneic V_T, yet neither adult treatment ($p=0.31$) nor the interaction between neonatal-adult treatments ($p=0.68$) had main

effects on adult eupneic V_T. There were no pairwise differences between treatments (p=0.102, **Fig. 7C**). Neonatal treatment (p=0.023) had a significant effect on adult male eupneic V_E. However, neither adult treatment (p=0.056), nor the interaction between neonatal-adult treatment (p=0.77) did not have a significant effect on adult male eupneic V_E. Pairwise differences showed no differences between treatments (p>0.080, **Fig. 7D**). In summary, no combination of neonatal and adult treatments affect eupneic male breathing, 24 hours after adult treatments.

No combination of neonatal and adult treatments affected female eupneic breathing frequency, as there were no changes in female eupneic minute ventilation (**Fig. 7E**). Eupneic breathing frequency was significantly affected by the interaction between neonatal-adult treatments (p=0.0091). However, neither neonatal treatment (p=0.12), nor adult treatment (p=0.093) had significant main effects on female eupneic breathing frequency. When assessing pairwise differences, neonatal saline + adult polyIC increased eupneic breathing frequency increased compared to neonatal saline + adult saline (p=0.024) and neonatal saline + adult LPS (p=0.026), suggesting adult polyIC alone increases adult female eupneic breathing frequency. However, there were no other significant differences between treatments (p>0.069, **Fig. 7F**). For adult female eupneic V_T, neither neonatal treatment (p=0.79), adult treatment (p=0.22), nor the interaction between neonatal-adult treatment (p=0.84) had main effects on adult female eupneic V_T, and there were no pairwise differences (p>0.99, **Fig. 7G**). For adult female eupneic V_E, neither neonatal treatment (p=0.94), adult treatment (p=0.14), nor the interaction between neonatal-adult treatments (p=0.37) were significantly different and there were no differences between any treatments (p>0.19, **Fig. 7H**). In summary, female eupneic breathing frequency was affected by adult polyIC treatment alone; however, these changes did not affect female eupneic minute ventilation.

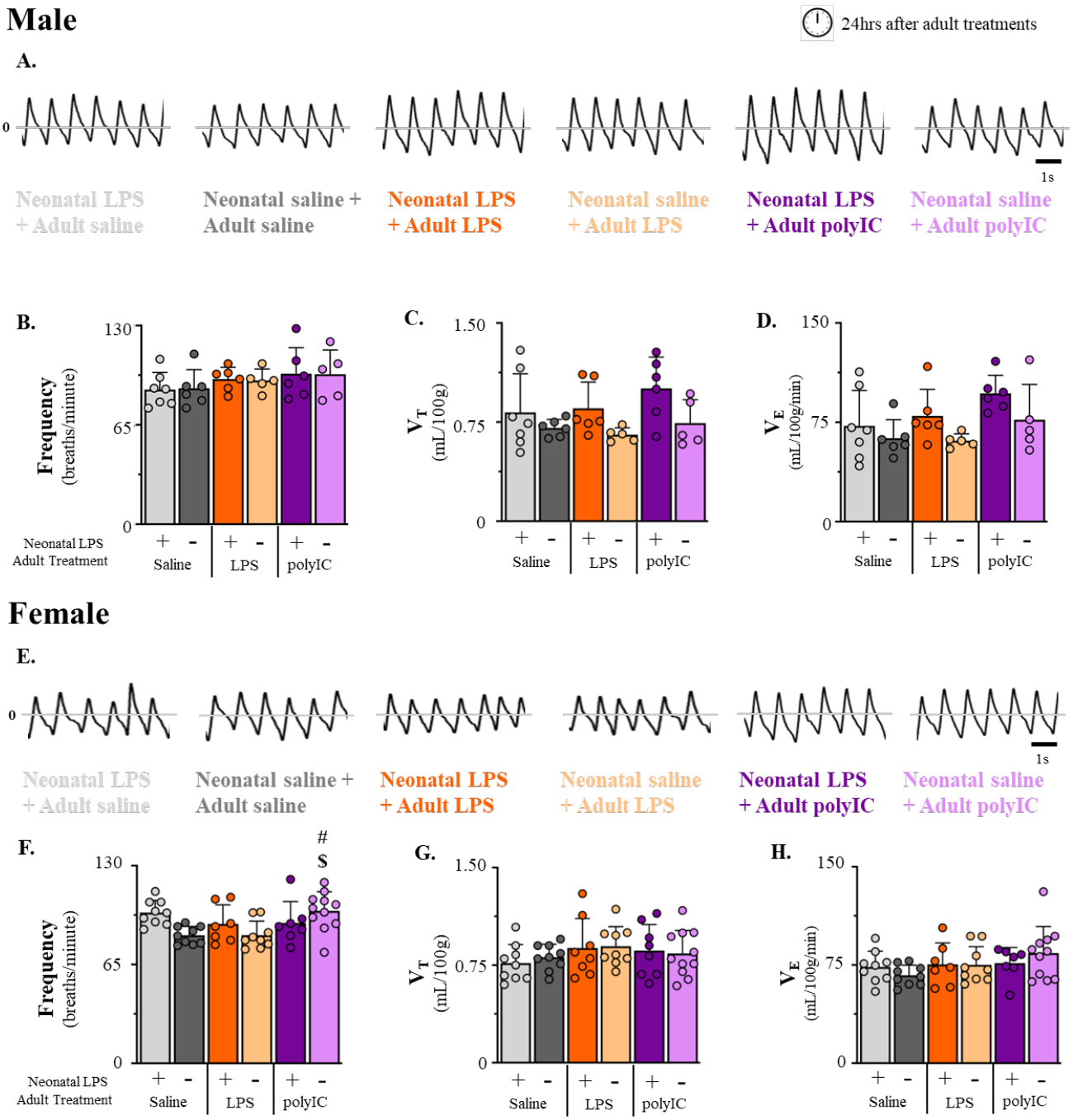


Figure 7. Sex-specific effects of neonatal and adult treatments on eupneic breathing, 24hrs after adult treatments. Plethysmography traces show breathing 24 hours after adult treatments were unchanged in adult males regardless of neonatal and adult treatment (**A.**). Adult male eupneic breathing frequency (breath/min, **B.**), tidal volume (V_T, mL/100g, **C.**), and minute ventilation (V_E, mL/100g/min, **D.**) were unchanged by any combination of neonatal and adult treatment. Female eupneic breathing (**E.**) was unchanged by any combination of neonatal and adult treatments. However, eupneic breathing frequency (**F.**) increased in females after neonatal LPS + adult saline compared to females after neonatal saline + adult saline and in females after neonatal saline + adult polyIC compared to females after neonatal saline + adult saline and neonatal saline + adult LPS was unchanged by neonatal and adult inflammation Tidal volume (**G.**) and minute ventilation (**H.**) were changed by any combination of neonatal and adult treatment. (# $p<0.05$ different from neonatal saline + adult saline; $ $p<0.05$ different from neonatal saline + adult LPS; Two-way ANOVAs, Bonferroni post-hoc.)

Male spontaneous apneas, sighs and post-sigh apneas during eupnea were not affected by any combination of neonatal and adult treatment, 24hrs after adult treatments. During eupnea,

neither neonatal treatment (p=0.54), adult treatment (p=0.99) nor the interaction between neonatal and adult treatment p=0.52) had main effects on male spontaneous apneas and there were no pairwise differences between treatment groups (p>0.99, **Table 5.**). For male sighs, neither neonatal treatment (p=0.53), adult treatment (p=0.91) nor the interaction between neonatal and adult treatment (p=0.67) had main effects on male sighs during eupnea and there were no pairwise differences between treatment groups (p>0.95, **Table 5.**). Similarly, male post-sigh apneas during eupnea were not significantly affected by neonatal treatment (p=0.67), adult treatment (p=0.76) or the interaction between neonatal and adult treatment (p=0.32) and there were no pairwise differences between treatment groups (p>0.99, **Table 5.**). Thus, the combination of neonatal and adult treatments did not affect male apneas or sighs during eupnea.

Female spontaneous apneas, sighs and post-sigh apneas during eupnea were not affected by any combination of neonatal and adult treatment, 24hrs after adult treatments. During eupnea, neither neonatal treatment (p=0.98), adult treatment (p=0.71) nor the interaction between neonatal and adult treatment p=0.96) had main effects on female spontaneous apneas and there were no pairwise differences between treatment groups (p>0.99, **Table 5.**). For female sighs during eupnea, neither neonatal treatment (p=0.24), adult treatment (p=0.12) nor the interaction between neonatal and adult treatment (p=0.34) had main effects on female sighs during eupnea and there were no pairwise differences between treatment groups (p>0.15, **Table 5.**). For female post-sigh apneas, neither neonatal treatment (p=0.16), adult treatment (p=0.070) or the interaction between neonatal and adult treatment (p=0.65) had significant main effects on female post-sigh apneas and there were no pairwise differences between treatment groups (p>0.15, **Table 5.**). Thus, the combination of neonatal and adult treatments did not affect female apneas or sighs during eupnea.

Table 5. Spontaneous apneas, sighs and post-sigh apneas during eupnea, 24hrs after adult treatments.							
		Number of spontaneous apneas		Number of sighs		Number of post-sigh apneas	
	Treatment groups	mean	SD	mean	SD	mean	SD
Male	Neonatal LPS + adult saline	0.43	0.54	1.43	1.27	0.71	0.76
	Neonatal saline + adult saline	0.33	0.52	2.00	1.41	0.67	0.82
	Neonatal LPS + adult LPS	0.50	0.55	1.67	1.21	0.67	0.82
	Neonatal saline + adult LPS	0.20	0.45	1.40	1.34	0.40	0.55
	Neonatal LPS + adult polyIC	0.33	0.52	1.50	0.84	0.17	0.41
	Neonatal saline + adult polyIC	0.40	0.55	2.00	1.22	0.80	0.84
Female	Neonatal LPS + adult saline	0.22	0.44	0.29	0.49	0.44	0.53
	Neonatal saline + adult saline	0.22	0.44	1.50	1.05	0.89	0.78
	Neonatal LPS + adult LPS	0.33	0.50	1.67	1.37	0.13	0.35
	Neonatal saline + adult LPS	0.38	0.52	2.20	1.30	0.25	0.46
	Neonatal LPS + adult polyIC	0.30	0.48	1.67	1.63	0.38	0.52
	Neonatal saline + adult polyIC	0.25	0.46	1.40	1.14	0.50	0.71

Neonatal LPS + adult saline augmented male, but not female, hypercapnic ventilatory responses 24hrs after adult treatments.

Time ($p<0.0001$), treatment (0.047), and the interaction between treatment-time ($p=0.035$) had main effects on adult male HCVR. Male V_E during hypercapnia increased from baseline at all timepoints and in all treatments (neonatal LPS + adult saline: 0-2mins, $p=0.0091$; 2-4mins, $p=0.0056$; 4-6mins, $p=0.0030$; 6-8mins, $p=0.00119$; 8-10mins, $p=0.0012$; neonatal saline + adult saline: 0-2mins, $p=0.0006$; 2-4mins, $p=0.0041$; 4-6mins, $p=0.0007$; 6-8mins, 0.0004; 8-10mins, $p=0.0005$; neonatal LPS + adult LPS: 0-2mins, $p=0.0007$; 2-4mins, $p=0.0012$; 4-6mins, $p=0.0051$; 6-8mins, $p=0.0049$; 8-10mins, $p=0.0067$; neonatal saline + adult LPS: 0-2mins $p=0.032$, 2-4mins $p=0.010$, 4-6mins $p=0.014$, 6-8mins $p=0.0039$, 8-10mins $p=0.0041$; neonatal LPS + adult polyIC: 0-2min $p=0.030$, 2-4mins $p=0.016$, 4-6min $p=0.0023$, 6-8mins $p=0.0067$, 8-10mins $p=0.0064$; neonatal saline + adult polyIC: 2-4mins $p=0.019$, 4-6mins $p=0.0003$, 6-8mins $p=0.0004$, 8-10mins

p=0.0090, **Fig. 8B**), except in neonatal saline + adult polyIC from 0-2mins (p=0.14). Thus, all males have typical breathing responses to hypercapnia. Comparing between treatment groups within a timepoint showed no between treatment differences (p>0.22, **Fig. 8B**), demonstrating no combination of neonatal and adult treatments impaired adult male breathing during HCVR, 24 hours after adult treatments.

Focusing on peak HCVR (gray box in **Fig. 8B**), male breathing frequency during peak HCVR was not affected by neonatal treatment (p=0.43), adult treatment (p=0.31), nor the interaction between neonatal-adult treatments (p=0.98), and there were no pairwise differences (p>0.99, **Fig. 8C**). For male V_T during peak HCVR, neither neonatal treatment (p=0.082), adult treatment (p=0.090), nor the interaction between neonatal and adult treatment (p=0.19) had significant main effects on male V_T during peak HCVR and there were no pairwise differences between treatment groups (p>0.053, **Fig. 8D**). Thus, no combination of neonatal and adult treatments affected male V_T during HCVR, 24hrs after adult treatments.

Similarly, neither neonatal treatment (p=0.074), adult treatment (p=0.051) and the interaction between neonatal and adult treatment (p=0.062) had main effects on male V_E during peak HCVR. When assessing pairwise differences, neonatal LPS + adult saline increased male V_E during peak HCVR compared males after neonatal saline + adult saline (p=0.047) and neonatal LPS + adult polyIC (p=0.041), with no other differences between treatment groups (p>0.060, **Fig. 8E**). Thus, neonatal inflammation increased adult male minute ventilation during HCVR, but this effect is not maintained after subsequent adult inflammatory challenges, regardless of adult treatment.

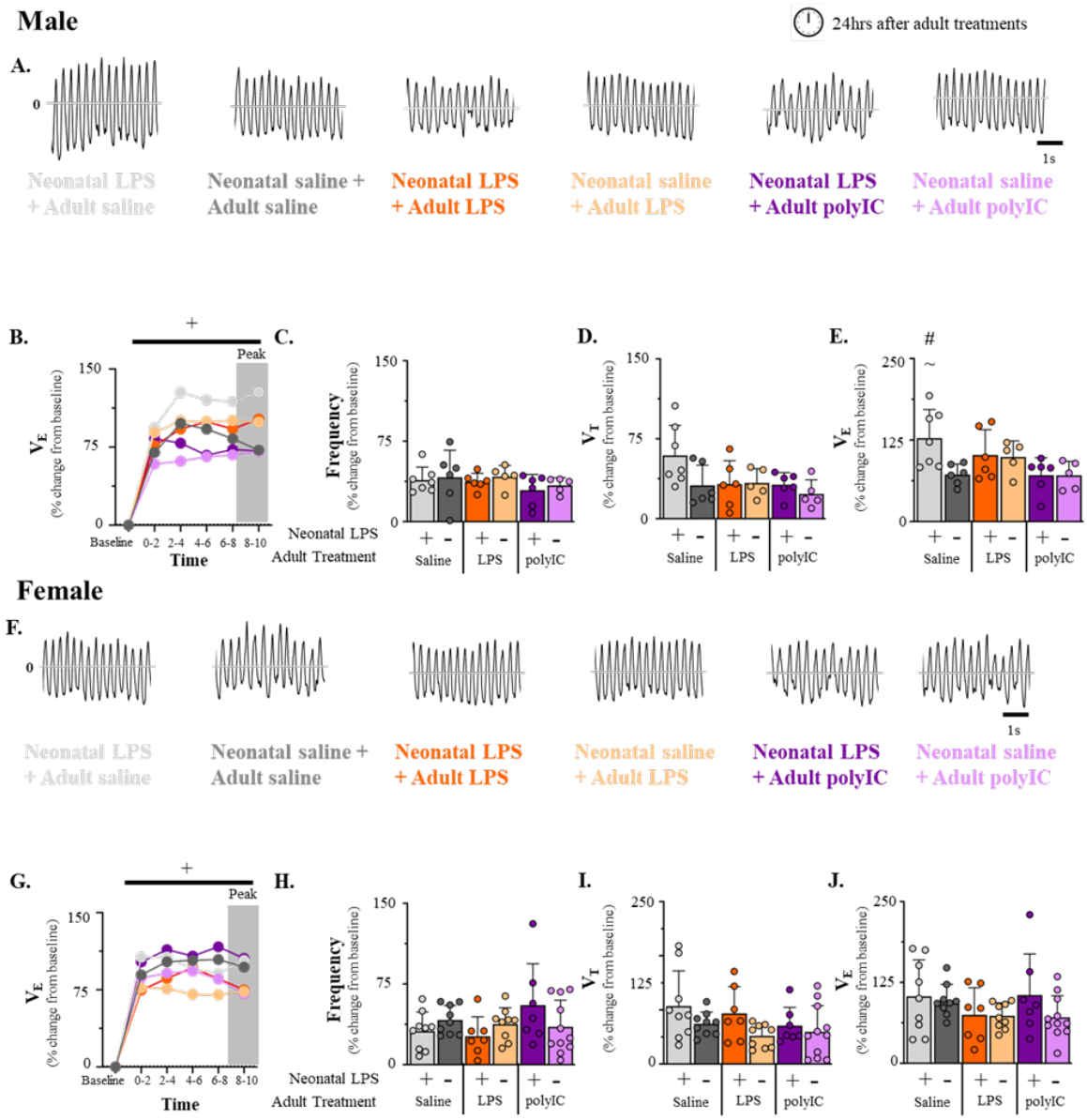


Figure 8. Neonatal LPS + adult saline augmented male, but not female, hypercapnic ventilatory responses, 24hrs after adult treatments. Representative plethysmography traces show breathing during the peak hypoxic ventilatory response (HCVR) was augmented in males after neonatal LPS + adult saline compared to neonatal saline + adult saline, neonatal LPS + adult polyIC, neonatal saline + adult polyIC (**A.**). Minute ventilation during hypercapnia (**B.**) increased from baseline at all timepoints and in all treatment groups, except from 0-2mins in males after neonatal saline + adult polyIC (**B.**). Male breathing frequency (**C.**) and tidal volume (**D.**) during peak HCVR was unchanged by any combination of neonatal and adult treatment. Minute ventilation (**E.**) during peak HCVR increased in males after neonatal LPS + adult saline compared to males after neonatal saline + adult saline, neonatal LPS + adult polyIC. Representative plethysmography show breathing in females was unchanged by any combination of neonatal and adult treatment (**F.**). Minute ventilation during HCVR (**G.**) increased from baseline at all timepoints and in all treatment groups. Breathing frequency (**H.**), tidal volume (**I.**) and minute ventilation (**J.**) during peak HCVR were unchanged by any combination of neonatal and adult treatment (+ $p<0.05$ different from baseline; # $p<0.05$ different from neonatal saline + adult saline; ~ $p<0.05$ different from neonatal LPS + adult polyIC; Two-way RM ANOVAs, Bonferroni post-hoc.)

In contrast to males, adult female minute ventilation was unaffected by any combination of neonatal and adult treatments (**Fig. 8E**). Time ($p<0.0001$) had a significant effect on female breathing during HCVR. However, neither treatment ($p=0.36$) nor the interaction between

treatment-time (p=0.49) had a main effect on female HCVR. Female V_E during hypercapnia increased from baseline at all timepoints in all treatment groups (neonatal LPS + adult saline: 0-2mins, p=0.0011; 2-4mins, p=0.0005; 4-6mins, p=0.0007; 6-8mins, p=0.0003; 8-10mins, p=0.0024; neonatal saline + adult saline: 0-2mins, p=0.0001; 2-4mins, p=0.0001; 4-6mins, p=0.0001; 6-8mins, p=0.0002; 8-10mins, p=0.0001; neonatal LPS + adult LPS: 0-2mins, p=0.027; 2-4mins, p=0.0032; 4-6mins, p=0.0023; 6-8mins, p=0.010; 8-10mins, p=0.016; neonatal saline + adult LPS: 0-2mins, p=0.0002; 2-4mins, p=0.0001; 4-6mins, p=0.0001; 6-8mins, p=0.0001; 8-10mins, p=0.0001; neonatal LPS + adult polyIC: 0-2mins, p=0.0088; 2-4mins, p=0.0046; 4-6mins, p=0.012; 6-8mins, p=0.013; 8-10mins, p=0.023; neonatal saline + adult polyIC: 0-2mins, p=0.0001; 2-4mins, p=0.0001; 4-6mins, p=0.0003; 6-8mins, p=0.0032; 8-10mins, p=0.0028), demonstrating all females increased breathing during hypercapnia. Further, there were no pairwise differences between treatment groups at any timepoint (p>0.43, **Fig. 8F&G**), demonstrating no combination of neonatal and adult treatments affected female V_E during HCVR, 24 hours after adult treatments.

Focusing on the peak HCVR, female breathing frequency during peak HCVR was not significantly affected by neonatal treatment (p=0.43) adult treatment (p=0.31) or the interaction between neonatal-adult treatment (p=0.98) and there were no pairwise differences between treatment groups (p>0.99, **Fig. 8H**). During peak HCVR, neonatal treatment (p=0.024) had a main effect on adult female V_T during peak HCVR. However, neither adult treatment (p=0.21) nor the interaction between neonatal-adult treatments (p=0.59) had main effects on adult female V_T during peak HCVR and there were no pairwise differences between any groups (p>0.15, **Fig. 8I**). For female V_E during peak HCVR, neither neonatal treatment (p=0.23), adult treatment (p=0.20), or the interaction between neonatal-adult treatments (p=0.47) had main effects and there were no

pairwise differences between treatment groups (p>0.99, **Fig. 8J**). Thus, no combination of neonatal and adult treatments affected female V_E during HCVR, 24 hours after adult treatments.

The combination of neonatal and adult treatments did not affect spontaneous apneas, sighs or post-sigh apneas during HCVR in either sex. There were no spontaneous and post-sigh apneas during HCVR in any animals (**Table 6.**). Male sighs during HCVR were not significantly affected by neonatal treatment (p=0.85), adult treatment (p=0.42), or the interaction between neonatal and adult treatment (p=0.63) and there were no pairwise differences between treatment groups (p>0.99, **Table 6.**). Female sighs during HCVR were not significantly affected by neonatal treatment (p=0.85), adult treatment (p=0.80), or the interaction between neonatal and adult treatment (p=0.82) and there were no pairwise differences between treatment groups (p>0.99). Thus, spontaneous apneas, sighs or post-sigh apneas during HCVR were not affected in either sex by any combination of neonatal and adult treatment, 24hrs after adult treatments.

Table 6. Spontaneous apneas, sighs and post-sigh apneas during hypercapnia, 24hrs after adult treatments.

		Number of spontaneous apneas		Number of sighs		Number of post-sigh apneas	
	Treatment groups	mean	SD	mean	SD	mean	SD
Male	Neonatal LPS + adult saline	0.00	0.00	4.14	0.90	0.00	0.00
	Neonatal saline + adult saline	0.00	0.00	3.67	1.21	0.00	0.00
	Neonatal LPS + adult LPS	0.00	0.00	3.83	1.17	0.00	0.00
	Neonatal saline + adult LPS	0.00	0.00	4.20	0.84	0.00	0.00
	Neonatal LPS + adult polyIC	0.00	0.00	3.50	1.05	0.00	0.00
	Neonatal saline + adult polyIC	0.00	0.00	3.40	1.14	0.00	0.00
Female	Neonatal LPS + adult saline	0.00	0.00	3.56	0.88	0.00	0.00
	Neonatal saline + adult saline	0.00	0.00	3.78	1.30	0.00	0.00
	Neonatal LPS + adult LPS	0.00	0.00	3.75	1.04	0.00	0.00
	Neonatal saline + adult LPS	0.00	0.00	3.89	1.05	0.00	0.00

	Neonatal LPS + adult polyIC	0.00	0.00	4.00	0.93	0.00	0.00
	Neonatal saline + adult polyIC	0.00	0.00	3.80	1.03	0.00	0.00

Male hypoxic ventilatory responses were unchanged by any combination of neonatal and adult treatments, 24 hours after adult treatments.

Male breathing during hypoxia phase I and II was similar between all treatment groups, 24hrs after adult treatments (**Fig. 9A**). Time (p=0.0001) had a main effect on V_E during hypoxia. However, neither treatment (p=0.25) nor the interactions between treatment-time (p=0.17) had main effects on adult V_E during hypoxia. V_E did not increase from baseline during hypoxia in Neonatal LPS + adult saline at any timepoint (0-2mins, p=0.23; 2-4mins, p=0.14; 4-6mins, p=0.063; 6-8mins, p=0.20; 8-10mins, p=0.21). Neonatal saline + adult saline increased V_E from baseline during hypoxia after 2 mins (0-2mins, p=0.12; 2-4mins, p=0.016; 4-6mins, p=0.0022; 6-8mins, p=0.0028; 8-10mins, p=0.0049). Neonatal LPS + adult LPS increased V_E from baseline during hypoxia after 2 mins (0-2mins, p=0.090; 2-4mins, p=0.014; 4-6mins, p=0.017; 6-8mins, p=0.023; 8-10mins, p=0.019). Neonatal saline + adult LPS increased V_E from baseline during hypoxia from 2-6 mins (0-2mins, p=0.068; 2-4mins, p=0.013; 4-6mins, p=0.019; 6-8mins, p=0.14; 8-10mins, p=0.15). Neonatal LPS + adult polyIC did not increase V_E from baseline during hypoxia at any timepoint (0-2mins, p=0.99; 2-4mins, p=0.26; 4-6mins, p=0.24; 6-8mins, p=0.60; 8-10mins, p=0.20). Neonatal saline + adult polyIC did not increase V_E from baseline during hypoxia at any timepoint (0-2mins, p=0.99; 2-4mins, p=0.70; 4-6mins, p=0.46; 6-8mins, p=0.61; 8-10mins, p=0.52). In summary, males after neonatal LPS + adult saline, neonatal LPS + adult polyIC, and neonatal saline + adult polyIC did not increase V_E in response to hypoxia, males after neonatal

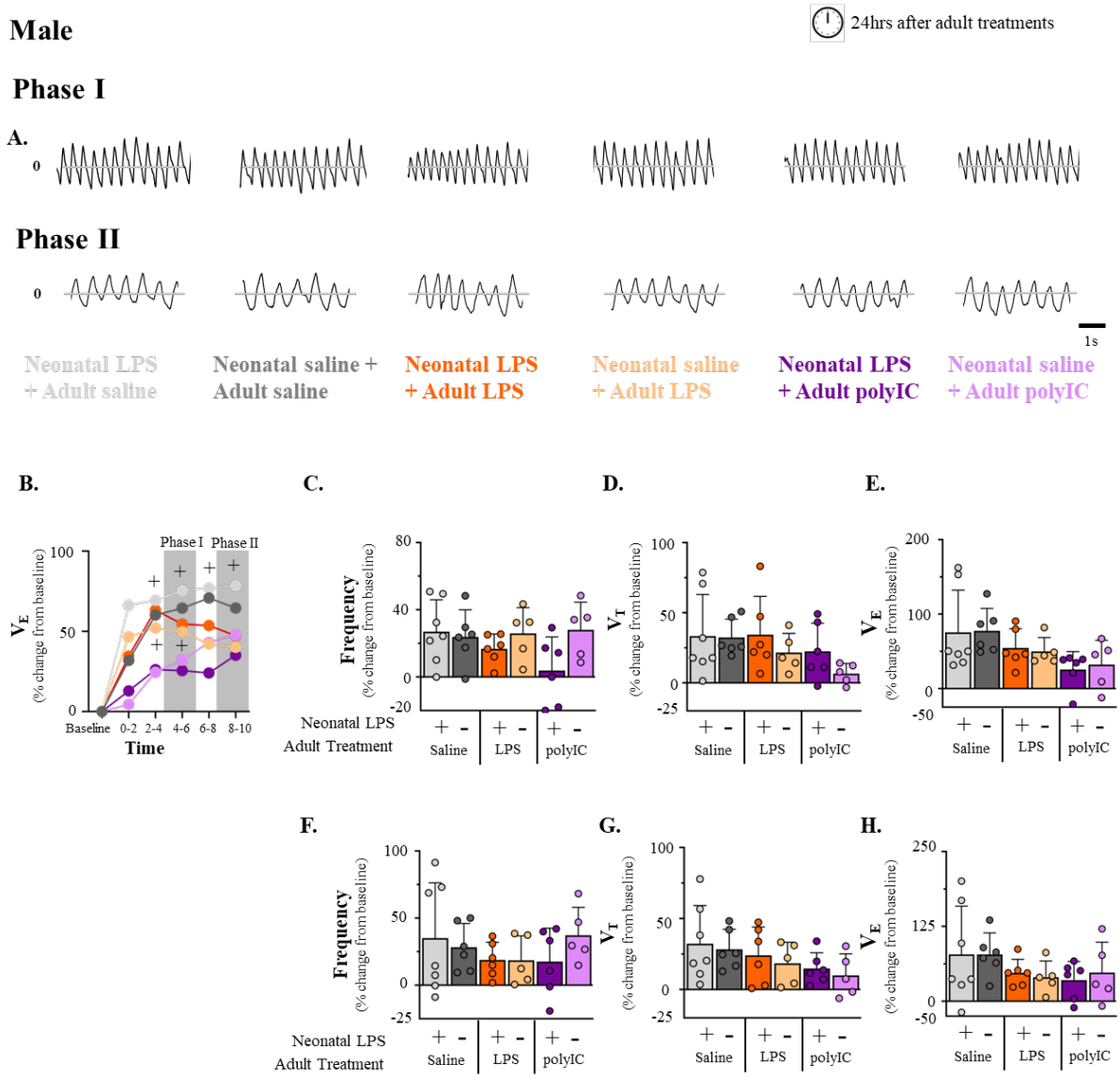


Figure 9. Male hypoxic ventilatory responses were unchanged by any combination of neonatal and adult treatments, 24 hours after adult treatments. Representative breathing traces show male breathing during phase I and phase II of the hypoxic ventilatory response (HVR) were similar between all treatment groups (**A.**). Minute ventilation during hypoxia (**B.**) increased from baseline after 2mins in males after neonatal saline + adult saline and neonatal LPS + adult LPS, and from 2-6mins in males after neonatal saline + adult LPS, but was unchanged from baseline in males after neonatal LPS + adult saline, neonatal saline + adult polyIC and neonatal LPS + adult polyIC. Breathing frequency (**C.**), tidal volume (**D.**), and minute ventilation (**E.**) during HVR phase I were unchanged by any combination of neonatal and adult treatments. During HVR phase II, breathing frequency (**F.**), tidal volume (**G.**), and minute ventilation (**H.**) were unchanged by any combination of neonatal adult treatments. (+ $p<0.05$ different from baseline; Two-way RM ANOVAs, Bonferroni post-hoc.)

saline + adult LPS increased V_E during hypoxia from 2-6 mins, while males after neonatal saline + adult saline and neonatal LPS + adult LPS had typical ventilatory responses to hypoxia.

When focusing on HVR phase I, neither neonatal treatment (p=0.082), adult treatment (p=0.39) nor the interaction between neonatal and adult treatment (p=0.15) had main effects on male breathing frequency during HVR phase I and there were no pairwise differences between groups (p>0.32, **Fig. 9B**). Similar to male breathing frequency, neither neonatal treatment (p=0.18), adult treatment (p=0.11), nor the interaction between neonatal-adult treatments (p=0.66) had significant main effects on male V_T during HVR phase I, and there were no pairwise differences between treatment groups (p>0.56, **Fig. 9C**). In contrast to V_T, adult treatment (p=0.021) had a main effect on male V_E during HVR phase I. However, neither neonatal treatment (p=0.79) nor the interaction between neonatal-adult treatments (p=0.82) had significant main effects on male V_E during HVR phase I and there were no pairwise differences between treatment groups (p>0.19, **Fig. 9D**). Thus, 24hrs after adult treatments, male V_E during HVR phase I was unchanged by any combination of neonatal and adult treatments.

Similar to HVR phase I, male breathing during HVR phase II was not impaired by any combination of neonatal and adult treatments. For male breathing frequency during HVR phase II, neither neonatal treatment (p=0.63), adult treatment (p=0.46), nor the interaction between neonatal-adult treatments (p=0.44) had significant main effects on male breathing frequency during HVR phase II, and there were no pairwise differences between treatment groups (p>0.99, **Fig. 9E**). For male V_T during HVR phase II, neither neonatal treatment (p=0.45), adult treatment (p=0.074), nor the interaction between neonatal-adult treatments (p=0.99) had significant main effects on male V_T during HVR phase II. Further, there were no pairwise differences between treatment groups (p>0.69, **Fig. 9F**). Neither neonatal treatment (p=0.86), adult treatment (p=0.22), nor the interaction between neonatal-adult treatments (p=0.78) had significant main effects on male V_E during HVR phase II, and there were no pairwise differences between treatment groups

(p>0.99, **Fig. 9G**). In summary, male breathing during HVR phase II was not changed by any combination of neonatal and adult treatments.

Female hypoxic ventilatory responses were not affected by any combination of neonatal and adult treatments, 24 hours after adult treatments.

Female breathing during hypoxia was not impaired by any combination of neonatal and adult treatment (**Fig. 10A**). Time (p<0.0001) had a main effect on Ve during hypoxia. However, neither treatment (p=0.82) nor the interactions between treatment-time (p=0.38) had main effects on female V_E during hypoxia. Neonatal LPS + adult saline increased female V_E from baseline during hypoxia at all timepoints (0-2mins, p=0.0024; 2-4mins, p=0.0070; 4-6mins, p=0.0067; 6-8mins, p=0.042; 8-10mins, p=0.015). Neonatal saline + adult saline increased female V_E from baseline during hypoxia at all timepoints (0-2mins, p=0.011; 2-4mins, p=0.0002; 4-6mins, p=0.0024; 6-8mins, p=0.029; 8-10mins, p=0.039). Neonatal LPS + adult LPS increased female V_E from baseline during hypoxia after 4 mins (0-2mins, p=0.74; 2-4mins, p=0.27; 4-6mins, p=0.0068; 6-8mins, p=0.0043; 8-10mins, p=0.011). Neonatal saline + adult LPS increased female V_E from baseline during hypoxia at all timepoints (0-2mins, p=0.049; 2-4mins, p=0.0005; 4-6mins, p=0.0001; 6-8mins, p=0.0001; 8-10mins, p=0.038). Neonatal LPS + adult polyIC increased female V_E from baseline during hypoxia after 4 mins (0-2mins, p=0.99; 2-4mins, p=0.095; 4-6mins, p=0.0087; 6-8mins, p=0.047; 8-10mins, p=0.076). Neonatal LPS + adult polyIC increased female V_E from baseline during hypoxia at all timepoints (0-2mins, p=0.0043; 2-4mins, p=0.016; 4-6mins, p=0.0043; 6-8mins, p=0.026; 8-10mins, p=0.011). In summary, c However, there were no differences between treatments at any timepoints (p>0.45, **Fig. 10B**), demonstrating no combination of neonatal and adult treatments affected female HVR.

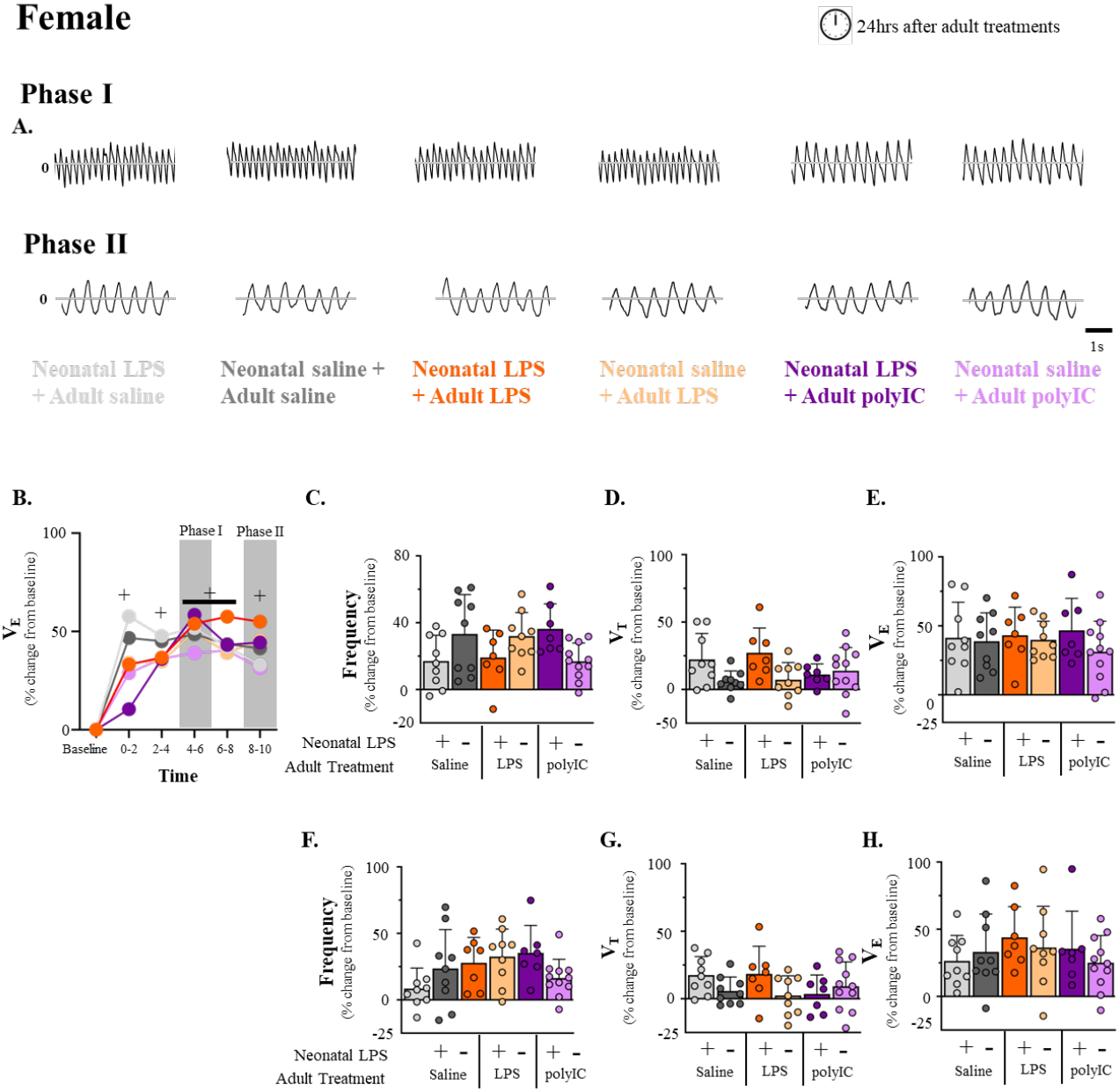


Figure 10. Female hypoxic ventilatory responses were not affected by any combination of neonatal and adult treatments, 24 hours after adult treatments. Representative plethysmography traces show females breathing during the hypoxic ventilatory response (HVR) was similar between all treatment groups (**A.**). Minute ventilation during hypoxia (**B.**) increased from baseline in all timepoints in females after neonatal LPS + adult saline, neonatal saline + adult saline, neonatal saline + adult LPS, neonatal saline + adult polyIC. Minute ventilation during hypoxia (**B.**) increased from baseline after 4mins in females after neonatal LPS + adult LPS, and from 4-8mins in females after neonatal LPS + adult polyIC. Breathing frequency (**C.**) during HVR phase I was similar between all treatment groups. However, tidal volume (**D.**) increased in females after neonatal LPS + adult polyIC compared to females after neonatal saline + adult saline, neonatal saline + adult LPS, neonatal saline + adult polyIC. Minute ventilation (**E.**) during HVR phase I increased in females after neonatal LPS + adult polyIC compared to neonatal saline + adult saline, neonatal saline + adult LPS, and neonatal LPS + adult LPS. During HVR phase II, breathing frequency (**F.**), tidal volume (**G.**) and minute ventilation (**H.**) was similar between all treatment groups. (+ $p<0.05$ different from baseline; Two-way RM ANOVAs, Bonferroni post-hoc.)

During peak HVR phase I, female breathing frequency was significantly affected by the

interaction between neonatal-adult treatments (p=0.0038). However, neither neonatal treatment (p=0.48) nor adult treatment (p=0.96) had significant main effects on female breathing frequency during HVR phase I and there were no pairwise differences between treatment groups (p>0.26, **Fig. 10C**). For female V_T during HVR phase I, neonatal treatment (p=0.010) had significant main effects on female V_T during HVR phase I. However, neither adult treatment (p=0.068), nor the interaction between neonatal-adult treatment (p=0.66) had significant main effects on female V_T during HVR phase I and there were no differences between treatment groups (p>0.103, **Fig. 10D**). For V_E during HVR phase I, neither neonatal treatment (p=0.24), adult treatment (p=0.94), nor the interaction between neonatal-adult treatments (p=0.61) had main effects on adult V_E during HVR phase I and there were no pairwise differences between treatment groups (p>0.99, **Fig. 10E**). Thus, female breathing during HVR phase I was not affected by any combination of neonatal and adult treatment.

During HVR phase II, female breathing frequency was significantly affected by the interaction between neonatal-adult treatments (p=0.048). However, neither neonatal treatment (p=0.96) nor adult treatment (p=0.12) had significant main effects on female breathing frequency during HVR phase I and there were no pairwise differences between treatment groups (p>0.18, **Fig. 10F**). For female V_T during HVR phase II, neither neonatal treatment (p=0.097), adult treatment (p=0.58), nor the interaction between neonatal-adult treatments (p=0.11) had significant main effects on female V_T during HVR phase II and there were no pairwise differences between treatment groups (p>0.65, **Fig. 10G**). For female V_E during HVR phase II, neither neonatal treatment (p=0.59), adult treatment (p=0.42), nor the interaction between neonatal-adult treatment (p=0.56) had significant main effects on female V_E during HVR phase II, and there were no

pairwise differences between treatment groups (p>0.63, **Fig. 10H**). Thus, female HVR phase II was unaffected by any combination of neonatal and adult treatment.

In males, spontaneous apneas, sighs and post-sigh apneas during HVR phase I or II were not affected by any combination of neonatal and adult treatment. There were no spontaneous apneas during HVR in any animal (**Table 7.**). During HVR phase I and II, neither neonatal treatment (phase I p=0.92, phase II p=0.83), adult treatment (phase I p=0.95, phase II p=0.98) nor the interaction between neonatal and adult treatment (phase I p=0.85, phase II p=0.97) had main effects on male sighs and there were no pairwise differences between treatment groups (phase I p>0.99, phase II p>0.99, **Table 7.**). In contrast, adult treatment (p=0.017) had a significant main effect on male post-sigh apneas during HVR phase I. However, neither neonatal treatment (p=0.33) nor the interaction between neonatal and adult treatment (p=0.66) had main effects on male post-sigh apneas during HVR phase I and there were no differences between treatment groups (p>0.15, **Table 7.).** Post-sigh apneas during HVR phase II were not significantly affected by neonatal treatment (p=0.28), adult treatment (p=0.15) or the interaction between neonatal and adult treatment (p=0.80) and there were no pairwise differences between treatment groups (p>0.74, **Table 7.**). Thus, the combination of neonatal and adult treatments did not affect male apneas or sighs during HVR, 24hrs after adult treatments.

In females, spontaneous apneas, sighs and post-sigh apneas during HVR phase I or II were not affected by any combination of neonatal and adult treatment. There were no spontaneous apneas during HVR in any animal (**Table 7.**). During HVR phase I and II, neither neonatal treatment (phase I p=0.83, phase II p=0.82), adult treatment (phase I p=0.74, phase II p=0.52) nor the interaction between neonatal and adult treatment (phase I p=0.80, phase II p=0.78) had main effects on female sighs and there were no pairwise differences between treatment groups (phase I

p>0.99, phase II p>0.99, **Table 7**). For female post-sigh apneas during HVR phase I, neither neonatal treatment (p=0.29), adult treatment (p=0.83), nor the interaction between neonatal and adult treatment (p=0.60) had main effects on female post-sigh apneas during HVR phase I and there were no differences between treatment groups (p>0.99, **Table 7).** In contrast, female post-sigh apneas during HVR phase II were significantly affected by neonatal treatment (p=0.0069). However, neither adult treatment (p=0.85) nor the interaction between neonatal and adult treatment (p=0.82) had main effects on female post-sigh apneas during HVR phase II and there were no pairwise differences between treatment groups (p>0.47, **Table 7**). Thus, the combination of neonatal and adult treatments did not affect female apneas or sighs during HVR, 24hrs after adult treatments.

Table 7. Spontaneous apneas, sighs and post-sigh apneas during hypoxia, 24hrs after adult treatments.

		Number of spontaneous apneas		Number of sighs		Number of post-sigh apneas	
	Treatment groups	mean	SD	mean	SD	mean	SD
	Neonatal LPS + adult saline	0.00	0.00	10.71	2.87	5.57	1.51
	Neonatal saline + adult saline	0.00	0.00	11.17	3.19	4.83	1.47
	Neonatal LPS + adult LPS	0.00	0.00	11.17	4.02	6.67	1.03
	Neonatal saline + adult LPS	0.00	0.00	10.20	2.39	6.00	1.58
	Neonatal LPS + adult polyIC	0.00	0.00	11.00	2.61	4.67	0.82
Phase I	Neonatal saline + adult polyIC	0.00	0.00	11.20	3.56	4.80	0.84
	Neonatal LPS + adult saline	0.00	0.00	11.71	2.56	4.00	1.41
	Neonatal saline + adult saline	0.00	0.00	11.83	3.60	3.67	0.82
	Neonatal LPS + adult LPS	0.00	0.00	12.00	3.46	5.20	0.84
	Neonatal saline + adult LPS	0.00	0.00	11.60	1.67	4.33	1.37
	Neonatal LPS + adult polyIC	0.00	0.00	12.17	2.48	4.00	1.41
Phase II	Neonatal saline + adult polyIC	0.00	0.00	11.80	3.42	3.80	1.30
	Neonatal LPS + adult saline	0.00	0.00	10.40	2.22	6.20	1.93
	Neonatal saline + adult saline	0.00	0.00	10.56	2.70	6.44	1.88
	Neonatal LPS + adult LPS	0.00	0.00	10.75	2.25	5.38	1.41
	Neonatal saline + adult LPS	0.00	0.00	11.44	2.46	6.56	1.67
Phase I	Neonatal LPS + adult polyIC	0.00	0.00	11.13	2.75	6.13	1.46

	Neonatal saline + adult polyIC	0.00	0.00	10.70	2.36	6.20	1.81
	Neonatal LPS + adult saline	0.00	0.00	10.90	2.60	5.11	1.05
	Neonatal saline + adult saline	0.00	0.00	11.44	2.74	3.89	1.36
	Neonatal LPS + adult LPS	0.00	0.00	11.88	2.59	5.25	1.04
	Neonatal saline + adult LPS	0.00	0.00	12.33	2.06	4.22	1.72
	Neonatal LPS + adult polyIC	0.00	0.00	12.13	2.80	5.00	1.41
Phase II	Neonatal saline + adult polyIC	0.00	0.00	11.60	2.37	4.30	0.82

Discussion

Some adults are more susceptible to respiratory deficits during inflammatory challenges, such as illness and disease (Kiaghadi et al., 2020; Zala et al., 2008); however, factors contributing to why some adults are at higher risk for respiratory disruptions during illness and disease remains unclear. Our research identified neonatal inflammation causes lasting augmentation of microglial inflammatory responses to otherwise innocuous inflammatory stimuli, demonstrating neonatal inflammation stimulus-specifically primes adult male and female microglia in respiratory control regions. Augmented inflammatory responses in primed microglia priming contributes to multiple neurological pathologies, such as Alzheimer's disease and Parkinson's disease and multiple sclerosis (reviewed in Airas & Yong, 2022; Bivona et al., 2023; Hickman et al., 2018; Chowen & Garcia-Segura, 2020; Hickman et al., 2018), however, little is known about how primed microglia effect adult breathing after neonatal inflammation. Despite microglia priming, male and female breathing was not impaired by any combination of neonatal and adult subthreshold inflammation, suggesting neonatal inflammation did not increase adult susceptibility for breathing disruptions to subthreshold homotypic or heterotypic inflammatory challenges. In conclusion, our results demonstrate neonatal inflammation augments adult microglial inflammatory responses, without impairing adult breathing.

Acute increases in inflammatory signaling after systemic inflammation contribute to impairments in respiratory control, such as increased eupneic breathing (Huxtable et al., 2011), blunted chemoreflexes (Huxtable et al., 2011) and abolished respiratory motor plasticity (Huxtable et al., 2011, 2013). Thus, primed microglia after neonatal inflammation may contribute to breathing disruptions during more severe inflammatory challenges associated with illness and disease. We determined neonatal inflammation augments adult male and female microglial

inflammatory responses, demonstrating neonatal inflammation primes adult microglia in respiratory control regions. In non-respiratory control regions primed hippocampal microglia for augmented inflammatory responses and disrupted adult cognition during adult subthreshold inflammation (Bilbo, 2010; Bilbo & Schwarz, 2009, 2012). However, adults after neonatal and adult inflammation maintained adequate breathing despite primed microglia in respiratory control regions. Further, augmentation of adult male chemoreflexes after neonatal inflammation alone was not maintained after adult subthreshold inflammation, suggesting primed microglia may act to maintain respiratory responses to respiratory challenges during minor adult inflammatory insults, distinct from the effects of primed microglia in cortical circuits. While neonatal and adult inflammation primed microglia for augmented pro-inflammatory gene expression, neonatal intermittent hypoxia, another early life stressor, causes lasting increases in microglial anti-inflammatory gene expression (Kiernan et al., 2019). Thus, an increase in microglial anti-inflammatory signaling after neonatal inflammation may contribute to maintenance of adult breathing in response to subsequent inflammation and will be the focus of future research.

Primed microglia in respiratory control regions may contribute to breathing disruptions during more severe inflammatory challenges, such as those associated with disease and illness. While the adult dose of inflammation was subthreshold to impairing breathing in otherwise healthy adults (neonatal saline + adult LPS and neonatal saline + adult polyIC), higher doses of systemic inflammation disrupt central respiratory control and breathing behaviors (Huxtable et al., 2011, 2013). Low levels of acute systemic inflammation (0.1 mg/kg LPS, i.p.) abolish adult respiratory motor plasticity and increase spinal cord inflammatory signaling, demonstrating neuroinflammatory signaling likely impairs adult central respiratory control during low levels of inflammation that are below the threshold to produce a severe inflammation, a febrile response or

septic shock (Teeling et al., 2007; Yamawaki et al., 2010). Higher doses of adult systemic inflammation (LPS, 5 mg/kg i.p.) increase eupneic breathing, blunt chemoreflexes, and increase inflammatory gene expression in respiratory control regions, in addition to abolishing respiratory motor plasticity (Huxtable et al., 2011), demonstrating neuroinflammatory signaling likely contributes to breathing impairments during severe inflammatory challenges such as those associated with severe infection and neurodegenerative disorders. Further, increases in peripheral IL-1β and IL-6 gene expression are used clinically to predict which patients will need breathing support during illness, such as H1N1 virus infection (Chiaretti et al., 2013) and COVID (Broman et al., 2021). Thus, neonatal and adult homotypic inflammation primed microglial IL-1β and IL-6 gene expression, suggesting adults after neonatal inflammation may be predisposed to have disrupted breathing during more severe homotypic inflammatory insults. Determining the role of microglial IL-1b and IL-6 signaling in adult breathing after neonatal and adult homotypic inflammation and the impact of primed microglia on adult breathing during more severe inflammatory challenges will be the subject of future investigations.

Our research here demonstrated neonatal and adult homotypic inflammation increased some similar genes (IL-6 and IL-1β) in male and female microglia, suggesting these pro-inflammatory genes are likely a part of the neuroinflammatory signaling in respiratory control regions in both sexes. However, TNFα gene expression specifically increased in male microglia after neonatal and adult homotypic inflammation, suggesting a potential role for microglial TNFα expression in contributing to sex differences between males and females after neonatal inflammation. Further, males are at increased risk for ventilatory control disorders, such as sleep apnea (reviewed in Bock et al., 2023; Chen et al., 2022) and at increased risk for breathing disruptions during infection and illness (Corica et al., 2022; Mauvais-Jarvis et al., 2020; Thomas et al., 2021), highlighting the need

for further investigation into the role of TNFα in male breathing vulnerabilities. For instance, increases in TNFα are causally linked to increases in obstructive sleep apnea (Yi et al., 2022). Additionally, increased circulating levels of TNFα increased eupneic breathing and blunted hypoxic ventilatory responses (Aleksandrova et al., 2021), demonstrating systemic TNFα contributes to respiratory effects of acute systemic inflammation. However, less is known about the role of microglial TNFα in respiratory effects after inflammation. Our data suggest showing lasting increases in microglial TNFα gene expression after neonatal inflammation, suggest TNFα may also play a role in the lasting augmentation of adult male hypercapnic responses after neonatal inflammation. Yet, neonatal and adult inflammation primed microglial TNFα gene expression to further increase, but hypercapnic ventilatory responses returned to control levels, raising intriguing questions about dose and/or location. The respiratory effects of systemic increases in TNFα may be distinct from respiratory effects of central microglial TNFα and TNFα may have non-linear effects on respiratory control due to cross-talk in inflammatory signaling. Thus, our research highlights the need to advance our understanding of how this cytokine disrupts respiratory circuitry and contributes to adult breathing vulnerabilities, particularly in males.

Investigating adult ventilation in response to hypoxia showed no differences between treatment groups; however, neither adults after neonatal inflammation alone (neonatal LPS + adult saline) nor adult male heterotypic treatment groups increased ventilation in response to hypoxia. These results contrast with our previous study that showed adults after neonatal inflammation responded to hypoxia (Beyeler et al., *in preparation*). This difference could be due to increased variability in ventilation in this study compared to the previous study (Beyeler et al., *in preparation*). Adult male ventilation was unchanged from baseline in neonatal LPS + adult polyIC and neonatal saline + adult polyIC, suggesting the adult polyIC treatments may have depressive effects on male HVR.

In contrast to males, females after LPS + adult polyIC and neonatal saline + adult polyIC increased breathing in response to hypoxia, suggesting adult polyIC treatments may have sex specific effects on HVR. This is the first study to investigate female respiratory control in responses to viral inflammatory challenges, and our results suggest male and female responses to subthreshold viral challenges may differ, highlighting the need for further investigation into how respiratory control is sex-specifically effected by viral challenges. Further, since males are at increased risk for respiratory distress during viral challenges, such as COVID (Thomas et al., 2021), understanding mechanisms protecting females will likely yield novel insights needed to develop better therapeutic strategies to protect adults at high risk for respiratory distress during viral challenges. In distinction to adult heterotypic inflammation, adults after neonatal LPS + adult LPS and neonatal saline + adult LPS increased breathing in response to hypoxia in both sexes and timepoints (3hrs and 24hrs after adult treatments), demonstrating adult LPS treatments did not impair HVR. In conclusion, these results suggest that adult treatments had stimulus- and sex-specific effects on HVR and likely have implications for adult risk of respiratory distress during inflammation.

In females, no combination of neonatal and adult inflammation disrupted breathing, despite primed female microglia in respiratory control regions, suggesting primed female microglia after neonatal inflammation may have protective effects on female breathing and risk for breathing impairments during subsequent inflammation. Advancing our understanding of the cellular mechanisms underlying how females after neonatal inflammation are potentially protected from disruptions in breathing is needed to identify novel signaling pathways to utilize therapeutically to support breathing in both sexes.

Our research is the first to identify that neonatal and adult heterotypic inflammation causes lasting augmentation of adult male medullary microglia and priming of male microglial medullary IL-18 gene expression and spinal IL-6 gene expression, suggesting neonatal inflammation likely reprograms microglia to respond differently to multiple adult stressors, highlighting a need to further investigate stimulus-specific responses in males after neonatal inflammation. While neonatal and adult heterotypic inflammation primed microglia in respiratory control regions, neonatal inflammation did not change adult peripheral immune responses or sickness behaviors after neonatal and adult heterotypic inflammation (Ellis et al., 2006; Kentner & Pittman, 2010), highlighting differences between how peripheral immune cells and microglial are reprogrammed by neonatal inflammation. Further, this study is the first to study female respiratory responses to viral inflammatory challenges and identified adult polyIC alone increased female spinal microglia and increased female breathing frequency, suggesting females may have increased responses to viral challenges. Thus, neonatal inflammation may reprogram microglia to respond differently to future stressors, highlighting a need to study epigenetic mechanisms that may underly how neonatal inflammation reprograms microglia in respiratory control regions.

Neonatal and adult inflammation increased microglial pro-inflammatory gene expression (TNFα, IL-6, IL-1β, IL-18) in a stimulus-, sex- and region-dependent manner, but this does not preclude the involvement of anti-inflammatory signaling in primed microglia after neonatal inflammation. In another early life stress, neonatal intermittent hypoxia (modeling sleep disordered breathing in neonates) caused lasting attenuation of adult microglial inflammatory responses by increasing anti-inflammatory gene expression (Kiernan et al., 2019), which may represent a protective effect of microglia priming, whereby neonates are able to adapt stressors pathological to adults. Additionally, gestational intermittent hypoxia (modeling sleep disordered breathing

during pregnancy) lead to attenuation of neonatal pro-inflammatory responses specifically in females (Johnson et al., 2018). This highlights the importance of disentangling the effects of sex on microglia after early life stressors, as it will likely reveal unique cellular mechanisms by which respiratory circuitry is able to compensate and adapt to maintain sufficient breathing. Thus, primed microglia after neonatal inflammation may have protective effects on adult breathing and chemoreflexes.

Our research highlights multiple areas for future investigations on how neonatal inflammation effects adult breathing. While no combination of neonatal and adult subthreshold inflammation disrupted breathing, primed microglia may act to disrupt breathing during non-inflammatory stressors, such as during pregnancy, when in low oxygen environments such as at altitude or during severe emotional stress. Further, our research did not identify significant disruptions in breathing at 3 or 24hrs after adult inflammatory treatments; however, disruptions in breathing may occur during chronic inflammation in adult breathing after neonatal inflammation, such as those associated with ventilatory control disorders, such as sleep apnea (Gaines et al., 2015; Hirotsu et al., 2017).

Overall, this research significantly advances our understanding of the lasting consequences of neonatal inflammation on adult inflammatory responses in respiratory control regions. Neonatal inflammation sex-specifically primes adult microglial inflammatory responses to subthreshold adult homotypic and heterotypic inflammation. However, since adult breathing was maintained after neonatal and adult subthreshold inflammation, adult microglia priming after neonatal inflammation likely does not increase susceptibility to breathing deficits to low levels of adult inflammation. Instead, primed microglia after neonatal inflammation may increase breathing deficits after more severe adult inflammatory challenges or have protective effects on breathing

during adult inflammation. While more research is needed to identify whether microglia priming is maladaptive or protective to adult breathing during adult inflammation, our research provides key insights into how microglia priming in respiratory regions after neonatal inflammation contributes to adult neuroinflammatory responses to subsequent adult inflammation.

CHAPTER IV

MATERNAL OPIOIDS AGE-DEPENDENTLY IMPAIR NEONATAL RESPIRATORY CONTROL NETWORK.

This chapter includes material previously published in *Frontiers in Physiology* and was co-authored with Robin Naidoo, Nina R. Morrison, MS, Emilee A. McDonald, David Albarran and Adrianne G. Huxtable. I designed, collected and analyzed data in the *in vitro* electrophysiology studies, provided guidance and analyzed the immunohistochemistry experiments, and wrote the manuscript. Nina Morrison and I designed, collected and analyzed data in the *in vitro* electrophysiology studies. Emilee McDonald and David Albarran designed and collected data for the immunohistochemistry experiment, as well as contributed to writing the corresponding methods section. Robin Nadioo contributed to writing the immunohistochemistry methods and results section. Dr. Adrianne Huxtable designed, contributed to writing, provided guidance and editorial assistance.

Introduction

Maternal opioid use is increasing, thereby increasing the number of infants born exposed to opioids *in utero* >6 fold (Batra et al., 2021). Approximately 80 infants are born daily exposed to *in utero* opioids (CDC, 2022), with an infant being born approximately every 15 minutes exposed to maternal opioids (NIDA, 2019). Yet, these infants remain an understudied population in the opioid crisis (Kocherlakota, 2014). Maternal opioids cross the placenta (Nanovskaya et al., 2008), directly disrupting *in utero* fetal development (including neural tube formation; Williams et al., 1991), with the most severe effects occurring late in gestation when opioids accumulate in the fetus (Condradt et al., 2018). Infants born after *in utero* opioid exposure are often diagnosed

with Neonatal Abstinence Syndrome (NAS), which presents as a diverse set of symptoms, including sleep disturbances, temperature instability, tremors, seizures, high pitched crying, excessive yawning, tachypnea (McQueen & Murphy-Oikonen, 2016), apneas, and respiratory deficits (Fältmarch et al., 2019). Although these symptoms are thought to result from dysfunctions in the central and autonomic nervous systems, and gastrointestinal system, their etiology remains poorly understood (McQueen & Murphy-Oikonen, 2016). Infants with NAS often require exogenous opioids to manage withdrawal symptoms (Mangat et al., 2019), though these additional opioids may have further disruptive effects on infant health. Respiratory deficits in these infants are a significant clinical problem and require additional clinical interventions for infant survival (Fältmarch et al., 2019). However, the etiology of these respiratory deficits in infants with NAS remains unclear, restricting the ability to develop treatments to facilitate infant breathing.

The direct effect of maternal opioids on neonatal breathing are difficult to study in humans, as maternal polysubstance use, poor nutrition, and stress often accompany maternal opioid use and may contribute to symptomology in infants with NAS, including respiratory deficits (reviewed in Farid et al., 2008; Conradt et al., 2018). The rodent represents an ideal model to directly study the impact of maternal opioids since it allows for control of these confounding factors and facilitates the use of more invasive techniques to identify the central origins of breathing deficits. In a rat model of maternal opioids starting at conception, maternal opioids induced reorganization of neonatal central respiratory networks (Gourévitch et al., 2016), yet the impact on neonatal breathing behaviors was not assessed. We developed a novel, late gestation opioid model in rodents to test the impact of maternal opioids on respiratory network maturation without disrupting early developmental processes (such as neurogenesis, gliogenesis, myelination, and synaptogenesis; reviewed in Farid et al., 2008; Hauser and Knapp, 2018). In this late gestation

model, opioid exposure begins at the onset of respiratory rhythm *in utero*, after other critical components of the central nervous system have developed (Hocker, Morrison et al., 2021).

Using this model, maternal opioids acutely destabilized neonatal breathing (increased apneas, increased breathing variability and blunted the hypoxic ventilatory decline) and blunted opioid-induced respiratory depression immediately after birth, before breathing normalized with age (Hocker, Morrison et al., 2021). Given the similarities in breathing deficits in neonates after late gestational maternal opioids (Hocker, Morrison et al., 2021) and human infants with NAS (Fältmarch et al., 2019), maternal opioids may disrupt perinatal maturation of the central respiratory system and contribute to breathing deficits. Yet, where within the heterogeneous and anatomically distributed respiratory system maternal opioids directly influence perinatal maturation remains unknown. To identify the central origins of breathing deficits in neonates after maternal opioids, we first assessed isolated neonatal central respiratory networks known to control breathing, independent of peripheral influences. We tested the hypothesis that maternal opioids impair neonatal central respiratory networks.

Methadone was the opioid of choice since it is commonly prescribed to mothers with opioid use disorder (Kocherlakota, 2014; Davis et al., 2018), in part due to its long half-life (Kraft et al., 2016). Others have demonstrated impaired ontogenesis in the rat with this same dose and type of opioid (reviewed in Farid et al., 2008) and it mimics aspects of the high/rush associated with opioid use (Hauser and Knapp, 2018). Further, infants with NAS show improved neonatal outcomes with methadone treatment (Patrick et al., 2014; Young et al., 2015; Davis et al., 2018). Here, we are extending our previous work (Hocker et al., 2021) to triangulate on the central deficits contributing to impaired neonatal breathing in this model of late gestation opioids.

Collectively, this study demonstrates maternal opioids age-dependently impair isolated neonatal central respiratory networks, suggesting central impairments may contribute to neonatal breathing deficits after maternal opioids. A component of the central mechanism contributing to these impairments involves opioid receptors. Opioid receptor antagonists suggest lingering opioids in neonatal central respiratory networks contribute to reduced fictive respiratory activity. Yet, isolated central respiratory networks from neonates after maternal opioids had blunted responses to acute opioids, similar to breathing responses shown previously (Hocker et al., 2021). This blunted response to acute opioids may be due to reduced expression of opioid receptors in a key respiratory rhythm generating region in the neonatal medulla, the preBötzinger complex (preBötC), after maternal opioids. However, central respiratory network impairments extend beyond the preBötC, since further isolated neonatal central respiratory networks showed no impairments. Emergence of a distinct respiratory pattern in neonates after maternal opioids further supports impairments to regions beyond the preBötC and highlights the need to further investigate regions associated with respiratory pattern. In summary, maternal opioids impair neonatal central respiratory networks and these central impairments likely contribute to neonatal breathing deficits after maternal opioids (Hocker et al., 2021). This study advances our understanding of the etiology of respiratory deficits in infants after maternal opioids and is a needed step toward developing novel treatments to support breathing in infants with NAS.

Methods

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Oregon and conformed to the policies of the National Institutes of Health, Guide for the Care and Use of Laboratory Animals. Timed pregnant Sprague-Dawley dams (E17) were purchased in pairs from commercial vendors (Envigo, colony 231 and 202; Charles River, stock H41) and monitored daily until giving birth. Rats were housed under standard conditions (12:12h light/dark cycle) with food and water *ad libitum*.

Maternal opioid exposure

Maternal opioid exposure was administered as described previously (Hocker, Morrison et al., 2021). This opioid exposure model facilitates investigating the effects of maternal opioids on maturation of the respiratory control system, without impacting the known effects of maternal opioids on critical neurodevelopmental processes, such as cell genesis, myelination and synaptogenesis (reviewed in Boggess and Risher, 2022). In brief, maternal opioid exposure (methadone hydrochloride in sterile saline, 5mg/kg, subcutaneous, Sigma-Aldrich, cat#1095905) began at embryonic day 17 (E17), the onset of respiratory rhythm generation *in utero* (Greer et al., 1992; Pagliardini et al., 2003). From E17 onward, dams were injected with methadone daily and maternal health monitored for at least 1hr post-injection. Maternal opioid exposure continued until postnatal day 5 (P5). To control for maternal care, litters were balanced and culled to 12 or fewer pups per dam. Since no differences were seen in neonatal breathing in neonates after maternal no treatment or maternal saline injections (Hocker, Morrison et al. 2021), neonates after maternal no treatment were used as controls.

Brainstem-spinal cord (BSSC) preparations

To study central deficits in isolated respiratory networks from neonates (postnatal day 0 to 5, P0-5) after maternal opioids, we isolated neonatal brainstems and spinal cords, which contain essential components of central respiratory networks, and recorded spontaneous respiratory-related motor activity (Greer et al., 1991; reviewed in Johnson et al., 2012). Fictive respiratory activity from isolated brainstem-spinal cords (BSSCs) was assessed in neonates after maternal no treatment (P0-1=3 male, 2 female, P2=2 male, 3 female, P3-5=3 male, 2 female) and neonates after maternal opioids (P0-1=3 male, 3 female, P2=3 male, 3 female, P3-5=2 male, 3 female). The responses to opioid receptor antagonism and agonism were measured in a separate set of experiments. Respiratory activity was assessed in isolated BSSCs after mu-opioid receptor antagonism (naloxone 10μM, 2hrs, Sigma-Aldrich, cat#N7758) in neonates after maternal no treatment (P0-1=3 male, 3 female, P2=3 male, 3 female, P3-5=3 male, 3 female) and neonates after maternal opioids (P0-1=3 male, 3 female, P2=2 male, 3 female, P3-5=3 male, 3 female). Respiratory activity was also assessed in response to acute, exogenous opioids in isolated BSSCs after mu-opioid receptor agonism (methadone 10μM, 2hrs, Sigma-Aldrich, cat#1095905) in neonates after maternal no treatment (P0-1=3 male, 3 female, P2=2 male, 3 female, P3-5=3 male, 3 female) and neonates after maternal opioids (P0-1=3 male, 3 female, P2=3 male, 3 female, P3-5=2 male, 2 female).

Brainstem-spinal cord (BSSC) preparations were prepared from neonates (P0-5), as described previously (Greer et al., 1992; Morrison et al., 2019; Suzue, 1984). In brief, neonates were anesthetized with isoflurane and decerebrated. The thoracic and cervical spinal cord regions were isolated and placed in artificial cerebrospinal fluid (aCSF), containing the following (in mM): 120 NaCl, 3 KCl, 1.25 NaH_2PO_4, 1.0 $CaCl_2$, 2.0 $MgSO_4$, 26 $NaHCO_3$, 20 D-glucose, equilibrated

with 95% O_2/5% CO_2. A dorsal laminectomy revealed the spinal cord before removal of the lungs and heart. A ventral laminectomy isolated BSSCs and brainstems were transected at the pontomedullary junction. Isolated BSSCs were pinned ventral side up in a recording chamber (8.2 ml volume, 28 °C) with aCSF (continuously bubbled with 95% O_2/5% CO_2) and circulated via a peristaltic pump (8–10 ml/min, MINIPULS3, Gilson, Inc., Middleton, WI). Respiratory activity was recorded from the 4th or 5th cervical nerve rootlets using glass suction electrodes (internal diameter 70–80 µm). Recordings were amplified (x1000–10k), bandpass-filtered (0.1 Hz to 1kHz; Model 1700 Differential AC amplifier, A-M Systems, Carlsburg, WA), integrated (τ=50 ms) and rectified (LabChart, Version 8.1, ADInstruments). Preparations equilibrated (40–50 minutes) prior to recording baseline activity. Neurograms illustrate respiratory activity from baseline through 120 minutes, whereby changes over time are compared within treatment at baseline (30-minute averages, baseline = 0-30 min) and 120 min (30-minute averages = 90-120 min), as described in Olsson et al. (2003), Tsuzawa et al. (2015) and Kotani et al. (2018). Data are presented as the percent change from within treatment baseline (% change from baseline: $\frac{\text{120 min average} - \text{baseline average}}{\text{baseline average}} * 100$), as described in Gumnit et al. (2022) and Cook-Snyder et al. (2020). Throughout the manuscript, asterisk (*) symbols denote significant differences from baseline within a treatment group, pound (#) signs highlight significant differences between treatment groups, and ampersand (&) symbols represent significant differences between ages. For experiments with the mu-opioid receptor antagonist (naloxone, 10µM) or opioid receptor agonist (methadone, 10µM), BSSCs equilibrated (40-50 minutes) and antagonists or agonists were bath-applied following baseline (0-30 min = baseline). Responses to antagonists and agonists were monitored for 120 min, whereby changes from baseline were analyzed at 120 min (90-120min = 120 min average). The percent change from baseline was calculated as described above.

Immunohistochemistry

Given the changes in respiratory activity after acute, exogenous opioids, we next sought to view neonatal opioid receptor expression in a key respiratory rhythm generating region, the preBötC (Smith et al., 1991), after maternal opioids. Opioid receptor expression in the neonatal preBötC were characterized at three postnatal ages: at a critical period immediately post-birth when neonatal breathing is acutely destabilized and opioid-induced respiratory depression is blunted after maternal opioids (P0), during the first week of life when breathing normalized after maternal opioids and neonates continued receiving opioids through breast milk (P4), and during the second week of life after maternal opioid exposure ceased (P11). Before determining the effect of maternal opioids on opioid receptor expression, mu-opioid receptors in the neonatal preBötzinger Complex (preBötC) during typical postnatal development were first characterized using immunohistochemistry in neonates (maternal no treatment neonates: P0=3 male, 3 female; P4=3 male, 3 female; P11=3 male, 3 female). In a separate set of experiments, the impact of maternal opioids on expression of mu-opioid receptors in the preBötC was determined with immunohistochemistry at the same three ages in neonates after maternal no treatment (P0=3 male, 3 female; P4=3 male, 3 female; P11=3 male, 3 female) and neonates after maternal opioids (P0=3 male, 3 female; P4=3 male, 3 female; P11=3 male, 3 female).

Immunohistochemistry experiments were performed similar to our previous work (Hocker et al., 2019), but were optimized for neonatal medullary tissue. In brief, neonates (P0, P4 and P11) after maternal no treatment or maternal opioids were perfused (transcardiac) with cold phosphate-buffered saline (PBS, pH 7.4) and 4% paraformaldehyde (pH 7.4 in PBS). Brain tissue was extracted and immersed in paraformaldehyde for 24hrs before being transferred to PBS until vibratome sectioning (Leica VT 1200S vibratome). The medullary slices containing the preBötC

were selected from -0.35mm to -0.45mm from the rostral edge of the inferior olive (Ruangkittisakul et al., 2006). We further identified medullary slices containing the preBötC using 10x images with the semi-compact nucleus ambiguous and bright expression of NK1R in the ventral lateral region, as described previously (Gray et al., 1999). Transverse medullary sections (40 μm) were washed in PBS, blocked with PBS, 0.3% Triton, and 1% BSA (2hrs, room temperature) to prevent non-specific antibody binding. Medullary slices were incubated in a buffer solution (PBS with 0.3% triton, 0.01% BSA at room temperature for 16hrs) with the following primary antibodies: (1) Guinea pig anti-substance p receptor (1:1000, Millipore AB15810) to identify preBötzinger Complex (preBötC) neurons and (2) Rabbit anti-mu-opioid receptor (1:250, Millipore, AB1774) to label mu-opioid receptors. After primary antibody incubation, medullary sections were rinsed (3 times with PBS) and incubated in a buffer solution (PBS with 0.3% Triton, and 0.01% BSA at room temperature for 2hrs) with the following secondary antibodies: (1) Goat anti-guinea pig 647 IgG (1:1000, Invitrogen, A21450; used when studying typical postnatal expression of mu-opioid receptors) or Goat anti-guinea pig 488 IgG (1:1000, Invitrogen, A11073; used to study changes in mu-opioid receptors after maternal opioids) to label NK1R primary antibodies, and (2) Donkey anti-rabbit 555 IgG (1:1000, Invitrogen, A31572) to label mu-opioid receptor primary antibodies. Binding of secondary antibodies to nonspecific epitopes was assessed in medullary sections as described above, but with the omission of primary antibodies. Background tissue fluorescence was assessed in medullary sections as described above, but with the omission of secondary antibodies. Cross reactivity of Guinea pig anti-substance p receptor primary antibody with Donkey anti-rabbit 555 IgG secondary was determined in medullary sections incubated as described above, but with only the Guinea pig anti-substance p receptor primary antibody (no Rabbit anti-mu-opioid receptor primary antibodies) and all relevant secondaries (either Goat anti-

guinea pig 647 IgG or Goat anti-guinea pig 488 IgG, and Donkey anti-rabbit 555 IgG). Cross reactivity of the Rabbit anti-mu-opioid receptor primary antibody with Goat anti-guinea pig 647 IgG or Goat anti-guinea pig 488 IgG secondary antibodies was identified in medullary sections incubated as described above, but with only the Rabbit anti-mu-opioid receptor primary antibody (no Guinea pig anti-substance p receptor primary antibodies) and all relevant secondaries (either Goat anti-guinea pig 647 IgG or Goat anti-guinea pig 488 IgG, and Donkey anti-rabbit 555 IgG). Medullary sections were washed and mounted onto charged microscope slides, air-dried, and covered with Prolong Gold (Life Technologies, cat#P10144) to preserve fluorescence. A glass coverslip was placed over the sections, sealed with clear nail polish and stored at 4°C until imaged.

All immunofluorescent images (1024 x 1024 pixels) were acquired using a Zeiss LSM 880 confocal microscope (40x, z-stacks, 0.5μm increments). To determine the typical expression of mu-opioid receptors in the preBötC during postnatal development, medullary sections from neonates after maternal no treatment (P0, P4, and P11) were all stained and imaged concurrently under identical microscope settings. To assess the effect of maternal opioids on mu-opioid receptor expression in the preBötC, medullary sections from neonates after maternal opioids (P0, P4, and P11) and age-matched neonates after maternal no treatment were stained and imaged concurrently under identical microscope settings. All images were pseudo-colored to visualize NK1 receptors in green and mu-opioid receptors in red. All images for sections run concurrently were taken using identical laser and gain settings, identically adjusted for contrast/brightness, and collapsed into maximum intensity projections using ImageJ open-source software to allow for comparisons between maternal treatments and all ages.

Rhythmically active medullary slice preparations

To further triangulate on where maternal opioids may impair neonatal respiratory networks, we evaluated respiratory activity in rhythmic slice preparations, which further isolate the preBötC. Activity was compared between neonates after maternal no treatment (P0-1=3 male, 3 female, P2=3 male, 3 female, P3-5=3 male, 3 female) and neonates after maternal opioids (P0-1=3 male, 3 female, P2=3 male, 3 female, P3-5=3 male, 3 female). Rhythmically active medullary slices containing the preBötC, hypoglossal motor nucleus, and hypoglossal nerve roots were isolated from P0-5 neonatal rats, as previously described (Morrison et al., 2019; Ruangkittisakul et al., 2006). In brief, isolated BSSCs (as described above) were pinned to a wax chuck and thin slices (200 μm) were cut using a vibratome (Leica VT 1200S vibratome) to visualize anatomical landmarks. Medullary slices were compared to the neonatal rat brainstem atlas and a single 700 μm slice was taken at −0.35 to −0.45 mm from the rostral edge of the inferior olive (Ruangkittisakul et al., 2006). Medullary slices were transferred to a recording chamber (8.2 ml volume, 28°C) with recirculated (10 ml/min) aCSF (continuously bubbled with 95% O_2/5% CO_2) via a peristaltic pump (MINIPULS3, Gilson, Inc., Middleton, WI). Extracellular potassium was elevated from 3 mM to 9 mM prior to the start of data collection (30–60 minutes) to offset a loss of tonic excitatory inputs and prevent the gradual slowing of respiratory activity in medullary slice preparations (Ballanyi et al., 2009; Ruangkittisakul et al., 2006; Smith et al., 1991). Respiratory activity was recorded from hypoglossal nerve rootlets using glass suction electrodes (internal diameter 70–80 μm). Recordings were amplified (x10k), bandpass-filtered (300Hz to 1kHz; Model 1700 Differential AC amplifier, A-M Systems, Carlsburg, WA), integrated (τ=50 ms) and rectified (LabChart v8.1, ADInstruments). Respiratory activity was assessed as described above in Section 2.2 (Brainstem-spinal cord preparations).

Burst-to-burst variation analysis

To characterize the effect of maternal opioids on respiratory pattern, Poincaré plots were generated in R from periods obtained from Peak Analyses in LabChart, as described previously (Morrison et al., 2019). Burst-to-burst variation in respiratory activity was revealed by graphing the period between two bursts (T_n) versus the subsequent period (T_{n+1}). To quantify the differences between maternal treatments, ratios of the width of the variation perpendicular (SD1) over the line of identity (SD2) were calculated from an ellipse encapsulating 95% of the bursts within a given age and maternal treatment group, as described previously (Brennan et al., 2001; Li & Nattie, 2006; Patrone et al., 2018).

Statistical analyses

GraphPad Prism software (version 9.3) was used for statistical analyses of *in vitro* electrophysiology experiments (BSSC and rhythmic slice experiments). Two-way ANOVAs were used to assess the effect of maternal treatment and age (maternal opioids, maternal no treatment) and age (P0-1, P2, P3-5) on respiratory burst frequency (**Fig. 1 & 7**) or SD1/SD2 (**Fig. 8**). Three-way ANOVAs were used to assess the effect of maternal treatment (maternal opioids, maternal no treatment), age (P0-1, P2, P3-5), and time (baseline average, 120 minute average) on percent changes in respiratory activity (**Fig. 1-3, 6**). To correct for multiple comparisons, a Bonferroni post-hoc test was used (α=0.05). No differences were observed between sexes in any *in vitro* electrophysiology experiments ($p>0.05$); thus, males and females were combined for all *in vitro* electrophysiology experiments. Values are reported as means ± SD and represent biological replicates.

Results

Litter size, neonatal mortality and neonatal weight gain after maternal opioids.

Maternal opioids decreased the number of neonates born per litter (12.8±3.2 neonates after maternal no treatment per litter, 25 litters; 10.6±2.5 neonates after maternal opioids per litter, 23 litters, p=0.03) and increased neonatal mortality at birth (0.6±0.4 neonatal mortality after maternal no treatment, n=215 neonates; 2.1±0.6 neonatal mortality after maternal opioids, n=215 neonates, p=0.04). As shown previously (Hocker, Morrison et al. 2021), neonates from both maternal groups gained weight significantly each day during development (P0-5), except for P2 to P3 in neonates after maternal no treatment. Between maternal treatment groups, P2 neonates after maternal opioids weighed less than P2 neonates after maternal no treatment (p=0.023), but weight was not significantly different between maternal treatments at any other age (**Table 1**).

Table 1. Maternal opioids delayed neonatal weight gain at P2, but not at other ages.

	P0	P1	P2	P3	P4	P5
Maternal opioids	5.1±0.2g	6.3±0.6g	7.0±0.4g$^{\#}$	8.1±1.2g	10.5±1.3g	11.4±1.0g
Maternal no treatment	5.2±0.5g	6.2±0.8g	8.2±1.1g	8.3±0.7g	9.7±1.5g	11.3±1.2g

# $p<0.05$ different from P2 neonates after maternal no treatment; Two-way ANOVA.

Maternal opioids age-dependently impair neonatal central respiratory control networks.

To determine the impact of maternal opioids on isolated central respiratory networks, we recorded respiratory-related motor activity from isolated brainstem-spinal cords (BSSCs) containing the neonatal respiratory control network. While maternal treatment and age had no main effect on baseline fictive respiratory burst frequencies ($p>0.05$), the interaction between maternal treatment and age had a significant main effect on burst frequency (p=0.0085). Pair-wise comparisons showed reduced baseline burst frequencies in P0-1 neonates after maternal opioids

(8.3±1.2 burst/min) compared to P0-1 neonates after maternal no treatment (12.2±1.8 burst/min, p=0.03; **Fig. 1A & B**), demonstrating maternal opioids impair central respiratory activity in neonates immediately after birth. These central impairments after maternal opioids were age-dependent, as baseline respiratory burst frequencies were similar in older neonates after maternal opioids (P2 neonates after maternal opioids=10.8±1.8 burst/min; P3-5 neonates after maternal opioids=11.8±1.6 burst/min) compared to age-matched neonates after maternal no treatment (P2 neonates after maternal no treatment=10.5±1.3 burst/min; P3-5 neonates after maternal no treatment=10.5±2.2 burst/min, p>0.9; **Fig. 1B**).

Maternal opioids also significantly impacted burst frequency changes over time. Group analysis demonstrated significant main effects of maternal treatment (p=0.001), time (p=0.0003) and the interaction between treatment and time (p=0.001). No significant main effects were evident on burst frequencies over time with age (p=0.9) or the interactions between maternal treatment and age (p=0.9), age and time (p=0.36), or maternal treatment, age and time (p=0.07). Pairwise comparisons demonstrated burst frequencies decreased from baseline in P2 neonates maternal no treatment (-20±5% change from baseline, p=0.03), while P0-1 neonates after maternal no treatment (-15%±10% change from baseline, p>0.9) and P3-5 neonates after maternal no treatment (-12±9% change from baseline, p>0.9) were unchanged from baseline (**Fig. 1C**). In contrast, burst frequencies decreased from baseline in all neonates after maternal opioids (P0-1 neonates after maternal opioids=-55%±34% change from baseline, p=0.01; P2 neonates after maternal opioids=-44±12% change from baseline, p=0.02; P3-5 neonates after maternal opioids=-26±15% change

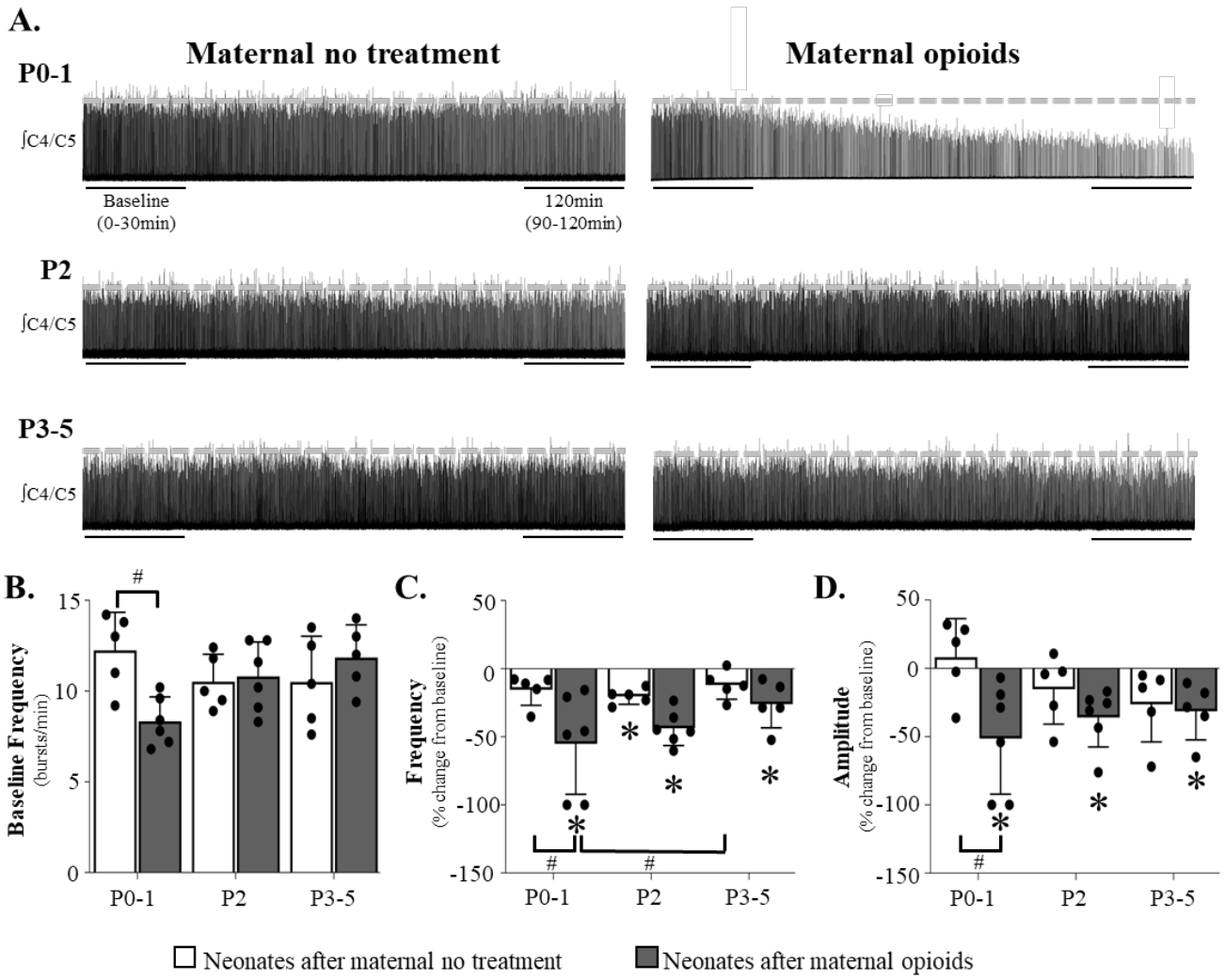


Figure 1. Maternal opioids age-dependently impair neural activity from isolated neonatal respiratory control networks. Representative neurograms (**A.**) from isolated respiratory control networks show fictive respiratory activity in neonatal brainstem-spinal cords were maintained in neonates after maternal no treatment at all ages and decreased in P0-1 neonates after maternal opioids. Baseline fictive respiratory burst frequencies (0-30min, **B.**) were similar in neonates after maternal no treatment (white bars) at all ages, reduced in P0-1 neonates after maternal opioids (grey bars), and unchanged in older (P2 and P3-5) neonates after maternal opioids. Fictive respiratory burst frequencies (**C.**) decreased from baseline in P2 neonates after maternal no treatment and in neonates after maternal opioids at all ages. Fictive respiratory burst frequency decreased in P0-1 neonates after maternal opioids compared to P0-1 and P3-5 neonates after maternal no treatment. Fictive respiratory burst amplitudes (**D.**) were maintained in all neonates after maternal no treatment and decreased from baseline in all neonates after maternal opioids. Fictive respiratory burst amplitudes decreased in P0-1 neonates after maternal opioids compared to P0-1 neonates after maternal no treatment. (* $p<0.05$ different from baseline within treatment; # $p<0.05$ different from neonates after maternal no treatment; Two- and Three-way ANOVA; Bonferroni post-hoc test.)

from baseline, p=0.03). Between maternal treatment groups, burst frequencies decreased in P0-1 neonates after maternal opioids compared to P0-1 neonates after maternal no treatment (p=0.04) and P3-5 neonates after maternal no treatment (p=0.02), but were not different from P2 neonates after maternal no treatment (p=0.2) or older (P2 and P3-5) neonates after maternal opioids (p>0.9; **Fig. 1C**). In contrast, respiratory burst frequencies were similar in older neonates (P2 or P3-5) after maternal opioids compared to all neonates after maternal no treatment (p>0.9). Thus, maternal

opioids impair neonatal respiratory motor output, demonstrating central respiratory networks within the brainstem and spinal cord are impaired in neonates after maternal opioids.

Similar to changes in frequency, neonatal burst amplitude changes over time were affected by maternal opioids (**Fig. 1D**). Maternal treatment ($p=0.0092$) and time ($p=0.0001$) and the interaction between maternal treatment and time ($p=0.003$) had a significant main effect on burst amplitudes over time. Age ($p=0.9$) or the interactions between maternal treatment and age ($p=0.9$), age and time ($p=0.85$), or maternal treatment, age and time ($p=0.056$) had no significant main effect. After maternal no treatment, burst amplitudes were unchanged from baseline in all neonates after maternal no treatment (P0-1 neonates after maternal no treatment=8%±25% change from baseline, $p=0.9$; P2 neonates after maternal no treatment=-15±23% change from baseline, $p=0.3$; P3-5 neonates after maternal no treatment=-26±25% change from baseline, $p=0.51$). However, burst amplitudes decreased from baseline in all neonates after maternal opioids (P0-1 neonates after maternal opioids=-51%±37% change from baseline, $p=0.009$; P2 neonates after maternal opioids=-36±20% change from baseline, $p=0.04$; P3-5 neonates after maternal no treatment=-31±19% change from baseline, $p=0.048$). Thus, maternal opioids impair respiratory activity over time, which further support central impairments in neonatal respiratory networks after maternal opioids, while highlighting a potential role for deficits in respiratory pattern generating nuclei.

Pairwise comparisons between groups showed burst amplitudes decreased in P0-1 neonates after maternal opioids compared to P0-1 neonates after maternal no treatment ($p=0.0287$), but burst amplitudes were not different from older (P2 and P3-5) neonates after either maternal treatment ($p>0.9$; **Fig. 1D**). Decreased burst amplitudes were age-dependent, as respiratory burst amplitudes were similar in older neonates (P2 or P3-5) after maternal opioids compared to age-matched neonates after maternal no treatment ($p>0.9$). Thus, maternal opioids age-dependently impair

neonatal central respiratory network activity, similar to destabilized breathing in neonates after maternal opioids (Hocker, Morrison et al., 2021).

Lingering neonatal opioids contribute to impaired central respiratory network activity in neonates after maternal opioids.

To investigate the contribution of continued opioid receptor activation to decreasing central respiratory activity in neonates after maternal opioids, we examined respiratory activity from isolated neonatal BSSCs in the presence of a mu-opioid receptor antagonist (naloxone, 10μM). Group data demonstrated no main effects of any factor (maternal treatment, age, time or the interactions between the three factors) on burst frequency over time after mu-opioid receptor antagonism ($p>0.9$) and no pairwise differences in any maternal treatment or age ($p>0.9$; **Fig. 2A & B**). Thus, reduced respiratory burst frequencies in P0-1 neonates after maternal opioids are unlikely to be due to continued neonatal opioid receptor activation.

While opioid receptor antagonism did not significantly impact burst frequencies, it had a significant effect on burst amplitudes. Maternal treatment ($p=0.0001$), age ($p=0.0001$), time ($p=0.01$), and interactions between treatment and age ($p=0.0001$), maternal treatment and time ($p=0.0001$), and maternal treatment, age and time ($p=0.001$) had significant main effects on burst amplitude. In neonates after maternal no treatment, burst amplitudes decreased from baseline after mu-opioid receptor antagonism in P2 neonates after maternal no treatment (-17%±5% change from baseline, $p=0.04$), while P0-1 neonates after maternal no treatment (-14%±4% change from baseline, $p=0.06$) and P3-5 neonates after maternal no treatment (-12%±4% change from baseline, $p=0.7$) were unchanged from baseline (**Fig. 2C**). In contrast, burst amplitudes after mu-opioid

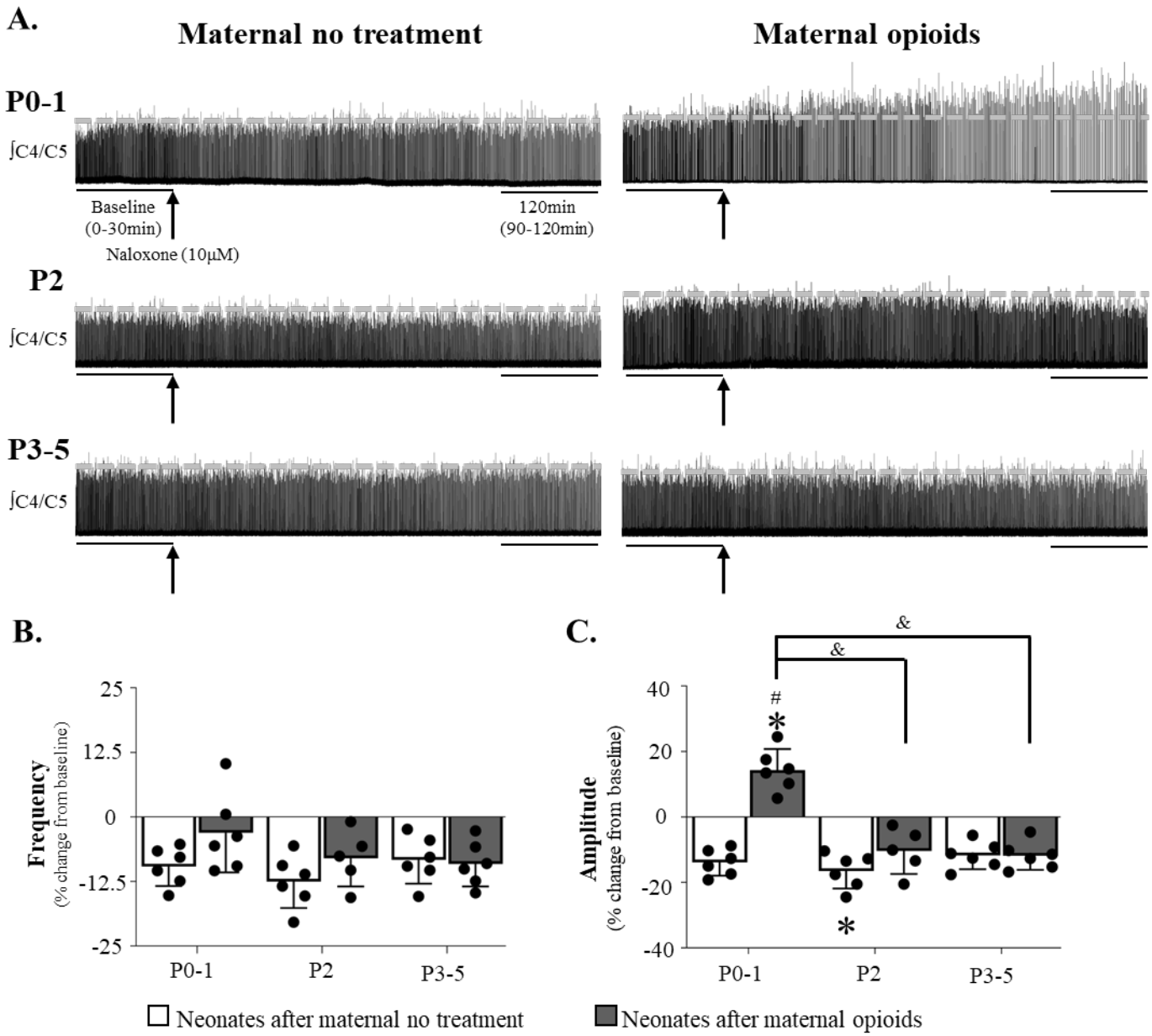


Figure 2. Mu-opioid receptor antagonism increased fictive respiratory burst amplitude, but not frequency, in neonates after maternal opioids. Respiratory neurograms (**A**.) show fictive respiratory activity in isolated neonatal brainstem-spinal cords were unchanged after mu-opioid receptor antagonism (arrow, naloxone, 10μM, bath application) in neonates after maternal no treatment at any age and increased in P0-1 neonates after maternal opioids. Fictive respiratory burst frequencies (**B.**) were unchanged from baseline after mu-opioid receptor antagonism in any maternal treatment or age. Fictive respiratory burst amplitudes (**C.**) decreased from baseline in P2 neonates after maternal no treatment and increased from baseline in P0-1 neonates after maternal opioids. Fictive respiratory burst amplitude increased in P0-1 neonates after maternal opioids compared to all neonates after maternal no treatment (white bars) and older (P2 and P3-5) neonates after maternal opioids (gray bars). (* $p<0.05$ different from baseline within treatment; # $p<0.05$ different from neonates after maternal no treatment at all ages; & $p<0.05$ different from neonates after maternal opioids; Three-way ANOVAs; Bonferroni post-hoc test.)

receptor antagonism increased from baseline in P0-1 neonates after maternal opioids (14%±6% change from baseline, p=0.03), while burst amplitude from older neonates after maternal opioids were unchanged (P2 neonates after maternal opioids=-10%±6% change from baseline, p=0.09; P3-5 neonates after maternal opioids=-12%±4% change from baseline, p=0.07). Thus, lingering

neonatal opioids may contribute to acute changes in burst amplitude, but not frequency, in neonates after maternal opioids.

Pairwise comparisons between groups demonstrated burst amplitudes increased over time after mu-opioid receptor antagonism in P0-1 neonates after maternal opioids compared to all neonates after maternal no treatment (P0-1: $p=0.0001$; P2: $p=0.0002$; P3-5: $p=0.0001$) and older (P2 and P3-5) neonates after maternal opioids (P2: $p=0.0009$; P3-5: $p=0.0004$; **Fig. 2C**). The effect of mu-opioid receptor antagonism on central respiratory activity in neonates after maternal opioids was age-dependent, as burst amplitudes were similar in older (P2 and P3-5) neonates after maternal opioids compared to all neonates after maternal no treatment ($p>0.9$). These acute increases in amplitude in response to opioid receptor antagonism suggest lingering opioids impact central nuclei generating or modulating respiratory pattern in neonates after maternal opioids.

Neonates after maternal opioids had blunted central respiratory responses to exogenous opioids immediately after birth.

To determine the contribution of opioid receptors to central respiratory responses to exogenous opioids after maternal opioids, we recorded respiratory activity in isolated BSSCs after mu-opioid receptor agonism (methadone, 10μM). Maternal opioids caused inter-preparation variability in the acute responses to exogenous opioids. Respiratory activity was maintained after acute opioids in younger neonates after maternal opioids (P0-1: 5/6 experiments; P2: 3/6 experiments), while activity was not maintained in older neonates after maternal opioids (P3-5: 0/4 experiments) nor neonates after maternal no treatment (P0-1: 0/6 experiments; P2: 0/5 experiments; P3-5: 1/6 experiments).

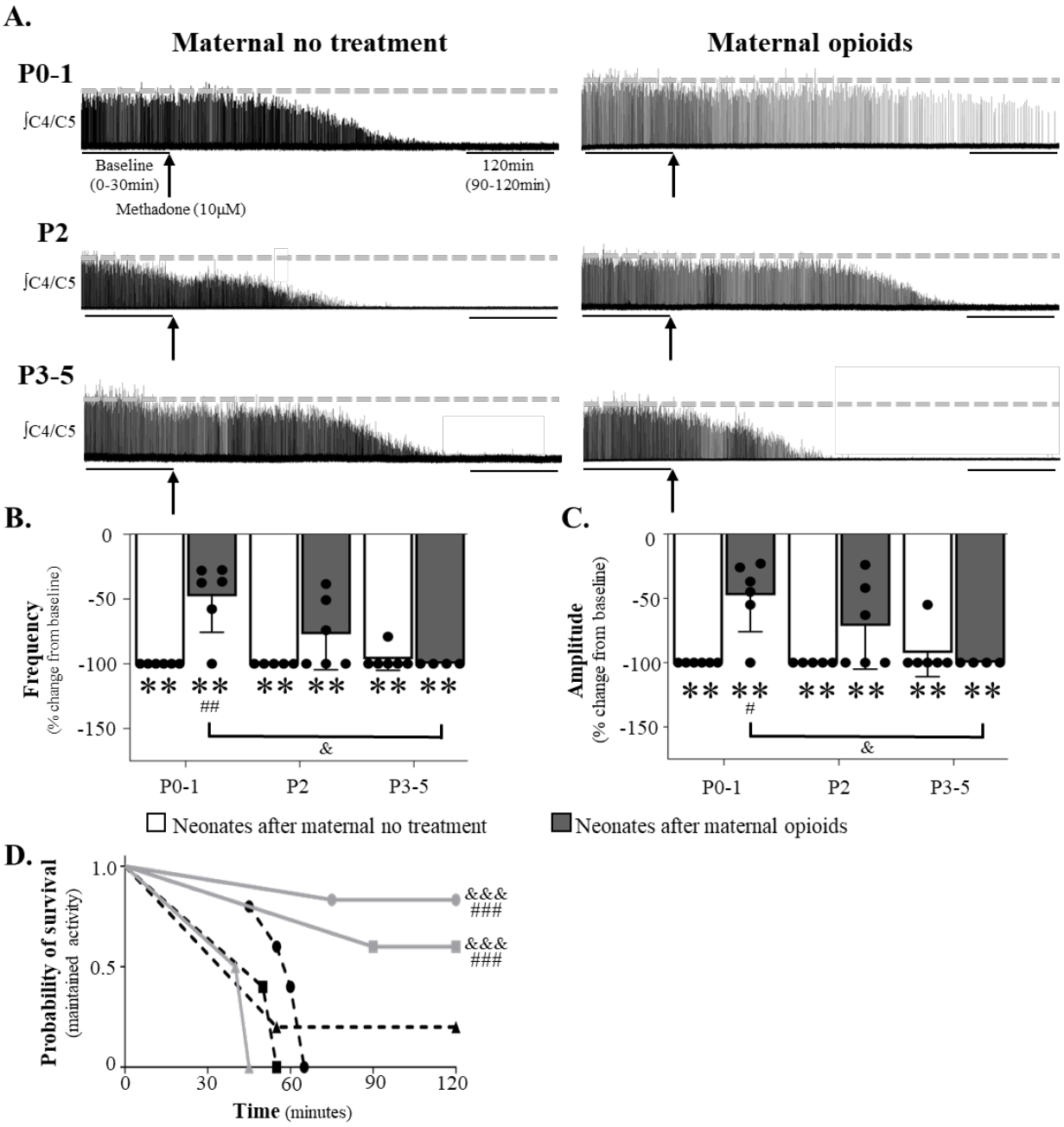


Figure 3. Neonates after maternal opioids age-dependently maintained respiratory activity after acute opioids. Representative neurograms from isolated brainstem-spinal cords (**A.**) show fictive respiratory activity was abolished with acute opioids (arrow, methadone, 10μM, bath application) in neonates after maternal no treatment at all ages, maintained in P0-1 neonates after maternal opioids, and abolished in older neonates (P2 or P3-5) after maternal opioids. Fictive respiratory burst frequencies (**B.**) and burst amplitudes (**C.**) were abolished in neonates after maternal no treatment at all ages and decreased from baseline in neonates after maternal opioids at all ages. Fictive respiratory burst frequencies and amplitudes were maintained in P0-1 neonates after maternal opioids compared to neonates after maternal no treatment at all ages and older neonates (P3-5) after maternal opioids, but not P2 neonates after maternal opioids. The probability of maintaining respiratory activity after acute opioids (**D.**) was greater in P0-1 (circles) and P2 (squares) neonates after maternal opioids (gray lines) than older neonates P3-5 (triangles) after maternal opioids and neonates after maternal no treatment (dotted lines) at all ages. (** $p<0.001$ different from baseline within treatment; # $p<0.05$, ## $p<0.001$, ### $p<0.0001$ different from neonates after maternal no treatment at all ages; & $p<0.05$, &&& $p<0.0001$ different from neonates after maternal opioids at P3-5; Three-way ANOVAs; Bonferroni post-hoc test.)

There was a main effect of maternal treatment (p=0.0005), age (p=0.0098), time (p=0.002), and the interactions between maternal treatment and age (p=0.0035), maternal treatment and time (p=0.009), age and time (p=0.006), and maternal treatment, age, and time (p=0.002) on respiratory burst frequency after mu-opioid receptor agonism. As expected, exogenous opioids abolished burst frequency in neonates after maternal no treatment at all ages (P0-1 neonates after maternal no treatment=-100%±0% change from baseline, p=0.001; P2 neonates after maternal no treatment=-100%±0% change from baseline, p=0.001; P3-5 neonates after maternal no treatment=-97%±7% change from baseline, p=0.001; **Fig. 3A & B**). In neonates after maternal opioids, burst frequencies decreased from baseline, but were not abolished, after exogenous opioids in P0-1 and P2 neonates (P0-1 neonates after maternal opioids=-48%±26% change from baseline, p=0.006; P2 neonates after maternal opioids=-77%±25% change from baseline, p=0.002). Activity was abolished in older neonates after acute opioids (P3-5 neonates after maternal opioids=-100%±0% change from baseline; p=0.001; **Fig. 3B**). Importantly, pairwise comparisons between groups showed burst frequencies were maintained in P0-1 neonates after maternal opioids compared to all neonates after maternal no treatment ($p<0.05$) and P3-5 neonates after maternal opioids (p=0.03), but were similar to P2 neonates after maternal opioids (p=0.8; **Fig. 3B)**. Thus, maternal opioids blunt central respiratory responses to exogenous opioids. Blunted central responses to exogenous opioids are age-dependent, as decreases in burst frequency in older neonates after maternal opioids (P2 and P3-5) were not significantly different from neonates after maternal no treatment at any age (p=0.9; **Fig. 3B)**.

Similar to changes in burst frequency, burst amplitude was age-dependently blunted by exogenous opioids (**Fig. 3A & C)**. Maternal treatment (p=0.002), time (p=0.0001), and the interactions between maternal treatment and time (p=0.002), maternal treatment and age

(p=0.008), and maternal treatment, age, and time (p=0.008) had significant main effects on burst amplitude after mu-opioid receptor agonism, with no main effect of age (p=0.055) or the interaction between age and time (p=0.96). As expected, exogenous opioids abolished burst amplitude in neonates after maternal no treatment at all ages (P0-1 neonates after maternal no treatment=-100%±0% change from baseline, p=0.0001; P2 neonates after maternal no treatment=-100%±0% change from baseline, p=0.0001; P3-5 neonates after maternal no treatment=-93%±16% change from baseline, p=0.0002; **Fig. 3C**). Similarly, burst amplitude decreased from baseline after exogenous opioids in all neonates after maternal opioids (P0-1 neonates after maternal opioids=-46%±26% change from baseline, p=0.005; P2 neonates after opioids=-72%±31% change from baseline, p=0.003; P3-5 neonates after opioids=-93%±16% change from baseline, p=0.0002; **Fig. 3C)**. Pairwise comparisons between groups demonstrated burst amplitudes were maintained after exogenous opioids in P0-1 neonates after maternal opioids compared to neonates after maternal no treatment at any age ($p<0.05$) and P3-5 neonates after maternal opioids (p=0.01), but were similar to P2 neonates after maternal opioids (p=0.9; **Fig. 3C)**. Blunted central responses to exogenous opioids were age-dependent, as exogenous opioids abolished burst amplitude in older (P2 and P3-5) neonates after maternal opioids, similar to neonates after maternal no treatment at all ages (p=0.9; **Fig. 3C)**. Thus, respiratory activity (frequency and amplitude) after exogenous opioids was age-dependently maintained in neonates after maternal opioids, supporting acute protection from significant opioid-induced respiratory depression immediately after birth (Hocker, Morrison et al., 2021).

Since changes in activity over time failed to capture the dynamic changes in activity in response to acute opioids, we assessed the probability of maintaining activity using survival curves. The probability of maintaining respiratory activity after mu-opioid receptor agonism was

significantly higher in P0-1 and P2 neonates after maternal opioids compared to neonates after maternal no treatment at any age ($p<0.001$) and P3-5 neonates after maternal opioids ($p<0.001$; **Fig. 3D**). Thus, central respiratory networks from P0-1 and P2 neonates after maternal opioids are less responsive to exogenous opioids, likely contributing to blunted opioid-induced respiratory depression in neonates after maternal opioids (Hocker, Morrison et al., 2021).

Mu-opioid receptor immunoreactivity in the preBötzinger Complex qualitatively decreased during postnatal development.

Results from opioid receptor antagonist and agonist experiments support a role for opioid receptors in changing respiratory activity in neonates after maternal opioids, highlighting the need to understand opioid receptor expression in neonatal central respiratory control networks. Further, decreased burst frequency in P0-1 neonates after maternal opioids also suggests the preBötzinger Complex (preBötC) may contribute to central impairments in respiratory networks. Thus, we investigated the effect of maternal opioids on mu-opioid receptor expression in an essential respiratory rhythm generating center, the preBötC. First, we characterized typical mu-opioid receptor expression in the preBötC during postnatal development (P0, P4, P11). Using immunohistochemistry, mu-opioid receptors and neurokinin-1 receptor positive (NK1R+) neurons were labeled in the ventral lateral medulla. Using anatomical landmarks, the preBötC was identified as a fusiform cluster of neurons ventral and lateral from nucleus ambiguous (**Fig. 4A & B**), based on previous work (Gray et al., 1999; Huxtable et al., 2010; Pagliardini et al., 2003). During typical postnatal development, mu-opioid receptor immunoreactivity appeared highest immediately after birth (P0) and decreased during postnatal development (**Fig. 4C**). Additionally, mu-opioid receptor immunoreactivity on NK1R+ preBötC neurons at P4 appeared greater than in

P11 neonates (**Fig. 4C**), supporting a progressive decrease in mu-opioid receptors during typical postnatal development.

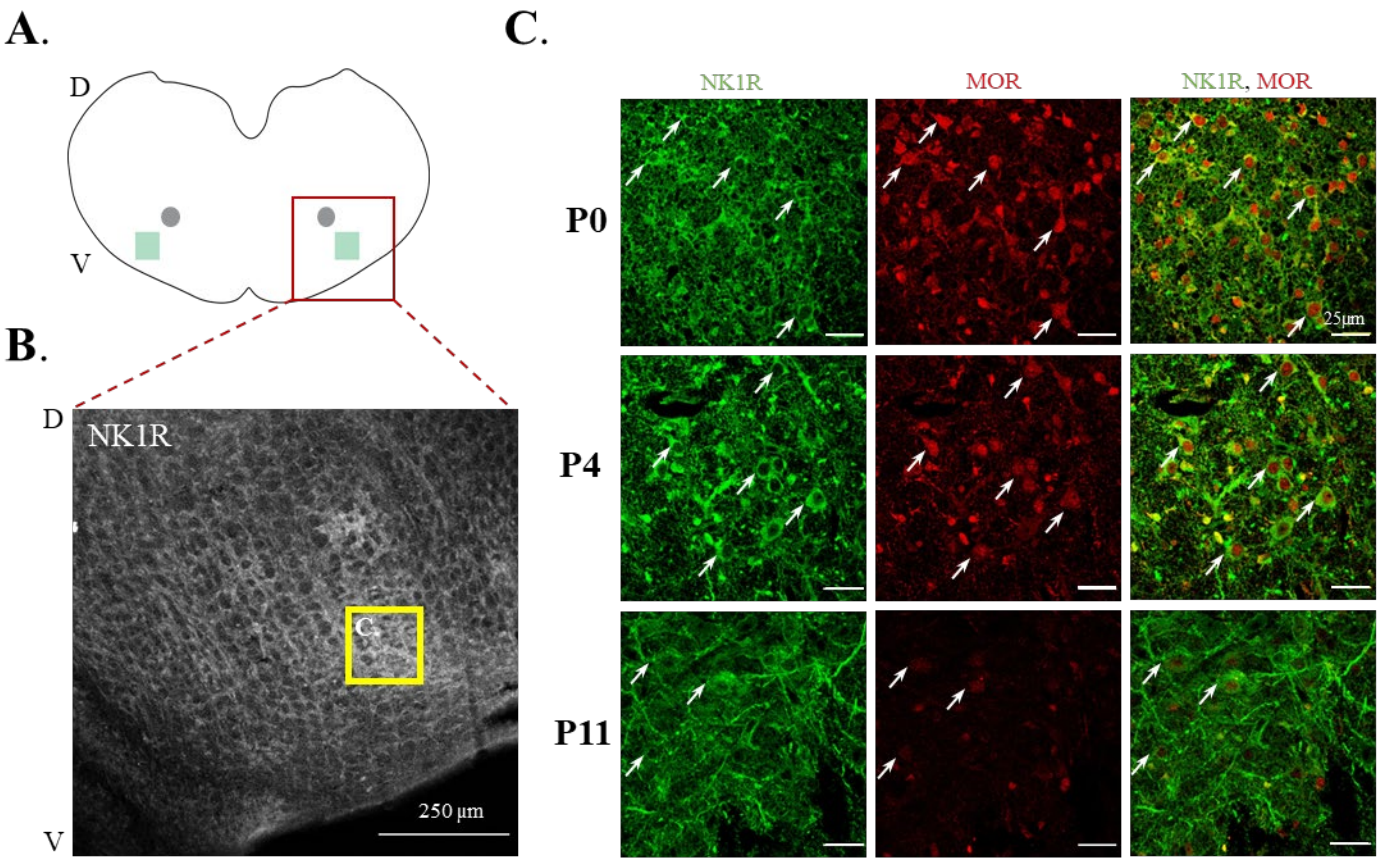


Figure 4. Mu-opioid receptor expression decreased in neonatal preBötzinger complex NK1R+ neurons during postnatal development. Schematic of transverse medullary slices (**A.**) highlights the preBötzinger Complex (preBötC, green square) located lateral and ventral to the semi-compact nucleus ambiguous (gray circle). Representative confocal images (**B.**, 10x) identify neurokinin-1 receptor positive (NK1R+) neurons in the preBötC (yellow box). Representative confocal images (**C.**, 40x) demonstrate NK1R+ preBötC neurons (green) express mu-opioid receptors (MOR) at all ages; however, P0 neonates appear to have the strongest expression of mu-opioid receptors in NK1R+ preBötC neurons (white arrows) compared to P4 neonates and mu-opioid receptor appeared to have the lowest expression in P11 neonates (n=6, 3 males, 3 females per age group).

Maternal opioids qualitatively decreased opioid receptor expression on neonatal preBötzinger Complex neurons.

After establishing the typical expression of mu-opioid receptors in the preBötC during postnatal development, we used immunohistochemistry on a separate group of neonates to determine the effect of maternal opioids on mu-opioid receptors in the neonatal preBötC. Male

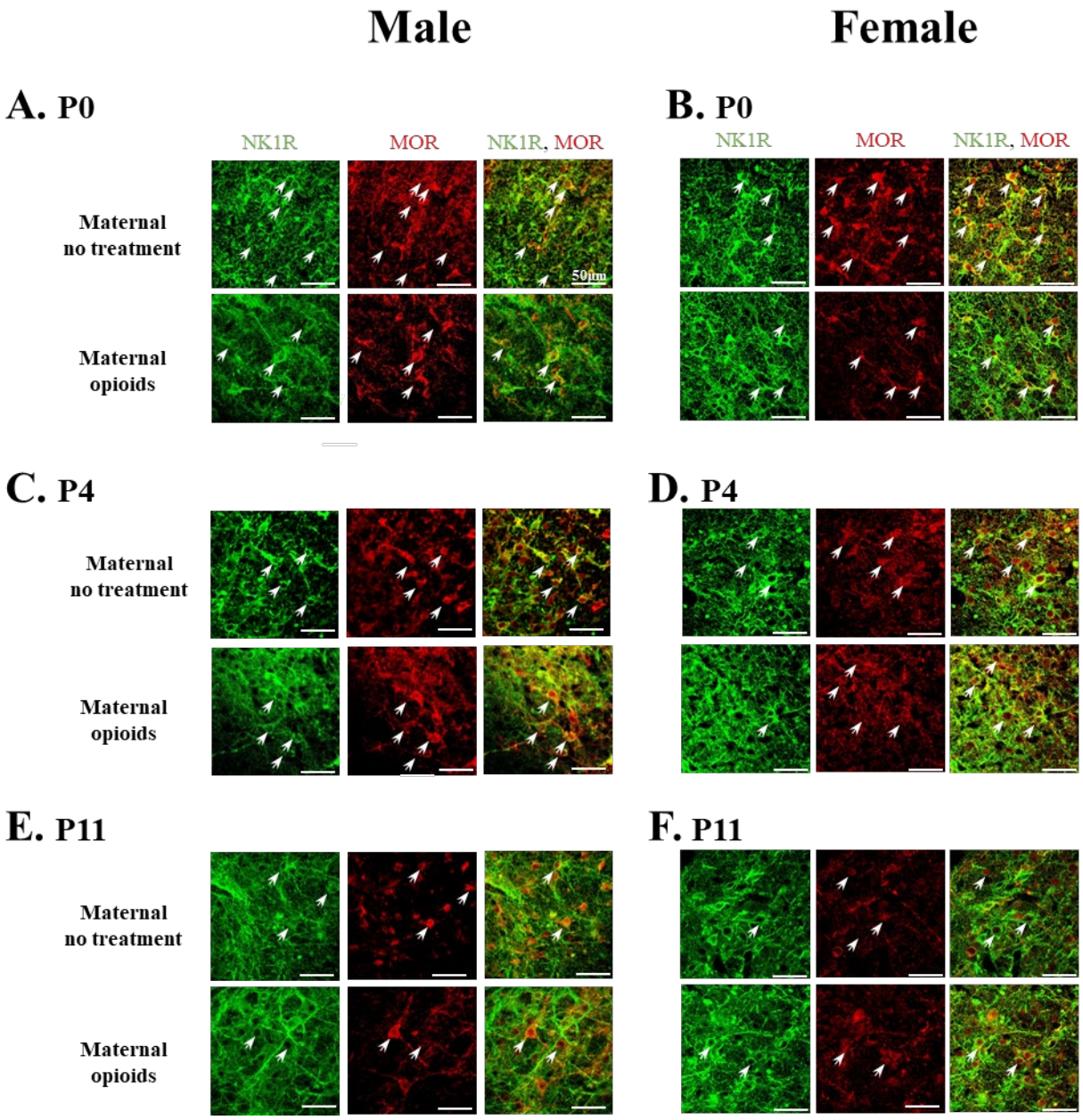


Figure 5. Mu-opioid receptor expression on preBötzinger Complex NK1R$^+$ neurons decreased in P0 neonates after maternal opioids, but not at P4 or P11. Representative confocal images (40x) show NK1R$^+$ preBötzinger Complex neurons (green) and mu-opioid receptors (red) colocalization (white arrows) decreased in P0 male (**A.**) and female (**B.**) neonates after maternal opioids compared to P0 sex-matched neonates after maternal no treatment. Mu-opioid receptors were similar in P4 male (**C.**) and female (**D.**) neonates after maternal opioids and P11 male (**E.**) and female (**F.**) neonates after maternal opioids compared to age- and sex-matched neonates after maternal no treatment. (n=3 per treatment, age and sex; scale bars 50µm.)

and female P0 neonates after maternal opioids appeared to have less mu-opioid receptor immunoreactivity in NK1R+ neurons compared to P0 neonates after maternal no treatment (**Fig.**

5A & B). However, male and female P4 and P11 neonates after maternal opioids had similar immunoreactivity to age-matched neonates after maternal no treatment (**Fig. 5C-F**). The immunohistochemistry results are consistent with changes in respiratory activity and further support similarities between the sexes after maternal opioids. Thus, opioid receptor expression decreases with typical postnatal development and decreases immediately after birth following maternal opioids.

Respiratory activity in neonatal medullary rhythmic slices were unaffected by maternal opioids.

To identify where within the brainstem-spinal cord networks maternal opioids impair respiratory activity, we utilized a further reduced preparation, consisting of the preBötC, hypoglossal motor nucleus, and hypoglossal nerve roots (Greer et al., 1991; Smith et al., 1991). In contrast to respiratory activity from BSSCs, maternal treatment, age or the interaction between maternal treatment and age did not have a significant main effect on baseline respiratory burst frequencies ($p>0.9$). Baseline burst frequencies were similar regardless of maternal treatment or age (P0-1 neonates after opioids=21±2 burst/min; P2 neonates after opioids=19±2 burst/min; P3-5 neonates after opioids=17±5 burst/min; P0-1 neonates after maternal no treatment=21±1 burst/min; P2 neonates after maternal no treatment=21±3 burst/min; P3-5 neonates after maternal no treatment=19±3 burst/min, $p>0.1$; **Fig. 6A & B**).

Maternal treatment also did not change burst frequency or amplitude over time. Neither maternal treatment, age, time, nor the interactions between the three factors had a significant main effect on respiratory burst frequencies ($p>0.9$). No pairwise differences exist in isolated medullary burst frequencies (P0-1 neonates after maternal no treatment=-11%±9% change from baseline, $p=0.3$; P2 neonates after maternal no treatment=-18%±7% change from baseline, $p=0.08$; P3-5

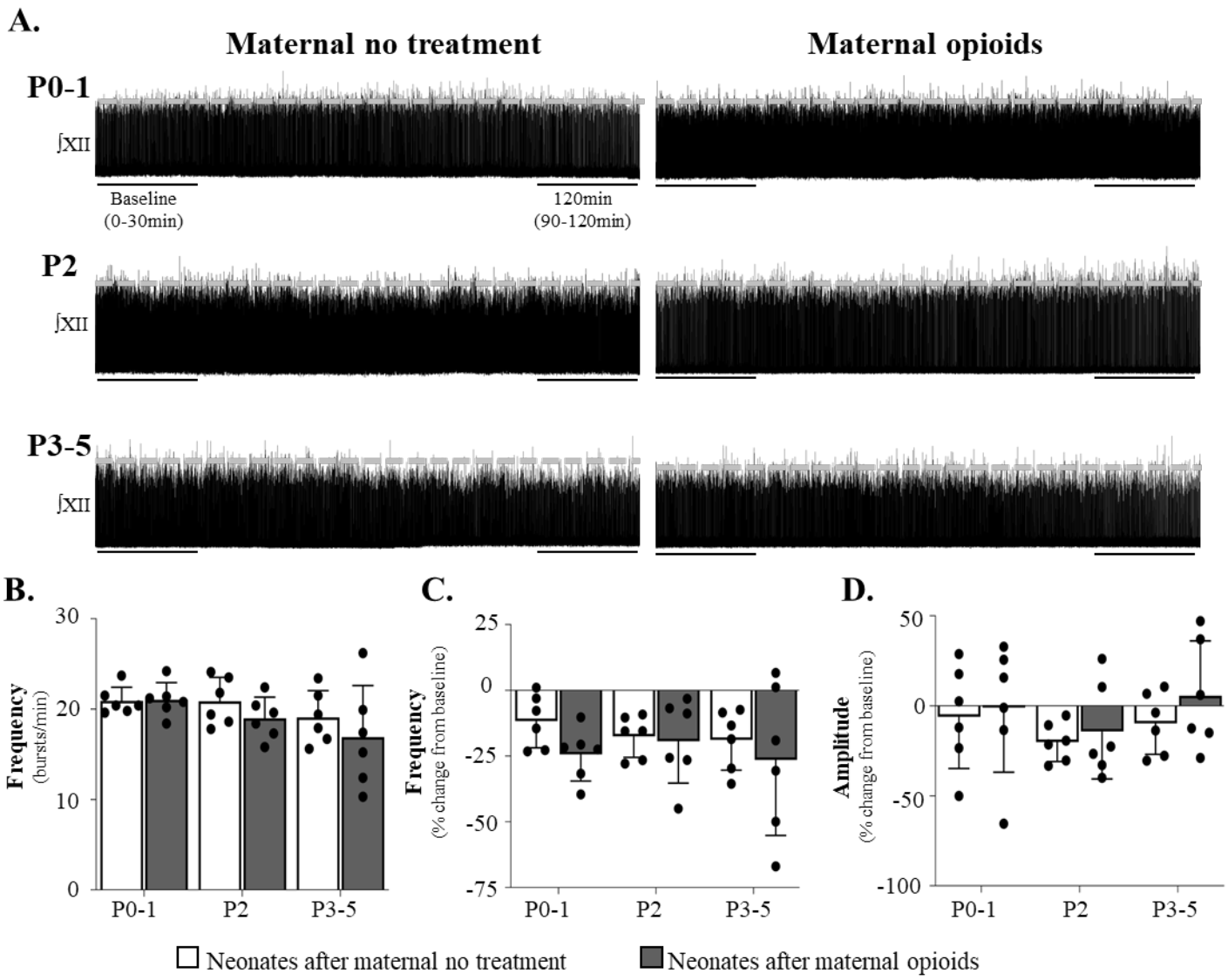


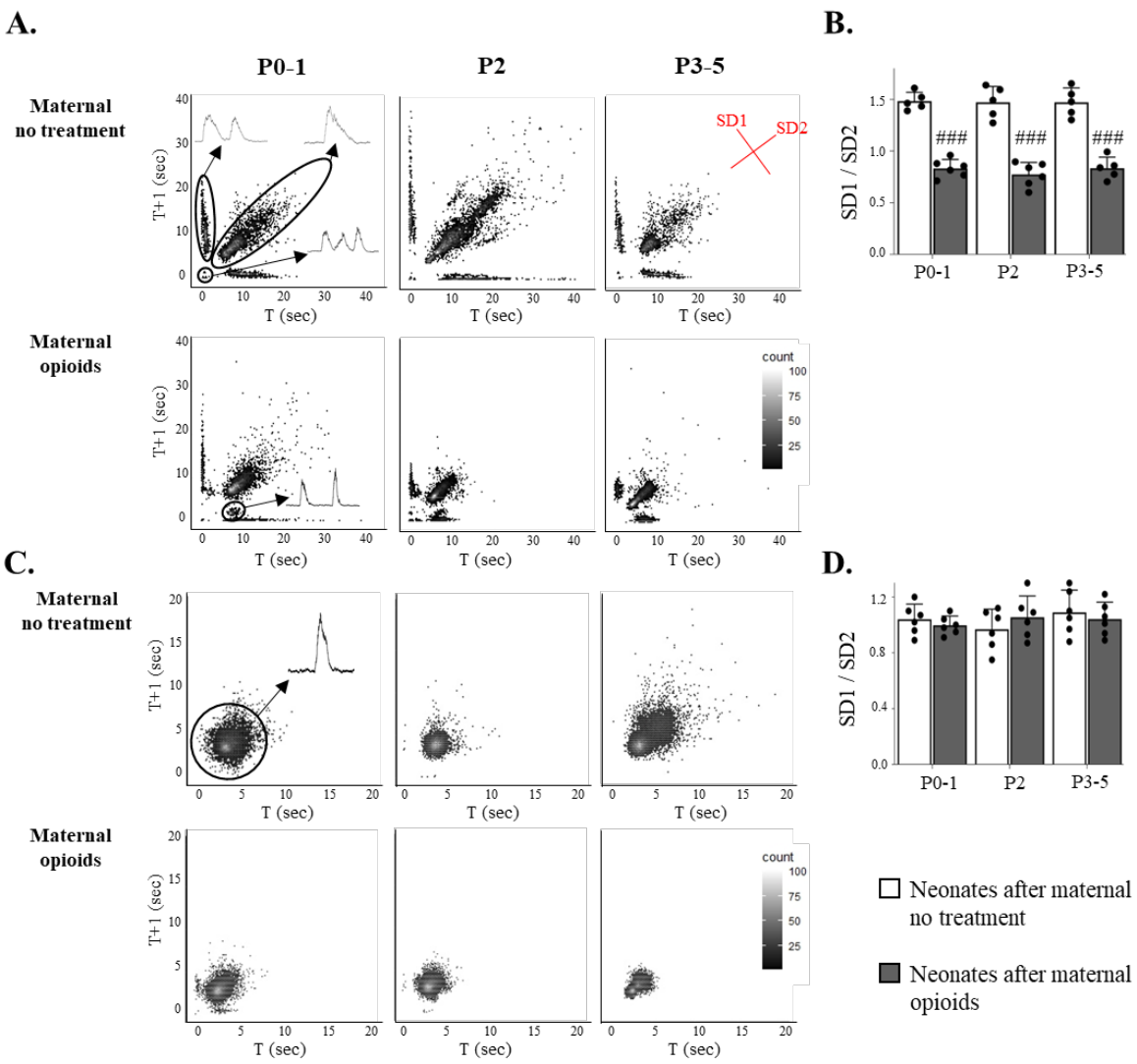
Figure 6. Isolated respiratory network activity was maintained in rhythmically active slices containing the preBötzinger Complex in neonates after maternal opioids. Representative neurograms (**A.**) show fictive respiratory activity from isolated rhythmic slices was maintained in neonates after maternal opioids at all ages. Baseline neonatal fictive respiratory burst frequencies (**B.**) were similar in neonates, regardless of maternal treatment or age. Over time, isolated rhythmic slice fictive respiratory burst frequencies (**C.**) and amplitudes (**D.**) were unchanged from baseline and similar between maternal treatments and ages. (Two- and Three-way ANOVAs; Bonferroni post-hoc test.)

neonates after maternal no treatment=-19%±11% change from baseline, p=0.07; P0-1 neonates after maternal opioids=-24%±9% change from baseline, p=0.07; P2 neonates after maternal opioids=-19%±15% change from baseline, p=0.08; P3-5 neonates after maternal opioids=-26%±26% change from baseline, p=0.07; **Fig. 6C**).

Similarly, neither maternal treatment, age, time, or interactions between the three factors had significant main effects on neonatal burst amplitudes ($p>0.9$), with no pairwise differences (P0-1 neonates after opioids=-1±32% change from baseline, $p=0.9$; P2 neonates after opioids=-14%±24% change from baseline, $p=0.09$; P3-5 neonates after opioids=6%±28% change from baseline $p=0.09$; P0-1 neonates after maternal no treatment=-6%±26% change from baseline, $p=0.8$; P2 neonates after maternal no treatment=-20%±10% change from baseline, $p=0.07$; P3-5 neonates after maternal no treatment=-10%±16% change from baseline, $p=0.1$; **Fig. 6D**). In summary, these data suggest central impairments in respiratory networks in P0-1 neonates after maternal opioids extend beyond the preBötC.

Maternal opioids impair neonatal respiratory pattern in isolated brainstem-spinal cords, but not medullary rhythmic slices, at any age.

Assessments of frequency and amplitude failed to capture intricate changes in respiratory burst pattern. Thus, to better describe the effects of maternal opioids on neonatal respiratory burst pattern, Poincaré plots were generated from neonatal respiratory activity in isolated brainstem-spinal cords (BSSCs) and medullary rhythmic slices. In isolated BSSCs, neonates after maternal no treatment displayed three respiratory burst patterns: singlets, doublets, and triplets (**Fig. 7A**, **top panels**). However, neonates after maternal opioids exhibited a unique respiratory pattern, a singlet burst with a decreased period to the next burst (**Fig. 7A, bottom panels**). When quantifying overall burst-to-burst variability with SD1/SD2, there was a main effect of maternal treatment ($p=0.0001$), but not age ($p=0.9$) or the interaction between maternal treatment and age ($p=0.9$), in respiratory activity from isolated BSSCs. Burst-to-burst variation in respiratory activity from isolated BSSCs decreased in all neonates after maternal opioids (SD1/SD2: P0-1 neonates after



opioids=0.78±0.12; P2 neonates after opioids=0.8±0.13; P3-5 neonates after opioids=0.82±0.1) compared to all neonates after maternal no treatment

Figure 7. A distinct respiratory pattern emerged in neonatal isolated brainstem-spinal cords, but not rhythmic slices, after maternal opioids. Poincaré plots (**A.**) show burst-to-burst variation in isolated brainstem-spinal cords (BSSCs). Three respiratory burst patterns were prominent after maternal no treatment: singlets, doublets, and triplets (inset). In neonates after maternal opioids, a distinct respiratory burst pattern emerged. SD1/SD2 measurements of burst-to-burst variation in isolated BSSCs decreased in neonates after maternal opioids compared to neonates after maternal no treatment (**B.**). In rhythmically active slices, singlet burst activity was prominent (inset) in all treatment groups and ages (**C.**) and burst-to-burst variation (**D.**) was unchanged in any treatment group or age. (### p<0.0001 different from neonates maternal no treatment at all ages; Two-way ANOVAs; Bonferroni post-hoc test.)

(SD1/SD2: P0-1 neonates after maternal no treatment=1.5±0.12; P2 neonates after maternal no treatment=1.4±0.18; P3-5 neonates after maternal no treatment=1.6±0.13, p<0.0001; **Fig. 7B**).

In contrast to respiratory activity from isolated BSSCs, rhythmic slices exhibited regular singlet patterns (**Fig. 7C**). SD1/SD2 burst-to-burst variation of respiratory activity in medullary rhythmic slices was unchanged in all neonates after maternal opioids compared to all neonates after maternal no treatment ($p>0.9$; **Fig. 7D**). Maternal treatment, age or the interaction between maternal treatment and age ($p>0.9$) had no a main effect on burst-to-burst variation from medullary rhythmically active slices and there were no pairwise differences between maternal treatment or age (SD1/SD2: P0-1 neonates after opioids=1±0.1; P2 neonates after opioids=0.96±0.13; P3-5 neonates after opioids=1.1±0.14; P0-1 neonates after maternal no treatment=1±0.06; P2 neonates after maternal no treatment=1.1±0.14; P3-5 neonates after maternal no treatment=1±0.11, $p>0.9$; **Fig. 7D)**. Thus, a diverse pattern of activity appears with more complete respiratory network circuitry and maternal opioids evoked a distinct pattern of a single burst with a decreased period to the next burst, not evident in more isolated respiratory networks, supporting maternal opioids may be impairing respiratory circuity beyond the preBötC networks.

Discussion

A clinical population of infants with NAS is continuing to grow (Krans, et al. 2015; Pryor et al. 2017) and these infants experience respiratory deficits (Fältmarch et al., 2019), highlighting a need to understand the etiology of breathing deficits in these infants. Here, we capitalize on our previous characterizations of neonatal breathing deficits after late gestational maternal opioids (Hocker, Morrison et al. 2021) and target central deficits in neonates after maternal opioids. Since breathing is controlled centrally by neural networks in the brainstem and spinal cord (reviewed in Del Negro et al., 2018), we began by studying the neural impairments in progressively more isolated central respiratory networks. We demonstrate maternal opioids age-dependently impair central respiratory networks; however, the deficits include regions beyond the preBötzinger Complex (preBötC), specifically those associated with respiratory pattern. Lingering opioids in neonatal central respiratory networks contribute to respiratory amplitude deficits immediately after birth, but not frequency. Since opioids likely remain in the neonatal brain for multiple days after maternal opioid exposure (Farid et al. 2008; Kongstorp et al., 2019), lingering neonatal opioids are likely of maternal origin. Yet, lingering opioids do not account for decreased respiratory frequency or blunted opioid-induced respiratory depression immediately after birth. Blunted respiratory responses to acute, exogenous opioids may, in part, be explained by decreased mu-opioid receptors in the preBötC, supporting diverse impairments after maternal opioids. Collectively, this study provides novel insights into the direct, central effects of maternal opioids on the neonatal respiratory system, further advancing our understanding the respiratory deficits in infants with NAS.

The respiratory network must be functional at birth to maintain homeostasis, but the respiratory network continues to mature postnatally (Patrone et al., 2018, 2020; Taxini et al., 2021; reviewed

in Wong-Riley et al., 2019), including refinement of respiratory rhythm generation (Bissonnette et al., 1991; Kobayashi et al., 2001), shifts in oscillator dominance (Hocker, Morrison et al., 2021; Onimaru & Dutschmann, 2012), and shifts in neurotransmitter/neuromodulator receptor expression (Gao et al., 2011; Liu & Wong-Riley, 2002; Wong-Riley et al., 2013). While lingering neonatal opioids contribute to reductions in respiratory amplitude in neonates after maternal opioids, we hypothesize that reduced respiratory frequency in neonates after maternal opioids is caused by disruption of perinatal maturation of respiratory networks after maternal opioids. We previously identified quantal slowing emerged at P2 in neonates, suggesting P2 is a key developmental age for shifting oscillator dominance (Hocker, Morrison et al., 2021). In support of this, central impairments in isolated neonatal respiratory control networks were most prominent in young neonates (P0-2), whereby older neonates demonstrated more rhythmic, consistent central activity. Interestingly, deficits in respiratory pattern continued in older neonatal ages, suggesting a potential for lasting impairment of respiratory networks beyond the deficits in breathing. None of these changes in respiratory activity were evident in neonates after maternal no treatment, with the exception of a decrease in activity over time acutely at P2. In fact, this finding may further support increased vulnerability during this window of network reorganization at P2 and decrease longevity of respiratory activity *in vitro*. Thus, our research provides insights into how maternal opioids impair perinatal maturation of the respiratory network, which is key to understanding the etiology of breathing deficits in neonates after maternal opioids (Hocker, Morrison et al., 2021).

Central respiratory networks consist of a diverse network of cells within the brainstem, whereby the medulla is the primary site for respiratory rhythm generation (reviewed in Del Negro et al., 2018). Essential neurons within these networks are born earlier in gestation (E10-E14), yet they remain immature even at birth (Akins et al., 2017; Gray et al., 2010; Kottick et al., 2017;

Pagliardini et al., 2003; Revill et al., 2015). The deficits here after late gestation maternal opioids were investigated in medullary networks, whereby maternal opioids directly impaired central medullary network activity. Deficits, however, extended beyond impairments to the preBötC and are consistent with previous research showing maternal opioids induce reorganization of neonatal respiratory control networks (Gourévitch et al., 2016). Increased amplitude and emergence of a distinct respiratory pattern support actions on opioid-sensitive respiratory regions generating and/or modulating respiratory burst pattern, such as the opioid-sensitive Post-Inspiratory Complex (PiCo; Ramirez & Baertsch, 2018), the Bötzinger Complex (Cinelli et al., 2020), premotor neurons (i.e. rostral ventral respiratory group; Lonergan et al., 2003), motoneurons (i.e. phrenic motor neurons; Xia & Haddad, 1991), the opioid-insensitive parafacial respiratory group/retrotrapezoid nucleus (pFRG/RTN; Onimaru et al., 1997; Onimaru & Homma, 2003, 2005; Onimaru & Dutschmann, 2012) and other modulatory regions (i.e. the nucleus tractus solitarius, NTS; Endoh, 2006; or the caudal medullary raphe neurons; Palkovic et al., 2022). Although pontine regions, such as the Kolliker-Fuse and the parabrachial complex, are opioid-sensitive and contribute to opioid-induced respiratory depression (Baldo, 2022; Bateman et al., 2021; Prkic et al., 2012; Saunders & Levitt, 2020), these regions do not contribute to central respiratory network impairments observed here, as the pons was removed. Findings from this study provide potential avenues for future investigation and suggest the importance of further analyses on respiratory pattern. Thus, maternal opioids directly impair central neonatal respiratory networks late in gestation after initial establishment of vital respiratory circuitry (Greer, 2015; Pagliardini et al., 2003; Ren & Greer, 2003).

Respiratory burst pattern is regulated by multiple distinct respiratory regions throughout the medulla and spinal cord, leading to the generation of inspiratory, post-inspiratory and pre-

inspiratory neural activity; all of which contribute to neonatal breathing at rest (reviewed in Del Negro et al., 2018). Here, we identified a distinct respiratory burst pattern of a single burst with a decreased period to the next burst in neonates after maternal opioids at all ages, suggesting maternal opioids may cause lasting alterations to neonatal respiratory pattern. After maternal opioids, the distinct burst pattern occurred in more complete respiratory networks (brainstem and spinal cords), but not in more isolated networks (medullary slices containing the preBötzinger Complex), suggesting changes in neonatal respiratory pattern extend beyond the preBötC. Three possibilities could explain the distinct respiratory pattern in neonates after maternal opioids. First, maternal opioids may reduce post-inspiratory activity from opioid-sensitive PiCo neurons (Anderson et al., 2016; reviewed in Ramirez & Baertsch, 2018) or impair recurrent excitatory or inhibitory connections between preBötC and PiCo neurons coordinating respiratory burst timing (reviewed in Ramirez & Baertsch, 2018). Second, a mismatch in pre-inspiratory activity from opioid-insensitive pFRG/RTN neurons and inspiratory activity from the opioid-sensitive preBötC neurons may contribute to the distinct respiratory burst pattern in neonates after maternal opioids. Third, maternal opioids may decrease activity in the opioid-sensitive NTS (Endoh, 2006) or disrupt excitatory or inhibitory recurrent connections between the NTS and preBötC (Yang et al., 2020) to influence respiratory burst pattern in neonates after maternal opioids. Breathing normalized in older neonates after maternal opioids (Hocker, Morrison et al., 2021); however, the distinct respiratory pattern occurred at all neonatal ages, suggesting the change in respiratory pattern is a lasting change in respiratory networks.

The distinct respiratory pattern induced by maternal opioids was similar to a pattern observed after neonatal inflammation (Morrison et al., 2019). Although TLR4 signaling activated by neonatal inflammation does not contribute to acute opioid-induced respiratory depression in

neonates (Zwicker et al., 2014), significant cross-talk between opioid receptors and inflammatory signaling pathways exists (reviewed in Zhang et al., 2020), which may explain some similarities in responses. In fact, perinatal opioid exposure increases neonatal cortical chemokines and cytokines, alters microglial morphology and upregulates cortical microglial TLR4 and MyD88 RNA expression (Jantzie et al., 2020). Thus, it remains possible that maternal opioids alter inflammatory signaling in neonatal respiratory networks to induce changes in neural activity.

Our results demonstrating maternal opioids decrease respiratory burst amplitude and emergence of a distinct respiratory pattern at all ages provide support for the hypothesis that maternal opioids disrupt perinatal maturation of central respiratory networks. Many early life stressors, such as gestational intermittent hypoxia (Gozal et al., 2003; Johnson et al., 2017), gestational ethanol exposure (Dubois & Pierrefiche, 2020), prenatal anxiety drug exposure (da Silva Junior et al., 2022), perinatal inflammation (Camacho-Hernández et al., 2022; Morrison et al., 2019), perinatal nicotine exposure (Cholanian et al., 2017; Ferng & Fregosi, 2015; Luo et al., 2004), perinatal anti-depressant drug exposure (Biancardi et al., 2022) and neonatal maternal separation (Kinkead et al., 2005; Kinkead & Gulemetova, 2010; Rousseau et al., 2017; reviewed in Tenorio-Lopes & Kinkead, 2021), acutely disrupt neonatal respiratory control. Some of these stressors even have enduring consequences on the adult respiratory system (Biancardi et al., 2022; Dubois & Pierrefiche, 2020; Genest et al., 2004; Hocker et al., 2019; Kinkead et al., 2009; Soliz et al., 2016). This research supports maternal opioids as a significant early life stressor, despite aspects of neonatal breathing normalizing over time after maternal opioids and has the potential to induce lasting impairments on central respiratory networks into adulthood.

Additional exogenous opioids are frequently given to human infants with NAS to curb withdrawal symptoms (Jansson & Velez, 2012), yet the impact of additional exogenous opioids

on central respiratory networks has not been studied. Similar to the effects we observed with neonatal breathing (Hocker, Morrison et al., 2021), responses of isolated central networks to exogenous opioids in neonates after maternal opioids were acutely blunted and correlate with decreased opioid receptor expression. Also consistent with previous research (Hocker, Morrison et al., 2021), mu-opioid receptor expression was similar between P4 neonates after maternal no treatment and maternal opioids, when breathing normalized after maternal opioids, despite neonates continuing to receive opioids through breast milk (Ito, 2018; Naumburg & Meny, 1988). Future research is needed to determine the mechanisms for normalized responses to exogenous opioids in older neonates (P3-5), despite this continued exposure to opioids. There were no lasting or rebound effects of ceasing maternal opioid exposure since mu-opioid receptor expression was similar at P11, after maternal opioid exposure ceasedClick or tap here to enter text.. These observations are consistent with those from the locus coeruleus, showing opioid receptor expression decreased during chronic adult opioid exposure and returned to pre-exposure levels after opioid exposure ceased (Dang & Christie, 2012; Quillinan et al., 2011). Developmental changes in responses to exogenous opioids (evident in survivability curves) is also consistent with clinical observations that infants with NAS have highly variable responses to additional opioids in the first week of life (Jansson & Velez, 2012). In summary, our research begins to investigate central mechanisms likely contributing to blunted opioid-induced respiratory depression in neonates after maternal opioids (Hocker, Morrison et al., 2021), which is needed to understand opioid sensitivities in infants with NAS during the first week of life (Jansson & Velez, 2012).

Many different central respiratory regions are involved in opioid-induced respiratory depression (Bachmutsky et al., 2020; Baertsch et al., 2021; Bateman et al., 2021; Bell et al., 2011; Emery et al., 2016; Greer et al., 1995; Mellen et al., 2003; Montandon et al., 2011; Pattinson, 2008;

Varga et al., 2020) and may contribute to blunted respiratory responses to exogenous opioids in neonates after maternal opioids. Candidate respiratory regions for future investigation of blunted central responses to exogenous opioids in neonates after maternal opioids include: the opioid-sensitive rostral ventral respiratory group (Cinelli et al., 2020; Lonergan et al., 2003; Miyawaki et al., 2002), the Bötzinger Complex (Cinelli et al., 2020), and the caudal medullary raphe (Palkovic et al., 2022) because these regions significantly contribute to acute opioid-induced respiratory depression. The opioid-insensitive pFRG/RTN may also contribute to blunted opioid-induced respiratory depression, since the pFRG/RTN is the dominant respiratory rhythm generator immediately after birth (Hocker, Morrison et al., 2021; Onimaru et al., 1989, 1995; Onimaru & Dutschmann, 2012). Maternal opioids may then increase reliance on the pFRG/RTN to maintain respiratory activity despite the ongoing presence of additional opioids, either of maternal origin or in response to additional exogenous opioids used to treat withdrawal symptoms (Kraft et al., 2016; Mangat et al., 2019). Understanding specific regions in the respiratory network contributing to blunted neural and breathing responses to exogenous opioids in neonates after maternal opioids is needed to determine the potentially unique mechanisms underlying opioid-induced respiratory depression in neonates after maternal opioids.

Significant research efforts are focused on how opioids depress respiratory control networks, primarily in adults (Bateman et al., 2021; Bell et al., 2011; Boom et al., 2012; Greer et al., 1995; Montandon et al., 2011, 2016; Palkovic et al., 2022; Prkic et al., 2012; Saunders & Levitt, 2020; Stucke et al., 2008, 2015; Takita et al., 1997); yet, less is known about postnatal development of opioid-induced respiratory depression and changes in opioid receptor expression. Early studies investigated opioid receptor expression in the brainstem during postnatal development used quantitative receptor autoradiography, showing opioids bind to many neonatal medullary

respiratory regions, such as dorsal parabrachial nucleus, NTS, Kölliker-Fuse nucleus, and the ventral parabrachial nucleus (Xia and Haddad, 1991). Opioid receptors in many respiratory control regions increased with age, until maximal expression between P10 and P21 before decreasing in adulthood (Xia and Haddad, 1991). Others identified mu-opioid receptor expression in a subset of NK1R positive preBötC neurons (Cinelli et al., 2020; Gray et al., 1999; Montandon et al., 2011; Pagliardini et al., 2003), yet the expression of mu-opioid receptors in the preBötC during early postnatal development remained unclear. Here, we characterized the expression of mu-opioid receptors in the putative preBötC during early postnatal development, demonstrating mu-opioid receptors appear highest at P0, decrease at P4, and decrease further from P4 to P11. This provides region-specific insights into opioid receptor expression in the preBötC across postnatal development and suggests neonatal breathing responses to exogenous opioids may shift during typical postnatal development as opioid receptor expression changes.

The type, dose, and dosing paradigm of opioids used to study the developmental effects of opioids remain controversial and complicated (reviewed in Faried et al., 2008). This is further complicated by species-specific differences in tolerance, withdrawal, and stress (reviewed in Farid et al., 2008). As such, the dose of maternal opioids used here (5mg/kg methadone, s.c, daily G17-P5) may lead to lower fetal and neonatal concentrations of opioids than human fetuses and infants exposed to maternal opioids (De Montis et al., 1983). It remains challenging to replicate the complex and extenuating factors influencing fetal opioid exposure in humans (Faried et al. 2008). However, substantial other work in rodent models support the dose of methadone used here (reviewed in Faried et al., 2008) and we have previously shown this dose paradigm impaired neonatal breathing (Hocker, Morrison et al. 2021). Future work should explore the effects of

different opioids at varying doses and developmental times to further mimic the complexity of NAS.

In conclusion, this research study provides insights into the etiology of breathing deficits in infants with NAS, by determining the direct effects of maternal opioids on central neonatal respiratory networks within the brainstem and spinal cord. Maternal opioids age-dependently disrupted neonatal respiratory network activity and circulating neonatal opioids contributed to central respiratory impairments in neonates after maternal opioids. Interestingly, central impairments extend beyond the preBötC and involve changes in respiratory pattern. Such central impairments in neonatal respiratory control after maternal opioids likely contribute to destabilized breathing (Hocker, Morrison et al., 2021); however, maternal opioids blunt central responses to exogenous opioids due, at least in part, to decreased opioid receptors in the preBötC. Our research suggests central changes to respiratory networks responses to exogenous opioids in neonates after maternal opioids likely contribute to variability seen in breathing responses to exogenous opioids in infants with NAS during the first week of life (Jansson & Velez, 2012). In summary, our research identifies the direct effects of maternal opioids on central neonatal respiratory networks, increasing our understanding the breathing deficits in infants exposed to maternal opioids.

CHAPTER V

OVERALL CONCLUSIONS & FUTURE DIRECTIONS

Overall, my dissertation significantly increases our understanding of how early life stressors impair neonatal and adult respiratory control, highlighting links between early life stressors and adult respiratory deficits. The second chapter of my dissertation identified adult microglia as key sources of inflammation in adult respiratory control regions after neonatal inflammation, which likely contribute to sex-specific impairment in the adult respiratory system. Furthermore, our results have broad implications for understanding how early life stressors have lasting impacts on adult respiratory control by identifying microglia as a key cell type causing lasting inflammation after neonatal inflammation. Many early life stressors, such as gestational intermittent hypoxia (Gozal et al., 2003; Johnson et al., 2017), neonatal intermittent hypoxia, gestational inflammation and neonatal maternal separation induce neuroinflammation early in life (Camacho-Hernández et al., 2022; Gozal et al., 2003; Johnson et al., 2017; Kinkead et al., 2005; Kinkead & Gulemetova, 2010; Rousseau et al., 2017; reviewed in Tenorio-Lopes & Kinkead, 2021), yet little is known about the lasting impact of early life stressors on adult microglia. Our research significantly increases our understanding of this underappreciated cell type and suggest long-term inflammatory signaling originating from adult microglia likely contribute to the lasting impacts of many early life stressors on adult respiratory control and may influence adult risk for developing ventilatory control disorders.

The second chapter of my dissertation significantly advances our understanding of the lasting consequences of neonatal inflammation on adult inflammatory responses in respiratory control regions. Neonatal inflammation primes adult microglial inflammatory responses to subsequent inflammation. Since adult breathing was maintained after neonatal and adult

subthreshold inflammation, adult microglia priming after neonatal inflammation likely does not increase susceptibility to breathing deficits to low levels of adult inflammation. Instead, primed microglia after neonatal inflammation may increase breathing deficits after more severe adult inflammatory challenges or have protective effects on breathing during adult inflammation. While more research is needed to identify whether microglia priming is maladaptive or protective to adult breathing during adult inflammation, our research provides key insights into how microglia priming in respiratory regions after neonatal inflammation contributes to adult neuroinflammatory responses to subsequent adult inflammation.

The third chapter of my dissertation provides key insights into how maternal opioids lead to neonatal respiratory control deficits after maternal opioids. These experiments are the first to characterize the impact of maternal opioids directly on the isolated neonatal respiratory control network and provide mechanistic insights into the etiology of neonatal breathing deficits after maternal opioids. This study has meaningful translational implications by determining central origins of breathing deficits in infants after maternal opioids and the impact of acute opioids on respiratory circuits. Rodent studies like these are necessary to determine how stressors, such as materal opioids, disrupt development of respiratory control and inform clinicans about how maternal opioids contribute to risk for neonatal mortality when providing acute neonatal opioids to treat these infants withdrawl symptoms.

In conclusion, this dissertation research substantially advances the respiratory field by investigating what types of cellular and molecular mechanisms contribute to respiratory control impairments after neonatal inflammation or maternal opioids and where within medullary and spinal respiratory networks impairments are located after neonatal inflammation or maternal opioids. This research lays the foundation for future studies to investigate how to restore

respiratory motor plasticity in adults after neonatal inflammation, determine the consequences of neonatal inflammation on adult risk for breathing instability during subsequent adult inflammation and determine how to enhance infant breathing after maternal opioids.

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