Sleep Disordered Breathing: Contribution of Abdominal Muscle Activation and Hormonal Intervention in Healthy and Pathological Conditions

by

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ABSTRACT

Breathing and sleep are both imperative and intricate physiological processes that require development in the perinatal period to sustain life and adapting to changes in postnatal life. Inspiration and expiration are generated by oscillatory microcircuits located in the ventrolateral medulla and work in unison to produce a normal breathing pattern. Inspiratory rhythm is generated by the preBötzinger complex that drives muscle activity of the diaphragm and other inspiratory muscles. The lateral parafacial region drives active expiration through contraction of the abdominal muscles. Respiration is an obligatory ongoing process that may be temporarily interrupted by ongoing behaviours or by apneas that occur in both healthy and pathological conditions.

Sleep is characterized by different states which are generated and modulated by multiple regions throughout the brain with considerable developmental changes in early postnatal life. The breathing pattern varies with changes in brain states, and this may contribute to the development of sleep disordered breathing. Infants are a particularly impacted population as breathing and sleep are simultaneously undergoing dramatic changes during development and they spend a considerable amount of time sleeping, during which breathing is vulnerable.

In this thesis I was interested in characterizing the developmental changes in sleep, breathing parameters and expiratory abdominal activity in rodents. Previous work in our laboratory in adult rodents demonstrated the occurrence of potent expiratory abdominal muscle recruitment mostly during rapid eye movement sleep. Abdominal activation periods in adult rats were more likely to occur after an episode of unstable breathing like an apnea, suggesting that active expiration may be involved in stabilizing breathing when it becomes irregular. Given the ongoing development of the brain and the respiratory system, neonates can be prone to greater

respiratory disturbances, so I aimed to investigate if neonatal rodents from postnatal day 0 to15 displayed expiratory activity during sleep and how this activation altered respiration. I hypothesized that abdominal activation was associated with a stabilization in ventilation. I instrumented rats with EMG electrodes in respiratory muscles and recorded their behavior to characterize the duration and pattern of sleep states and occurrence of apneas and hypopneas throughout postnatal development, focusing on the activation of abdominal muscles and its relationship to sleep states. Our results suggest that the occurrence of abdominal activation during quiet sleep did not alter respiratory parameters and occurrence decreased with age, whereas active expiration during active sleep was present across development and associated with an increase in minute ventilation and a decrease in respiratory variability across development.

Given our results in infant rodents, I wanted to characterize the pattern of abdominal muscle recruitment in polysomnography recordings obtained from children suspected of sleep disordered breathing. I aimed to investigate whether children also displayed abdominal muscle activation during sleep and how this correlated with respiratory events such as apneas and hypopneas. I hypothesized that activation of the abdominal muscles in human infants occurred during both quiet and active sleep as seen in infant rodents. My results indicated that children also exhibited an expiratory abdominal activation pattern during sleep in both regular sleep and in conjunction with respiratory events, with no specific differences between infants at 0-2 years of age.

In the last experimental chapter of my thesis, I used a model of impaired central chemoreception to investigate the effects of a potent progestin drug on respiration and chemoreflex responses. A fortuitous study reported a chemoreflex response recovery following

implementation of an oral contraceptive etonogestrel, in two female patients affected by congenital central hypoventilation. Here I investigated the hypothesis that etonogestrel would increase ventilation in adult female rodents that were healthy and those with lesions to the retrotrapezoid nucleus, a central chemosensitive region of the brain. My findings indicated that etonogestrel did not affect baseline breathing nor CO_2 chemoreflex in healthy female rats. Interestingly, in lesioned rats, respiratory parameters were restored to control levels, indicating a potent stimulatory role of sex hormones on ventilation.

The final chapter of this thesis is the conclusion which covers the future directions of study on the abdominal activation investigations in mammals, and on the use of etonogestrel as a potential respiratory stimulant in conditions of an impaired chemoreflex. It also addresses the limitations of the present studies that were addressed in subsequent studies in our laboratory.

PREFACE

This thesis is an original work by Jasmeen Saini. The research projects, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "NEURAL CONTROL OF BREATHING IN VIVO", No. AUP461, 2012-Present Chapter 2 of this thesis is published as J.K. Saini, S. Pagliardini, "Breathing During Sleep in the Postnatal Period of Rats: The Contribution of Active Expiration," *Sleep*, vol. 40, issue 12, 1-13. I was responsible for the data collection, analysis and manuscript preparation. Dr. S. Pagliardini assisted with the manuscript preparation, edits and concept formation.

Chapter 3 of this thesis has been published as J.K. Saini, T.A. Janes, J.E. MacLean, S. Pagliardini, "Expiratory Activity During Sleep in Children" *Journal of Sleep Research*, e13539, 1-12. The research conducted in chapter 3 is a collaborative project between Dr. J. MacLean at the University of Alberta, and Dr. S. Pagliardini. This study is a secondary analysis of data previously collected by Dr. J. MacLean. I was responsible for the data analysis and Drs. S. Pagliardini, T. Janes and I were responsible for manuscript composition. Dr. S. Pagliardini and Dr. J. MacLean were the supervisory authors and were involved in concept.

The data collection and analysis in chapter 4 is unpublished work. I was responsible for the data collection and analysis, Dr. S. Pagliardini assisted with concept formation and chapter composition. I was assisted by summer student, L.A. DeHoog as part of his individual research project (PHYSL467; 2017-2018) and summer internship (2016, 2017). This research contributed preliminary results to the paper published as S. Cardani, T.A. Janes, J.K. Saini, S.Di Lascio, R. Benfante, D. Fornasari, S. Pagliardini, "Etonogestrel Administration Reduces the Expression of PHOX2B and Its Target Genes in the Solitary Tract Nucleus" *International Journal of Molecular Sciences*, no. 9, 4816, 1-21.

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LIST OF ABBREVIATIONS

AASM: American Academy of Sleep Medicine

ABD: Abdominal Muscles

ABD^{+} : Abdominal Electromyography Onset

ABD^{W}: Abdominal Electromyography Present

$ABD^{W/O}$: Abdominal Electromyography Absent

ALTE: Apparent Life-Threatening Events

AS: Active Sleep

bpm: Breaths Per Minute

BötC: Bötzinger Complex

CHS: Central Alveolar Hypoventilations

ChAT: Anti-Choline Acetyltransferase

COPD: Chronic Obstructive Pulmonary Disease

CTR: Control Values

CVRP: Coefficient of Variation of the Respiratory Period

Cy2-DAR: Cy2 Conjugated-Donkey Anti-Rabbit

Cy3-DAGt: Cy3 Conjugated-Donkey Anti-Goat

Cy5-DAM: Cy5 Conjugated-Donkey Anti-Mouse

Dbx1: Developing Brain Homeobox Protein 1

DIA_{EMG}: Diaphragmatic Electromyography

DRN: Dorsal Raphe Nucleus

DV: Dorsoventral

E2: 17 Beta-Estradiol

EMG: Electromyography

EOG: Electrooculography

ETO: Etonogestrel

FBM: Fetal Breathing Movements

GABA: Gamma Aminobutyric Acid

GPR4: G-Protein Coupled Receptor 4

HcVR: Hypercapnic Ventilatory Response

HVR: Hypoxic Ventilatory Response

INT: Intercostal

IS: Indeterminate Sleep

LC: Locus Coeruleus

LSD: Least Significant Difference

MCH: Melanin-Concentrating Hormone

ML: Mediolateral

MnPO: Median Preoptic Nucleus

NA: Nucleus Ambiguus

NDS: Normal Donkey Serum

NK1r: Neurokinin-1 Receptor

nREM: Non-Rapid Eye Movement Sleep

NTS: Nucleus Tractus Solitarius

OSA: Obstructive Sleep Apnea

PB: Parabrachial Nucleus

PBS: Phosphate Saline Buffer

P: Post-Natal Day

pF: Parafacial Region

pF_L: The Lateral Parafacial Regions

pF_V: Ventral Parafacial Regions

PHOX2B: Paired Like Homeobox 2

PiCo: Post-Inspiratory Complex

PMNs: Phrenic Motoneurons

POA: Preoptic Area

preBötC: PreBötzinger Complex

QS: Quiet Sleep

RC: Rostrocaudal

REM: Rapid Eye Movement Sleep

RIP: Respiratory Inductance Plethysmography

RN: Raphe Nucleus

RTN: Retrotrapezoid Nucleus

SDB: Sleep Disordered Breathing

sEMG: Submental Electromyography

SIDS: Sudden Infant Death Syndrome

SP-SAP: Substance-P Conjugated to Saporin

SpO_2: Oxygen Saturation

SST: Somatostatin

$TcCO_2$: Transcutaneous CO2

TH: Tyrosine Hydroxylase

TASK-2: TWIK Related Acid Sensitive K+ Channel 2

$\dot{V}e$: Minute Ventilation

VLPO: Ventrolateral Preoptic Area

vPAG: Ventral Periaqueductal Grey

VT: Tidal Volume

VRC: Ventral Respiratory Column

W: Wakefulness

Vglut2: Vesicular Glutamate Transporter 2

vlPAG: Ventrolateral Periaqueductal Grey

VT/ V_T: Tidal Volume

Chapter 1: General Introduction

1.1 Basics of Respiratory Control

Breathing is an imperative physiological process, facilitating oxygen intake and elimination of carbon dioxide necessary for proper tissue metabolic activity. Respiratory rhythm is generated in the brainstem by neurons in the ventrolateral medulla and involves at least two main oscillators, which produce inspiration and active expiration. Other respiratory neural subgroups in the brainstem play different modulatory roles in respiration (Feldman et al., 2013). Over the past few decades, studies have uncovered the anatomical location and function of the microcircuits involved in respiratory rhythmogenesis and are summarized in various reviews (Anderson & Ramirez, 2017; Del Negro et al., 2018; Feldman et al., 2013). Respiration is characterized by two fundamental components: the central rhythmogenesis that is generated in the microcircuits described below, and a central pattern generator that synchronizes the motor discharge to respiratory musculature as seen in figure 1.1a (Feldman et al., 2013; Marder & Bucher, 2001). These excitatory microcircuits are situated in the ventrolateral medulla, along the ventral respiratory column (VRC). They generate the phases of ventilation, neural activity and interactions between the microcircuits can be observed in figure 1.1c.

1.1a The preBötzinger Complex

The preBötzinger complex (preBötC) is a kernel of neurons that is necessary and sufficient for the generation of respiratory rhythm (Gray et al., 2001; Gray et al., 1999; Rekling & Feldman, 1998; Smith et al., 1991). The preBötC is located in the ventrolateral medulla and has been identified in rodents (figure 1.1c) (Feldman et al., 2003; Gray et al., 2001), cats (Schwarzacher et al., 1995), rabbits (Mutolo et al., 2002), goats (Wenninger et al., 2004), and humans (Schwarzacher et al., 2011).

In vitro investigations with serial transverse microdissections of the rodent medulla allowed for the first identification of the area responsible for generating the respiratory rhythmic activity, the preBötC (Smith et al., 1991). The preBötC is located in the medulla, ventral to the semi-compact nucleus ambiguus (NA) and caudal to the compact NA (Pagliardini et al., 2003). The neurons making up the preBötC are a heterogenous population of mostly excitatory glutamatergic neurons that express the

neurokinin-1 receptor (NK1r), somatostatin (SST) and are derived from progenitor cells expressing the developing brain homeobox protein 1 (Dbx1) (Gray et al., 2001; Gray et al., 1999; Pagliardini et al., 2003; Stornetta et al., 2003). Null Dbx1 mice do not display fetal breathing movements in utero and were unable to generate breaths at parturition (Bouvier et al., 2010). *In vitro* laser ablation experiments of Dbx1 neurons caused a complete loss of respiratory discharge in medullary slice preparations, as well as elimination of premotor activity in hypoglossal motor output (Revill et al., 2015; Song et al., 2016; Wang et al., 2014). These studies indicate Dbx1 is indeed a transcription factor that specifies preBötC neurons and play an essential role in both respiratory rhythm generation and in upper airway motor output of respiratory pattern.

The necessary role of preBötC neurons was initially demonstrated with bilateral injections of substance-P conjugated to saporin (SP-SAP), a toxin that induces cell death in NK1r expressing cells in the preBötC. When > 80% of preBötC neurons were eliminated, ataxic breathing occurred (Gray et al., 2001; McKay et al., 2005), while less substantial lesions produced a disrupted breathing pattern during sleep that mimics sleep disordered breathing (McKay & Feldman, 2008; McKay et al., 2005). Similarly, acute silencing of SST+ preBötC cells resulted in persistent apnea (Tan et al., 2008). Although this study indicates the importance of SST+ preBötC cells in respiratory output, subsequent studies have suggested that the SST subpopulation of preBötC neurons may have a role in regulating pattern formation, rather than rhythmogenesis (Cui et al., 2016; Montandon et al., 2016; Revill et al., 2015; Tan et al., 2010).

The preBötC also contains inhibitory glycinergic neurons, a population of cells which play a modulatory role in shaping inspiratory motor pattern but are not necessary for respiratory rhythm (Sherman et al., 2015), although some glycinergic neurons display rhythmogenic properties (Morgado-Valle et al., 2010). The contributions of different preBötC subpopulations are summarized in figure 1.2. For a long time, the role of glial cells were acknowledged to be primarily supportive and nutritive in the central nervous system and in the respiratory network. Recent research has shown that glia also play a role in informing preBötC cells of hypoxia through gliotransmission (Gourine & Funk, 2017; Huxtable et al., 2010; Rajani et al., 2018).

1.1b Post-Inspiratory Complex

The phase of respiration following inspiration is known as post-inspiration (Richter, 1996). During post-inspiration, laryngeal adductors and crural diaphragm muscles are recruited, and this activity is associated with different respiratory and non-respiratory-related behaviours such as sneezing, coughing, swallowing and vocalization (Dutschmann et al., 2014). Historically, multiple regions have been suggested to be involved in post-inspiratory activity, such as the nucleus tractus solitarius (NTS) (Costa-Silva et al., 2010), the Bötzinger Complex (BötC) (Richter, 1996; Smith et al., 2009) and pontine structures like the Kölliker Fuse (Dutschmann & Herbert, 2006; Dutschmann et al., 2014).

More recently, Anderson and colleagues proposed that a specific rhythmogenic population of cells is responsible for the generation of post-inspiratory rhythmic activity, the post-inspiratory complex (PiCo) (Anderson et al., 2016). PiCo is an excitatory network in the medulla composed of neurons co-expressing glutamate and acetylcholine located dorso-rostral to the preBötC and caudal to the facial nucleus (figure 1.1b) (Anderson et al., 2016). *In vitro* medullary slice preparations show that bursting and pharmacological properties of PiCo make this area spatially and functionally unique (Anderson et al., 2016). They proposed that PiCo is responsible for the generation of post-inspiratory activity in rodents. It has to be noted that research involving this microcircuit is limited to a few studies with controversial perspectives (Hulsmann, 2021). However, more recently this area has been implicated in both respiratory and non-respiratory behaviours like swallowing and crying (Oliveira et al., 2021; Toor et al., 2019; Wei et al., 2022).

1.1c The parafacial region

Another microcircuit important in respiratory control is located in the parafacial (pF) region, which is subdivided in the lateral parafacial (pF_L) and ventral parafacial (pF_V) regions. The region equivalent to the pF_L was previously known as parafacial respiratory group (pFRG), while the pF_V identifies the anatomical region containing the chemosensitive neurons of the retrotrapezoid nucleus (RTN).

The pF region sits ventral to the facial nucleus in the rostral medulla (figure 1.1b) (Huckstepp et al., 2015; Thoby-Brisson et al., 2009). Originally, pF_L and pF_V (previously pFRG and RTN) were grouped together due to their proximity, the functional overlap between regions and the limited understanding about their anatomical and functional distinctions. Recent investigations suggest that they are distinct, with the pF_L (pFRG) being an expiratory oscillator and the pF_V (RTN) being a key chemosensitive region that affects both inspiratory and expiratory activity (Abbott et al., 2011; Abbott et al., 2009; Huckstepp et al., 2015; Huckstepp et al., 2018; Magalhaes et al., 2021; Pagliardini et al., 2011; Pisanski & Pagliardini, 2019; Stornetta et al., 2006).

The pF_L (also referred as pFRG in the literature from the 2000-2020 period) has been shown to act as a conditional expiratory oscillator: silent during restful breathing and active under high respiratory drive, like exercise or chemical stimuli (e.g., hypercapnia or hypoxia), or following release of inhibition (de Britto & Moraes, 2017; Huckstepp et al., 2015; Huckstepp et al., 2016; Janczewski & Feldman, 2006a; Moraes et al., 2012; Pagliardini et al., 2011). Upon activation, the pF_L becomes rhythmically active and recruits expiratory abdominal muscles. Abdominal recruitment increases intra-abdominal pressure and heighten expiratory airflow, with a consequent potentiation of minute ventilation (O'Halloran, 2018; Pagliardini et al., 2011).

In adult rodents during resting conditions, pF_L neurons are silent, possibly due to synaptic inhibition, since removal of gamma-aminobutyric acid (GABA) inhibition activates the pF_L and induces active expiration via recruitment of abdominal muscles (Flor et al., 2020; Huckstepp et al., 2015; Huckstepp et al., 2016; Magalhaes et al., 2021; Pagliardini et al., 2011). The source of this tonic inhibition is currently unknown although it has been proposed to originate from neurons in the adjacent Bötzinger Complex and preBötC, the NTS, the RTN and the medullary raphe, as well as regions of the pons including the locus coeruleus, Köllifer-Fuse and the pedunculopontine tegmental nucleus (Barnett et al., 2018; Biancardi et al., 2021; de Britto & Moraes, 2017; Flor et al., 2020). Furthermore, pF_L neurons can be activated by acetylcholine through M3 muscarinic receptors (Boutin et al., 2017) or by glutamate through AMPA receptors (Huckstepp et al., 2018; Magalhaes et al., 2021).

The effect of pF_L activity on ventilation is linked to the activation of ABD muscle activity during late expiration, leading to increaseed tidal volume and heightened diaphragm and genioglossus EMG activity and minute ventilation (Boutin et al., 2017; Huckstepp et al., 2015; Huckstepp et al., 2016; Huckstepp et al., 2018; Pagliardini et al., 2011). While activation of pF_L neurons generates active expiration and affects the subsequent inspiratory efforts, pF_L neurons are not necessary for respiratory rhythm generation at rest as hyperpolarization of pF_L neurons *in vivo* does not affect breathing during rest or under hypoxic or hypercapnic stimuli, and activation of pF_L is not sufficient to reinstate breathing when preBötC is inhibited (Huckstepp et al., 2015; Huckstepp et al., 2016; Huckstepp et al., 2018). Regarding the main objectives of this thesis, it is important to note that pFRG/ pF_L may also function in a state-dependent fashion, since expiratory ABD activity has been observed in adult rats in sleep (in particular in REM sleep) and the hyperpolarization of pFRG/ pF_L neurons depressed occurrence of expiratory activity during sleep while depolarization of pFRG/ pF_L enhanced the recruitment of ABD activity (Andrews & Pagliardini, 2015; Pisanski et al., 2020).

The neurons in the RTN / pF_V lie ventromedially to the facial nucleus (Feldman et al., 2003; Guyenet et al., 2005; Huckstepp et al., 2015). The pF_V contains central chemoreceptors that are pH sensitive neurons. Once activated, neurons of the pF_V stimulate the central pattern generator and modulate inspiratory and expiratory motor activity to increase the respiratory drive and enhance ventilation (Abbott et al., 2011; Abbott et al., 2009; Guyenet et al., 2010; Mulkey et al., 2004; Stornetta et al., 2006). Anatomical markers of RTN neurons include NK1r, the transcription factor PHOX2B and the vesicular glutamate transporter 2 (Vglut2) (Onimaru et al., 2008; Stornetta et al., 2006), in addition to galanin (Stornetta et al., 2009) and most importantly, neuromedin B (Li et al., 2016; Shi et al., 2017). The firing frequency of these neurons is increased with lowering of pH in both juvenile and adult rats (Marina et al., 2010; Mulkey et al., 2004) and the pH sensitivity is thought to be mediated by both g-protein coupled receptor 4 (GPR4) and TWIK related acid sensitive K^+ channel 2 (TASK-2) (Kumar et al., 2015).

Silencing, depletion, or hyperpolarization of RTN/pF_V neurons attenuates basal breathing, the CO_2 response and abdominal activity (Huckstepp et al., 2015; Marina et

al., 2010; Nattie & Li, 2002; Souza et al., 2018). In addition to the primary role in chemoreception of RTN (Guyenet et al., 2008, 2010), other sites involved in chemoreception include the NTS (Fu et al., 2019; Nattie & Li, 2002), orexin neurons of the lateral hypothalamus and the PB, serotonergic neurons in the medullary raphé (Kaur & Saper, 2019; Nattie, 2000; Nattie & Li, 2009; Porzionato, Stocco, et al., 2018; Saper & Kaur, 2018; Wang et al., 2021). The relationship between the pF region and the preBötC is summarized in Column D of figure 1.3.

1.1d Ontogenesis of respiratory rhythms

Periodic diaphragmatic contraction (i.e. foetal breathing movements) begins early in utero in both rodents (Kobayashi et al., 2001) and humans (Cosmi et al., 2003). Coincidentally, rhythmic activity in the preBötC neurons commences during embryonic development as early as embryonic (E) age 16.5 in rats (Pagliardini et al., 2003) and E15 in mice (Thoby-Brisson et al., 2005), with an overall activity that strengthens and becomes more regular with increasing age (Pagliardini et al., 2003; Thoby-Brisson et al., 2005).

Developmental anatomical studies showed that NK1r cells of the preBötC are born in rats on E12.5-13.5 and settle into the ventrolateral medulla by E17 (Pagliardini et al., 2003). This is also the age at which inspiratory motor discharge activity can be observed in rat hypoglossal neurons via electrophysiology (Pagliardini et al., 2003). Similarly, in mice, preBötC neurons are born on E11, settle and are rhythmically active by E15.5 (Thoby-Brisson et al., 2005).

The developmental origin of the embryonic paraFacial region (epF), the proposed precursor of the pF_L / pF_V has also been investigated in perinatal mice. These neurons originate from rhombomere segments 3 and 5 and express the Erg-2 transcription factor, NK1r, PHOX2B and VGlut2 (Thoby-Brisson et al., 2005). The e-pF originates and is functionally active prior to the onset of preBöC activity, it entrains preBötC early in development until preBötC neurons become the primary microcircuit for respiratory rhythm generation. The e-pF has been proposed to be the precursor of pF_L / pF_V neurons (figure 1.3 Column A) (Mellen et al., 2003; Oku et al., 2007; Pagliardini et al., 2003; Smith et al., 1991; Thoby-Brisson et al., 2009; Thoby-Brisson et al., 2005).

1.1e Interactions between pFRG and preBötC

As mentioned in the previous section, rhythmic activity of the e-pF precedes the inception of preBötC activity (Thoby-Brisson et al., 2009). Both oscillators develop independently, but the e-pF couples and entrains to the preBötC early in development (Thoby-Brisson et al., 2009). Once the preBötC becomes rhythmic and functional, it becomes the primary respiratory oscillator and drives respiratory activity throughout life. While epF in the postnatal period is thought to develop into both the pF_L /pF_V according to the current hypotheses, which has yet to be demonstrated (Huckstepp et al., 2016; Oku et al., 2007; Pagliardini et al., 2003; Thoby-Brisson et al., 2009; Thoby-Brisson et al., 2005).

The interaction between inspiratory (preBötC) and expiratory (pFRG/ pF_L) oscillators has been investigated in both juvenile and adult rats. For example, in both *in vitro* brainstem preparations (Mellen et al., 2003) and *in vivo* juvenile rats (Janczewski & Feldman, 2006a), opioids exhibited differential sensitivity in preBötC and pFRG/pF_L . These studies showed that depressing preBötC activity with opioids led to the uncoupling of inspiratory and expiratory activities through the process of quantal slowing of inspiratory rhythms and demonstrated the persistence of the coupled oscillators in the postnatal period.

The independence of the two oscillators has also been tested in adult anesthetized rats (Huckstepp et al., 2015). Differential silencing of each oscillator demonstrated the key role of preBötC in generating respiratory rhythm and the secondary role of pFRG/pF_L in driving active expiration, to enhance ventilation in conditions of high respiratory drive. Figure 1.3 illustrates developmental changes in the interaction and state dependent changes in the preBötC, e-pF and pF_L.

1.2 Basics of Sleep Control

Like breathing, sleep is also a physiologically essential behaviour. Changes in brain activity across sleep states contribute to the development of the central nervous system in early infancy and can influence cognitive and physical health later in life (Cirelli & Tononi, 2015; Kayser & Biron, 2016; Kurth et al., 2015; Weber & Dan, 2016). Sleep can be characterized into two general states: non-rapid eye movement (nREM) and

rapid eye movement (REM) sleep, with nREM sleep further organized in three stages: N1, N2 and N3, according to the American Academy of Sleep Medicine Manual for the Scoring of Sleep and Associated Events (Iber et al., 2007a). In the early postnatal period, the precursor states to nREM and REM sleep are termed quiet sleep (QS), active sleep (AS) and indeterminate sleep (IS), which cannot be identified as either QS or AS (Davis et al., 2004).

1.2a Characteristics of Sleep Stages

The classical measurement criteria to define human sleep states include behaviour, cardiorespiratory parameters, electrooculography (EOG), muscle tone, and most importantly, brain activity measured with electroencephalography (EEG) (Jouvet-Mounier et al., 1970; MacLean et al., 2015; Mirmiran et al., 2003). Similar to infants, the EEG activity in rodents is not fully developed in its typical activity pattern until postnatal day (P) 11, therefore sleep states in young rodents can be classified as AS and QS based on behavioural activity and neck muscle electromyography (EMG), whereas in adulthood sleep states are classified based on EEG and EMG activity (Andrews & Pagliardini, 2015; Pisanski et al., 2020; Seelke & Blumberg, 2008).

Infant sleep varies from adult sleep. Newborns begin their sleep cycles with AS followed by QS and spend majority of their time sleeping in AS (Colten, Altevogt, Institute of, et al., 2006). Despite spending 65% of their time asleep, newborns' sleep cycles are relatively short due to a developing circadian rhythm (Davis et al., 2004; Sheldon, 2006). Significant changes occur to sleep characteristics during the first year of life in humans. The circadian rhythm is synchronized with external environmental cues like daylight and darkness, resulting in a more consolidated sleep pattern, characterized by extended sleep periods at night and increased wakefulness during the day. As infants mature, the stages of nREM sleep become distinguishable and the proportion of REM decreases, predominantly occurring towards the end of the sleep cycle (Carskadon & Dement, 2005; Colten, Altevogt, Institute of, et al., 2006; Davis et al., 2004; Sheldon, 2006).

In adults, nREM is characterized by cortical oscillations that display slow, synchronized delta waves in the range of 0.5-4 Hz activity (Luppi & Fort, 2019; Trinder

et al., 1997). Additional features of nREM sleep include a decrease in heart rate, blood pressure, muscle tone and ventilation compared to wakefulness (Colten, Altevogt, Institute of, et al., 2006; de Andres et al., 2011; McCormick & Bal, 1997; Weber & Dan, 2016). Typically, as nREM progresses into each substages, breathing becomes more stable with an increase in tidal volume and a decreased respiratory rate. As a consequence, P_{CO2} increased and P_{O2} decreased (Buchanan, 2013). The overall reduction in tonic activity during sleep in the upper airways also increases upper airway resistance (Trinder et al., 1997).

In adult humans, nREM sleep is further classified into stages N1, N2 and N3, which correspond to the continuum of depth in nREM sleep (Carskadon & Dement, 2005; Colten, Altevogt, Institute of, et al., 2006; Iber et al., 2007b). The first stage of sleep, N1, is a transitionary state between wakefulness and the onset of sleep with a low arousal threshold. The second stage, N2, is deeper than N1 and corresponds to the greatest proportion of nREM sleep time (Colten, Altevogt, Institute of, et al., 2006), in this stage sleep spindles and K complexes are present in the EEG recordings. Slow wave sleep (SWS) or N3 corresponds to the deepest stage of nREM sleep that features low frequency and high voltage EEG activity, typical of SWS.

The cortical EEG during REM sleep is characterized by a low voltage, high frequency, and desynchronized activity, while hippocampal activity presents with theta oscillation within a 4-12 Hz frequency range. REM sleep is also characterized by a loss of muscle tone in the postural muscles, and rapid movements of the eyes (Arrigoni et al., 2016; Colten, Altevogt, Institute of, et al., 2006; Siegel, 1990; Weber & Dan, 2016). REM sleep is associated with fluctuations and increases in heart rate and blood pressure compared to nREM sleep (Colten, Altevogt, Institute of, et al., 2006; Siegel, 1990). Ventilatory changes also display a similar trend, with an increase in breathing frequency, variability and occurrence of respiratory disturbances during REM compared to nREM (Buchanan, 2013; Colten, Altevogt, Institute of, et al., 2006).

REM represents a paradoxical state characterized by a overall behavioural depression combined with increased brain activation and the occurrence of dreams. REM related activity has been proposed to be a major contributor to maturation of the central nervous system, in particular development of the visual network (Peirano & Algarin,

2007; Weber & Dan, 2016). The impact of nREM and REM sleep on cardiovascular, respiratory, sympathetic nerve activity, cerebral blood flow and on the renal and endocrine systems is presented in table 1.1.

1.2b Sleep Generation

Sleep is regulated by two processes: the sleep/wake dependent homeostatic system and the system that governs circadian rhythms (Borbely, 2022; Borbely et al., 2016). Wake-promoting neurons are located in the lateral and posterior hypothalamus, as well as and in brainstem regions such as the dorsal raphe nucleus (DRN), locus coeruleus (LC), ventral periaqueductal grey (vPAG), and in the parabrachial nucleus (PB). Most of these are inhibited by sleep-on neurons of the preoptic area (POA) (Luppi & Fort, 2019; Weber & Dan, 2016). Additional rostral areas implicated as wake-on regions include the basal forebrain and the tubermammillary nucleus (Gompf & Anaclet, 2020; Luppi & Fort, 2019; Weber & Dan, 2016). The laterodorsal and pedunculopontine tegmentum (LDT/PPT), which are involved in modulation of REM sleep, have also been implicated in wakefulness. The above-mentioned areas have vast projections throughout the cortex and trigger wake-on brain pattern that is high frequency and low amplitude. These areas and their projections are illustrated in figure 1.4.

Suppression of wakefulness by sleep-on neurons is followed by alternations between nREM and REM sleep (Weber & Dan, 2016). The length of nREM and REM sleep cycles in rodents and humans last about 10-20 minutes and 90-120 minutes, respectively (Weber & Dan, 2016). Transitions between nREM and REM sleep are also controlled by a reciprocal system, where one neural population of cells ceases firing and another becomes active. The regions involved in the generation of nREM and REM sleep states are discussed below.

Sleep is initially driven by cells that turn off arousal systems, known as sleep-on neurons, which are located in the POA. One of the initial demonstrations of POA involvement in sleep came from post mortem results in patients that experienced insomnia and displayed tissue injury in the POA (Economo, 1930). These findings were supported by studies with bilateral lesions of the POA in cats and rats that induced

insomnia (Fort et al., 2009; McGinty & Szymusiak, 2001; McGinty & Sterman, 1968; Nauta, 1946; Weber & Dan, 2016).

GABAergic cells of the ventrolateral preoptic area (VLPO) and the median preoptic nucleus (MnPO) have specifically been implicated in sleep, particularly in SWS (Gong et al., 2004; Luppi & Fort, 2019; McGinty & Szymusiak, 2001; Sherin et al., 1996; Weber & Dan, 2016). The VLPO is recognized as a predominant region responsible for generation of nREM sleep; lesions in this area cause significant decreases in both the delta oscillations and the time spent in nREM (Lu et al., 2000). The basal forebrain, which contains many neural cell types some of which are wake-on, and others such as SST neurons that are sleep-on (Gompf & Anaclet, 2020; Weber & Dan, 2016). These regions are considered sleep enhancing structures due to the inhibitory influence they have on wake-on regions and the VLPO and MnPO are responsible for the generation and maintenance of SWS sleep, respectfully (Fort et al., 2009; Luppi & Fort, 2019; Sherin et al., 1996).

Pharmacological activation of cells from the NTS, and the dorsolateral pons, GABA neurons of the parafacial zone, GABA neurons of the thalamic reticular nucleus and the ventrolateral periaqueductal grey (vlPAG) encourages a SWS sleep state and simultaneously depresses both REM and wake states (Gompf & Anaclet, 2020; Weber & Dan, 2016).Overall, generation of SWS sleep is dependent on several regions of the brain from the cerebral cortex, thalamus, hypothalamus, basal forebrain and brainstem and cerebellar structures (de Andres et al., 2011). The transitions between sleep and wake, and between SWS and REM are also reciprocal, where one system is turned off and the other is turned on. Neurons that are SWS-active maintain SWS by suppressing REM-on neurons, whereas REM-on neurons work to prompt the transition from SWS to REM sleep (Weber et al., 2015). The proposed mechanism involved in nREM and RWM sleep circuits is depicted in figure 1.5 and figure 1.6 respectively.

Different areas in the brainstem contribute to the generation of REM sleep and contain REM-on and REM-off neurons and the induction of REM muscle atonia (Jouvet, 1962). A major player for both REM sleep generation and maintenance are the GABAergic neurons of the ventral medulla. Optogenetic stimulation activating these GABAergic cells in mice demonstrated quick onset of REM sleep and lengthened the

time spent in REM, whereas silencing of the same GABAergic neurons results in decreased length of REM sleep (Weber et al., 2015).

Cells that are REM-on are also observed in sublateral dorsal nucleus (SLD), and are either glutamatergic or GABAergic (Arrigoni et al., 2016; Lu et al., 2006; Luppi et al., 2012; Luppi & Fort, 2019). The application of $GABA_A$ receptor antagonists on SLD neurons induces typical features of REM sleep like desynchronized brain activity, loss of muscle tone and absence of response to stimuli (Boissard et al., 2002). Chemogenetic activation of glutamatergic SLD cells enhances REM sleep (Erickson et al., 2019) while lesions of the SLD showed a reduction in REM sleep (Clement et al., 2011).

Activation of a neural population that expresses melanin-concentrating hormone (MCH) and lies in the lateral hypothalamus is observed during REM sleep (Vyazovskiy & Delogu, 2014). Also, application of MCH into the SLD increased REM sleep (Torterolo et al., 2009). The PPT and LDT contain cholinergic neurons that display activity during both wake and REM (Boucetta et al., 2014). Optogenetic activation of cholinergic neurons of the PPT/LDT during nREM sleep induced REM sleep and elevated REM sleep episodes (Van Dort et al., 2015).

These data indicate that generation and maintenance of REM sleep generation and maintenance involves what Peever and colleagues refer to as a '*distributed network of circuits'* that involves the brainstem, midbrain and hypothalamus (Peever & Fuller, 2017). Numerous cells contribute to the generation of sleep through SWS/REM-on (sleep on) or SWS/REM-off (wake on) mechanisms.

1.2c Respiratory Control in Sleep

Momentary pauses and shallow breaths are defined as apneas and hypopneas, respectively, and they occur most frequently during sleep. These events cause changes in blood gas levels (O_2 desaturation, hypercarbia) that can affect health in both the short and long term. Sleep represents a behaviorally depressed state, with specific sleep states having variable impacts on breathing (seen in table 1.1).

At the onset of nREM sleep, breathing may become periodic and eventually stabilize with progression into deeper nREM stages (Buchanan, 2013). During nREM sleep, minute ventilation is reduced compared to wakefulness, airway resistance is

augmented, but muscle tone is unaltered from wakefulness (Buchanan, 2013; Colten, Altevogt, Institute of, et al., 2006).

One defining physiological feature of REM sleep is the complete loss of tone in the postural and orofacial muscles withoutimpact to the diaphragm, as well as the appearance of phasic muscle twitches, (Arrigoni et al., 2016). The loss of muscle tone during sleep significantly contributes to obstructive breathing disorders that are exacerbated during sleep (Arrigoni et al., 2016). Unlike nREM, where respiration progressively becomes regular, REM sleep is associated with irregularities in breathing frequency and amplitude (Buchanan, 2013; Colten, Altevogt, Institute of, et al., 2006; Phillipson, 1978). Despite its indispensable nature, sleep has consequential effects on breathing, specifically during the neonatal period when a significant amount of time is spent sleeping in both rodents and humans and both the respiratory system and the respiratory neural networks have not fully matured (MacLean et al., 2015; Peirano et al., 2003; Saini & Pagliardini, 2017b). Several changes induced by nREM and REM sleep in other physiological processes are outlined in table 1.1.

1.3 Rodent and Human Development Parallel Comparisons

Both sleep and respiration are processes that begin developing in the prenatal period and dramatically change postnatally. The embryonic preBötC is responsible for breathing movements in utero, which contribute to the development of motoneurons innervating respiratory muscles and the growth of the lungs that will accomplish the pivotal task of gas exchange (Fortin & Thoby-Brisson, 2009). Similarly, evidence of sleep is observed in utero in both humans and rodents (Cosmi et al., 2003; Kobayashi et al., 2001) and both processes undergo developmental changes from the prenatal to postnatal period. The developmental processes and changes that occur through development are discussed below.

1.3a Respiration

Evidence of fetal breathing movements can be observed in rats beginning on E17 (Greer et al., 1992; Kobayashi et al., 2001), in mice at E15 (Thoby-Brisson et al., 2005; Viemari et al., 2003) and in humans in the second trimester (Cosmi et al., 2003;

Kitterman, 1996). This suggests that although the development of the respiratory network continues postnatally, the rhythmic activation of respiratory muscles begins in the prenatal period.

The phrenic motoneurons (PMNs) control the activity of the diaphragm and they are responsible for the generation of FBMs (Greer et al., 2006). The period between E12 and E17 plays an important role in the development and migration of PMNs as well as the onset of diaphragmatic contractions and FBMs in embryonic rats (Greer et al., 2006).

The human homologue of the preBötC has been anatomically identified in the ventrolateral region of the medulla by the same anatomical markers present in rodents and other species (Schwarzacher et al., 2011), although no studies have identified the preBötC in the early neonatal or postnatal period. The RTN/pFv, which has been identified by the expression the PHOX2B transcription factor in rodents (Guyenet et al., 2008; Mulkey et al., 2004; Stornetta et al., 2006; Takakura et al., 2008) has been also identified in adult humans (Levy et al., 2021; Rudzinski & Kapur, 2010).

Changes in neurochemical and physiological processes occur with postnatal brain development. For example, switch of neurotransmitters and receptor subunits from excitatory to inhibitory can be observed during the postnatal period and correlates with a critical period during which neonates are in a vulnerable state and may fall victim to external stressors (more below) (Wong-Riley & Liu, 2008). In the neonatal rat, this critical period is observed at ~P12-13 which is suggested to correspond with 4-6 months of life in postnatal human infants (Moon et al., 2007; Wong-Riley & Liu, 2008).

1.3b Sleep

The predominant behavioural state in the neonatal period of rodents and humans is sleep (Jouvet-Mounier et al., 1970; Roffwarg et al., 1966), a physiological state that has been proposed to contribute to brain development (Anders & Keener, 1985; Roffwarg et al., 1966). In the first few months of life, REM sleep, also known as paradoxical sleep (Roffwarg et al., 1966) is the predominant state. The term paradoxical sleep comes from the expression of high neural activity coupled with a behaviourally suppressed state (Mirmiran et al., 2003).

In humans, sleep can be first identified between gestational ages 28-32 weeks (Graven, 2006; MacLean et al., 2015; Mirmiran et al., 2003; Suwanrath & Suntharasaj, 2010). Startles are one of the first behaviours observed in humans in the *in-utero* period; interestingly, startles are also exhibited by rodents but occur in the postnatal window (Blumberg et al., 2014; Peirano et al., 2003; Seelke & Blumberg, 2008).

For altricial mammals like rodents and humans, full brain development occurs postnatally, which correlates with time spent in the neonatal precursor for REM sleep, AS (Mirmiran et al., 2003). At term the human brain is only 27% of the adult human brain weight, whereas at birth the rat brain is only 12% of the weight of an adult human brain (Dobbing & Sands, 1979) and 15% of the final rodent brain (Cirelli & Tononi, 2015). Thus, at birth the rat brain is equivalent to that of a premature human brain, and by around post-natal day (P)10 rats have undergone a large degree of neural development including neurogenesis, myelination and synaptic connections for a relatively more matured brain (Cirelli & Tononi, 2015; Clancy et al., 2007; Gramsbergen, 1976; Saini & Pagliardini, 2017b; Seelke & Blumberg, 2008). The immature pattern of activity of the rodent brain in the first days of life makes EEG an implausible measure of sleep states, leading to the need for behavioural/physiological criteria (MacLean et al., 2015; Seelke et al., 2005). In rodents, EEG becomes reliable approach to identify sleep states in the second postnatal week. Similarly, in human newborns, EEG alone is not sufficient to distinguish between sleep states and video is required alongside polysomnography (Dereymaeker et al., 2017)

In both rodent and human neonates, sleep can be classified as QS and AS, and mostly in humans an IS (indeterminate sleep) that shares features of both QS and AS, can be identified (MacLean et al., 2015; Seelke & Blumberg, 2008). The criteria defining QS includes regular breathing and heart rate, behavioural quiescence and in humans an EEG pattern marked by variable frequency and large amplitude (MacLean et al., 2015; Seelke & Blumberg, 2008). Respiratory frequency and heart rate become variable and increase during AS compared to QS. AS is associated with behavioural quiescence that occurs on a background of myoclonic twitches, rapid movements of the eyes and an EEG pattern in humans that displays low frequency and an assorted frequency (MacLean et al., 2015). In rodent, EEG activity does not show evidence of slow wave oscillations and delta or theta

wave patterns until P11 (Cirelli & Tononi, 2015; Feng & Vogel, 2000). After P11 EEG activity during sleep is similar in rodents and humans, where nREM sleep presents as slow wave activity or delta waves and during REM a hippocampal theta pattern (Cirelli & Tononi, 2015; Feng & Vogel, 2000).

In rodents, the average sleep cycle ranges from 1-3 minutes during the first two postnatal weeks and increases to 10-20 minutes in adulthood (Saini & Pagliardini, 2017b; Weber & Dan, 2016). In humans, the sleep cycle ranges from 6-9 minutes in infants to 90-120 minutes as adults (Curzi-Dascalova et al., 1988; Palm et al., 1989; Weber & Dan, 2016). In neonatal rats aged P0-P15 the length of AS cycle varies from 104 seconds to 32 seconds and decreases with increasing age (Saini & Pagliardini, 2017b).

It has been proposed that in the neonatal period, development of the brain activity, specifically of the sensory systems is promoted by the large amount of time spent in AS in both humans and rodents (Anders & Keener, 1985; Graven & Browne, 2008; Peirano et al., 2003; Peirano & Algarin, 2007). The amount of time that humans spend in AS declines from 75% to 10% in the first 4 weeks (Jouvet-Mounier et al., 1970) and in rats declines from 50% to 13% in the second postnatal week (Saini & Pagliardini, 2017b). Studies in which animals were specifically deprived of AS caused a reduction in brain size and many cognitive disorders such as anxiety, hyperactivity (Peirano & Algarin, 2007). Literature on both neonatal humans and rodents propose that sleep is a vulnerable but necessary behaviour that contributes to better psychological and physiological health in later life (Anders & Keener, 1985; Peirano et al., 2003).

1.4 Sleep and Breathing

The term “sleep disordered breathing” (SDB) describes a wide variety of respiratory syndromes that occur during sleep. Sleep induces changes in respiratory stability and dampens chemoreception. The drive to breathe is regulated by central and peripheral chemoreceptors that respond to fluctuating blood gas levels of oxygen and carbon dioxide (Goridis et al., 2010). The brain areas responsible for central chemoreception have been identified and it has been suggested that distinct brain regions may play different roles based on arousal state, or stimulus intensity (Nattie, 1999).

1.4a Sleep Disordered Breathing

Sleep disordered breathing conditions consist of an array of pathologies that can manifest as snoring, central, obstructive, or mixed sleep apneas, sleep related hypoventilation, hypoxemia (Foldvary-Schaefer & Waters, 2017; Ivanhoe et al., 2007; Katz et al., 2014; Marcus, 2001; Owens, 2009). In Canada and United States approximately 1-5% of children are afflicted by SDB although this may be an underestimation with increasing number of kids with childhood onset obesity (Gipson et al., 2019; Marcus et al., 2012).

In the pediatric population, SDB is linked to many comorbidities, such as cardiovascular, psychological, cognitive and behavioural impairments that may influence attention, memory capacity and academic performances (Gipson et al., 2019; Ivanhoe et al., 2007; Marcus et al., 2012; Owens, 2009; Wildhaber & Moeller, 2007). Certain respiratory conditions occur exclusively during sleep, like sleep apnea, whereas other ventilatory disorders may be exacerbated by sleep, such as in central congenital hypoventilation syndrome (CCHS) (Marcus, 2001; Trang et al., 2005). In the neonatal population, SDB also encompasses apnea of prematurity, sudden infant death syndrome (SIDS) and apparent life-threatening events (ALTE) (Ross & Rosen, 2014).

The occurrence of SIDS is most common in human infants between 2-4 months of age, a period suggested to correlate with significant changes in neurotransmitters and receptors expression in the brain, i.e., the critical period mentioned above (Moon et al., 2007). The Triple Risk Model proposed by Filiano and Kinney suggests that three circumstances converge to create a specifically unstable environment for infants (Filiano & Kinney, 1994). The three factors are 1) an at-risk infant, that experiences: 2) external stressors during a 3) critical period window in postnatal maturation (Filiano & Kinney, 1994). Many studies investigated the developmental changes in different regions of the brain in infants with SIDS and several changes in neurochemical and receptor systems have been identified (Huang et al., 2017; Kinney et al., 1995; Kinney et al., 2001; Machaalani & Waters, 2014; Porzionato, Macchi, et al., 2018).

The relevant developmental changes in sleep occurring in the postnatal life include: 1) a progressive increase in time spent in QS, 2) a transition in sleep that commences with AS in early neonatal life to QS and 3) the duration of sleep occurring

during the night is the longest (Mirmiran et al., 2003). The postnatal period is important for brain development and correlates closely to the time during which there is concurrent heightened vulnerability to SIDS or ALTE. SDB can occur at any age and developmental changes throughout life and state dependent changes can impose conditions that make individuals more vulnerable.

1.4b Central Congenital Hypoventilation Syndrome and Central Chemoreception

CCHS is genetic disorder that affects the autonomic control of breathing and the ability to respond to changes in arterial CO_2 (Amiel et al., 2003; Chen & Keens, 2004; Ginsburg et al., 2021; Goridis et al., 2010; Marcus, 2001). Hypoventilation in CCHS patients is generally worsened with sleep, but in more severe cases it can also occur during wakefulness (Ginsburg et al., 2021; Goridis et al., 2010; Weese-Mayer et al., 2005). CCHS is associated with a mutation in the PHOX2B gene. PHOX2B is a transcription factor that plays a pivotal role in the neural development of the autonomic nervous system (Pattyn et al., 1999). The most typical mutation causing CCHS is a polyalanine repeat expansion in the PHOX2B gene and the severity of the disease is proportional to the polyalanine expansion (Amiel et al., 2009; Amiel et al., 2003; Goridis et al., 2010; Weese-Mayer et al., 2005).

Rodent models of CCHS in which the mutated PHOX2B gene is inserted in their genome, show lack of chemosensitive response to hypercapnia and present with fatal central apneas within the first few hours after birth (Amiel et al., 2009; Dauger et al., 2003; Dubreuil et al., 2008; Durand et al., 2005). Anatomical analysis of these mutants indicates that the PHOX2B mutation alters the development and function of the RTN and selective expression of the mutant PHOX2B in RTN neurons replicates the chemoreflex impairment observed in mutant mice and in CCHS patients (Amiel et al., 2009; Goridis et al., 2010).

1.5 Current Treatments and Sex Steroid Hormones

1.5a Current Treatments and Limitations

The adverse effects of SDB on health start in the early neonatal period and have lasting effects into adulthood. Late onset of SDB is also common and may have important cardiovascular and metabolic consequences in the adult population.

In order to reduce the occurrence of SIDS, the "back to sleep campaign" encouraged infants sleeping in supine position rather than side or prone position to favour airflow and was associated with a significant decline in SIDS amongst infants (Trachtenberg et al., 2012; Wildhaber & Moeller, 2007). Additional campaigns to reduce risk factors such as smoking during pregnancy, infants smoke exposure, excessive room and body temperature, covering of the infants head, feeding with a bottle, poor sleeping environment and bed sharing have also reduced the occurrence of SIDS (Wildhaber & Moeller, 2007).

Premature infants are also at risk of developing sleep apneas (Marcus et al.) due to an underdeveloped respiratory network and mechanics (Di Fiore et al., 2013). For half a century, xanthine's have been utilized as a treatment for apnea of prematurity (Di Fiore et al., 2013). Xanthine's and their derivatives like caffeine are the treatment of choice for respiratory potentiation (Bairam et al., 2015). They exert their stimulant effects on ventilation through adenosine receptors mediated effect that downstream increased neural output (Di Fiore et al., 2013; Joseph et al., 2013). Long-term use and side effects at different age groups of methylxanthines remain unclear (Orff, 2014). Zhao and colleagues, suggest that a high dose of methylxanthines elevates the risk of tachycardia, cardiac dysrhythmia and provokes occurrence of seizures in premature infants (Zhao et al., 2011). Because some infants do not respond to methylxanthine treatment, it has been propose to use alternate methods and, given the known role of progesterone as respiratory stimulant (Loiseau et al., 2018), the use of a combination of caffeine and progesterone has been proposed (Bairam et al., 2015; Uppari et al., 2016; Uppari et al., 2017).

While the treatment with methylxantines offers a treatment option for a large proportion of infants with apnea of prematurity, no effective treatment have been

proposed in conditions of central hypoventilation, such as CCHS and the only management option is with mechanical intervention (Loiseau, 2017).

1.5b Sex Steroid Hormones and Male and Female Differences

Steroid hormones are produced predominantly in the adrenal glands, gonads and the placenta, but they can also be produced centrally in the brain. Cholesterol is the precursor of sex steroid hormones and is converted to sex hormones via intricate enzymatic processes (Behan et al., 2003). Progesterone can elicit its effects including those on ventilation through nuclear or membrane progesterone receptors (Behan et al., 2003; Joseph et al., 2013). More rapid non-genomic effects of progesterone can be observed through interactions with membrane receptors, whereas slower effects are exerted through genomic nuclear receptors (Behan et al., 2003). A series of studies performed in different male and female animal models including guinea pigs (Hosenpud et al., 1983), rats (Brodeur et al., 1986; Tatsumi et al., 1991), cats (Bayliss & Millhorn, 1992), and humans (Straus et al., 2010) indicate administration of progesterone or its synthetic derivatives have stimulating effects on respiration.

Some of the first studies suggesting that female sex steroid hormones may be contributing to ventilation occurred over a century ago (Fitzgerald & Haldane, 1905; Hasselbalch & Gammeltoft, 1915) and, to date, many investigations aimed to explore the mechanisms and regions through which progesterone may elicit its effects (Bairam et al., 2019; Boukari et al., 2017; Boukari et al., 2015; Loiseau, 2017; Marcouiller et al., 2014). Investigations in animal models suggest that sex steroid hormones effects vary based on many factors including sex, receptor type, age, and estrus cycle phase (Bairam et al., 2019; Bairam et al., 2015; Boukari et al., 2016; Joseph et al., 2020; Joseph et al., 2018; Lefter et al., 2007; Marques et al., 2017). The central and peripheral regions potentially implicated in progesterone-induced respiratory augmentation include the serotonergic neurons in the raphe nuclei, the NTS, the ventral tegmental area, the hypoglossal nucleus, the locus coeruleus, the PB, the hypothalamus and the peripheral chemoreceptors in the carotid bodies (Behan & Wenninger, 2008; Behan et al., 2003; Curran-Rauhut & Petersen, 2002; Hannhart et al., 1990; Loiseau et al., 2018).

Many studies investigating respiratory disturbances during sleep in adult men and women conclude that men experience SDB syndromes more frequently than women, and women do so after menopause, indicating a protective role for female sex steroid hormones (Block et al., 1979; Boukari et al., 2017; Cistulli et al., 1994; Lozo et al., 2017; Orff, 2014; White et al., 1983). To test this hypothesis, Skatrud and colleagues administered exogenous medroxyprogesterone acetate in healthy adult males to find that it increased ventilation in rest and in exercise (Skatrud et al., 1978).

Both pregnancy and the luteal phase of the estrous cycle correlate with heightened endogenous progesterone levels (Behan et al., 2003). Interestingly, pregnant women exhibit periods of hyperventilation (Behan et al., 2003; Brodeur et al., 1986; Skatrud et al., 1978) and maintenance of healthy oxygenation (Saaresranta & Polo, 2002). Also, premenopausal women during the luteal phase of their menstrual cycle display reductions in arterial P_{CO2} and have reduced upper airway resistance compared to the follicular phase (Driver et al., 2005; England & Farhi, 1976; Goodland & Pommerenke, 1952; Griffith, 1929; White et al., 1983). A reduction in serum levels of female sex hormones in postmenopausal was associated with an increase in OSA symptoms such as snoring, abnormal breathing and gasping (Saaresranta & Polo, 2002; Sigurðardóttir et al., 2022), and a reduction in genioglossal activity that was restored with hormone replacement therapy (Driver et al., 2005).

Investigation of hormone replacement therapy with progesterone and estrogen in women with postmenopausal SDB onset displayed a decrease in number of events and duration of abnormal breathing episodes (Pickett et al., 1989), although various studies gave variable results in terms of ventilatory potentiation and improvement of SDB events (Cistulli et al., 1994; Joseph et al., 2013; Lindberg et al., 2020; Loiseau et al., 2014; Saaresranta & Polo, 2002).

1.5c Etonogestrel

Etonogestrel (ETO) is a synthetic progestin that belongs to the gonane family and binds to progesterone receptors with a 25-times greater affinity than progesterone itself (Bergink et al., 1981; Loiseau et al., 2014). A fortuitous study demonstrated a recovery in the ventilatory response to CO2 of two women affected by CCHS upon administration of

desogestrel (75 µg/day) (Straus et al., 2010). Desogestrel is metabolized to its active ingredient ETO and it is used for contraceptive purposes.

The serendipitous findings with the two CCHS patients was the first study to indicate a partial recovery in the chemoreflex response upon administration of etonogestrel (Straus et al., 2010). Follow up investigations further demonstrated potentiation of basal breathing in CCHS patients as well as respiratory stimulation of respiratory function in *in vivo* and *in vitro* perinatal rodents (Joubert et al., 2016; Straus et al., 2010) possibly through supramedullary structures and the serotonergic systems (Casciato et al., 2022; Joubert et al., 2016; Loiseau et al., 2014). *In vitro* work in neuroblastoma cell lines expressing PHOX2B and progesterone receptors indicates that administration of etonogestrel alters expression of PHOX2B and its target genes (Cardani et al., 2018), therefore linking the effects of ETO on activation of nuclear progesterone receptors and changing the expression of PHOX2B gene and its genomic targets.

Collectively, these investigations prompted our lab to explore the relationship between ETO administration, PHOX2B and progesterone receptors in regions of the brain that control breathing (Cardani et al., 2022). We showed that ETO administration in healthy female rats caused an hyperventilatory response due to the reduction on metabolic activity in both normoxia and hypercapnia. Further analysis of gene and protein expression in the brain pointed to the effect of ETO on the NTS since expression of PHOX2B and some of its known target genes were selectively reduced in this brain structure, further supporting previous *in vitro* results (Cardani et al., 2018). More recently, Casciato et al. used an *in vitro* rodent model of CCHS, with a 20-residue polyalanine stretch in Phox2B, to show that ETO recovered chemosensitivity and increased activity in serotoninergic neurons in the raphe obscurus nucleus (Casciato et al., 2022). The recent research in this area has sparked interest to understand how ETO induces changes in gene expression and affect breathing. My contribution to this research are reported in Chapter 4.

1.6 Thesis Aims and Hypothesis

The brainstem is the central command center for respiration, it receives inputs from chemoreceptors and upper levels of the brain, and in turn sends output signals to

respiratory motoneurons that innervate the respiratory muscles and facilitate movement of air in and out. Respiration occurs incessantly, and although subjected to significant developmental, environmental and state dependent changes, it remains a resilient process. Despite its general stability, breathing is prone to irregularities like apneas and hypopneas that are more frequently observed during sleep. Such disturbances can occur due to developmental defects of the respiratory system, neural networks, a perturbation in the rhythmic or central pattern generation and/or the development of pathological conditions that influence neural circuits involved in the control of breathing (Di Fiore et al., 2013). Onset of SDB can occur right at birth or begin later in life and poses imminent and life-long health risks. In my thesis, I focused on how activation of abdominal muscles and generation of expiratory activity contribute to ventilation in the postnatal period of rats and humans. I also explored the effect of ETO on ventilation in adult female rats that have a perturbation to central chemoreception.

Breathing and sleep change dramatically in the early postnatal period and breathing is most fragile in infancy during sleep, especially in prematurity. Sleep irregularities and respiratory events (apnea, O_2 desaturation or a combination thereof) are often present in the infant population. While inspiration is the main active process in the act of breathing, expiration is generally thought to occur passively. Although commonly considered as quiet during sleep, expiratory abdominal muscles have been proposed to be recruited to promote ventilation, facilitate gas exchange and reduce the work of breathing during conditions of increased respiratory drive, exercise or airway obstruction.

In the first project of my thesis, I characterized the respiratory parameters across the first two weeks of postnatal development and across sleep states in rats. In this study, I also investigated the relationship between respiration and expiratory muscle recruitment during sleep. This aspect of the analysis was motivated by research from our laboratory, which demonstrated that despite REM induced atonia of the skeletal muscles, adult rats exhibited periods of abdominal muscle activation during REM (Andrews & Pagliardini, 2015). Based on these data we hypothesized that infant rodents would spend a significant amount of time in sleep and, during that time, they would also display expiratory modulated abdominal muscle activation. Further analysis was performed to assess how respiratory rhythm changed with abdominal recruitment and across both development and

sleep states. To accomplish this, I instrumented neonatal rats from P0-P14 with EMG electrodes in neck abdominal and intercostal muscles. The nuchal muscle tone along with the detection of behaviour via video recording allowed me to determined sleep states (AS and QS), and EMG electrodes in the intercostal and abdominal muscles allowed me to identify inspiration and activation of expiratory muscles through the respiratory cycle.

In the second project of my PhD thesis, I extended the investigation to the activation of abdominal muscle in human infants during sleep. I investigated the occurrence of expiratory modulated abdominal muscle activity from polysomnographic studies of infants (0-2 years old) suspected of SDB. My objective was to determine whether human infants also exhibited activation of the abdominal muscles during sleep and how this correlated with respiratory events. I hypothesized that like infant (Saini & Pagliardini, 2017b) and adult rodents (Andrews & Pagliardini, 2015), human infants will experience periods of abdominal activation during sleep.

I performed a retrospective study on 72 polysomnography recordings provided by our collaborator, Dr. Joanna MacLean, pediatric respirologist at the University of Alberta. The dataset included subjects aged 0-2 years old that underwent polysomnographic study because they were suspected of obstructive sleep apnea in absence of other cofounding factors. The polysomnography captured data that measured EEG, EOG, and submental EMG which was unique for our study as they this dataset also measured abdominal muscle activity.

In the fourth chapter of my PhD thesis, I was motivated by the results of the ETO administration in CCHS patients to investigate the respiratory effect of ETO in healthy rats and in a rat model of central chemoreflex impairment.

I hypothesized, that like adult women, female rats with an impaired CO_2 chemoreflex would also show improvement in their hypercapnic response after ETO administration. Impairment of the CO_2 chemoreflex response was achieved through microinjection of substance-P saporin into the RTN, a key component of the CO_2 chemoreflex. The ventilatory responses to normoxia, hypercapnia and hypoxia were tested within a whole-body plethysmograph during baseline and gas challenges before and following ETO delivery.

Table

Table 1.1 Physiological Changes During nREM and REM Sleep.

Physiological Process	*nREM*	*REM*
Brain activity	Decreases from wakefulness	Increases in motor and sensory areas, other areas similar to nREM
Heart rate	Reduced from wakefulness	Increases and varies from nREM
Blood pressure	Reduces from wakefulness	Increases and varies from nREM
Respiration	Decreases from wakefulness	Increases and varies from nREM, may be brief pauses
Muscle Tone	Similar to wakefulness	Absent
Airway resistance	Increases from wakefulness	Increases and varies from wakefulness
Blood flow to brain	Reduces from wakefulness	Increases from nREM, depending on brain region
Sympathetic nerve activity	Decreases from wakefulness	Increases significantly from wakefulness
Body temperature	Regulated, reduced from wakefulness	Not regulated

Table delineating the physiological changes from wakefulness to nREM and then from nREM to REM. *Adapted with permission from Colten 2006*

Figures

1.1

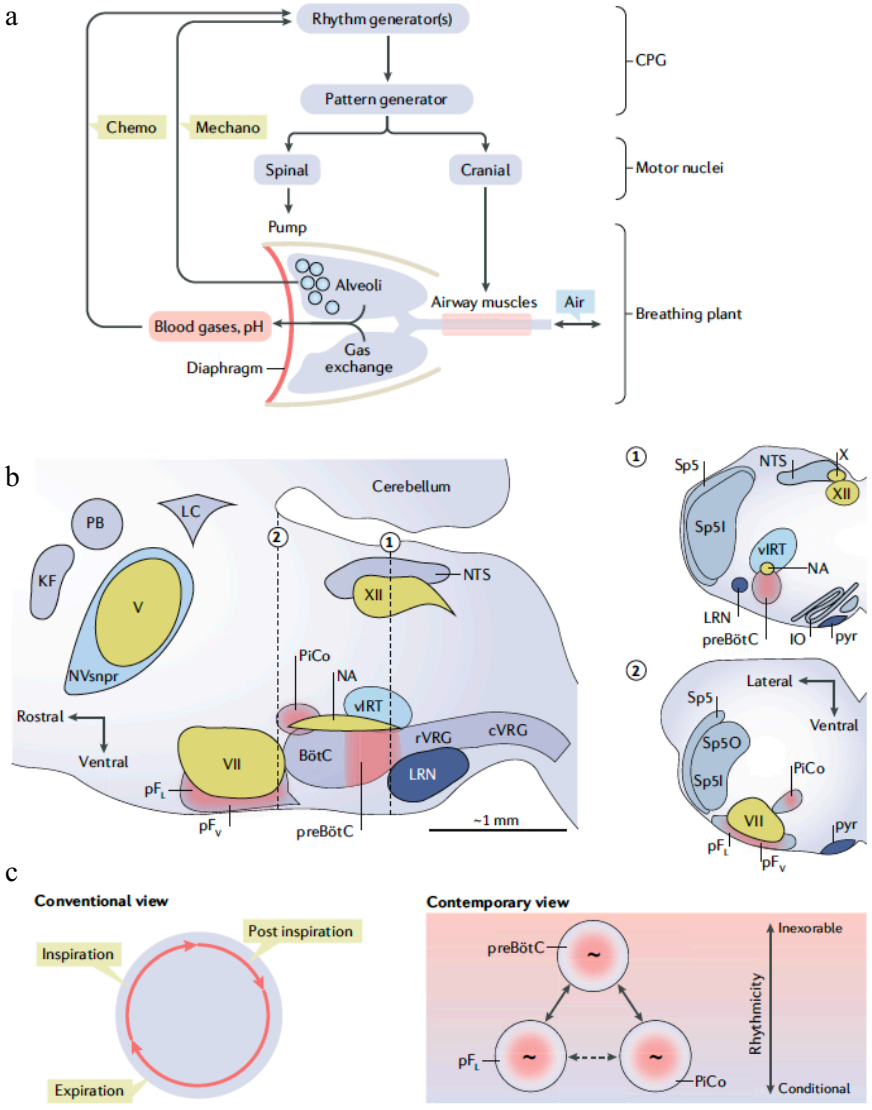

Figure 1.1. **Components of the breathing central pattern generator.** a) Diagram of the central pattern generator (CPG) that contains the rhythm and pattern generating components, the motor nuclei involved in transmitting central and spinal signals to the structures that facilitate movement of air in and out of the lungs. The breathing plant is inclusive of the lungs, diaphragm, airway muscles and blood gases which send chemosensory feedback whereas the alveoli send mechanosensory feedback to the rhythm generator.b) Parasagittal visual of the brainstem that comprises the CPG. Red indicates the rhythmogenic respiratory areas, which include the preBötzinger Complex

(preBötC; inspiratory), the pF_L (expiratory) and the more medial chemosensitive ventral parafacial respiratory group (pF_V; rhythmogenic in the perinatal period only), as well as the 'postinspiratory complex'(PiCo; hypothesized to underlie postinspiration). In green the cranial motor nuclei that govern the airway resistance muscles are illustrated, this includes the hypoglossal motor nucleus (XII) and the nucleus ambiguus (NA), as well as facial muscles, the facial motor nucleus (VII) and the trigeminal motor nucleus (V). The regions that are involved in regulating the respiratory motor pattern or sensorimotor integration are displayed in grey and include the rostral ventral respiratory group (rVRG) containing inspiratory (phrenic and external intercostal), premotor neurons and the caudal ventral respiratory group (cVRG) containing expiratory premotor neurons, also the pontine Kölliker- Fuse nucleus (KF) and parabrachial nucleus (PB), the nucleus of the solitary tract (NTS) and the expiratory BötC. The two supplementary transverse views 1 and 2 are sliced at the preBötC (dotted line 1) and pF (dotted line 2). c) The conventional representation of respiration, inspiration, postinspiration and expiration which create a single breathing cycle. Alternatively, the contemporary interpretation, inspiration is the necessary part of the respiratory cycle, and the conditional components are postinspiration and expiration. These three microcircuits are facilitated through three unique and coupled oscillators. *Used with permission from Del Negro et al., 2018.*

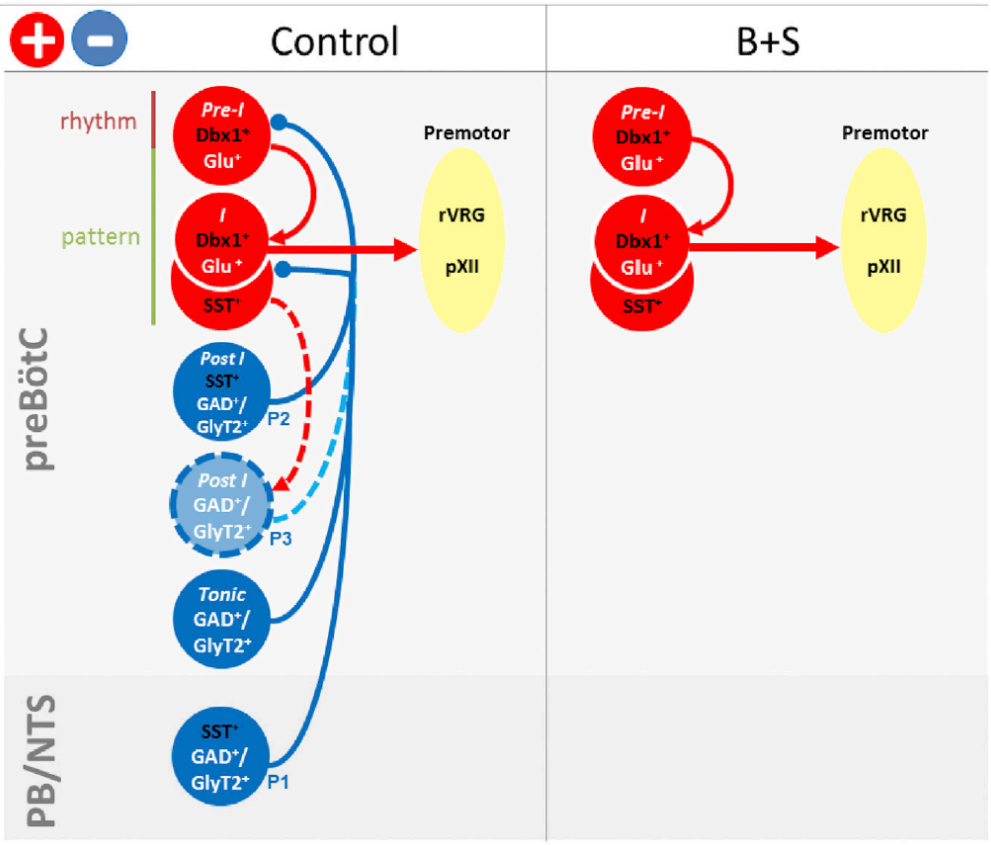

Figure 1.2. **Subpopulations of preBötC neurons involved in the respiratory rhythm and pattern generation model and their functional roles.** Schematic shows the connections within neural subpopulations in control conditions (Lefter et al.) and after administration of bicuculline and strychnine to the preBötC (right). Cui et al. (2016) suggest that preBötC burst (pattern) generation is a two-stage process consisting of a low-amplitude rhythmogenic preinspiratory component (Pre-I) and a high-amplitude pattern-generating inspiratory burst (I). The figure describes the functional roles in the preBötC, the parabrachial nucleus and the nucleus of the solitary tract and how they contribute to either: rhythm or pattern generation through excitatory inputs or moduatle these processes through inhibitory mechanisms. *Used with permission from Cui et al., 2016.*

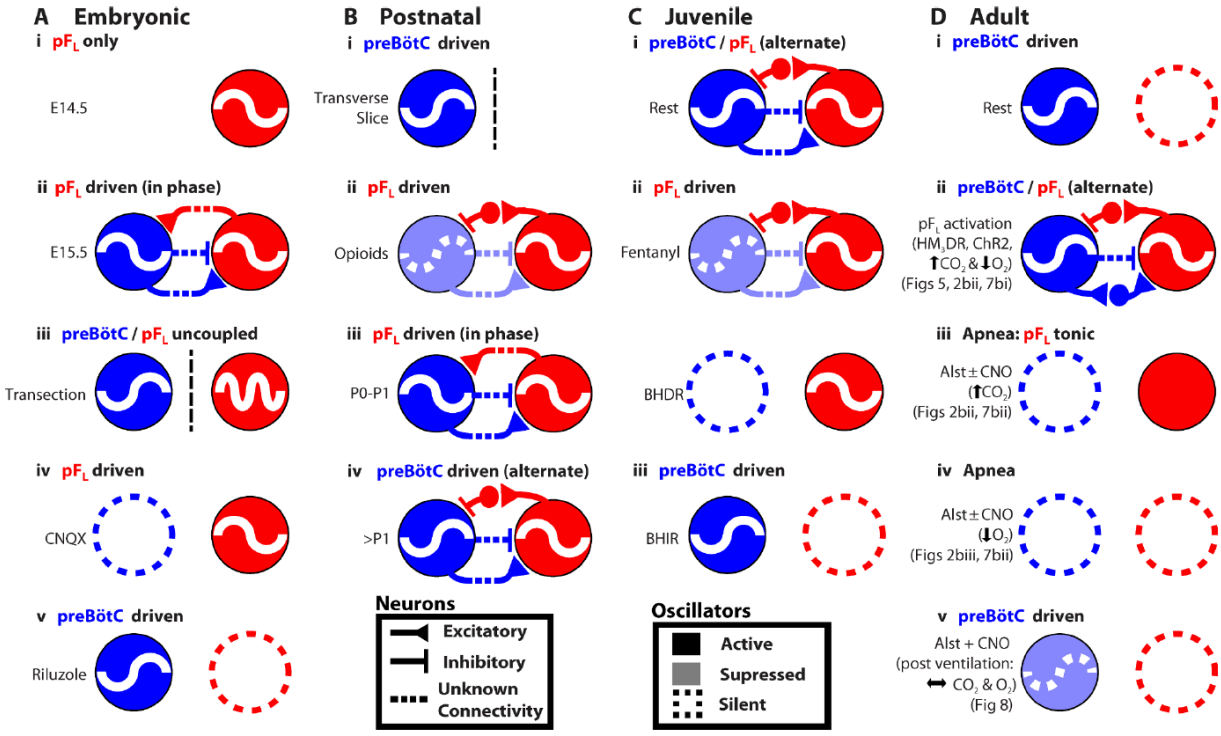

Figure 1.3. Developmental and state-dependent changes in coupling between pF_L and preBötC. The unknown connectivity is displayed as broken lines. The pF_L neurons are excitatory and are absent of inhibitory markers, but can send inhibitory connections to the preBötC directly. A-D depict the relationship between the preBötC and the pF_L at different phases from embryonic (A), postnatal (B), juvenile (C) and in adult (D) rodents. Each component of the columns (i-v) demonstrate different manipulations to demonstrate if the oscillator is active, suppressed or silent and if the connectivity between the preBötC and the pF_L is excitatory, inhibitory or undetermined.

Of notable consideration is Aii and Bii Cii) a period in the embryonic, postnatal and juvenile stage of development where activity is driven by the pF_L. *Used with permission from Huckstepp et al., 2016.*

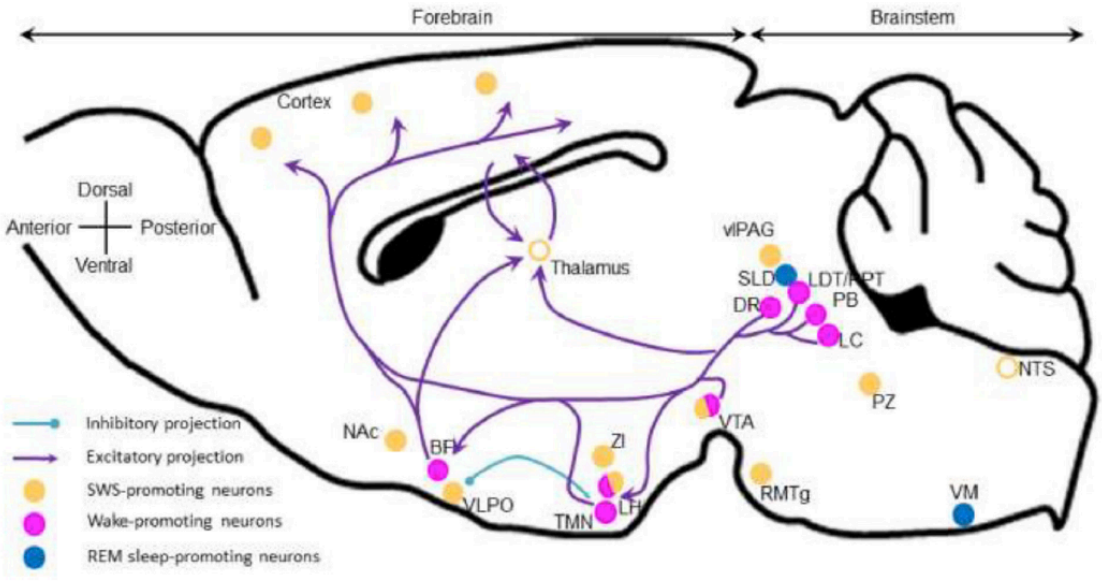

Figure 1.4. Saggital view of mouse brain forebrain and brainstem illustrating wake-promoting systems and their projections. The wake promoting neural populations can be found in the brainstem and the forebrain, and project to the cortex to induce activity in the cortex and result in wakefulness. The wake promoting neurons in the tuberomammillary nucleus inhibit the ventrolateral preoptic area which is a predominate sleep promoting area. The open circles are regions in the brain that contribute to sleep control but are not sleep promoting. *Used with permission from Gompf et al., 2020.*

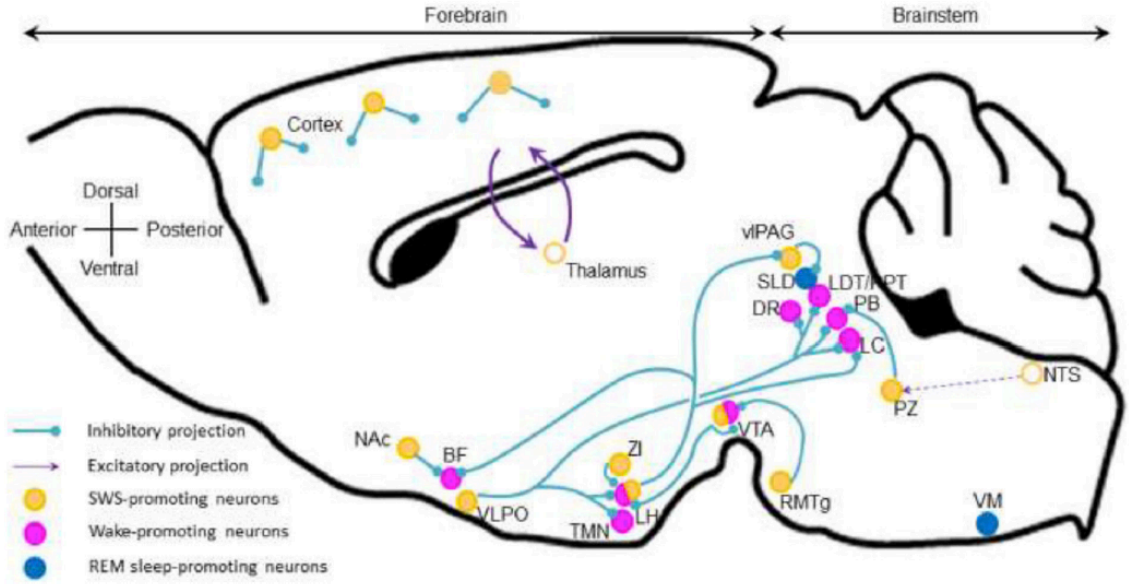

Figure 1.5 Saggital view of mouse brain forebrain and brainstem illustrating nREM-promoting systems and their projections. The neurons that are nREM promoting are spread throughout the neural axis and in the cortex. These cells are primarily GABAergic and they inhibit wake-promoting neurons. The ventrolateral preoptic area is a primary nREM promoting area that sends inhibitory projections to many wake-promoting systems in the forebrain and the brainstem. The open circles are regions in the brain that contribute to sleep control but are not sleep promoting. *Used with permission from Gompf et al., 2020.*

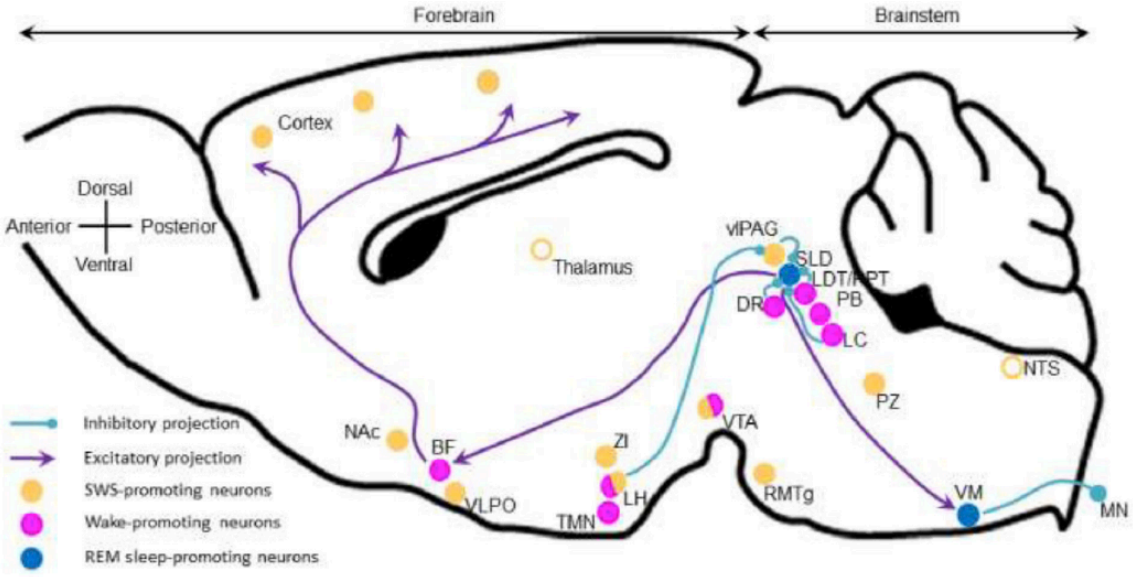

Figure 1.6 Saggital view of mouse brain forebrain and brainstem illustrating REM-promoting systems and their projections. REM sleep is facilitated through the sublaterodorsal nucleus which projects rostrally to activate cortical activity and projects caudally to the ventral medulla to inhibit muscle atonia. The REM-promoting cells of the SLD are glutamatergic and get inhibitory projections from the pontine wake-promoting neurons. The open circles are regions in the brain that contribute to sleep control but are not sleep promoting. *Used with permission from Gompf et al., 2020.*

Chapter 2: Breathing during sleep in the postnatal period of rats: the contribution of active expiration

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2.1 Introduction

Breathing is an essential physiological process under neural control that must be functional at birth and be able to adjust to a host of physiological challenges in the newborn period, such as postnatal lung and neuronal development, changes in pattern and dynamics of sleep/wake cycles and circadian rhythms, and the progressive development of the hypoxic and the hypercapnic ventilatory responses.

In the first days and weeks of life both human and rodent infants spend a large amount of time asleep. Active sleep (AS), the equivalent of adult rapid eye movement (REM) sleep, (Cirelli & Tononi, 2015; MacLean et al., 2015) dominates in the newborn, but it decreases progressively through development as more time is spent in quiet sleep (QS), the equivalent of adult nonREM sleep, and wakefulness (Blumberg et al., 2014; Blumberg et al., 2005; Cirelli & Tononi, 2015; Karlsson et al., 2004; MacLean et al., 2015).

Breathing in premature, and occasionally full-term human infants is characterized by frequent respiratory disturbances during sleep, in particular during active or REM sleep. Sleep-related breathing disorders in infants are characterized by an irregular breathing pattern that includes frequent depressions (hypopneas) or interruptions (apneas) of breathing that are obstructive, central, or mixed, in origin and are often associated with blood oxygen desaturation (Di Fiore et al., 2013). Sleep-related breathing disorders not only pose immediate risks for survival, but are also associated with serious and life-long health consequences, such as poor weight gain, abnormal motor skills, adverse behavioral changes, decreased cognitive function, maladaptive cardiovascular changes, and sympathetic activation (Baroni, 1992; Einspieler, 1994; Franco et al., 2003; Freezer et al., 1995; Liao et al., 2010; Loscher et al., 1990; O'Driscoll et al., 2009; Perfect et al., 2013).

In rodents, several studies have investigated developmental changes in respiratory and metabolic rate, respiratory stability, hypoxia-induced arousal from sleep, and development of the ventilatory responses to hypoxia and hypercapnia within the first three weeks of postnatal life (Abu-Shaweesh et al., 1999; Cameron et al., 2000; Carroll, 2003; Darnall et al., 2010; Darnall et al., 2016; Eden & Hanson, 1987; Gleed & Mortola, 1991; Huang et al., 2004; Liu et al., 2009; Liu et al., 2006; Liu & Wong-Riley, 2013; Putnam et al., 2005; Stunden et al., 2001). These developmental analyses of respiratory

behavior have established that ventilation becomes more stable with development, and the function of both peripheral and central chemoreceptors and the neuromodulatory systems that control breathing mature over the first postnatal weeks. Semiquantitative immunohistochemical analysis of neurotransmitransmitters and receptors expression have also identified a critical respiratory period at post-natal day (P) 13 where excitability appears to fall briefly (Wong-Riley & Liu, 2008). Around the same time in development, ventilatory and metabolic responses to hypoxia appear to be the weakest (Liu et al., 2009; Liu et al., 2006; Liu & Wong-Riley, 2013).

Importantly, the majority of the developmental studies involving physiological measurements were performed in animals that were either awake or in an unknown brain stat , or, alternatively, with limited (mostly observational) evaluation of sleep/wake state. This is understandable given the challenge of recording EEG activity from newborn pup,s and that EEG alone is not sufficient to define sleep state due to the lack of clear sleep state-defining cortical EEG features prior to P11 in rats (Cirelli & Tononi, 2015). However, given the impact of sleep state on respiratory control and the evidence that virtually all clinical disorders of breathing that involve the CNS manifest primarily in sleep, brain state information in respiratory studies is critical.

Observational methods for defining sleep state in neonatal rats are a significant improvement (Darnall et al., 2010; Darnall et al., 2016). However, the systematic analysis of nuchal EMG activity in combination with overt behavioral markers has increased our capacity to detect state-specific behaviors/control mechanisms and it has greatly advanced our understanding of the postnatal development of sleep dynamics in rodents (Blumberg et al., 2005; Cirelli & Tononi, 2015; Karlsson & Blumberg, 2002; Karlsson et al., 2005; Seelke et al., 2005). Further, the ability to record EMG activity directly from respiratory muscles (current study) gives us the opportunity to determine state dependent activity and modulation of muscles that control ventilation.

The first objective of this study was therefore to apply these tools to characterize how breathing pattern changes in rats across sleep/wake cycles in the first two postnatal weeks. The second objective of this study was based on our recent demonstration in freely moving adult rats that recruitment of expiratory muscles both reduced respiratory variability and increased minute ventilation in REM sleep (Andrews & Pagliardini,

2015). We therefore set out to determine if and when during postnatal development expiratory abdominal (ABD) muscles were recruited as well as the impact of this recruitment on ventilation across sleep/wake cycles.

Our developmental analysis of sleep states indicates a progressive consolidation of sleep toward the adult pattern by the end of the second postnatal week; a reduction in the time spent in AS in the second postnatal week was associated with extension of wake and QS time. Similar to adult rats and human infants, postnatal rats displayed increased respiratory variability in AS compared to QS at all age groups examined. Expiratory ABD activity was also present in all age groups and its occurrence and intensity progressively decreased with age in both QS and AS. Although ABD recruitment was not associated with changes in respiratory frequency or variability in QS, onset of expiratory ABD activity was associated with a reduction in respiratory variability in >P1 rats during AS and increased ventilation in P0-1 and P8.

2.2 Materials and Methods

Experimental procedures were approved by the Health Science Animal Policy and Welfare Committee of the University of Alberta according to the guidelines established by the Canadian Council on Animal Care.

Animals

A total of 41 Sprague-Dawley perinatal rats of post-natal (P) day P0 (n=6; within 10 hours of birth), P1 (n=6), P3-4 (n=10), P7-8 (n=10), P14-15 (n=9) were used in this study; for simplicity, the latter groups are indicated as P4, P8 and P15. An additional 12 rats were used to compare changes in respiratory rate and body temperature in non-instrumented conditions (n=3/age at P1, P4, P8 and P15). Pregnant rats were received from Charles River breeding facility (Montreal, Canada) at gestational day (E) 13-16 and housed at the University of Alberta. Dams provided care for their offspring in standard size cages, in which food and water were available *ad libitum* under a regular 12 hour light-dark schedule with lights on at 0800.

Surgery

Postnatal rats weighing between 5 (P0) and 55 (P15) grams were removed from the litter and anesthetized in a chamber saturated with isoflurane and maintained under 2% isoflurane anesthesia for the duration of the acute surgical procedure. Rats were maintained warm during instrumentation procedure with an electrical heating pad set at the lowest setting. Five-mm to one-cm long skin incisions were made in the region of the neck, thorax and abdomen. Paired electromyography (EMG) electrodes comprised of hook needles (Fine Science Tools, Canada) and multistranded, Teflon-coated, stainless-steel wires (AM-Systems, USA) were implanted in the nuchal, intercostal (INT; in ≥P4 rats) and ABD muscles to measure postural, inspiratory and expiratory muscle activity, respectively. The skin was sutured with a 6/0 non-absorbable nylon monofilament suture (Havel's, USA). The long-term acting local anesthetic bupivacaine (Marcaine) was applied subcutaneously (50-100 μL at 5mg/ml) at the incision sites to eliminate discomfort following instrumentation procedures. The surgical procedure on average lasted less than 30 minutes.

Cortical delta (0.5-4 Hz) activity, indicative of slow wave sleep emerges in rats at P11 (Seelke & Blumberg, 2008), thus, only P15 rats were instrumented with paired electroencephalogram (EEG) electrodes made of stainless steel screws (#00-96 x 1/16, diameter 1.19mm;Plastics One, USA) connected with Teflon coated stainless-steel wires (AM-Systems, USA). Screws were inserted in the skull, above the left frontal and parietal cortices as well as on top of the cerebellum to serve as a ground electrode (Seelke & Blumberg, 2008). Screws and wires were then fixed with dental acrylic to the rats' skull.

Recording of Respiratory Parameters and EMG/EEG Activity

Following EMG/EEG instrumentation, rats were placed in prone position inside a size-adjusted whole body plethysmographs (P0-1= 50ml; P4-8=500ml;P15= 260ml) directly warmed by a servo-controlled heating pad positioned beneath the plethysmograph and set at temperature of 37 ± 1°C (Harvard Apparatus, Canada). Ambient room temperature was set at 25°C. Body temperature was measured using a non-contact infrared thermometer (BeBetter, model #11-927, Canada) before and after plethysmograph recordings in the interscapular region. Surface body temperatures prior to recording procedures were: 35.5±0.4, 34.8 ± 0.3, 35.9±0.4, 35.6±0.4, and 35.1±0.3°C

in P0, P1, P4, P8 and P15 rats respectively. At the end of the experiment recorded surface body temperatures were 35.5±0.4, 35.0±0.2, 36.5±0.1, 35.9±0.6 and 35.4±0.3 °C in P0, P1, P4, P8 and P15 rats respectively. No significant difference in temperature between the two time points was observed for any age group investigated ($p>0.05$), suggesting a limited effect on body temperature during the recording session.

Rat pups at all ages were always in the prone position for the duration of the recording. Electrodes were channeled outside the recording chamber and connected to differential amplifiers (AM-Systems, USA). EMG/ EEG signals were recorded at 1 kHz using the Powerlab 16/30 data acquisition system (AD Instruments, United States). EMG and EEG signals were amplified at a gain of 10,000 and filtered between 100-500 Hz and 0.1-500 Hz, respectively. A constant flow of air was delivered through the plethysmograph chamber, respective to the size of the plethysmograph (80ml/min flow in 50ml plethysmographs, 450ml/min flow in 260 ml plethysmographs and 800ml/min flow in 500ml plethysmographs) and pressure fluctuations were recorded via a differential pressure transducer connected to a carrier demodulator (Validyne, USA). The pressure fluctuations resulted in a signal indicative of inspiratory and expiratory events. The airflow signal was high pass filtered with a frequency cut off of 0.01 Hz to eliminate small signal drifts during the recording session. Also, a video camera was set up to simultaneously record overt behavior in order to allow for analysis of sleep/wake states (Karlsson & Blumberg, 2002). A total recording session of two hours was collected for all age groups with the exception of P15 rats, which underwent a five-hour recording session in order to obtain sufficient periods of active sleep for analysis.

Data Analysis

The first hour of each recording session was excluded from analysis to ensure adequate recovery from the surgery and from the effects of anesthesia. Sleep states were confirmed using video recording (to observe overt behavior and myoclonic twitching), nuchal EMG (to measure muscle tone and body movements) and EEG activity (to measure cortical activity changes in P15 rats). Through video monitoring we were able to identify occurrence of myoclonic twitches against a background of nuchal muscle atonia, which are a defining feature of active sleep (AS), whereas, behavioral quiescence and

low nuchal activity were indicative of quiet sleep (QS) (**video S1**). Periods of coordinated motor movements like yawning, kicking and stretching were indicative of wakefulness (Karlsson & Blumberg, 2002). In P15 rats, EEG recordings provided further classification for sleep states as the convergence of low nuchal activity and fast low voltage cortical EEG are typical of AS whereas high power delta waves (0.5-4 Hz bandwidth) are a characteristic feature of QS (Seelke & Blumberg, 2008).

Within each sleep state, a breath-by-breath analysis was performed on respiratory flow traces to calculate breaths per minute (bpm) and the coefficient of variation of the respiratory period (CV_{RP}; obtained by calculating the ratio between the standard deviation and the mean BPM), a measure used to quantify respiratory variability (Andrews & Pagliardini, 2015). Given the limitation in plethysmograph readings associated with signal drifts, moving and gas compression artefacts, the analysis of changes in relative tidal volume (VT) and minute ventilation (VE) was limited to brief AS epochs that displayed ABD recruitment in order to determine differences in VT and VE with ABD onset.

INT and ABD_{EMG} signals were rectified and integrated (time constant decay 0.08s) and used, together with respiratory flow data, to determine inspiratory and expiratory phases and relative contribution of ABD activity to respiration. ABD_{EMG} activity was classified as being either tonic (non-respiratory modulated), weakly expiratory modulated or robustly recruited (peak amplitude >50% of preceding baseline activity) based on at least three consecutive respiratory cycles.

Analysis of respiratory parameters within each age group was initially performed in QS and AS irrespective of the observed pattern of ABD_{EMG} activity. Further analysis was performed in order to compare respiratory parameters within sleep states in which robust ABD_{EMG} was either present (ABD^{W}) or absent ($ABD^{W/O}$). AS epochs that displayed recruitment of ABD_{EMG} activity were further analyzed to compare breathing characteristics (bpm, CV_{RP}, VT and VE) in six respiratory cycles preceding ABD_{EMG} onset (ABD^{-}) with the breathing pattern observed with ABD_{EMG} onset (ABD^{+}). Pressure volume artefacts in the plethysmograph signal and large swings in INT and ABD_{EMG} activity occurring together with myoclonic twitches were eliminated from our $ABD^{+/-}$ analysis.

In order to further characterize the relationship between the occurrence of twitches and INT and ABD_{EMG} activity in AS, we performed twitch-triggered analysis of ∫INT and $\int ABD_{EMG}$ amplitude in the 2 second interval preceding and following the occurrence of twitches. Twitches at AS onset and occurring within a 4sec inter-twitch interval were eliminated from the analysis to avoid overlap.

Average values for each age group were calculated, normalized and compared (ANOVA, Tukey test and post-hoc t-test) to test for significance between means of respiratory variables within age groups and across age groups. Data are presented as mean ± standard error of the mean. P values less than 0.05 were considered significant.

2.3 Results

All rats used in this study were the result of spontaneous deliveries and were tested to determine sleep states, ventilatory parameters and the contribution of ABD muscle recruitment to ventilation during normal "healthy" postnatal development. P0 rats were tested within 10 hours of delivery to investigate ABD recruitment in presence of an immature and often irregular breathing pattern (Ren et al., 2015). In contrast, P1 rats displayed a more regular breathing pattern and therefore were analyzed separately. Other groups were tested within a 48-hour interval: P3-4; P7-8 and P14-15, for simplicity, these groups are indicated as P4, P8 and P15.

Sleep characteristics in the postnatal period

The percentage of time spent in wakefulness (W), QS and AS for the five age groups examined is displayed in table 2.1. Similar to human infants,(MacLean et al., 2015) and in agreement with previous work done in rodents, (Jouvet-Mounier et al., 1970; Seelke & Blumberg, 2008) neonatal rats spent most of their time asleep. Developmentally, the amount of time spent sleeping increased significantly from P0 to P1 (ANOVA, Tukey test $P<0.05$), did not change between P1 and P8 and then and decreased at P15 relative to the P1 and P4 rats (ANOVA, Tukey test $P<0.05$).

By using $nuchal_{EMG}$ traces combined with video monitoring of overt behavior we classified epochs of QS and AS according to sleep scoring criteria developed by

Blumberg and colleagues for infant rats (Karlsson & Blumberg, 2002). We extended this sleep scoring approach for the first time to P0 rodents.

The amount of time spent in QS increased in P15 rats compared to P0 rats (ANOVA, Tukey test, $P<0.01$). In contrast, the amount of time spent in AS decreased significantly during the second week of postnatal development (ANOVA, Tukey test, $P<0.01$; table 2.1).

Table 2.1 also indicates the total number of W, QS and AS events/hour and their average duration in seconds. W epochs were most frequent in P8 compared to P0-1 and P15 rats (ANOVA, Tukey test, $P<0.01$; table 2.1). Their duration was highest at P0 and P15 when rats spent a large part of their time awake. QS epochs increased in number from P0-4 to P8 and then fall precipitously at P15 relative to P4-P8 (ANOVA, Tukey test, $P<0.05$). The duration of QS events increased in P15 compared to P0 and P8 rats (ANOVA, Tukey test, $P<0.05$). Frequency of AS events increased in P0 compared to P8 (ANOVA, Tukey test, $P<0.05$), and decreased at P15 compared to the first postnatal week (ANOVA, Tukey test, $P<0.01$). Mean duration of AS events decreased in P8-P15 relative to P0 (ANOVA, Tukey test, $P<0.05$).

Respiratory pattern in the postnatal period

Respiratory frequency, when calculated across the entire recording session of instrumented rats without consideration of sleep state, averaged 130±11 bpm at P0 (n=6), 132±12 bpm at P1 (n=6), 116±5 bpm at P4 (n=10), 126±8 bpm at P8 (n=10), and 142±10 bpm in P15 rats (n=9), with no significant difference in breathing rate across age groups (ANOVA, Tukey test, $P>0.05$). Frequency values tended to be numerically less compared to rats that did not undergo anesthesia and surgery (132±25, 196±1, 195±13, 153±12 bpm at P1, P4, P8 and P15, respectively), but the there was no significant difference in comparison to values non-instrumented rats and across age groups (ANOVA, Tukey test, $P>0.05$).

Respiratory frequency was then specifically examined in AS and QS and are plotted in figure 2.1A. Respiratory rate tended to be numerically greater in AS compared to QS, with differences being significant at P4 ($p= 3.15\times10^{-2}$), P8 ($p=2.615\times10^{-2}$) and P15 ($p=5.57\times10^{-4}$).

Respiratory variability (calculated as coefficient of variation of respiratory period) across the entire recording session was 0.64±0.06 at P0, 0.31±0.03 at P1, 0.36±0.04 at P4, 0.30±0.03 at P8 and 0.35±0.04 at P15, where variability was significantly higher at P0 relative to all other age groups (ANOVA, Tukey test, $P<0.01$). Respiratory variability was not different in non-instrumented rats (0.39±0.08, 0.19±0.02, 0.28±0.06, 0.43±0.07 at P1, P4, P8, P15 respectively) compared to instrumented rats (ANOVA, Tukey test, $P>0.05$).

Analysis of respiratory variability across sleep states showed that CV_{RP} was significantly greater in AS compared to QS at all ages (figure 2.1B) and the ratio between the CV_{RP} of AS/QS was greater in P15 rats compared to P0-P8 rats (ANOVA, Tukey test, $P<0.01$). Further, when CV_{RP} in both AS and QS sleep were compared to the CV_{RP} obtained through the recording session (no sleep states considered) these values were significantly lower at all ages with the exception of AS in P15 rats.

The occurrence of central apneas, defined as an interruption in respiratory flow lasting longer than the average duration of two missed breaths (Mendelson et al., 1988), was greatest at P0 (table 2.2). Apneas were more frequent during AS compared to QS; 75±23%, 63±16%, 86±42%, 44±26% and 60±29% of apneas occurred in AS at P0, P1, P4, P8 and P15, respectively. Post-sigh apneas were rare compared to central apneas in all age groups (table 2.2).

Recruitment of ABD_{EMG} activity in the postnatal period

Analysis of ABD_{EMG} signals through development and across sleep/wake cycles revealed that ABD_{EMG} activity was either tonic (i.e., not expiratory modulated), weakly expiratory modulated (i.e., characterized by phasic activity during expiration and silent during inspiration), or robustly recruited during expiration (i.e., active expiration). Multiple patterns of ABD_{EMG} activity could be observed in any single QS or AS epoch across the different age groups (figure 2.2-2.4), although bouts of high amplitude, expiratory-modulated ABD_{EMG} recruitment occurred most frequently in AS compared to QS throughout development (table 2.3).

Figures 2.2 and 2.3 displays $nuchal_{EMG}$ and ABD_{EMG} activities (raw and integrated signals) and airflow traces recorded across wake/sleep alternations in P0, P1, P4 (figure

2.2) and P8 rats (figure 2.3A). Analysis of EMG activity and behavioral data indicate that rats transitioned from W, characterized by large body movements and high nuchal tone, to QS, characterized by low nuchal tone and behavioral quiescence, to AS, characterized by intermittent muscle twitches.

Myoclonic twitches could be followed by lasting bouts of high amplitude ABD_{EMG} activity in AS. These high-amplitude events were preceded by twitches within a 2 second interval in 51.4±13.3% of occurrences at P0, 57.7±11.3% at P1, 62.0±22.5% at P4, 73.9±10.9% at P7 and 49.1±12.3% at P15. The remainder of these events, although occurring in AS, were not preceded by myoclonic twitches. As shown in figure 2.3B for P8 rats, we observed a very brief (~500ms on average) and small amplitude increase in both INT_{EMG} and ABD_{EMG} associated with twitch occurrence. These small increases did not affect our analysis of changes in INT_{EMG} and ABD_{EMG} activation since the immediate time window surrounding twitch events were eliminated from our analysis. Further, twitch-triggered analysis of changes in ABD_{EMG} (P0-P15) and INT_{EMG} (P8-P15) peak amplitude activity preceding and following a twitch event (figure 2.3C) indicated that, with the exception of P0 rats (+13±0.3% increase in ABD_{EMG} activity following a twitch compared to pre-twitch activity; $p=0.01$; $n=6$) respiratory muscle activity in both INT and ABD muscles was not significantly influenced by occurrence of twitches ($p>0.05$).

Figure 2.4 displays a sleep/wake cycle from a P15 rat in which sleep pattern was evaluated not only by $nuchal_{EMG}$ and behavior, but also by cortical EEG activity. QS was characterized by low $neck_{EMG}$ activity, behavioral quiescence and high power low frequency cortical EEG activity. Similar to previous reports, (Seelke & Blumberg, 2008) we often observed transitions from QS to AS and back to QS within the same sleep cycle in P15 rats. The occurrence of twitches throughout AS events and high amplitude ABD bursts following a twitch were less frequent in P15 compared to the younger age groups.

Recruitment of ABD_{EMG} activity in QS is not associated with significant changes in respiratory variability

Recruitment of high amplitude expiratory ABD_{EMG} activity during QS epochs was most common in P0 to P4 rats and declined thereafter (P8-15). Bouts of high amplitude ABD_{EMG} activity occurred in 11.4± 3.7% of QS epochs in 5 out of 6 P0 rats, 6.3± 1.3% of

QS epochs in 4 out of 6 P1 rats and 5.5±3.5% of QS epochs in 6 out of 10 P4 rats (table 2.3). High amplitude ABD_{EMG} recruitment was observed in only 3.0±1.2% of QS epochs in 4 out of 10 P8 rats and in only a few QS epochs in 1 out of 9 P15 rats.

We further characterized breathing pattern by separating QS epochs that did (ABD^{W}) or did not ($ABD^{W/O}$) display high amplitude ABD_{EMG} recruitment. Our results indicate that respiratory frequency in QS was not affected by the presence of ABD recruitment in the first postnatal week (figure 2.5A).

Figure 2.5B illustrates the distribution of CV_{RP} in QS epochs that displayed ABD recruitment (ABD^{W}) or not ($ABD^{W/O}$). The CV_{RP} in ABD^{W} increased compared to $ABD^{W/O}$ in QS only at P0 (0.37±0.06 and 0.23±0.04; p=2.25x10-3, n=5), whereas CV_{RP} did not change at P1 (0.16±0.04 vs 0.12±0.01; p=0.20, n=4), P4 (0.21±0.05 vs 0.16±0.02; p=0.09, n=6) and P8 (0.27±0.06 vs 0.17±0.03; p=0.09, n=4). Only one P15 rat recruited ABD_{EMG} during QS. Thus, these data were not analyzed further.

Occurrence of apneas was also investigated in ABD^{W} and $ABD^{W/O}$ QS epochs across development. 82.3%, 40%, 71.4%, 100% and 89.5% of apneas occurred in $ABD^{W/O}$ in P0, P1, P4, P8 and P15 rats, respectively. Post sigh apneas in QS was present only in P4-P15 rats, with 100% of them being in $ABD^{W/O}$ at P4 and 66.7% of them being in $ABD^{W/O}$ at P8 and P15.

Recruitment of ABD_{EMG} activity in AS is associated with increased breathing variability

AS epochs were also analyzed according to the presence (ABD^{W}) or the absence ($ABD^{W/O}$) of high amplitude ABD_{EMG} activity. The proportion of AS epochs that displayed bouts of high amplitude ABD_{EMG} activity increased within P0-P8 rats and was present in all rats examined at each age group (table 2.3). 40.0 ±4.2% of the AS epochs displayed ABD_{EMG} recruitment at P0, 48.4±8.2% at, 40.8±9.2% at P4, and 39.3±5.8% at P8. Contrary to P4-P8, the percentage of ABD^{W} events in AS decreased to 22.7±3.8% of AS epochs at P15 (ANOVA, Tukey test $P<0.01$). This value is comparable to what we observed previously in adult rats (Andrews & Pagliardini, 2015; Pagliardini et al., 2012).

While we did not observe differences in respiratory rate with occurrence of bouts of high amplitude ABD_{EMG} recruitment at P0,-P1, and P8-P15 (figure 2.6a), respiratory

rate significantly decreased with ABD_{EMG} recruitment only in P4 rats (109±7.8 bpm in $ABD^{W/O}$ vs 104±7.7 bpm in ABD^{W}; $p=2.2x10^{-2}$).

When CV_{RP} was compared between $ABD^{W/O}$ and ABD^{W} events in AS epochs, we observed an increase in respiratory variability in ABD^{W} events compared to $ABD^{W/O}$ events in all age groups, with the exception of P0 (figure 2.6b). CV_{RP} increased from 0.17±0.01 to 0.23±0.01 with ABD recruitment at P1 ($p=3.6x10^{-4}$), from 0.19±0.01 to 0.24±0.02 at P4 ($p=1.8x10^{-3}$), from 0.21±0.03 vs 0.25±0.03 at P8 ($p=2.0x10^{-2}$) and from 0.33±0.04 to 0.41±0.04 at P15 ($p=2.3x10^{-3}$).

Apneas occurring in AS were more frequent in ABD^{W} compared to $ABD^{W/O}$ events in the first postnatal week (70.6%, 100%, 82.4% and 60.0% of apneas were in ABD^{W} at P0, P1,P4,P8), whereas at P15 apneas occurred more frequently in $ABD^{W/O}$ events (36.4%). Post sigh apneas occurred in P0-P4 and P15 rats. At P0 40.0% of post-sigh apneas occurred in $ABD^{W/O}$, at P1 100% of post-sigh apneas occurred in $ABD^{W/O}$ and at P4 and P15 66.7% of post-sigh apneas occurred in $ABD^{W/O}$.

The occurrence of ABD recruitment was investigated in relation with the timing of apnea episodes (within a 10s period). In P0 rats, 44.7% of apneas occurred following ABD_{EMG} recruitment and 28.9% of apneas were both preceded and followed by respiratory cycles that displayed ABD_{EMG} activity within 10 seconds from the apneas, whereas only 2.6% of apneic events were followed by ABD_{EMG} recruitment. In P1 rats, 42.9% of apneas were preceded by ABD_{EMG} recruitment, whereas 28.6% of apneas were both preceded and followed by forced expiratory activity. In P4 rats, 22.2% of apneas were followed by expiratory ABD recruitment, 37.0% of apneas were preceded by expiratory ABD recruitment and 14.8% of apneas were both preceded and followed by forced expiratory activity. In P8 rats, 100% of apneas were both preceded and followed by forced expiratory activity, whereas in P15 rats 50% of apneas were preceded by ABD recruitment and 16.7% of apneas were followed by forced expiratory activity.

The onset of ABD muscle recruitment in AS is associated with a decrease in respiratory variability and an increase in minute ventilation

We further analyzed breathing characteristics within ABD^{W} events occurring in AS. Six respiratory cycles preceding ABD recruitment (ABD^{-}) and following the onset of

ABD recruitment (ABD^+) were analyzed to determine changes in respiratory rate, respiratory variability, relative tidal volume and minute ventilation with ABD recruitment across all age groups.

With the onset of high amplitude ABD_{EMG} recruitment in ABD^W AS (ABD^+), there was no change in respiratory frequency compared to the preceding respiratory cycles that did not display ABD_{EMG} recruitment (ABD^-; figure 2.7A), with the exception of a modest but significant increase in respiratory rate measured at P1 (+11.1±0.3%, p=0.02). As shown in figure 2.7B, CV_{RP} significantly decreased in ABD^+ with the onset of ABD_{EMG} recruitment compared to preceding ABD^- events at P0 (0.29±0.04 vs 0.18±0.03; $p=1.8x10^{-3}$, n=6), at P1 (0.13±0.02 vs 0.07±0.01 ; $p=0.02$, n=6), at P4 (0.14±0.02 vs 0.06±0.01; $p=2.4x10^{-3}$, n=10), at P8 (0.13±0.02 vs 0.10±0.01; $p=0.01$, n=10), and at P15 (0.19±0.05 vs 0.08±0.01 ; $p=0.03$, n=9). These data suggest that variability in respiratory rhythm decreases with the onset of high amplitude ABD_{EMG} recruitment in AS.

Although tidal volume tended to increase in all age groups with recruitment of ABD activity, the increase was only significant at P0 (+100±18% ;$p=6.6x10^{-3}$, figure 2.8A). Similarly, in older rats (P4-P15) that were also instrumented with INT_{EMG} electrodes, the peak amplitude of $\int INT_{EMG}$ activity indicated a tendency to increase with onset of ABD recruitment, but the changes were not significant (+8.1±24.6% at P4, n=4; +7.7±33.1% at P8, n=4; +14.5±16.2%, P15, n=3).

However, due to the combined changes in rhythm and amplitude that were associated with active expiration, minute ventilation increased significantly in all ages except P4 and P15 (P0=+146±37%, $p=8.2x10^{-3}$; P1=+36±16%, p=0.03; +P8=8.9±2.6%, $p=6.9x10^{-3}$; figure 2.8B).

2.4 Discussion

In this study we investigated respiratory changes occurring in the postnatal period of rodents across QS and AS states using electromyogram and behavioral criteria previously developed and validated by others (Jouvet-Mounier et al., 1970; Karlsson & Blumberg, 2002, 2005). We confirmed that changes in sleep pattern occurs with

development and we reported differences in breathing characteristics associated with AS and QS through the first two weeks of postnatal life in rats. Further, for the first time, we analyzed the recruitment of expiratory muscle activity across sleep/wake cycles in postnatal rats. We reported the occurrence of bouts of high amplitude expiratory ABD muscle activity during AS and QS and we demonstrated that the onset of recruitment of expiratory muscle activity was associated with an increase in ventilation and an improvement in respiratory stability in AS. These results indicate that active expiration contributes to ventilation in the first post-natal weeks of rats and its occurrence is associated with increased respiratory stability and improvement of ventilation. The ability of increasing active ventilation during unstable breathing may have important clinical implication for sleep-related breathing disorders in the perinatal period.

Sleep changes across development

Our findings confirmed that sleep pattern changes through development, with a large amount of time spent in AS in the first post-natal week followed by a dramatic AS reduction in favor of QS and W times during the second postnatal week.

Because of absence of cortical delta activity in rats younger than P11, we used criteria developed by Blumberg and colleagues (Karlsson & Blumberg, 2002) to determine sleep states. These criteria established that low nuchal muscle tone and behavioral quiescence in <P11 rats are indicative of QS in absence of a fully developed cortical delta activity. The occurrence of myoclonic twitches on top of the low nuchal muscle tone was indicative of AS. These standards have been extensively validated by further analyses of cortical and hippocampal activity (Seelke et al., 2005) in addition to studies that have further investigated mechanisms associated with development of extraocular muscle activity and sleep muscle atonia in the first two post-natal weeks (Karlsson & Blumberg, 2005; Seelke et al., 2005).

Based on these criteria, we observed an increased amount of time spent in wakefulness both in P0 (few hours after birth) and in P15 rats. The high prevalence of awake time in P0 rats is in agreement with what was previously observed in humans, where infants spent 30 to 50% of time awake in the first hours post-partum (Theorell et al., 1973). In accordance with previous studies, an increase in awake time and a decrease

in AS time occurring in the second post-natal week (compared to the first postnatal week) is evidence of progressive brain development and organization of brain dynamics that regulate brain sleep state alternations and cortical activity (Gramsbergen et al., 1970; Jouvet-Mounier et al., 1970; Seelke & Blumberg, 2008, 2010).

Respiratory changes across development

In this study, we systematically analyzed respiratory parameters and recruitment of expiratory activity in active and quiet sleep by using $nuchal_{EMG}$ and behavioral activity to rigorously assess wake/sleep cycles in the first two post-natal weeks. This approach has given us the opportunity to determine in each sleep state (active and quiet sleep), respiratory frequency and variability, apnea occurrence, and expiratory muscle recruitment across development.

Previous studies have investigated breathing and its variability across development and its response to different ventilatory challenges regardless of brain or behavioural states (Abu-Shaweesh et al., 1999; Cameron et al., 2000; Carroll, 2003; Eden & Hanson, 1987; Gleed & Mortola, 1991; Huang et al., 2004; Liu et al., 2009; Liu et al., 2006; Liu & Wong-Riley, 2013; Putnam et al., 2005; Stunden et al., 2001). Compared to previous studies, we observed a lower respiratory rate in both AS and QS at each age considered (Cameron et al., 2000; Liu et al., 2006). We attribute the higher respiratory frequency (Liu et al., 2006) to the measurement of breathing not only during sleep but also during wakefulness and active behaviors. In order to verify this observation we analyzed our data regardless of the sleep states and reported higher values that are comparable to previous studies (Eden & Hanson, 1987; Liu et al., 2006). Because respiratory disorders in the perinatal period occur mostly during sleep, in particular active sleep, (MacLean et al., 2015) we believe that an attempt investigation of breathing in specific sleep states may give important contribution to understanding respiratory control in both physiological and pathological conditions.

Analysis of breathing characteristics in developing rats indicates that respiratory rate in baseline conditions was consistent in AS and QS during the first post-natal week and changes in respiratory rate occurred only in P8 and P15 rats, with a significant increase in respiratory rate occurring in AS compared to QS. Nonetheless, in each age

group respiratory variability, as indicated by the coefficient of variation of the respiratory period, was consistently higher in AS compared to QS.

Two age groups displayed the largest difference in coefficient of variability between AS and QS: P0 and P15. In P0, we observed high respiratory variability in both AS and QS, with CV in AS being 1.4 times higher than in QS. In the immediate time following birth, respiration is usually more variable in mammals, (Fisher et al., 1982; Mortola, 1987; Ren et al., 2015) with frequent apneas and respiratory irregularities. Post-natal respiratory variability has been attributed to rapidly occurring neonatal adjustments in respiratory mechanics, blood oxygenation, metabolic rate and airway receptors input function associated with air breathing, in addition to the necessary and progressive reabsorption of lung fluids. These adjustments usually occur within the first hours after birth and by P1 respiratory rhythms in mammals become more stable. In support of this, we observed the highest frequency of apneas immediately following birth (P0) and a progressive reduction in respiratory variability and respiratory disturbances in ≥ P1 rats.

Sleep states in P15 rats were analyzed based on their EEG signal and behavioral criteria. Although EEG activity is not as fully developed as it is in adult rats, clear cortical delta activity helped us differentiate the occurrence of QS from AS (Seelke & Blumberg, 2008). The age around P12-15 has been proposed to be a critical respiratory period in rats because of changes in the expression of neurotransmitters and receptors within the respiratory network and variations in ventilatory and metabolic responses to hypoxia (Liu et al., 2009; Liu et al., 2006; Liu & Wong-Riley, 2013; Wong-Riley & Liu, 2008). Here, we show that breathing variability is highest during AS sleep at P15. Although we did not observe the peak of respiratory frequency (~300bpm) that Liu and Wong Riley reported in their study at P15, (Liu et al., 2006) in either instrumented or non-instrumented rats, we demonstrated that respiratory variability was highest in P15 rats compared to other age groups ≥ P1 and the highest variability was observed in AS compared to QS.

ABD recruitment across development.

An objective of our study was to determine if expiratory ABD muscle activity was recruited across sleep/wake cycles in the first two postnatal weeks of rats. Although

resistant to hypoxic challenges, at this time of development, critical changes in ventilation, chemosensitivity, lung development and lung compliance occur (Mortola, 1987; Wong-Riley & Liu, 2005, 2008) and are source of respiratory variability and increased frequency of respiratory disturbances.

Because robust recruitment of expiratory activity was shown to be associated with increased minute ventilation and more stable breathing in REM sleep of adult rats, (Andrews & Pagliardini, 2015) an in depth analysis on the occurrence of this activity across development was therefore performed. Similar to our previous results in adult rats, (Andrews & Pagliardini, 2015) we observed clear recruitment of expiratory ABD_{EMG} activity in all age groups in multiple sleep/wake cycles. Expiratory ABD activity displayed bursts of high amplitude expiratory modulated activity that was associated with expiratory flow. Occurrence of ABD activity was often preceded by myoclonic twitches, although twitches did not influence activity of either inspiratory or expiratory muscles following twitches. Most significantly, recruitment of high amplitude ABD_{EMG} activity was associated with a reduction in respiratory variability and an increase in ventilation in AS, when the majority of respiratory disturbances occur in infancy. From the results of this study we conclude that in postnatal rats the occurrence of high amplitude ABD_{EMG} activity is consistently present in sleeping rats and it is associated with increased minute ventilation and reduction in respiratory frequency variability in AS. It will now be vital to determine the contribution of expiratory ABD_{EMG} activity in pathological conditions given the association of active expiration with increased ventilation and respiratory stability in AS and REM sleep (Andrews & Pagliardini, 2015).

The source of the state dependent excitatory drive to expiratory ABD muscle is currently unknown. The retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG) is the area of the brain that has been proposed to be key for the generation of active recruitment of ABD muscles (forced expiration) (Abbott et al., 2011; Huckstepp et al., 2016; Janczewski & Feldman, 2006b; Marina et al., 2010; Pagliardini et al., 2011). In vitro and in vivo studies across perinatal development of the expiratory oscillator (epF in embryonic stage and pFRG in juvenile and adult rats) (Janczewski & Feldman, 2006b; Mellen et al., 2003; Thoby-Brisson et al., 2009) suggest that the expiratory oscillator is crucial for the onset of respiratory rhythms in rodents embryos (Thoby-Brisson et al.,

2009) and may still be rhythmically active in the first post-natal period to pace and drive respiration (Janczewski & Feldman, 2006b; Mellen et al., 2003). In vivo studies in adult rodents further suggest that with development, pFRG becomes a conditional oscillator that is only active in presence of an increased respiratory drive (Huckstepp et al., 2015; Huckstepp et al., 2016; Janczewski & Feldman, 2006b; Pagliardini et al., 2011).

These current results demonstrate that ABD muscle recruitment in the postnatal period is associated with potentiation of minute ventilation and with a reduction in respiratory variability. Even though the cellular and network mechanisms underlying the activation of expiratory activity during AS in the postnatal period are still unknown, our data support that hypothesis that recruitment of forced expiration contributes to breathing in the postnatal period of behaving rats.

Tables

Table 2.1. Distribution of sleep states in P0-P15 rats

	Wakefulness			Quiet Sleep			Active Sleep		
Age	**% Time**	**Epochs/hr**	**W length (s)**	**% Time**	**Epochs/hr**	**QS length (s)**	**% Time**	**Epochs/hr**	**AS length (s)**
P0	41.9±3.2 *,#	27.6±5.2 ‡	80±30 *,#,‡	11.1±1.8 ⁂	25.0±5.1 ‡	15±1 ⁂	47.0±2.9 ⁂	19.8±4.2 ‡,⁂	104±26 ‡,⁂
P1	21.9±2.6 †,⁂	42.2±7.9 ‡	18±2 †	24.4±6.7	39.7±5.5 ‡	19±4	53.7±6.6 ⁂	25.4±3.7 ⁂	83±22
P4	23.6±2.9 †,⁂	57.2±6.7 ⁂	17±2 †	22.1±1.7	54.4±6.9 ‡,⁂	18±3	54.3±3.2 ⁂	28.9±3.3 ⁂	85±16
P8	30.4±3.8 ⁂	89.8±12.5 †,*,⁂	13±2 †	27.6±3.5	88.9±11.7 †,*,#,⁂	11±1 ⁂	42.0.4±6 .6 ⁂	34.2±2.8 †,⁂	44±8 †
P15	47.8±5.3 *,#,‡	14.5±2.8 #,‡	42±7	39.2±5.8 †	15.1±3.0 #,‡	29±3 †,‡	13.0±1.6 †,*,#,‡	4.1±0.4 †,*,#,‡	32±4 †

The percentage of time spent in each state, the frequency of epochs (wakefulness; quiet sleep QS, active sleep AS) and the length of these events (in seconds) are described for each age group. Symbols indicates statistical significance using One Way ANOVA and the Tukey test, † indicates statistical significance ($P<0.05$) with respect to P0, * indicates significance with respect to P1, # significant with respect to P4, ‡ significant with respect to P8, and ⁂ significant with respect to P15.

Table 2.2. Distribution of central and post-sigh apneas in P0-P15 rats

	Central Apneas			Post-Sigh Apneas		
Age	**Apneas/hr**	**Apnea/hr QS (s)**	**Apnea/hr AS (s)**	**Apneas/hr**	**Apnea/hr QS (s)**	**Apnea/hr AS (s)**
P0	11.5±3.6 *,#,‡,⁂	25.3±9.3 *,#,‡,⁂ (3.5±0.3s)	19.6±6.6 *,‡ (3.8±0.4s)	1.4±0.7	0	3.3±1.9 (4.0±1.0s)
P1	1.9±0.6 †	2.8±2.5 † (2.7±0.6s)	2.0±0.8 † (2.5±0.1s)	0.2±0.2	0	0
P4	4.3±1.8	3.0±1.6 † (3.7±0.5s)	7.8±3.9 (2.7±0.2s)	0.5±0.2	0.8±0.6 (2.9±0.3s)	0.7±0.4 (2.4±0.2s)
P8	1.3±1.0 †	0 †	1.1±0.6 † (3.6±0.6s)	0.3±0.2	0.6±0.4 (3.2±0.6s)	0
P15	1.6±0.4 †	1.6±0.8 † (3.3±0.4s)	9.5±3.4 (2.8±0.1)	0.2±0.1	0.2±0.2 (4.6±1.5s)	0.8±0.4 (3.2±0.3s)

Frequency of apneas and their average duration (in seconds) were calculated for the entire recording session (apneas/hr and in QS and AS within each age group. Symbols indicates statistical significance using One Way ANOVA and the Tukey test, † indicates statistical significance ($P<0.05$) with respect to P0, * indicates significance with respect to P1, # significant with respect to P4, ‡ significant with respect to P8, and ⁂ significant with respect to P15.

Table 2.3. Distribution of QS and AS epochs that display (ABD ^{W}) or do not display (ABD $^{W/O}$) recruitment of high amplitude ABD_{EMG} across the first two weeks of rat development

	Quiet Sleep			Active Sleep		
Age	**ABD $^{W/O}$ epochs/hr**	**ABD ^{W} epochs/hr**	**# rats**	**ABD $^{W/O}$ epochs/hr**	**ABD ^{W} epochs/hr**	**# rats**
P0	25.3±6.3	3.3±1.1	5/6	12.1±4.0	7.7±0.8	6/6
P1	43.7±10.6	3.0±0.6	4/6	13.0±1.8	12.2±2.1	6/6
P4	50.3±8.7	2.9±1.9	6/10	17.2±1.9	11.8±2.7	10/10
P8	106.4±21.8	3.3±1.3	4/10	20.6±3.6	13.4±2.0	10/10
P15	-	-	1/10	3.1±0.4	0.9±0.2	9/10

The number of ABD $^{W/O}$ and ABD ^{W} epochs/hr is indicated for rats that displayed both ABD $^{W/O}$ and ABD ^{W} in the same recording session. # rats indicate the fraction of rats that displayed ABD_{EMG} recruitment within each sleep state.

Figures

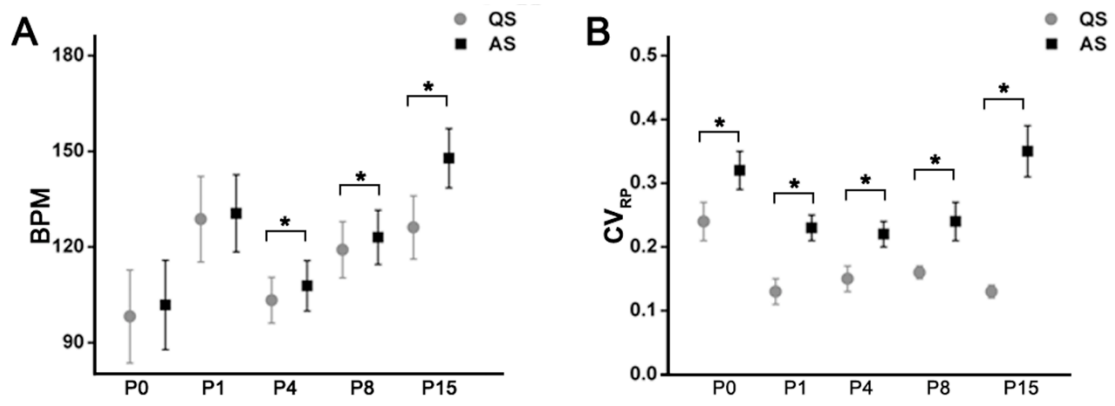

Figure 2.1. Respiratory frequency and variability in Active Sleep (AS) and Quiet Sleep (QS) across development. AS events display greater respiratory variability compared to quiet sleep QS events throughout development. Pooled average data of breaths per minute (BPM) and respiratory variability (coefficient of variation of the respiratory period, CV_{RP}, in P0-P15 rats. **A)** P4-P15 rats displayed a significant increase in BPM in AS compared to QS ($p<0.05$). **B)** AS events display a greater respiratory variability during AS across all age groups examined ($p<0.05$). The ratio of CV_{RP} between QS and AS variability was greater in P1 rats compared to P4 and P8. QS/AS CV_{RP} ratio in P15 rats was significantly greater than P0-P8 rats ($P<0.05$). Asterisks (*) indicate significance between QS and AS values within the same age group.

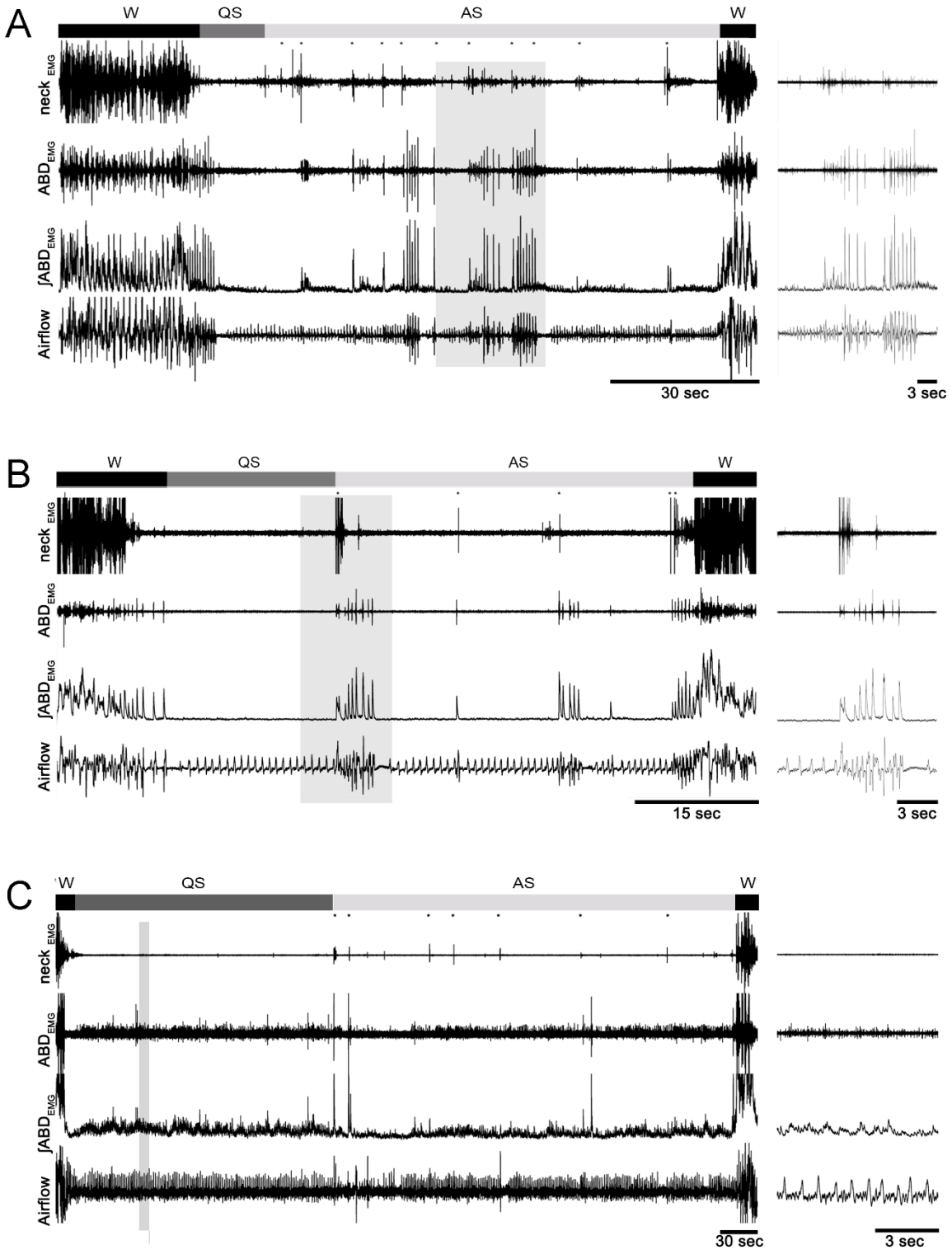
A
W
QS
AS
W
neck EMG
ABD EMG
∫ABD EMG
Airflow
30 sec
3 sec
B
W
QS
AS
W
neck EMG
ABD EMG
∫ABD EMG
Airflow
15 sec
3 sec
C
W
QS
AS
W
neck EMG
ABD EMG
∫ABD EMG
Airflow
30 sec
3 sec

Figure 2.2. Plethysmograph, EMG and EEG traces across a sleep cycle in P0-P4 rats. Rat plethysmographic recordings obtained at P0 (**A**), P1(**B**), and P4(**C**). Traces display $neck_{EMG}$, abdominal $(ABD)_{EMG}$, its integrated trace ($\int ABD_{EMG}$) and airflow in rats transitioning from wakefulness (**W**) into quiet sleep (**QS**) and active sleep (**AS**). Abdominal activity is recruited during AS, most frequently following a twitch event (indicated by *). Details of traces during AS in the grey boxes are displayed in the right panels.

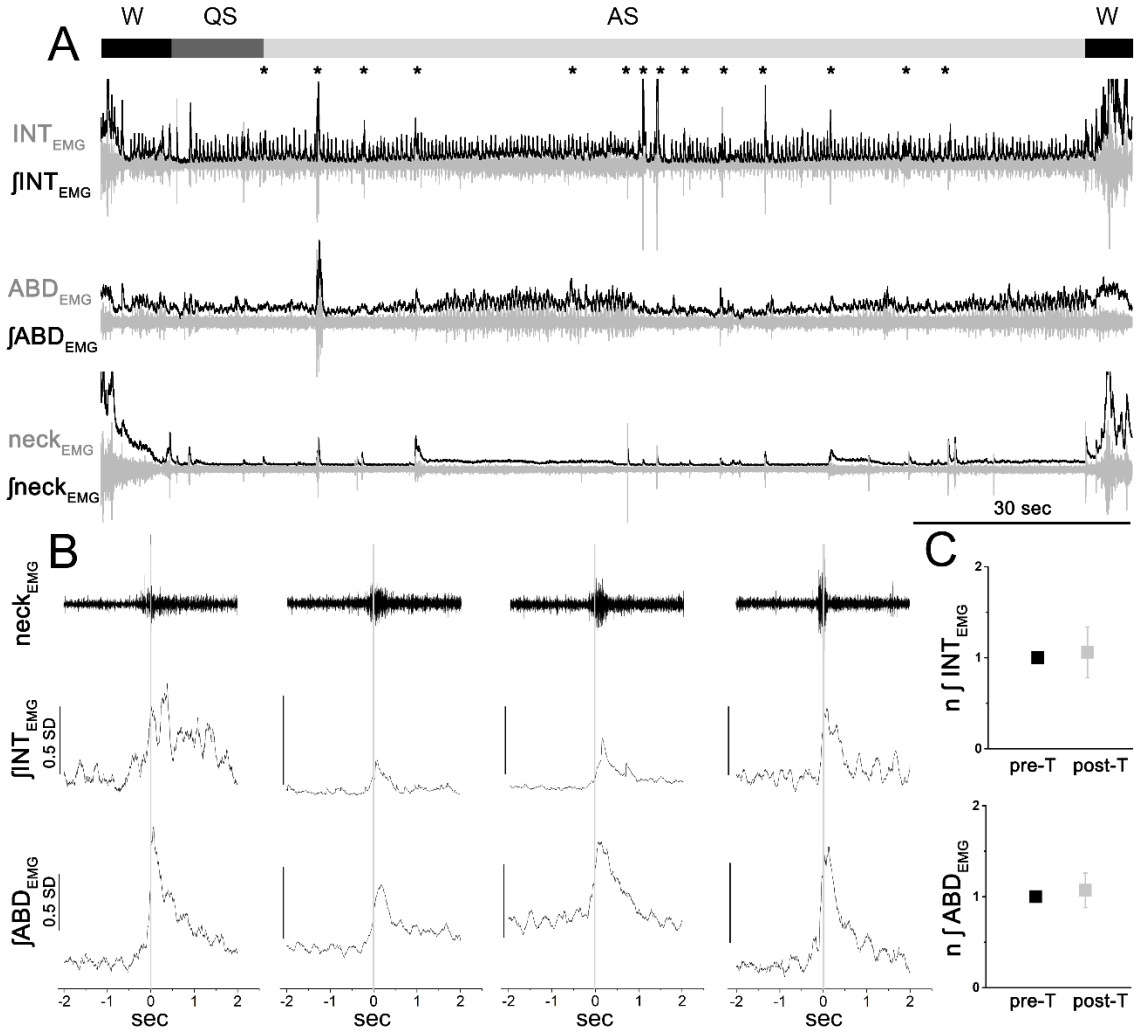

Figure 2.3. Plethysmograph, EMG and EEG traces across a sleep cycle (P8 rat). **A)** Traces display intercostal $(INT)_{EMG}$ and abdominal $(ABD)_{EMG}$ (grey), their integrated traces (black), $neck_{EMG}$, and airflow in rats transitioning from wakefulness (**W**) into quiet sleep (**QS**) and then active sleep (**AS**). **B)** Twitch-triggered averaged traces of neck EMG, $\int INT_{EMG}$ and $\int ABD_{EMG}$ in 4 P8 rats indicating only a transient (<500ms) increase of amplitude in both INT and ABD_{EMG}

directly associated with myoclonic twitches. **C)** Analysis of averaged normalized peak amplitude for ∫INT_{EMG} and ∫ABD_{EMG} 2 sec before and after myoclonic twitches indicate that there is no significant effect of myoclonic twitches per se on respiratory muscle activation.

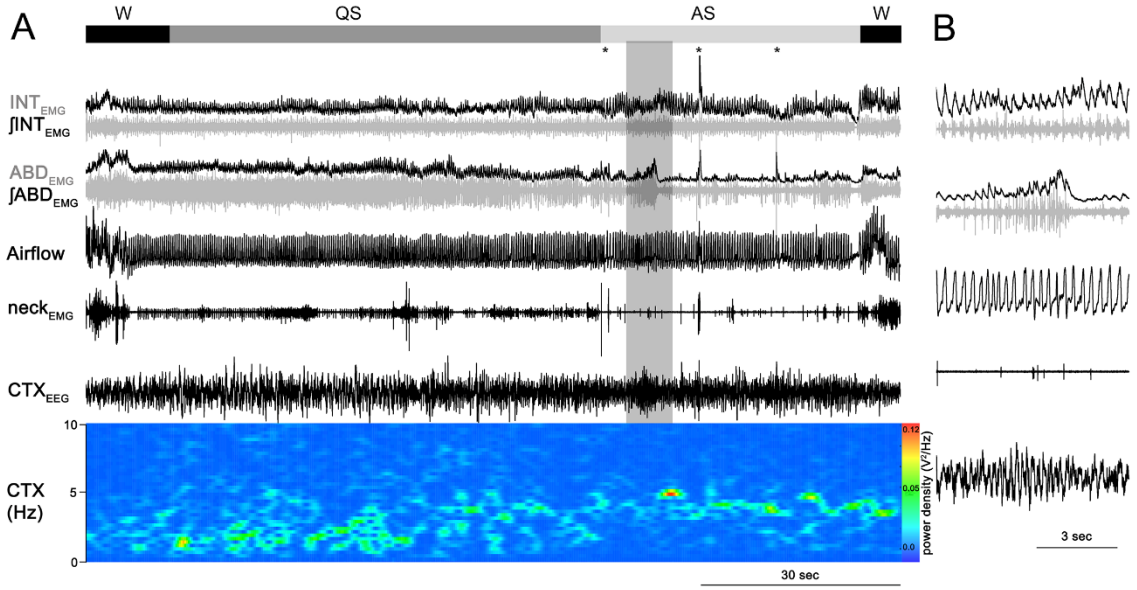

Figure 2.4. Plethysmograph, EMG and EEG traces across a sleep cycle in P15 rats. Traces display intercostal $(INT)_{EMG}$, abdominal $(ABD)_{EMG}$ (grey), and their integrated traces (black), airflow, $neck_{EMG}$, in addition to cortical (CTX) EEG signal and its power spectrogram (bottom). Twitch events occurring during AS are indicated by and asterisk (*). Details of traces during AS in the grey box are displayed in the right panel.

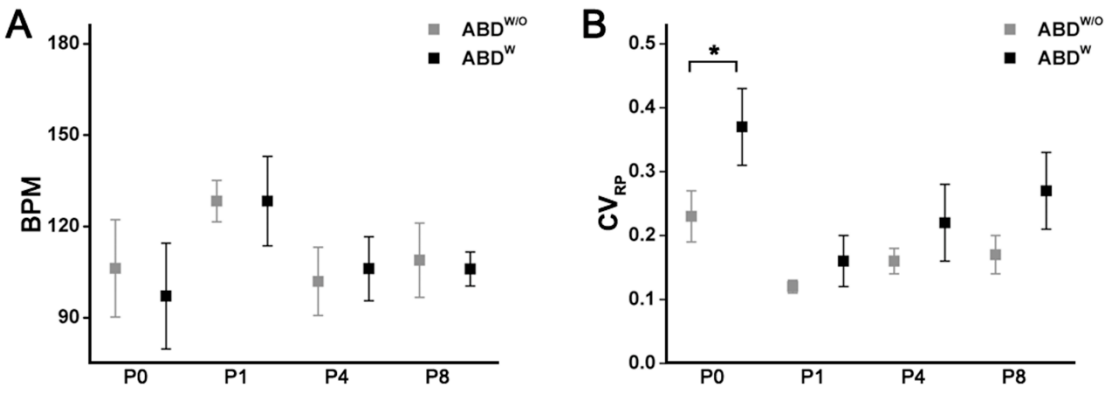

Figure 2.5. Occurrence of Abdominal recruitment (ABD^W) in QS increases respiratory variability. Pooled average data for breaths per minute (BPM) and respiratory variability (coefficient of variation of the period CV_{RP}) in P0-P15 rats. **A)** The respiratory frequency does

not change between ABD^{W} and $ABD^{W/O}$ events at any age group considered. **B)** The CV_{RP} however, displays a significant increase with abdominal recruitment (ABD^{W}; $P<0.05$) in P0 rats while other age groups show only a tendency to increase. Asterisks (*) indicate statistical significance between QS and AS values within the same age group.

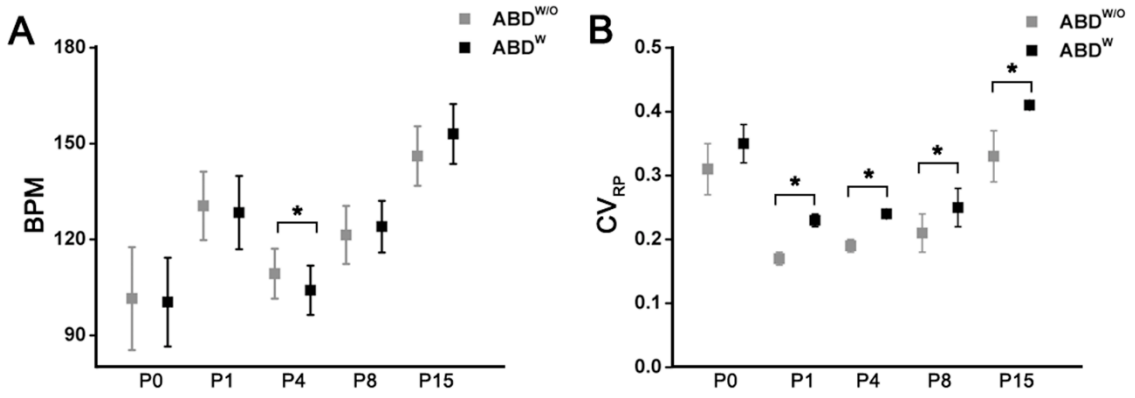

Figure 2.6. Occurrence of Abdominal recruitment (ABD^{W}) during AS events increases respiratory variability. Pooled average data of breaths per minute (BPM) and respiratory variability (coefficient of variation of the period CV_{RP}) in P0-P15 rats in AS events. **A)** The BPM is significantly different between ABD^{W} and $ABD^{W/O}$ events in P4 rats only. **B)** The CV_{RP} however, displays a significant increase ($P<0.05$) in ABD^{W} in P1-P15 rats. Asterisks indicate statistical significance between QS and AS values within the same age. Asterisks (*) indicate statistical significance ($p<0.05$) between ABD^{W} and $ABD^{W/O}$ within each age.

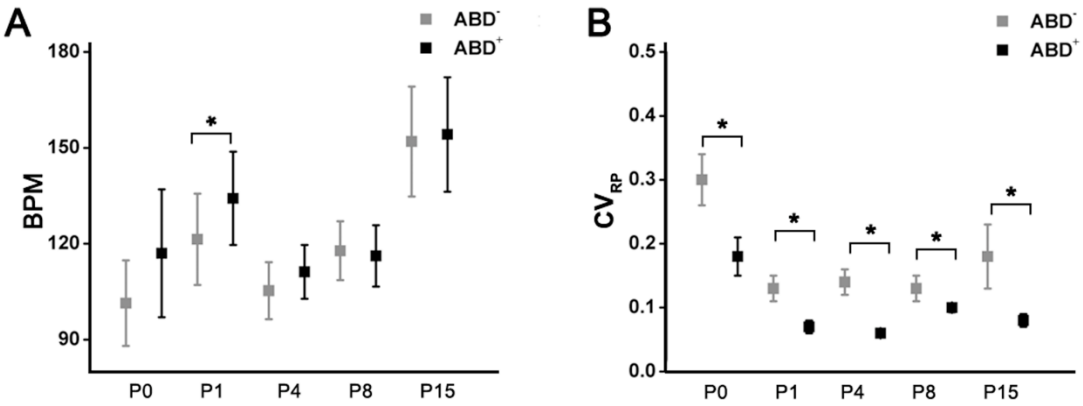

Figure 2.7. Respiratory frequency and variability changes with the onset of high amplitude expiratory ABD recruitment in AS epochs. Pooled average data of the breaths per minute (BPM) and the respiratory variability (CV_{RP}) in P0-P15 rats. The average of six respiratory cycles preceding ABD_{EMG} recruitment (ABD^-) was compared to six respiratory cycles occurring at the onset of recruitment (ABD^+). **A)** Respiratory frequency increased only in P1 rats with the onset of ABD recruitment. **B)** The CV_{RP} however, significantly decreased in all age groups. Asterisks (*) indicate statistical significance ($p<0.05$) between ABD^- and ABD^+ within each age.

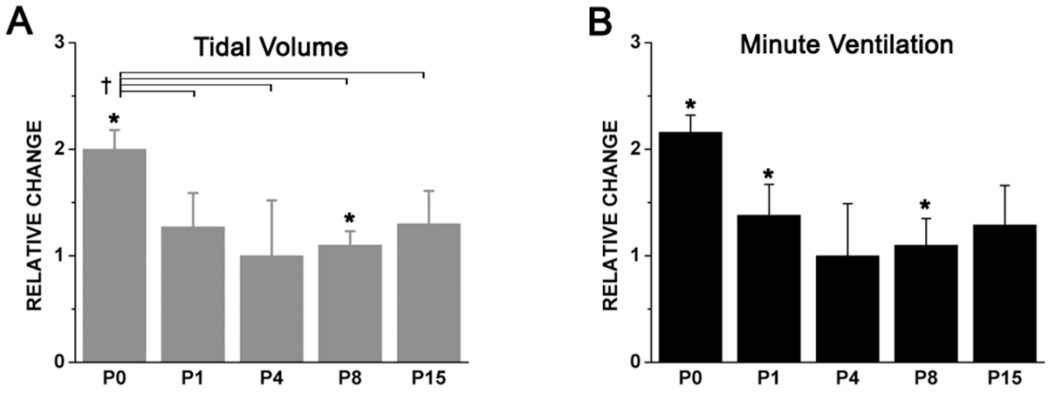

Figure 2.8. Tidal Volume and Minute ventilation changes with the onset ABD recruitment in AS events. Relative measurements of tidal volume (A) and minute ventilation (B) indicate that there is an overall trend for tidal volume to increase with the onset of recruitment (ABD^+) but this change is significant only in P0 and P8 rats. **B)** The minute ventilation increased with ABD recruitment at all age groups except in P4-P15 rats. Asterisks (*) indicate significant change ($p<0.05$) in values between ABD^- and ABD^+ within each age group.

Chapter 3: Expiratory Activity During Sleep in Children

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3.1 Introduction

Respiration is a dynamic and adaptable physiological function that requires fine coordination of ventilation with multiple oromotor activities from swallowing to coughing, to speech and vocalization. The respiratory cycle commences with the active process of inspiration, it is usually followed by a post-inspiratory phase, and terminates with an expiratory phase in which elastic lung recoil allows for passive expiratory flow during restful breathing. However, under conditions of increased respiratory drive (e.g., exercise, hypercapnia, hypoxemia) the abdominal wall musculature is recruited to augment effective gas exchange by enhancing minute ventilation.

Despite being a highly regulated physiological process, respiration is prone to irregularities, especially during sleep (Carroll & Agarwal, 2010; Ivanhoe et al., 2007). Sleep disordered breathing occurs most often during sleep transitions and rapid eye movement (REM) sleep, and is classified as obstructive (OSA) in which reduction of upper airway muscle tone results in decreased upper airway patency and obstruction, or central (CSA), where decreased central drive produces apneas and alveolar hypoventilation, or a combination thereof (Ivanhoe et al., 2007; Marcus, 2001). Recurrent apneas and hypopneas induce intermittent hypoxemia with repeated bouts of arterial O_2desaturation. Clinical outcomes in adults with OSA include inflammation, hypertension, and cardiovascular and metabolic dysfunction, whereas in infants OSA results in poor growth and cognitive impairment (Kaditis et al., 2017).

Respiratory disturbances are magnified in the newborn period when infants spend most of their time sleeping, have an immature respiratory control network, and undergo extensive developmental changes to the sleep and respiratory regulatory networks as well as the respiratory system (Carroll & Agarwal, 2010; Darnall, 2010; MacLean et al., 2015; Peirano et al., 2003). The incidence of respiratory events in newborns is common especially in the pre-term population and are typically characterized by apneas, periodic breathing, and bouts of arterial O_2desaturation, occurring most often during REM sleep (de Weerd & van den Bossche, 2003; DeHaan et al., 2015; Di Fiore et al., 2013; Ivanhoe et al., 2007; MacLean et al., 2015).

Compared to humans, rodents are born with a relatively ‘premature’ respiratory control. Insights gained from rodents studies show that during the first postnatal weeks, sleep transitions to a more mature state (Cirelli & Tononi, 2015; Saini & Pagliardini, 2017a; Seelke & Blumberg, 2008) and breathing also becomes more regular with progressive development of chemoreflex

responses (Carroll & Agarwal, 2010; Darnall, 2010; Marcus, 2001). Interestingly, we recently observed that postnatal rodents display expiratory abdominal muscle (ABD) recruitment during both quiet and active sleep (QS and AS, respectively) combined with stabilization of ventilation and increased tidal volume (Saini & Pagliardini, 2017a). Similar observations were made during REM sleep in adult rats, (Andrews & Pagliardini, 2015; Sherrey et al., 1988b), suggesting a potential involvement of the expiratory neuronal networks in sleep-related ventilatory control through life (Leirao et al., 2017; Pisanski & Pagliardini, 2019).

Since the classic dogma of REM-induced motor atonia posits that ABD muscles are silent during REM sleep (Iscoe, 1998), we investigated the occurrence of ABD activity during sleep in human infants, when breathing is more fragile and potentially compromised. Thus, we revisited polysomnographic traces from infants and children aged 0-2 years old suspected of OSA to determine if our observations in rodents extend to the human population, and whether the occurrence and pattern of the ABD recruitment is correlated with respiratory events.

Our results indicate that a portion of patients also exhibit ABD recruitment during sleep, primarily during nREM stages N1+N2 and REM sleep compared to nREM stage N3 (slow wave sleep, SWS). Although the significant majority of ABD recruitment is not associated with any identified respiratory event, when the two did co-occur, respiratory disturbances often preceded or coexisted with ABD recruitment. Our investigation indicates that up to 70% of all O_2desaturation events recovered during the abdominal recruitment period, raising the possibility that ABD recruitment may contribute to ventilation during sleep and facilitate recovery to physiological O_2 levels following desaturation events.

3.2 Methods

Study population

This study is a secondary analysis of deidentified data collected as part of a retrospective chart review approved by the Health Research Ethics Board - Health Panel at the University of Alberta (Pro70932). The original study included 205 subjects (0-2 years of age) who underwent polysomnography in a pediatric sleep laboratory from 2008 to 2010 inclusive (DeHaan et al., 2015). For this sub-study, the dataset was limited to those patients whose primary indication for polysomnography was suspected OSA (n=72). This sub-population was chosen as it was the largest single group of subjects from the original study population and excluded confounding

risk factors for OSA such as pre-term birth, known neuromuscular disorders, or other known conditions that might impact muscle activity. From the total of 72 polysomnography recordings, we identified 27 recordings that had abdominal electromyography signals (ABD_{EMG}) of sufficient quality for the assessment of ABD activation.

Polysomnography recording and analysis

Sleep laboratory records provided demographic information, referral information, and polysomnography results. The polysomnogram was performed according to the sleep laboratory's standard protocols. Sleep states were determined using electroencephalogram (10-20 system EEG; coordinates: C4-M1, C3-M2, O1-M2, O2-M1, F4-M1, F3-M2), electrooculargram (EOG; ROC/M1, LOC/M2), and submental electromyography (sEMG). Respiratory status was evaluated using nasal/oral air flow by thermistor, nasal pressure, and respiratory inductance plethysmography (RIP) to monitor thoracic and abdominal wall movements. Oxygen saturation and carbon dioxide levels were measured from pulse oximetry (SpO2; Oximax, sampling frequency 4Hz; averaging time 2-3s) and transcutaneous CO_2 monitoring ($TcCO_2$; TCM 4/40, version 2.14.1, Radiometer Medical Aps, Brønshøj, Denmark; sampling frequency: 4Hz; Range: 5-100 mmHg). Cardiac monitoring included electrocardiogram (ECG; ECG1 and ECG2). Diaphragm and abdominal muscle activities were monitored using diaphragmatic and abdominal electromyography (DIA_{EMG} and ABD_{EMG}). Costal DIA_{EMG} electrodes were positioned on the chest, in line with the nipple, with electrodes placed in the two lowest intercostal spaces. The ABD_{EMG} electrodes were placed on either side of the umbilicus to detect signal from the rectus abdominus muscle. EEG and EMG signals were collected at a sampling rate of 1kHz.

Polysomnography data was scored by an experienced scorer according to the criteria of the American Academy of Sleep Medicine (AASM). Sleep staging varied from this standard with sleep in infants > 6 months old scored using infant criteria outlined by (Anders et al., 1971; Iber et al., 2007a) and the ASSM criteria for infants ≥6 months as per local laboratory protocol at the time of polysomnography collection. For analysis, AS in infants <6 months and REM in infants ≥6 months were combined as REM, QS (<6 months infants) and N3 (≥6 months of age) were combined as SWS, and indeterminate (INT) sleep (<6 months infants) and N1+N2 (≥6 months of age) were combined as N1+N2. Respiratory events were defined as apneas (cessation of airflow, <10% of baseline level, for ≥2 breaths) and hypopneas (10-50% decrease in baseline

airflow associated with an arousal, awakening, or ≥3% oxygen desaturation). Obstructive apneas and hypopneas included respiratory events with ongoing respiratory effort. Central apneas showed a cessation of effort followed by arousal, awakening or ≥3% oxygen desaturation, or a duration of ≥20 seconds. Mixed apneas included both central and obstructive components in the same event. Desaturation events were identified based on a reduction of ≥ 3% in SpO_2 levels and duration was calculated from the onset until SpO_2 levels began to rise. The apnea-hypopnea index (Hernandez-Miranda et al.) was calculated as the number of apneas and/or hypopneas observed during sleep, divided by total sleep time.

In this study we also identified sighs and post-sigh apneas, with sighs characterized by increased (>50% amplitude) airflow/nasal pressure signal in combination with increased DIA_{EMG} signal. While sighs are relatively common in infants, we considered these as respiratory events in our analysis when they were followed by ≥3% SpO_2 desaturation. Post-sigh apnea was not distinguished from spontaneous central apnea on the basis that infant sighs are commonly followed by apneas or hypoventilation that may destabilize breathing.

Data processing

Polysomnogram raw data were acquired using Sandman (Natus Neuro, USA) and converted to EDF format to import into Labchart (ADInstruments, USA). After identifiers were removed, the following channels were extracted: nasal/oral respiratory flow; chest and abdominal RIP; EEG channels: C4-M1 and L-EOG-M2; ECG channels ECG1 and ECG2; DIA_{EMG} and ABD_{EMG}; sEMG; SpO2; transcutaneous CO_2 ($TcCO_2$). Event data (respiratory events, start time, duration, upper and lower limits) were also exported to assist with the identification and classification of respiratory events.

The nature of the recording methods introduced heartbeat artefacts into the raw DIA_{EMG} and ABD_{EMG} signals. We utilized a custom macro available through ADInstruments (Colorado Springs, CO, USA) to remove the QRS complex from EMG data [https://www.adinstruments.com/support/videos/labchart-mastery-removing-ecg-artifacts-emg-data]. DIA_{EMG} and ABD_{EMG} data retrieved in the 10-20 millisecond range preceding the QRS complex was used to fill in the signal gap left by QRS complex removal. Rectified DIA_{EMG} and ABD_{EMG} signals were analyzed as root mean square (Armstrong et al.) to create an integrated and measurable signal.

Analysis of ABD muscle activity

Any DIA_{EMG} and ABD_{EMG} signals of insufficient quality were excluded from analysis (45/72 polysomnograms). Exclusion criteria included: a poor signal-to-noise ratio, poor signal quality due to signal interference (e.g., DIA_{EMG} activity in the ABD_{EMG} channel and vice versa) or erroneous electrode placement that gave no respiratory modulated signal. Recordings with less than one hour of consecutive data of sufficient quality were also eliminated.

The duration of time spent in each sleep stage was determined from EEG and EOG signals. Wakefulness periods were not analyzed, and ABD recruitment was assessed only during sleep states (N1+N2/INT, SWS/QS, REM/AS) from the rectified ABD_{EMG} (i.e. ABD_{RMS}) signal in which we could identify five or more consecutive bursts with amplitude 50% greater than baseline non-respiratory tonic ABD activity. These criteria are consistent with those applied to our previous rodent sleep studies (Andrews & Pagliardini, 2015; Saini & Pagliardini, 2017a). We also measured the duration of ABD_{RMS} events as the number of consecutive breaths concurrent with ABD recruitment. Records were categorized based on the absence or presence of ABD muscle recruitment. The latter category was further subdivided into categories where ABD recruitment was concurrent with: 1) respiratory apnea/hypopnea + O_2 desaturation events (Resp+Desat), 2) respiratory apnea/hypopnea only (Resp), 3) O_2 desaturation events only (Desat), 4) a sequential combination of the previous categories 1,2 and/or 3 (Complex) and 5) ABD recruitment that occurred in the absence of respiratory apnea/hypopnea or O_2 desaturation events (No Resp or Desat). For categories 1-4, only respiratory events that occurred within a 15-breath interval of the ABD events were considered. It was also noted whether the ABD recruitment preceded, was concurrent with, or followed apnea/hypopnea or arterial O_2 desaturation. A period of ABD recruitment that presented with more than one of the sub-categories 1-3, consecutively, was classified complex, as a combination of respiratory events (subcategory 4).

Statistics

Statistical analysis for comparison across groups was done using SPSS (13.0, IBM). A one-way ANOVA with a Least Significant Difference (LSD) was used to confirm that demographic variables did not differ between data that was included (ABD recruitment present

or absent) vs. excluded from the present analysis. Independent Samples T-tests (normality assumption tested) or Chi Square tests (sex) were used to determine if demographic or other measurements differed between patients with and without ABD recruitment (Table 3.1), where $p<0.05$ was considered statistically significant. The number of ABD events per hour were compared across sleep state with a Repeated Measures ANOVA. One-way (single factor) or Univariate ANOVAs (two independent factors) were used to test statistical significance in the remaining data. Bonferroni corrections were applied for multiple comparisons when necessary, otherwise the LSD statistic was used for post-hoc comparisons. If a One-way ANOVA violated the equal variance assumption, an alternative robust statistic was used in conjunction with the Games-Howell post-hoc test.

3.3 Results

The 27 polysomnogram recordings that had ABD_{EMG} signals of sufficient quality for further analysis included subjects ranging from 24 days to 21.5 months old (mean ± SEM = 10 months, 28 ± 42 days): 9 patients were <6 months (33%) and 18 were 6–22 months (67%) at the time of testing. Sex ratios showed a male predominance (70% of total cases). No significant differences in age, sex or anthropometry of patients were observed between the excluded (i.e., poor EMG signal quality) versus the included studies.

A sub-set of pediatric patients exhibits abdominal recruitment during sleep

Forty-four % of subjects (12/27) (3/9 < 6 months old, 33%; 9/18 = 6-22 months, 50%) exhibited occasional ABD recruitment during sleep. Figure 3.1 shows a representative polysomnogram trace in which a 15.3-month-old subject in SWS exhibited prolonged expiratory-related ABD recruitment (figure 3.1, left panel: ABD_{RMS}) during uninterrupted respiration (as indicated in Airflow and DIA_{RMS}) without significant O_2desaturation. In these traces (figure 3.1B), the cardiac QRS complex overlapping with the EMG signals in both DIA_{EMG} and ABD_{EMG} traces was eliminated in the RMS channels by the ECG signal processing approach (Methods – Data Processing). The raw DIA_{EMG} and ABD_{EMG} and their respective RMS channels display an antiphase pattern of activation, indicative of ABD recruitment in the expiratory phase (figure 3.1B, blue box).

Age, sex, and anthropometry were compared between subjects exhibiting ABD recruitment and those that did not (Table 3.1) indicating no differences between subject groups, except for weight for height z-scores (higher in subjects that exhibited ABD recruitment).

ABD recruitment events are associated with N1+N2 and REM sleep states

Since respiratory instability is associated to varying degrees with different sleep states, we initially questioned whether ABD recruitment occurred more frequently during N1+N2, REM or SWS. We analyzed a total of 174 ABD_{RMS} recruitment events from 12 subjects (424 ± 17 min of polysomnogram traces per subject) to determine their association with specific sleep states. On average, ABD recruitment occurred often during N1+N2 (74 events; 2.2±0.6/hr; P=0.07), but more frequently in REM (82 events; 2.5±0.6/hr; P<0.05) compared to SWS (18 events; 0.7±0.3/hr; figure 3.2A). As ABD recruitment was significantly associated with REM sleep, we next determined if this was a result of ABD recruitment subjects spending more time in REM sleep as compared to those subjects without ABD recruitment. The absolute time and the percentage of time each subject spent in N1+N2, REM and SWS was calculated from the portions of the analyzed polysomnogram (figure 3.2B) and we did not resolve any statistical difference between the two groups (no ABD: REM: 82±16 mins, 29%; N1+N2, 121±27 mins, 35% and SWS 82±12 mins, 36%; ABD: REM: 117±21 mins, 28%; N1+N2: 200±26 mins, 46%; SWS: 108±10 mins, 26%).

Respiratory instability leading to respiratory events is frequently associated with arousals and it is possible that ABD recruitment may also occur in association with sleep fragmentation and arousals. In both subjects with and without ABD recruitment, SWS bout times were significantly longer than their corresponding N1+N2 and REM bout times (P<0.001; figure 3.2C). Yet, for each sleep state, bout duration was comparable between subjects with ABD recruitment (in mins: N1+N2: 9±2, SWS: 19±2 and REM: 11±1) and those without (in mins: N1+N2: 9±2, SWS: 17±2, REM: 12±2). Arousal frequency did also not differ between groups (14.6±1.3 arousals/hr in subject with ABD recruitment; 12.3±2.4 arousals/hr in subject without ABD recruitment; p=0.44). Therefore, sleep time bout duration and arousal, on their own, did not account for the occurrence of ABD recruitment observed in our subjects during sleep. To determine the association between ABD recruitment and arousals, we quantified the fraction of ABD recruitment events that occurred within 15-breaths of an arousal during sleep in general,

and for each sleep state. Arousals occurred in association with ABD activation during sleep at a frequency of 38±7% (figure 3.2D, hashed bar). Specifically, ABD recruitment was associated most frequently with arousals occurring in the N1+N2 state (20±5%; $P<0.05$) as compared to SWS (4±2%) or REM (14±4%; figure 3.2D); this association was likely due to the preponderance of arousals in N1+N2 as compared to other sleep states. Furthermore, when all ABD recruitments were analyzed in relation to the occurrence of the subsequent arousal, we observed a wide temporal distribution within and across subjects, suggesting that arousals are not triggered by ABD events or the factors that would trigger ABD recruitment (figure 3.2E).

The frequency of respiratory events does not influence occurrence of ADB muscle activity

Based on the nature of the dataset (subjects with suspected OSA), many of the patients with and without ABD recruitment events exhibited varying incidence of respiratory events. The apnea hypopnea hindex (Hernandez-Miranda et al.) in the two groups were not different (with ABD, AHI=16.83±5.41; without ABD, AHI= 17.14±3.53, p=0.9) and there was no difference in the total amount of time spent in apnea (with ABD, 17.39±8.45 min ; without ABD, 16.21±3.60 min; p=0.89).

While the presence of respiratory events was not prerequisite for occurrence of ABD activity, it is plausible that when ABD recruitment does occur, it is triggered by or it is in association with apnea/hypopnea or desaturation events (Esquer, Claure, D'Ugard, Wada, & Bancalari, 2007, 2008). In support of this idea, we found that within the cohort showing ABD activation, two patients with the highest frequencies of ABD recruitment (6.4/hr and 4.3/hr, respectively) also had the highest number of apnoea/hypopneas (79 and 88 total events; figure 3.3A).

Expiratory ABD muscle activity occurs most frequently during regular breathing or complex respiratory events

To further determine the existence of any relationship between ABD activity and respiratory disturbances, we categorized abdominal recruitment events by their association with respiratory (apnea/hypopnea) and desaturation events: 1) ABD activity associated with respiratory events and O_2 desaturation (resp+desat), 2) ABD activity associated with respiratory events without O_2 desaturation (resp), 3) ABD activity associated with O_2 desaturation only

(desat), 4) a combination of multiple respiratory events and O_2 desaturation (complex) that occurred within a single ABD recruitment, and 5) ABD activity that was not associated with apnea/hypopnea or O_2 desaturation (no resp+no desat).

Out of a total of 2891 identified respiratory events, 231 occurred within a 15-breath window prior, during or following the 174 ABD recruitments. figure 3.3B summarizes the percentage of total ABD recruitment events associated with each of the categories (# 1-5), averaged across subjects. Out of the total number of ABD events per patient, 55±9% occurred in the absence of respiratory events (ANOVA $P<0.001$). ABD recruitment was observed to a similar degree in the various categories associated with respiratory events (categories 1-4): resp+desat (#1) = 12±4%; resp (#2) = 6±3%; desat (#3) = 13±3%; and complex (#4) = 14±4% (ANOVA, $P>0.05$). These results suggest that even in the absence of evident respiratory challenges (apneas, hypopneas or O_2 desaturation), ABD muscle activity is present during sleep in infants and young children.

ABD recruitment is initiated during or after apneas and O_2 desaturation

In cases where ABD recruitment was associated with respiratory events, we sought to determine the temporal relationship between the two. We reasoned that if ABD recruitment is a contributing factor to respiratory instability or disturbances, we would observe its onset more often prior to the onset of a respiratory event. In contrast, if ABD recruitment was part of the physiological response to respiratory disturbances, then ABD activation would be triggered by the respiratory events, and thus occurring during or following a respiratory event. Determination of the onset of respiratory events in relation to the onset of ABD activity revealed that respiratory events, with or without O_2 desaturation, and isolated O_2 desaturation events started most frequently prior to or in conjunction with ABD recruitment. On the contrary, ABD recruitment was rarely followed by a respiratory event, suggesting that ABD recruitment itself was not a triggering factor for respiratory events (figure 3.3C). The temporal distribution of such events in relation to the ABD recruitment is also illustrated in the figure 3.3D.

To quantify the duration of ABD activity from commencement to termination, we assessed it both by the number of breaths and its duration in time (seconds). In cases where respiratory events were coincident with arterial O_2 desaturation (category #1), ABD recruitment lasted an average of 42±20 consecutive breaths (89±33s). ABD recruitment occurring with

apneas and hypopneas (category #2), had a duration of 38 ± 12 breaths (74±17s), while O_2 desaturation events (category #3) were associated with ABD activation lasting 48±14 breaths (112±35s) and ABD recruitment associated with multiple respiratory and desaturation events (category #4) had a duration of 100±28 breaths (263±79s). In the absence of any respiratory event (category #5), ABD recruitment persisted for an average of 45±7 breaths (108 ± 17s).

We further analyzed the temporal association between ABD recruitment and the recovery of O_2 saturation to baseline levels. Given that we measured respiratory and desaturation events occurring within a 15-breath interval (before, during and after ABD recruitment), we classified the recovery from O_2 desaturation as occurring before, during, or after the onset of ABD recruitment. In 74±9% of instances, O_2 desaturation recovered during the expression ABD activity (ANOVA, $P<0.001$; figure 3.4). In contrast, only 9±5% of O_2 desaturation events recovered before the onset of the ABD recruitment, 8±3% of O_2 desaturation events recovered after the termination of ABD activity, and 5±2% began and ended after the termination of ABD activity (figure 3.3D). This observation suggests that recovery from O_2 desaturation often occurs in temporal association with ABD recruitment. Only a fraction (5/60) of O_2 desaturations not associated with respiratory events followed a sigh.

Since recovery from apnea and O_2 desaturation may involve arousal, we questioned whether preceding arousals might drive the expression of ABD recruitment, rather than the respiratory event *per se*. We found no tendency for arousals occurring spontaneously (i.e., no respiratory event) or associated with a respiratory event to precede ABD recruitment ($P>0.05$).

Expiratory ABD muscle activity is primarily associated with apneas originating from airway obstruction

The increased respiratory rate and volume of expiratory airflow that accompanies active expiration has been postulated to play a role in clearing the airways of obstruction. In order to determine if ABD recruitment was selectively associated with airway obstruction causing apnea, we classified apneic events associated with ABD recruitment as obstructive or central in origin. Out of 171 apneic events, 19.9% (34/171) were central apneas occurring either in N1+N2 (15) or REM sleep (19), but not in SWS, while 80.1% of events were either obstructive apneas or hypopneas (137/171) that occurred primarily in REM sleep (REM:81; N1+N2:29; SWS:27). Among the ABD events that were not associated with any respiratory event (69), the majority

occurred in N1+N2 (35), although some events occurred also in REM (25) and SWS (8). Intriguingly, although central and obstructive apneas/ hypopneas associated with ABD recruitment were observed in both REM and N1+N2 sleep, the incidence of respiratory events associated with ABD activity tended to occur primarily in the same sleep state for an individual subject. For example, one subject showed 100% of respiratory events during N1+N2, while another exhibited 87% of such events during REM. We further wanted to quantify if there was any relationship between desaturation events and sighs. We found that of the 60 isolated desaturations from 12 subjects, only 5 desaturations were likely a consequence of a sigh and not due to an obstruction. Thus, majority of the desaturation events were likely due to an obstruction of the upper airways.

3.4 Discussion

The current study analyzed the occurrence of ABD expiratory activity using a dataset previously employed to investigate a pediatric population suspected of OSA (DeHaan et al., 2015). Based on surface EMG signals from the ABD muscles, in combination with classic polysomnography, we identified instances of ABD recruitment occurring in all sleep states, with preponderance in REM and N1+N2 compared to SWS sleep. These findings contrast with the dogma of REM-induced muscle atonia and suggest that ABD muscles are indeed recruited across different sleep stages. In our dataset we also observed ABD activity most frequently occurring in either regular breathing or associated with complex respiratory events. Significantly, the onset of ABD activity followed the commencement of respiratory events and, in most cases, terminated with restoration of regular breathing and recovery to physiological O_2 levels. Our study therefore suggests that, in contrast to previous studies dismissing significant recruitment of expiratory activity during REM sleep in OSA children (Praud et al., 1989), ABD recruitment is frequently observed during sleep and, based on these findings, we speculate that it may contribute to counteracting airway resistance, O_2desaturation and maintenance of proper ventilation during early stages of postnatal development across different sleep states.

Respiration requires a coordinated activation of respiratory muscles to promote efficient gas exchange from the first breath until death. The neonatal period of both preterm and full-term infants represents a critical time in respiratory control as the body undergoes progressive changes both in terms of lung development, respiratory control, and sleep maturation. Sleep changes

dramatically in the first months of postnatal life, with progressive transition from more primitive AS and QS states to a more organized EEG sleep pattern, sleep consolidation increases, and sleep stage distribution progressively resembles the one present in the adult population (de Weerd & van den Bossche, 2003; MacLean et al., 2015; Peirano et al., 2003). Concurrent with postnatal maturation of brain states and sleep homeostasis, respiratory control progressively develops, respiration becomes more regular and acquires sleep stage-specific characteristics (Carroll & Agarwal, 2010; Di Fiore et al., 2013; MacLean et al., 2015). However, breathing in the postnatal period still often features irregularities and respiratory events that pose a challenge for supporting adequate O_2 delivery to the brain and the entire body, delays overall infant development and may be life-threatening.

Our interest in investigating the ABD recruitment across sleep states in 0–2-year-old subjects stemmed from experimental evidence showing that respiration is generated by a network in which two oscillators control alternating contraction of inspiratory and expiratory muscles, with active inspiration being the primary drive for respiratory function, and active expiration being recruited in conditions of high respiratory and metabolic drive (Pisanski & Pagliardini, 2019). Exercise, hypercapnia and chronic and acute hypoxia (da Silva et al., 2019; Praud et al., 1993; Zoccal et al., 2018b; Zoccal et al., 2008) drive recruitment of ABD muscle activity, with hypercapnia being an important stimulus to ABD recruitment also during nREM sleep (Leirao et al., 2017). In rodents, expiratory muscles recruitment is also observed in normoxia in nREM and, surprisingly, REM sleep (Andrews & Pagliardini, 2015; Pisanski et al., 2020; Saini & Pagliardini, 2017a; Sherrey et al., 1988b). Although the physiological significance and the modulatory mechanisms for ABD recruitment are currently unknown, the temporal relationship with respiratory events suggest that ABD activation may contribute to maintaining ventilation in conditions of respiratory instability, increased airway resistance, increased metabolic demand, or reduction of inspiratory drive (Pisanski et al., 2020; Pisanski & Pagliardini, 2019). While generally silent (and not respiratory modulated) in supine position in humans, ABD tonic activity may occur in standing posture (De Troyer et al., 1983; Mesquita Montes et al., 2017), during and following mechanical ventilation (Esquer et al., 2007, 2008), and activation of phasic ABD muscles is present with increased inspiratory and expiratory loading, hypercapnia, hypoxia, or exercise (Abe et al., 1999; Abraham et al., 2002; De Troyer et al., 1990; Mesquita Montes et al., 2016).

Expiratory ABD muscles are also active in disorders such as hypertension, chronic obstructive pulmonary disease (COPD) and heart failure, and their recruitment is suggested to improve respiratory mechanics, support ventilation and reduce the work of breathing (Giordano, 2005; Haupt et al., 2012; Ninane et al., 1992; Ninane & De Troyer, 1995). In infants and children, ABD recruitment occurs in OSA patients, both during normal breathing and in presence of respiratory events (Esquer et al., 2007, 2008; Praud et al., 1989; Praud et al., 1991). Interestingly, in neonates ABD activity was rarely observed in REM, even under hypercapnic challenges (Praud et al., 1991). Here, we show that in about half of our limited dataset, ABD activity was frequently recruited in sleep despite lack of specific differences in sleep pattern, duration, or in respiratory disturbances across the two groups. Interestingly, ABD muscle activation was associated not only with hypoxic or obstructive events that are known to stimulate recruitment of ABD muscles (Jeffries et al., 1984; Praud et al., 1989), but was also present during apparently regular breathing, suggesting that expiratory ABD activity may occur in the absence of challenging factors, and contribute to ventilation during sleep. Whether ABD activation commonly occurs also in infants not affected by OSA remains to be determined. In fact, our ability to detect these events over long polysomnogram recordings may be favoured by using surface EMG electrodes, an approach that is not commonly used in clinical polysomnogram studies. Additionally, the lack of randomization of subjects, which were selected among patients suspected of OSA, may have emphasized respiratory events, as the AHI and respiratory assessment confirmed. Furthermore, since on average only a fraction of recorded time was analyzed, we may have under or overestimated the probability and fraction of time in which ABD activity occurred across sleep states.

In instances in which ABD activity was linked to a respiratory event or drop in O_2 saturation, it most often followed the event onset and decayed with recovery from either O_2 desaturation or airway obstruction. Although this is only a temporal association between the events, we propose that ABD recruitment during expiration may contribute to respiratory recovery by means of counteracting airway obstruction, facilitating the next inspiration by optimizing the end-expiratory diaphragm position for the most efficient inspiratory contraction, and therefore reducing and sharing the work of breathing with inspiratory muscles in agreement with previous work (Iscoe, 1998; Jeffries et al., 1984; Praud et al., 1989). This hypothesis will require future interventional studies to demonstrate causality.

Because of the limited subject number and the pure observational (and not interventional) nature of this study we may only suggest that ABD activity present in both regular breathing and during both central and obstructive respiratory events appears not to be a cause for unstable breathing, but likely contributes to maintenance and recovery of breathing in infants. Based on our previous work in rodents (Pisanski et al., 2020), we conclude that, although not necessary for respiratory function, expiratory ABD activity may contribute to respiratory stability and may be recruited to prevent the worsening of an already compromised breathing function in sleep.

Table

Table 3.1. Description of the demographics, sleep and respiratory parameters in subjects with and without ABD recruitment based on chart data.

Parameter	ABD Absent	ABD Present	Significance
Study Demographics			
Age (Mo.)	9.6 ± 2.0 (15)	12.6 ± 1.9 (12)	0.52
Sex: % Male	73%	67%	0.63
Height (z score)	-0.51 ± 0.38 (11)	0.01 ± 0.55 (11)	0.45
Weight (z score)	-0.24 ± 0.36 (13)	0.98 ± 0.52 (11)	0.15
Weight for height (z score)	-0.26 ± 0.51 (12)	1.44 ± 0.47 (11)	0.02**
Head circumference (z score)	-0.27 ± 0.57 (12)	0.52 ± 0.56 (12)	0.52
Sleep Characteristics			
TST (Straus et al.)	405.7 ± 30.9 (15)	443.1 ± 16.3 (12)	0.52
Sleep Efficiency (%)	81.6 ± 3.4 (15)	83.9 ± 2.7 (12)	0.25
N3 Sleep (%)	30.3 ± 2.9 (15)	34.4 ± 4.1 (12)	0.67
REM Sleep (%)	28.2 ± 2.7 (15)	26.4 ± 3.7 (12)	0.81
Arousal Index (events/hr)	27.5 ± 6.5 (15)	26.5 ± 7.4 (12)	0.99
Respiratory Characteristics			
TST/AHI (events/hr)	17.1 ± 3.5 (14)	16.8 ± 5.4 (12)	0.99
MnSpO_2TST (%)	97.7 ± 0.4 (15)	97.2 ± 0.6 (12)	0.78
DesatTSTN (events/hr)	20.7 ± 5.4 (15)	26.9 ± 8.5 (12)	0.70
CO2over50mmHg	3.1 ± 1.9 (15)	1.0 ± 0.7 (12)	0.40
OMAHI (events/hr)	13.7 ± 2.9 (15)	13.8 ± 5.2 (12)	0.96
CAI (events/hr)	4.6 ± 1.9 (15)	3.6 ± 0.9 (12)	0.79

Replicates noted in brackets. Significance from One Way Anova. Abbreviations: TST, total sleep time; TST/AHI, total sleep time/ apnea-hypopnea index; MnSpO_2TST, mean SpO2 (%) for TST; DesatTSTN, oxygen desaturation index for TST (events/h); OMAHI, obstructive mixed apnea hypopnea index; CAI, central apnea index.

Figures

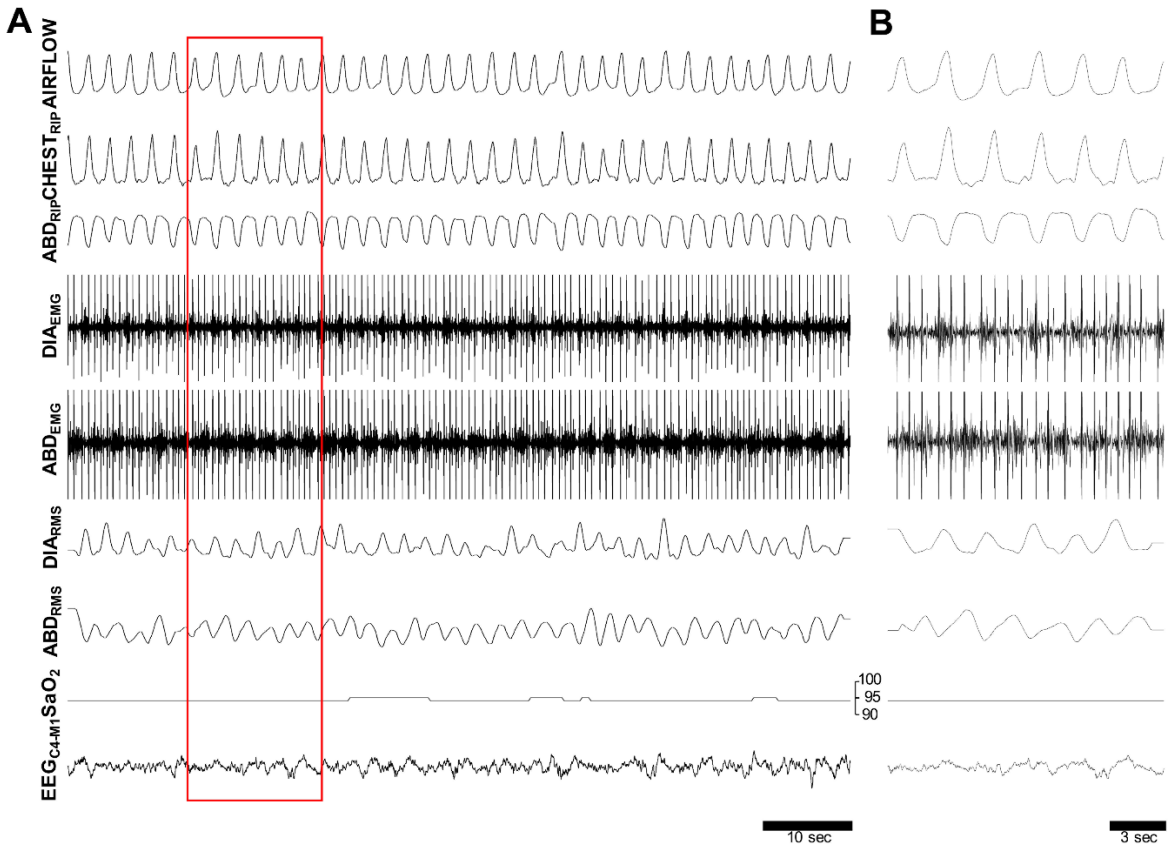

Figure 3.1. Expression of ABD muscle activity in infant polysomnogram recordings. A) A representative 70-second polysomnogram recording during SWS in which the abdominal (ABD) muscle showed continuous expiratory activity as illustrated by surface EMG recordings (ABD_{EMG}) and its integrated trace (ABD_{RMS}) during uninterrupted breathing. Respiratory activity was monitored by airflow measurement (top), respiratory inductance plethysmography in the chest and abdomen ($CHEST_{RIP}$, ABD_{RIP}) and surface EMG electrodes in the diaphragm (DIA_{EMG}, DIA_{RMS}). B) Expansion of the red boxed area in (A) illustrates the ECG artefact caused by the QRS complex, present in both DIA_{EMG} and ABD_{EMG} traces and eliminated in the RMS signal through filtering (see Methods for details). EEG_{C4-M1}: right central electroencephalogram; SaO_2: arterial O_2 saturation (%).

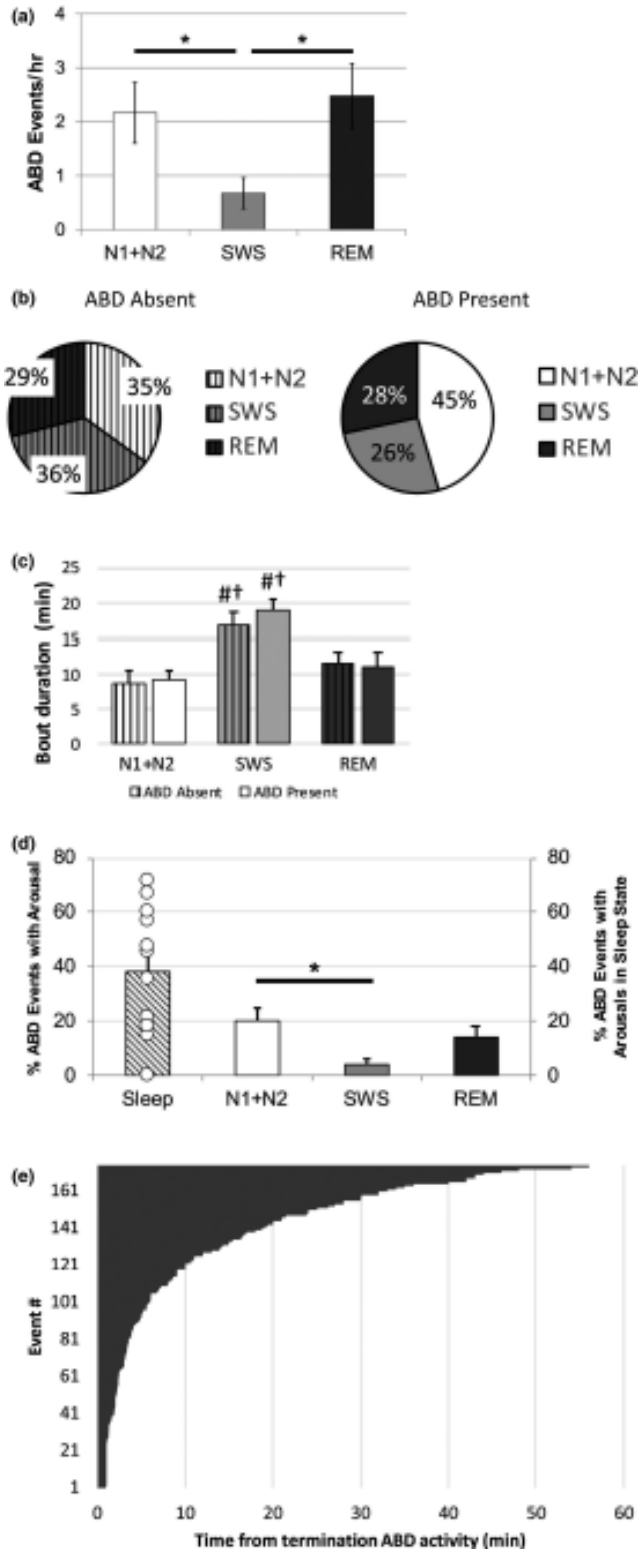

Figure 3.2. Expression of ABD activity across sleep states and sleep characteristics. A) Frequency (events/hr) of ABD events in N1+N2 (white), SWS (grey) and REM (dark grey) sleep. ABD events were more frequent in REM sleep compared to SWS. B) The percentage of time spent in N1+N2 (white), SWS (grey) and REM (dark grey) sleep was similar between subjects that did not exhibit ABD recruitment (striped) and those that showed ABD recruitment (solid). C) Sleep bout duration (in minutes) was similar for patients with ABD recruitment (striped) and without (solid). Bout duration was longest during SWS in subjects both with and without ABD recruitment compared to N1+N2 (#) and REM (†) sleep ($P<0.05$). D) Percentage of total ABD recruitment events associated with arousal in during any sleep stage (Sleep) and in association with a specific sleep state (N1+N2, SWS, REM). Mean ± SEM. Dots represent individual data points. E) The temporal association (in mins) between termination of ABD activity (time=0) and onset of subsequent arousal is shown for each ABD recruitment event.
*$P<0.05$

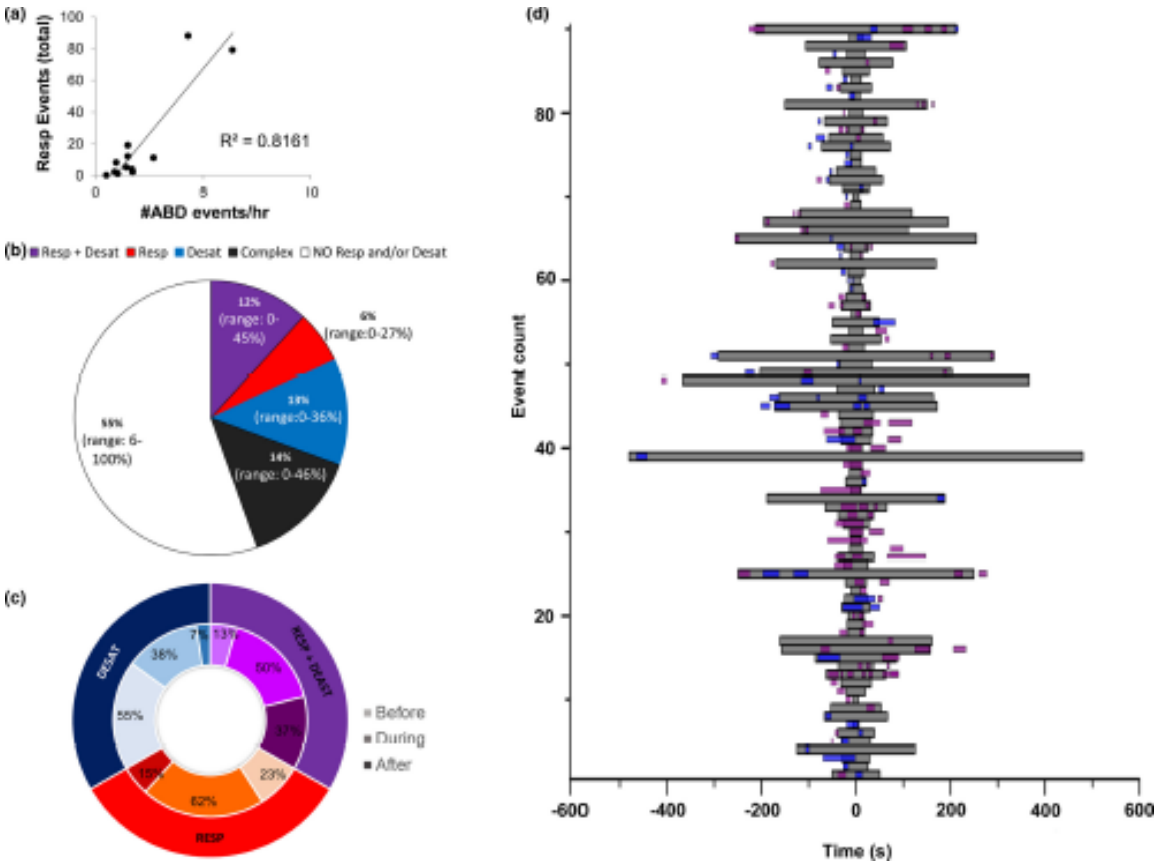

Figure 3.3. Occurrence and temporal relationship between respiratory events and ABD recruitment. A) The frequency of ABD recruitments/hr is plotted against the number of respiratory events for each subject (n=12). B) Mean percentage of ABD events occurring in association with defined respiratory events (categories #1-5 described in the results): respiratory events (resp; red), desaturation events (desat; blue), respiratory events with desaturation (RESP + DESAT; purple), complex respiratory events (COMPLEX; black) and ABD recruitment that occurred in the absence of respiratory disturbances (NO RESP/DESAT; white). "Range" indicates maximum and minimum values. C) Analysis of the temporal relationship between respiratory events indicated that in most cases, respiratory events occurred before (light shading) or during (medium shading) ABD events rather than following ABD events (dark shading); respiratory apnea (RESP; red), O_2 desaturation (DESAT; blue); combination (RESP + DESAT; purple). D) Floating bar graphs showing the temporal distribution of desaturation events in relation to ABD recruitment (gray bars; ABD recruitment, centered to time = 0; blue bars: desaturation only; purple bars: desaturation occurring in conjunction with respiratory events; n=90).

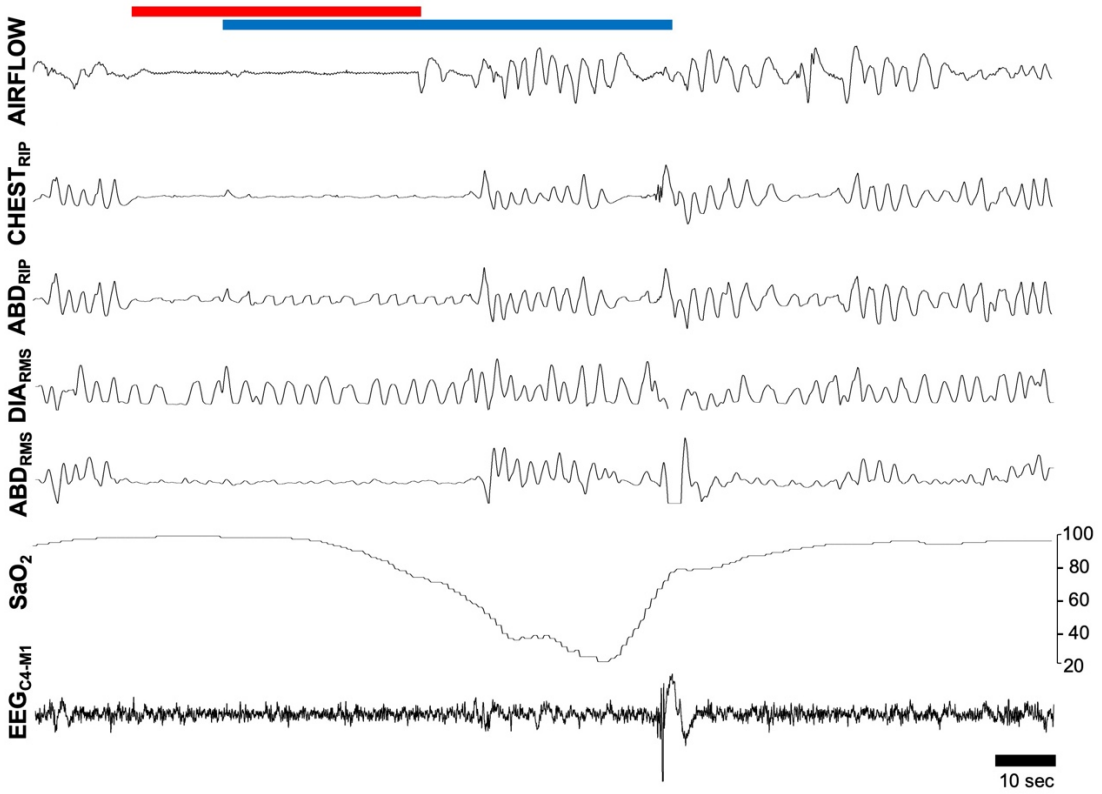

Figure 3.4. Abdominal muscle recruitment is associated with arterial O_2 desaturation. Representative polysomnogram trace of a 10.3 month-old subject in REM sleep exhibiting a single obstructive apnea (red bar; 50 sec duration) as indicated by the lack of AIRFLOW and $CHEST_{RIP}$ activity concurrent with activation of the diaphragm (DIA_{EMG}, DIA_{RMS}). The respiratory event was associated with significant arterial O_2 desaturation (blue bar; SaO_2 decreased from 99% to 23%; 77 sec duration). Abdominal muscle recruitment (ABD_{RMS}, red trace) was initiated during the desaturation event and terminated upon recovery of the SaO_2. EEG_{C4-M1}: right central electroencephalogram; SaO_2: arterial O_2 saturation (%).

Chapter 4: Ventilatory recovery by Etonogestrel in Central Chemoreflex-impaired Adult Female Rats

Jasmeen K. Saini-Rai, Landon A. De Hoog, Silvia Pagliardini

Not published. The study constitutes the foundation for two subsequent studies performed in the Pagliardini lab.

One study has been published as:

Etonogestrel Administration Reduces the Expression of PHOX2B and Its Target Genes in the Solitary Tract Nucleus.

Silvia Cardani, Tara A. Janes, Jasmeen K. Saini, Simona Di Lascio, Roberta Benfante, Diego Fornasari and Silvia Pagliardini (2022)

International Journal of Molecular Science 23(9):1-21

The other study is going through the peer review process as:

Etonogestrel promotes respiratory recovery in an in vitro rat model of central chemoreflex impairment

Tara A. Janes, Silvia Cardani, Jasmeen K. Saini, Silvia Pagliardini

4.1 Introduction

Progesterone has been implicated in breathing augmentation during pregnancy (Behan & Wenninger, 2008; Saaresranta & Polo, 2002) and the luteal phase of the menstrual cycle (England & Farhi, 1976; Goodland & Pommerenke, 1952; Slatkovska et al., 2006; White et al., 1983). Additionally, the reduction in circulating progesterone levels in women is proposed to be a main factor affecting the increased occurrence of SDB following menopause (Saaresranta & Polo, 2002), although studies investigating the use of progesterone and synthetic progestins to counteract respiratory disorders have given mixed results (Andersen et al., 2006; Cistulli et al., 1994; Collop, 1994; Cook et al., 1989; Driver et al., 2005; Pickett et al., 1989; Saaresranta & Polo, 2002; Strohl et al., 1981).

Studies in experimental models have identified multiple sites and mechanisms of action of progesterone, its metabolites, and synthetic progestins. Central and peripheral areas potentially involved in the potentiation of respiration by progestins are peripheral chemoreceptors in the carotid bodies, serotonergic neurons in the RN, the NTS, the ventral tegmental area, the hypoglossal nucleus, the LC, the PB, and the hypothalamus (Behan & Wenninger, 2008; Behan et al., 2003; Curran-Rauhut & Petersen, 2002; Hannhart et al., 1990; Hannhart et al., 1989; Loeppky et al., 2001; Loiseau et al., 2018). Furthermore, progestins may act through alternate pathways via modulation of GABAergic, glutamatergic, nicotinic, serotonergic, and oxytocinergic receptors in addition to modulation of various ion channels (Behan et al., 2003; Loiseau et al., 2018).

Recently, the stimulatory effects on ventilation by a subclass of progestins (gonanes) have been reported in patients affected by Congenital Central Hyperventilation Syndrome (CCHS; (Straus et al., 2010), a rare genetic disorder caused primarily by a polyalanine expansion repeat mutation in the Paired Like Homeobox 2 (*PHOX2B*) gene that causes severe hypoventilation and impairment in the CO_2 chemoreflex, particularly during sleep (Amiel et al., 2003; Trang et al., 2005). A serendipitous finding in two CCHS patients indicates potentiation of baseline respiratory frequency and associated reduced $PETCO_2$ (Joubert et al., 2016), and partial recovery of CO_2 chemosensitivity with administration of a potent progestin contraceptive, desogestrel (Straus et al., 2010), although these results were not confirmed in a subsequent single-case study (Li et al., 2013), likely due to differences in drug composition, genotype and severity in the disease (Loiseau et al., 2014; Mahesh et al., 1996). Since the number of cases in

both studies is limited, further preclinical and clinical studies are necessary to determine the efficacy of this treatment in improving ventilation in CCHS patients (Straus & Similowski, 2011; Straus et al., 2010).

Nonetheless, *in vivo* and *in vitro* studies in perinatal rodents suggest that desogestrel and its active metabolite, etonogestrel (3-ketodesogestrel; ETO) may have stimulatory effects on respiratory frequency *in vivo* and on the response to metabolic acidosis *in vitro* (Joubert et al., 2016) possibly due to ETO stimulation of pontine and midbrain areas that are rich in progesterone receptors, such as locus coeruleus, hypothalamus or periaqueductal gray (Loiseau et al., 2014) in addition to the raphe nuclei (Joubert et al., 2016).

The partial recovery of CO_2 chemosensitivity with desogestrel in CCHS patients and the *in vitro* stimulatory ETO effect in perinatal rodents prompted us to investigate the effects of long-term systemic ETO administration in healthy and central chemoreflex-impaired (RTN lesioned) (Mantyh et al., 1997) adult female rats. We hypothesized that ETO would stimulate breathing during ventilatory challenges in healthy and pathological conditions and would provide us with the experimental model to further investigate the mechanisms of ventilatory potentiation elicited by ETO in healthy and pathological conditions.

In this study, we tested baseline breathing and the respiratory response to hypercapnic and hypoxic challenges following instrumentation of healthy and chemoreflex-impaired female rats with slices of Nexplanon® rods (Merck, MSD Corp) to continuously deliver ETO over a four-week period. We assessed baseline breathing and the chemoreflex on a weekly basis. At the end of the treatment period, chemosensitivity was also tested under isoflurane anesthesia to eliminate potential confounding behavioural responses.

Our results indicate that long-term ETO administration does not alter baseline breathing and chemoreflex responses in healthy conditions. A potentiation in the secondary response to hypoxia was only observed under isoflurane anesthesia. In contrast, ETO supplementation in RTN lesioned rats increased normoxic tidal volume and reverted minute ventilation to pre- lesion values. Furthermore, long-term ETO administration promoted a recovery of the hypercapnic ventilatory responses despite significant RTN lesion and impairment. Our results thus indicate that ETO is effective in promoting recovery of respiratory function and chemosensitivity in conditions in which the central chemoreflex is impaired.

4.2 Materials and Methods

Ethical Approval

A total of 60 adult female Sprague-Dawley rats (Charles River, Montreal, Canada) at ~ 5-6 weeks of age and weighing between 200-250 grams were used in this study. The Health Science Animal Committee at the University of Alberta approved all the experimental procedures according to the guidelines established by the Canadian Council on Animal Care (AUP#461). Rats were housed with food and water *ad libitum* under a regular 12-hour light-dark schedule with lights turned on at 0800.

Vaginal Swabs

Rats underwent daily vaginal swabs to observe cytology of fluctuations in hormone levels during the estrus cycle (Long JA, 1922; Marcondes et al., 2002). Vaginal smears were collected daily at 9:00 AM using 60 µl of saline (0.9% NaCl) with a plastic pipette and examined on a glass slide under a light microscope (Zeiss, Primo Star, Jena, Germany). Rats were considered to be in proestrus when nucleated epithelial cells dominated the vaginal smear morphology. The cytology of estrous was 75% nucleated cells and 25% cornified cells (Lohmiller JJ, 2006; Marcondes et al., 2002). Rats were used for experiments only if they demonstrated regular progression through the estrous cycle.

Hormonal Interventions

Female Sprague-Dawley rats (200-250g; n=60) were tested in proestrus until the beginning of hormonal treatments. Etonogestrel (DeHaan et al.) was delivered implantation of Nexplanon® rods (MERCK, Kenilworth, NJ). In order to potentiate progesterone receptor expression, and therefore ETO effect on ventilation, we chronically delivered 17β-estradiol (E2; 50µg/day; Sigma Aldrich, Oakville, Canada) through osmotic pumps (2ML4; Alzet, Cupertino, CA).

A pilot study was initially conducted on 22 rats to determine the appropriate thickness of the Nexplanon® rods that would elicit comparable ETO serum levels observed in women instrumented with Nexplanon® rods for contraceptive regimen (Bennink, 2000; Palomba et al., 2012). Different thicknesses of Nexplanon® rod slices were tested: 250, 500 and 1000µm and ETO serum levels were analyzed 4 weeks after instrumentation.

Rats used for chemosensitivity testing in healthy conditions were separated into four treatments groups: 1) sham operated (n=9); 2) ETO (1000μm thick Nexplanon® rod, estimated to deliver ETO 1.75μg/day, subcutaneous; n=10); 3) 17β-estradiol (E2; 50μg/day, subcutaneous; n=6) and 4) ETO+E2 (1000μm thick Nexplanon® rod +50μg/day E2; n=6). In RTN impaired rats two groups were analyzed: SHAM SP-SAP (n=4) and ETO SP-SAP (n=5; 1000μm thick Nexplanon® rod, estimated to deliver ETO 1.75μg/day) rats.

Osmotic Pump Implantation

Priming animals with estrogen (E2) has been proposed to increase expression of progesterone receptors in various tissues and to potentiate progesterone effects on ventilation (Bayliss & Millhorn, 1992; Brodeur et al., 1986; Hosenpud et al., 1983; Kato et al., 1978; MacLusky & McEwen, 1978, 1980; Monks et al., 2001). We therefore tested the effects of ETO in conjunction with E2 administration. Rats were implanted with an osmotic pump delivering 17β-estradiol (50μg/day) dissolved in polyethylene glycol 300 for four weeks in two experimental groups: E2 (n=6) and ETO + E2 (n=6).

In order to subcutaneously insert the osmotic pump, rats were briefly anesthetized with 2% isoflourane and surgery was conducted in aseptic conditions. A 2-centimeter incision was made in the dorsal region below the neck, the osmotic pump was inserted, and the skin was then sutured back. Rats were treated with Marcaine (0.5%, subcutaneously) and Metacam (1mg/Kg, orally) to alleviate post-operative pain.

Etonogestrel therapy and Nexplanon® rod instrumentation

Nexplanon® rods were kindly donated by MERCK through the Investigator Initiated Studies Program (IIS#: 55869) and were adapted to use in rodents by cutting them at the appropriate size to adjust levels of ETO release in rats. The Nexplanon® rods were initially cut into 250, 500 and 1000μm thick slices using a vibrating microtome (VT1000S, Leica, Concord, Canada).

Rats were anesthetized with 2% isoflourane and slices of Nexplanon® rods were placed subcutaneously in between the shoulder blades. After surgery, Marcaine (0.5%; 0.5ml) was subcutaneously injected around the incision site to alleviate post-operative pain.

Ablation of retrotrapezoid nucleus (RTN) chemosensitive neurons

An additional group of rats was used to impair CO_2 chemoreflex by lesioning neurons in the retrotrapezoid nucleus (RTN), a chemosensitive area that plays a key role in the CO_2 chemoreflex (Abbott et al., 2009; Bodineau et al., 2000; Hernandez-Miranda et al., 2018; Nattie & Li, 2002; Stornetta et al., 2006) and is impaired/deleted in CCHS animal models (Dubreuil et al., 2008; Durand et al., 2005). We selectively killed RTN neurons by injecting saporin conjugated to substance P (SP-SAP, Advanced Targeting Systems, San Diego, CA), a ribosome-inactivating agent that is internalized by neurokinin-1 receptors expressing neurons (Mantyh et al., 1997). Adult female rats (~300-350 gram) were anesthetized with intraperitoneal injections of ketamine (90 mg/kg) and xylazine (10 mg/kg) in aseptic conditions. Rats were positioned on a stereotaxic frame with bregma and lambda positioned at a 30° angle and 2 bilateral injections of SP-SAP solution (150nl; 43.3 ng/ml; IT-11) were performed according to the following coordinates relative to obex (in mm): dorsoventral (DV) -3.5; mediolateral (ML) ±1.8, and rostrocaudal (RC) +2.0 and DV-3.6; ML ±1.8; RC+2.4. Following each injection, the glass pipette (30μm in diameter) was left in place for 5-minutes to avoid backflow of the toxin along the electrode track. At the end of the surgery, neck muscles and skin were sutured, and rats were treated with Marcaine (0.5%, 0.5ml; subcutaneously) and Metacam (1mg/Kg, oral) to alleviate post-operative pain. Rats recovered for one week before vaginal swabbing and testing resumed.

Hypoxic and Hypercapnic challenges in freely behaving rats

Rats underwent two days of acclimatization (3-hours/day) in 4-liter whole body plethysmographs (PY4311, Data Sciences International, St. Paul, MN) in normoxia before the beginning of the experiments. Air composition in the chambers was regulated using a gas mixer (GSM-3, CWE, PA) and flow related pressure changes were measured with a pressure transducer connected with a carrier demodulator (model CD15, Valydine, Los Angeles, CA) and recorded with PowerLab acquisition system in LabChart 7 software (AD Instruments – Data Acquisition Systems for Life Science, Sydney, Australia).

Rats were acclimatized for 1.25 hours (or until restful) in normoxic air (1.8 L/min in 21% O_2 balanced with N_2) followed by a 15-minute challenge with either hypoxia (10% O_2 balanced with N_2; n=15) or hypercapnia (5% CO_2, 21% O_2 balanced with N_2; n=17). After a 1.5-hour recovery period, rats were challenged with a subsequent hypercapnic or hypoxic challenge for 15 minutes.

At least two sets of respiratory challenges in control conditions were obtained during the proestrus phase before acute DMSO or ETO injections. After acute testing and Nexplanon rod instrumentation, rats were tested weekly for the subsequent four weeks.

In RTN lesioned rats (n=9) 4 weekly plethysmograph recordings were performed during proestrus following SP-SAP injection (W1,2,3,4) and prior to hormonal treatment. Following Nexplanon (n=5)/sham (n=4) instrumentation, RTN-lesioned rats were again tested weekly for four subsequent weeks. Because no significant different in ventilatory parameters were observed across the 4 weeks (both pre and post Nexplanon implantation) an average of the 4 weekly recordings is reported in the results section.

Results from rats that underwent hypoxia first were compared with the ones that underwent hypoxia following hypercapnia, in order to ensure there were no order differences in the two groups. The same analysis was performed for hypercapnia and no differences were observed in the order of either respiratory challenge.

Hypoxic and hypercapnic challenges under isofluorane anesthesia

At the end of the 4-week testing period rats underwent a final acute surgery under isofluorane anesthesia (2-2.5%). Rats were instrumented with paired electrodes in the diaphragm muscle (DIA), and a tracheal tube was connected to a pneumotach in order to measure airflow and deliver gases and anesthesia. Signals from the DIA_{EMG} electrodes were connected to a differential amplifier (AM-Systems, Sequim, WA) and sampled at 1kHz on a PowerLab acquisition system.

The anesthetic machine (Somnosuite, Kent Scientific, Torrington, CT) was connected to the GSM-3 gas mixer in order to deliver the appropriate anesthetic levels and to change gas composition through the experimental procedure. Exhaust air from the rats were continuously analyzed through a gas analyzer (ML206, ADInstruments) to assess End tidal CO_2.

An initial hyperoxic (40% O_2) gas mixture was delivered for 20 minutes or until breathing rate was stable for 15 consecutive minutes. Rats were then challenged with either hypoxia or hypercapnia for 15 minutes and allowed to recover to baseline breathing (>15 minutes) before the second challenge (either hypercapnia or hypoxia). In these experiments, hypercapnia was delivered in stepwise intervals, increasing percent CO_2 delivered every 3 minutes from 3, 5, 7, 9

to 11% CO_2 (in 40% O_2 balanced with N_2). In RTN impaired rats (SHAM SP-SAP and ETO SP-SAP), a stepwise increase in hypercapnia was always delivered first, followed by hypoxia.

At the end of the experiment, blood was collected from the heart to determine ETO and E2 serum levels and rats were transcradially perfused with 4% paraformaldehyde dissolved in phosphate buffer.

Etonogestrel and Estradiol serum levels

In an initial set of rats (n=22) blood serum was collected after 4 weeks of ETO administration with variable thicknesses of the Nexplanon® rod slices (250, 500, 1000μm thick slices). Rats were anesthetized with urethane (1.5g/kg, IP), blood samples were collected from the heart, and collected sample tubes were left in the upright position at room temperature for 30 minutes. The samples were then centrifuged at 2000xg for 15-minutes to separate the serum and stored at -20° Celsius until further analysis. Serum concentration of ETO was determined by a liquid-liquid extraction method performed by Q2 lab solution (Q2 Solutions, New York). Both blood samples and the internal standards underwent reverse phase high-performance liquid chromatography and tandem mass spectrometric detection (SCIEX, API6500, Framingham, MA). This mass spectrometry utilizes a turbo ion spray interfase in the positive ion mode.

Estrogen levels were analyzed in E2 rats (n=12). E2 serum concentration was determined by an electrochemiluminescence immunoassay (ECLIA, Elecsys Estradiol III Assay). The assay involved a four-step process including two incubations steps, the first with the sample and two monoclonal anti estradiol-biotinylated antibodies and the second involving an interaction of biotin and streptavidin coated microparticles. In the third step microparticles were magnetically captured to the surface of the electrode and then voltage applied to the electrode generated a chemiluminescent emission that was evaluated by a photomultiplier. The results were quantified with a standard calibration curve (Modular analytics E170).

Immunohistochemistry and cell counting procedure

Perfused RTN lesioned brains and aged matched controls were sliced on a vibrating microtome (VT1000S, Leica). Fifty-micron thick serial sections were immunoreacted for detection of specific markers to determine the degree of SP-SAP induced ablation in the RTN. The primary antibodies used were the following: anti-PHOX2B (raised in mouse; Santa Cruz

Biotechnology, Cat# sc-376997, Lot# J0218, RRID: AB_2813765, dilution 1:100, Dallas, TX), anti- receptor for Substance-P (NK-1 receptor; raised in rabbit, EMD Millipore, Cat# AB5060, Lot# 3030163, RRID:AB_2200636, dilution 1:500, Burlington, MA) anti-choline acetyltransferase (ChAT; raised in goat, EMD Millipore, Cat# AB144P, Lot# 3047032, RRID:AB_2079751, dilution 1:500, Burlington, MA,) and anti-Tyrosine hydroxylase (TH; EMD Millipore, Cat# AB152, Lot#: 2159637,RRID:AB_390204, dilution 1:1000, Burlington, MA).

Free floating sections were rinsed in phosphate saline buffer (PBS; Fisher Bioreagents, Geel, Belgium) and incubated with 10% normal donkey serum (NDS, Jackson ImmunoResearch Labs, Cat# 017-000-121, Lot#: 142410, RRID:AB_2337258, West Grove, PA) and 0.3% Triton X-100 (MP Biomedicals, Illkirch, France) in PBS for 60 min. Sections were incubated overnight with primary antibodies diluted in PBS containing 1% NDS and 0.3% Triton X-100. After three, five-minute washes in PBS, sections were incubated with secondary antibodies conjugated to the fluorescent probes Cy2 conjugated-donkey anti-rabbit (Cy2-DAR, Jackson ImmunoResearch Labs, Cat# 711-225-152, Lot#: 139999, RRID:AB_2340612, dilution 1:200, West Grove, PA); Cy3 conjugated-donkey anti-goat (Cy3-DAGt, Jackson ImmunoResearch Labs, Cat# 705-166-147, Lot#: 142875, RRID:AB_2340413, dilution 1:200, West Grove, PA) and Cy5 conjugated-donkey anti-mouse (Cy5-DAM, Jackson ImmunoResearch Labs , Cat# 715-175-151, Lot#: 130540, RRID:AB_2340820, dilution 1:200, West Grove, PA) diluted in PBS and 1% NDS for 2 hours. Sections were further washed in PBS, incubated with and NucBlue (DAPI; Thermofisher Scientific, Waltham, MA) for 30 minutes and washed in PBS before being mounted on slides and cover slipped with Fluorsave mounting medium (EMD Millipore, Burlington, MA).

PHOX2B and TH positive nuclei in the RTN region were counted under an inverted microscope (EVOS FL, Thermofisher Scientific) to determine the degree of cell ablation in SP-SAP injected rats (Paxinos G, 2005) . Phox2B and TH positive cells were counted in 1 every 4 section (200µm interval) in the ventral region surrounding the facial nucleus (identified by ChAT immunoreactivity) beginning 600µm from the caudal tip of the facial nucleus and going 600µm rostral.

Data Analysis

A breath-by-breath analysis was performed on traces acquired for both freely behaving (whole body plethysmography) and anesthetized (pneumotach and DIA_{EMG}) experiments to

calculate breaths per minute (BPM) and the relative changes in tidal volume (V_T) and minute ventilation ($\dot{V}e$). Relative V_T and $\dot{V}e$ were normalized to CTR with respect to body weight**.** Respiratory parameters were averaged from three 3-min intervals in normoxia. During hypoxia and hypercapnia, analysis was performed in 3 minutes of quiet breathing during the last 10 minutes of the challenge.

All behavioural plethysmography data is presented in box and whiskers plots as either a percent change from the control (baseline responses before hormonal treatment) or percent of change from baseline during hypoxic/hypercapnic ventilatory responses. Under isoflurane anesthesia the hypoxic ventilatory response is presented as a percent of change relative to normoxic breathing over the 15-minute challenge, whereas the hypercapnic ventilatory response is presented over a stepwise increase in CO_2 concentration.

Statistics

To test the hypothesis that hormonally implanted rats in healthy and pathological conditions would display an increase in ventilatory parameters we averaged and normalizedvalues per treatment group for both baseline and challenge conditions compared by either two-way repeated measures (data from freely behaving experiments and HcVR under anesthesia) and one-way analysis of variance (hypoxia under anesthesia) [ANOVA] using Sigmaplot (Sigmaplot, RRID:SCR_003210). The post-hoc tests were used to assess statistical significance between means of different treatment groups. All the data tested for statistical changes were normally distributed.

In RTN lesioned rats, recordings from SP-SAP, ETO SP-SAP and SHAM SP-SAP were averaged across 4 weeks post lesion and the 4 weeks of ETO/SHAM treatment since no significant differences were observed from W1 to W4 in each treatment.

The majority of the data is presented either as a percent change or as the mean ± standard deviation. Only breathing frequency in RTN impaired rats is presented as absolute value. P values less than 0.05 were considered statistically significant. In figures, asterisks (*) represent significant changes between groups at a given time, theta (Φ) represents changes within a group relative to control, gamma (Colten, Altevogt, & Institute of Medicine (U.S.). Committee on Sleep Medicine and Research.) represents significant decrease in SHAM SP-SAP and ETO SP-SAP rats compared to control, and delta (Colten, Altevogt, & Institute of Medicine (U.S.).

Committee on Sleep Medicine and Research.) represents significant decrease in ETO SP-SAP rats relative to both CTR and SHAM SP-SAP.

4.3 Results

Etonogestrel serum levels in Nexplanon® instrumented rats

Human grade Nexplanon® rods were used in this study. Because the full rod is designed to continuously provide ~200pg/ml ETO in women's serum for a 3 year period (Bennink, 2000; Palomba et al., 2012), we initially size-adjusted Nexplanon® rods for adult female rats and measured ETO serum levels 4 weeks after instrumentation. 1000µm-thick Nexplanon® rods produced ETO levels (236.8±50.0pg/mL; n=5), while thinner rods were below such levels (250µm=176.0±39.2pg/ml, n=5; 500µm=175.3±7.5pg/ml, n=4). Thus, 1000µm thick Nexplanon® rods were used for the remainder of the study.

Baseline breathing (normoxia)

Baseline breathing frequency (breath per minutes, BPM), relative tidal volume (V_T) and minute ventilation ($\dot{V}e$) were assessed weekly during the 4-week treatment period and compared to control values (CTR, before hormonal treatment; figure **1**). All rats displayed a regular estrous cycle until instrumented with the Nexplanon® rods, E2 osmotic pumps, or a combination of both. Rats in CTR conditions and SHAM rats were always tested in proestrus.

There was no difference in BPM, V_T, and $\dot{V}e$ between groups in CTR conditions. Figure **4.1A** illustrates the decrease in BPM relative to CTR at week 1 in SHAM (87.1±12.8%; P<0.001) and ETO rats (90.5±9.2%; P=0.013), at week 2 in SHAM (85.4±11.4%; P<0.001) and ETO rats (86.8±7.9%; P<0.001) and at weeks 3-4 in all groups.

Relative V_T and $\dot{V}e$ were normalized to CTR with respect to body weight**.** Relative V_T decreased at week 4 in ETO rats (86.6±18.7%; P=0.0091; figure 4.1B), while $\dot{V}e$ decreased in ETO rats at week 2 (75.0±19.6%; P<0.001) and week 3 (76.1±14.6%; P<0.001), and in every group at week 4 compared to their CTR (figure 4.1C).

Overall, these results indicate that hormonal treatments (ETO, E2, ETO+E2) had limited effects on baseline breathing compared to SHAM rats. Irrespective of the hormonal treatment, rats displayed a BPM decrease at weeks 3-4 and a small but significant reduction in $\dot{V}e$ was observed in all groups at week 4 compared to their own CTR.

Hypercapnic and hypoxic ventilatory responses in ETO treated rats

The hypercapnic ventilatory response (HcVR) and the hypoxic ventilatory response (HVR) prior to hormonal therapy (control conditions, CTR) and during the 4-week treatment are presented in figures 4.2 and 4.3. Each week, V_T and $\dot{V}e$ were normalized to baseline values (i.e. normoxia) obtained immediately prior to the challenge. Figure 4.2 illustrates the response in BPM, V_T and $\dot{V}e$ during a 15-minute hypercapnic challenge. In CTR conditions, no differences in HcVR were observed across the four treatment groups. The hypercapnic BPM response was consistently increased in ETO rats from week 1 (169.6±23.1%; P=0.001) to 4 (164.1±19.6%; P=0.02) compared to its own CTR (142.2±9.8%). In contrast, the hypercapnic BPM response increased in SHAM (143.6±18.5%) and E2 rats (130.7±3.2%) only at weeks 3-4 (W3 = 164.8±21.4%; P=0.043; W4 = 176.9±23.7%; P<0.001) and weeks 2-4 (W2=162.3±26.1%; P=0.0029; W3=164.0±9.0%; P=0.003; W4=158.8±18.0%; P=0.022), respectively. The hypercapnic V_T response did not vary within or between groups, however, $\dot{V}e$ response increased in E2 rats at week 3 (320.3±32.2%; P=0.043) and in ETO rats at both week 2 (326.5±112.5% P=0.006) and 4 (313.5±89.9%, P=0.033) compared to CTR.

In summary, these results indicate that in adult female rats, long-term ETO administration enhances the hypercapnic BPM (W1-4) and $\dot{V}e$ responses (W2,4) compared to their own CTR conditions, but this response was not significantly different from other treatment groups across the 4 weeks of hormonal treatment. Furthermore, E2 supplementation did not potentiate ETO response.

Figure 4.3 illustrates the ventilatory response in BPM, V_T and $\dot{V}e$ to a 15-minute hypoxic challenge in CTR and over the 4 weeks of hormonal treatment. Although some differences in frequency response were observed in CTR and week 1 between ETO, E2 and ETO+E2, the main effect was a hypoxic BPM response increase in SHAM (222.0±28.5%, P=0.011), E2 (155.1±23.8%, P=0.025) and ETO+E2 (201.3±26.5%, P=0.015) rats at week 4 compared to their own CTR (187.9±27.0%, 161.5±11.7%, 166.7±23.4%, respectively). We observed no changes in the hypoxic V_T response within and between groups, although $\dot{V}e$ response increased at week 3 in ETO+E2 (335.1±49.8%; P=0.015) rats compared to their own CTR (267.4±49.5%), and in ETO (354.7±72.7%; P=0.002) and E2 (316.0±57.0%, P=0.03) rats at week 4 compared to their own CTR (293.6±35.0%, 252.9±38.2%, respectively).

These results indicate that long-term ETO treatment and E2 supplementation do not consistently change the hypoxic ventilatory response compared to SHAM rats in healthy female rats.

Hypercapnic and hypoxic ventilatory response in anesthetized rats

In order to unmask respiratory effects that may be concealed by behavioural responses during hypercapnia or hypoxia in freely behaving rats, and to obtain accurate measurements of respiratory flow and diaphragm (DIA_{EMG}) activity during these challenges, at the end of the 4-week treatment rats were instrumented and challenged under isoflurane anesthesia.

Hypercapnia was delivered in a 3-minute stepwise manner, within a range of 3-11% CO_2 (3,5,7,9,11% CO_2) in hyperoxia (40% O_2) in order to eliminate the contribution of peripheral chemoreceptors. No differences in BPM, V_T and $\dot{V}e$ or peak $\int DIA_{EMG}$ activity (not shown) responses during the hypercapnic challenge were observed between groups (figure 4.4A-C).

The HVR results are presented in figure 4.4D-F. The initial peak in ventilation was observed 2 minutes after the commencement of the hypoxic exposure and no differences were observed between groups. During the secondary phase of HVR (i.e, in the last 5 minutes of hypoxia) the BPM response increased in ETO (122.6±23.3% of baseline; P<0.001), E2 (118.4±11.7%; P=0.006), and ETO+E2 (118.3±20.0%; P<=0.005) compared to SHAM (110.0±18.1%) rats. The hypoxic V_T response was reduced in ETO+E2 (106.9±10.5%) rats relative to SHAM (113.1±19.7; P=0.001) and E2 (113.4±24.8%; P=0.002) rats, although no differences were observed in the peak $\int DIA_{EMG}$ activity between groups (SHAM= 1.2±0.2, ETO= 1.1±0.1, E2=1.0±0.1 & ETO+E2=1.2±0.2). Overall, the hypoxic $\dot{V}e$ response in the secondary phase of the HVR was greater in ETO (134.9±26.3% of baseline) compared to SHAM (123.7± 29.3%; P=0.007) and ETO+E2 (125.4±13.4; P=0.022) rats, and in E2 (133.7±25.0%) compared to SHAM rats (123.7± 29.3%; P=0.023). To assess the ETO and E2 serum levels, blood was collected at the end of the experiments and ETO serum concentration (ETO: 236.8±50.0pg/mL, n=5; ETO+E2: 183.5±26.5pg/mL, n=6) as well as E2 serum concentration (E2:63.1±42.9pmol/L; ETO+ E2: 122.4 ± 82.1pmol/L) were analyzed.

Overall, the results under isoflurane anesthesia indicate that, due to an increase in breathing frequency, ETO and E2 treated rats displayed a small but significant increase in $\dot{V}e$ during the secondary phase of hypoxia.

Baseline breathing (normoxia) in ETO-treated SP-SAP injected rats

The fortuitous finding reported by Straus (Straus et al., 2010) with Desogestrel in CCHS patients prompted our investigation into the use of ETO as a respiratory stimulant in conditions of impaired CO_2 chemoreflex. We tested this hypothesis in female rats in which Phox2b/NK1 receptor expressing neurons of the RTN (Stornetta et al., 2006) were ablated by the use of a selective toxin, SP-SAP (Nattie & Li, 2002; Souza et al., 2018; Takakura et al., 2008). Figure 4.5 illustrates normoxic BPM (A), V_T (B) and $\dot{V}e$ (C) in control (CTR, prior to SP-SAP lesion; n=9), post RTN lesion (SP-SAP; n=9) and following either sham surgery (SHAM SP-SAP; n=4) or Nexplanon® instrumentation (ETO SP-SAP; n=5). After control recordings and RTN lesion surgery, rats were test weekly in proestrus for the subsequent four weeks, no differences were observed at week 1 to 4 post lesion in rats that were allocated for SHAM operation or ETO implantation. Because no differences in BPM, VT and $\dot{V}e$ were observed between weekly recordings, values were averaged between recordings in weeks 1 to 4 following RTN lesions for each rat. One to 4 weeks following SP-SAP injection, BPM (84.0±8.5 vs 74.3±5.0 bpm; P=0.018) and $\dot{V}e$ (100.0±9.8% vs 88.3±12.8%; P=0.011) were reduced compared to CTR (n=9).

Following sham surgery (4-weeks post RTN lesion and for the subsequent 4-weeks of testing), SHAM SP-SAP rats displayed a reduced BPM compared to their own CTR (62.4±3.8 vs 89.1±8.3 bpm; P=0.001) and SP-SAP conditions (62.4±3.8 vs 73.1±1.9 bpm; P=0.008; n=4), and a reduced $\dot{V}e$ compared to their own CTR (68.7±8.4% vs 100.0±9.2%; P<0.001) and SP-SAP conditions (68.7±8.4% vs 81.1±13.8%). These effects were observed on week 1 post ETO-instrumentation and persisted for the entire 4-week ETO treatment. Interestingly, in ETO SP-SAP rats BPM was not affected in normoxia, and V_T increased compared to their own CTR (113.0±12.5% vs 100.0±10.6%; P=0.007; n=5) and SP-SAP conditions (113.0±12.5% vs 100.0±10.6 vs 99.4±13.5%; P=0.005). Furthermore, $\dot{V}e$ in ETO SP-SAP rats also increased compared to SP-SAP (109.3±12.8% vs 94.0±9.8%; P=0.036) and was no different from CTR conditions. A comparison between SHAM SP-SAP and ETO SP-SAP rats further showed that ETO treatment increased both BPM (62.4±3.8 vs 76.8±4.6 bpm; P=0.02) and $\dot{V}e$ (68.7±8.4% vs 109.3±12.8%; P<0.001). These results indicate that in normoxia ETO treatment potentiated tidal volume and reverted both respiratory frequency and minute ventilation to control conditions (i.e., pre-lesion) over the four-week treatment period, despite RTN impairment.

Hypercapnic and hypoxic ventilatory responses in ETO-treated SP-SAP injected rats

In accordance to previously published work in adult male rats (Souza et al., 2018), the HcVR decreased following RTN lesions (figure 4.6), and SP-SAP rats displayed a persistent reduction in the frequency response compared to CTR (133.4±14.6 vs 103.7±8.3 bpm; P<0.001) and a significant reduction in $\dot{V}e$ response (100.0±20.5% vs 76.5±14.0%; P<0.001) that persisted in SHAM SP-SAP rats compared to their own CTR (100.4±8.1 vs 134.8±16.8 bpm; P<0.001; n=4). Both hypercapnic BPM and $\dot{V}e$ responses in ETO SP-SAP rats increased compared to their own SP-SAP conditions (101.1±9.7 vs 120.5±3.8 bpm, P=0.007; 104.7±14.0% vs 72.6±15.8%; P=0.01) and they were not different from their own CTR responses. Furthermore, the hypercapnic BPM and $\dot{V}e$ responses in ETO SP-SAP rats were higher compared to SHAM SP-SAP rats (120.5±3.8 vs 100.4±8.1, P<0.013; 104.7±14.1% vs 75.6±12.5%; P<0.019 respectively). The ETO effects on the hypercapnic response were observed 1 week after ETO instrumentation and persisted over the 4-weeks ETO treatment period. We conclude that ETO administration successfully promotes recovery of the CO_2 chemoreflex response in absence of a functional RTN.

Following SP-SAP injection, the hypoxic BPM and V_T responses were unaffected, however $\dot{V}e$ was slightly, but significantly reduced (figure 4.7; 100.0±9.5% vs 90.6±13.7%, P<0.038 n=9). The BPM response in SHAM SP-SAP rats was reduced compared to their own CTR (104.9±3.3 vs 154.4±14.2 bpm, P<0.001) and SP-SAP conditions (133.6±22.7 bpm; P=0.011). Although no changes were observed in V_T, the $\dot{V}e$ response to hypoxia was impaired in SHAM SP-SAP rats (60.4±11.5%) compared to their own CTR (100.0±3.9%; P<0.001) and SP-SAP (83.5±15.9%; P=0.011) conditions. This effect was observed on week 1 following SHAM surgery (5 weeks following RTN lesion) and persisted through the 4-weeks SHAM treatment period. Interestingly, the hypoxic response in ETO SP-SAP rats did not change compared to their own CTR and SP-SAP conditions, but rather BPM and $\dot{V}e$ responses increased compared to SHAM SP-SAP rats (145.8±9.3 vs 104.9±3.3 bpm, P<0.001; 99.2±16.7 vs 60.4±11.5%, P<0.001). These changes were observed in the first week post Nexplanon® rod instrumentation and persisted through the 4-week ETO treatment period.

These results indicate that ETO treatment is also effective in preserving the hypoxic ventilatory response despite RTN impairment.

Hypercapnic and hypoxic ventilatory responses in ETO-treated SP-SAP rats under isoflurane anesthesia

The hypercapnic and hypoxic ventilatory responses in ETO-SP-SAP and SHAM SP-SAP rats were also tested under isoflurane anesthesia 4 weeks following Nexplanon® instrumentation. Overall, a trend for a stronger HcVR in BPM and $\dot{V}e$ was observed in ETO-SP-SAP rats compared to SHAM-SP-SAP animals throughout the CO_2 challenges (figure 4.8A-C). However, this increase was only statistically significant in the $\dot{V}e$ response at 9% CO_2 (228.7±52.3 vs 176.0±28.5%; P=0.047).

The response to hypoxia is presented in figure 4.8D-F and it indicates a trend for an increased V_T and $\dot{V}e$ during the secondary phase of the HVR, although these differences were not significant between ETO SP-SAP and SHAM SP-SAP rats. From these results, we conclude that ETO implementation increases $\dot{V}e$ response at 9% CO_2 under isoflurane anesthesia and it also provides a trend, although not statistically significant, to potentiate the hypoxic ventilatory responses in RTN lesioned rats under isoflurane anesthesia.
Following the acute experiments, serum was collected and ETO levels in ETO SP-SAP rats were 234.8±67.0pg/ml (n=5).

Histological analysis of SP-SAP injected brain

Brain histological analysis confirmed that the SP-SAP ablation was limited to the region containing RTN chemosensitive neurons. Figure 4.9A illustrates the caudal to rostral distribution of PHOX2B-immunoreactive cells in the region ventromedial to the facial motor nucleus (VII) in CTR (naïve, n=4) SHAM SP-SAP (n=4) and ETO SP-SAP (n=5) rats. We observed a reduced number of $PHOX2B^+$ cells at the caudal tip of VII in both SHAM SP-SAP (40.6% decrease; P=0.018) and ETO SP-SAP rats (67.3% decrease; P<0.001) and the reduction extended rostrally, both in SHAM SP-SAP (78.4% decrease; P=0.002) and ETO SP-SAP (78.0%; P<0.001) rats compared to CTR rats. Caudal to the VII, $PHOX2B^+$ cell number was also reduced in ETO SP-SAP compared to CTR and SHAM SP-SAP (42.8%; P=0.015 and 54.6%; P<0.001 decrease, respectively), suggesting the presence of an even larger lesion in ETO SP-SAP rats compared to SHAM SP-SAP rats. In order to verify if SP-SAP injections affected also $PHOX2B^+$ cells in the C1 region (Souza et al., 2018) we immunolabelled for tyrosine hydroxylase (TH), a marker for cathecolaminergic C1 cells (figure 4.9B). The region caudal to VII (0-400μm caudal to the tip of

VII) contained 56.4±4.2, 74.4±17.5 and 45.6±12.7 TH+ cells in CTR, ETO SP-SAP, and SHAM SP-SAP rats, respectively. Although TH+ cell number was reduced in SHAM SP-SAP rats compared to both CTR and ETO SP-SAP rats, there was no difference in TH+ cell number between ETO SP-SAP and CTR rats. We conclude that in both SHAM SP-SAP rats and ETO SP-SAP rats we successfully destroyed of a large majority of RTN neurons with limited effects in the surrounding areas (figure 4.9C).

4.4 Discussion

This study investigated basal breathing and the chemoreflex response following long-term ETO administration through size-adjusted Nexplanon® rods in both healthy conditions and RTN-impaired female rats. While ETO administration did not significantly affect normoxic breathing in addition to hypoxic and hypercapnic chemoreflex responses in healthy female rats, the same ETO dose improved ventilation in normoxia and hypoxia, and successfully recovered the response to hypercapnia in rats in which RTN was partially destroyed and the CO_2 chemoreflex was impaired.

The use of ETO to potentiate ventilation and chemosensitivity has been recently investigated in a rare hypoventilation syndrome, CCHS, given that two CCHS patients displayed a partial recovery of CO_2 chemosensitivity (Straus et al., 2010) with the introduction of desogestrel (ETO is its active metabolite) as a contraceptive regimen. Due to the limited number of patients, and contradictory results from another study (Li et al., 2013), further preclinical and clinical investigations are required to assess the efficacy of ETO and determine the mechanism and sites of action of ETO.

ETO is a potent progestin of the gonane family (Boukari et al., 2015; Brinton et al., 2008) with an affinity to progesterone receptors that is 25-times greater than progesterone itself (Bergink et al., 1981). Its biological effects are associated with genomic and non-genomic actions through progesterone receptors in addition to modulation of other neurotransmitters and channels in the brain (Boukari et al., 2015; Brinton et al., 2008; Cardani et al., 2018; Loiseau et al., 2018; Marcouiller et al., 2014). The effects of progesterone and its synthetic derivatives on ventilation have been previously investigated with various outcomes depending on models used, dose, mode of administration, and sites of action (Bairam et al., 2019; Bayliss & Millhorn, 1992; Behan & Wenninger, 2008; Brodeur et al., 1986; Hosenpud et al., 1983; Mahesh et al., 1996).

Overall, there is general agreement that progesterone and its analogues act on various areas of the brain to potentiate ventilation, although the mechanistic details of these effects need to be further investigated (Boukari et al., 2015; Holley et al., 2012).

The specific effects of ETO on breathing have been explored in neonatal rodents (Joubert et al., 2016; Loiseau, 2019; Loiseau et al., 2014) and an increased respiratory frequency was observed *in vivo* and *in vitro*, possibly acting through modulation of GABAergic, glutamatergic and serotoninergic neurotransmission (Joubert et al., 2016). Furthermore, acute ETO administration enhanced ventilation *in vitro* and *ex vivo* under metabolic acidosis and this effect was attributed to activation of supramedullary structures (Loiseau et al., 2014), possibly orexinergic neurons (Loiseau, 2019).

In our study we investigated long-term ETO administration in sexually mature female rats in healthy and pathological (chemoreflex-impaired) conditions. In contrast to perinatal data (Joubert et al., 2016), we failed to observe any change in normoxic, hypoxic or hypercapnic ventilatory responses during proestrus in adult female rats. We additionally tested the possibility that E2 supplementation could increase progesterone receptor expression (Bayliss & Millhorn, 1992; Brodeur et al., 1986; Hosenpud et al., 1983; Kato et al., 1978; MacLusky & McEwen, 1978, 1980; Monks et al., 2001) and therefore potentiate ETO effects, but found no effects on ventilation in these conditions. The lack of a response may be due to different delivery methods, higher hormonal levels in mature rats compared to newborns (Dohler & Wuttke, 1975), different species, developmental changes in progesterone receptors expression (Chakraborty & Gore, 2004; Guerra-Araiza et al., 2003; Quadros & Wagner, 2008), or distinct mechanisms of actions of ETO through development (Behan & Wenninger, 2008; Ren & Greer, 2006).

To investigate the potential effect of an ETO-based contraceptive regimen on ventilation we opted to deliver ETO through a body weight-appropriate Nexplanon® rod section in order to obtain continuous ETO serum levels comparable to the ones reported in women using Nexplanon rods (Palomba et al., 2012). We opted not to use an ovariectomized rat model because our objective was to assess if ETO supplementation was effective compared to physiological, rather than hormonally impaired, conditions. In these experimental conditions, we were not able to identify significant respiratory changes in normoxia, hypercapnia or hypoxia. The only small, but consistently significant difference was the potentiation of hypercapnic BPM and $\dot{V}e$ responses in freely behaving rats, as well as in the secondary phase of hypoxia under isoflurane anesthesia

compared to SHAM rats (but not in behaving unanesthetized rats). The increase in the secondary phase of the hypoxic response was similarly observed in unanesthesized newborn male pups that chronically received progesterone (Hichri et al., 2012; Lefter et al., 2007) and may be an indication of ETO acting on both central and peripheral chemoreceptors to affect the central mechanisms that control the secondary phase of hypoxia (Hichri et al., 2012).

Because of the fortuitous results obtained with desogestrel in CCHS patients, we tested long-term ETO ventilatory effects in chemosensitive-impaired adult female rats. Altered development and function impairment of the central chemosensitive brain areas (i.e., RTN and other chemosenstive brain structures) have been proposed to be the main cause for the reduced CO_2 chemosensitivity in CCHS patients (Amiel et al., 2003; Dubreuil et al., 2008; Harper et al., 2015). Therefore, we selectively depleted the NK1R/Phox2b neurons of the RTN region (Nattie & Li, 2002; Souza et al., 2018; Takakura et al., 2008). Using a similar approach in adult male rats causes a long-lasting impairment in the CO_2 chemoreflex and limited effects on normoxic breathing and the HVR (Souza et al., 2018; Takakura et al., 2008). In our study, we observed a similar reduction in HcVR in female rats, and, most importantly, we demonstrated that long-term ETO administration successfully increased $\dot{V}e$ in normoxia and potentiated both the HcVR and HVR compared to SHAM SP-SAP rats. The mechanisms and sites of action where ETO exerts its function are currently unknown and will be further investigated in order to understand how ETO acts to promote recovery of the hypercapnic and hypoxic responses despite a significant lesion to the RTN and corresponding significant impairment of its function. We speculate that potential candidate sites are areas rich in PG receptors that are involved directly or indirectly in respiratory control (Boukari et al., 2015; Brinton et al., 2008; Cardani et al., 2018; Loiseau et al., 2018; Marcouiller et al., 2014). Candidate brain structures are the locus coeruleus, the periaqueductal gray, the hypothalamus, the raphe nucleus, the ventral respiratory group, in addition to peripheral carotid bodies.

Furthermore, in relation to CCHS pathology, but also of critical relevance in other hypoventilation syndromes and conditions of impaired CO_2 chemosensitivity, it will be key to further investigate the genomic effects that are associated with ETO stimulation on the respiratory network (Cardani et al., 2018).

In conclusion, our results in chemoreflex impaired rats demonstrate that ETO administration significantly improves breathing characteristics in normoxia, hypercapnia and hypoxia

throughout the 4-week treatment. Although further studies will be necessary to identify the mechanisms or sites of action of ETO in this process, these results support the serendipitous findings observed in CCHS patients and the use of potent progestins to ameliorate conditions of impaired central chemoreflex response.

Figures

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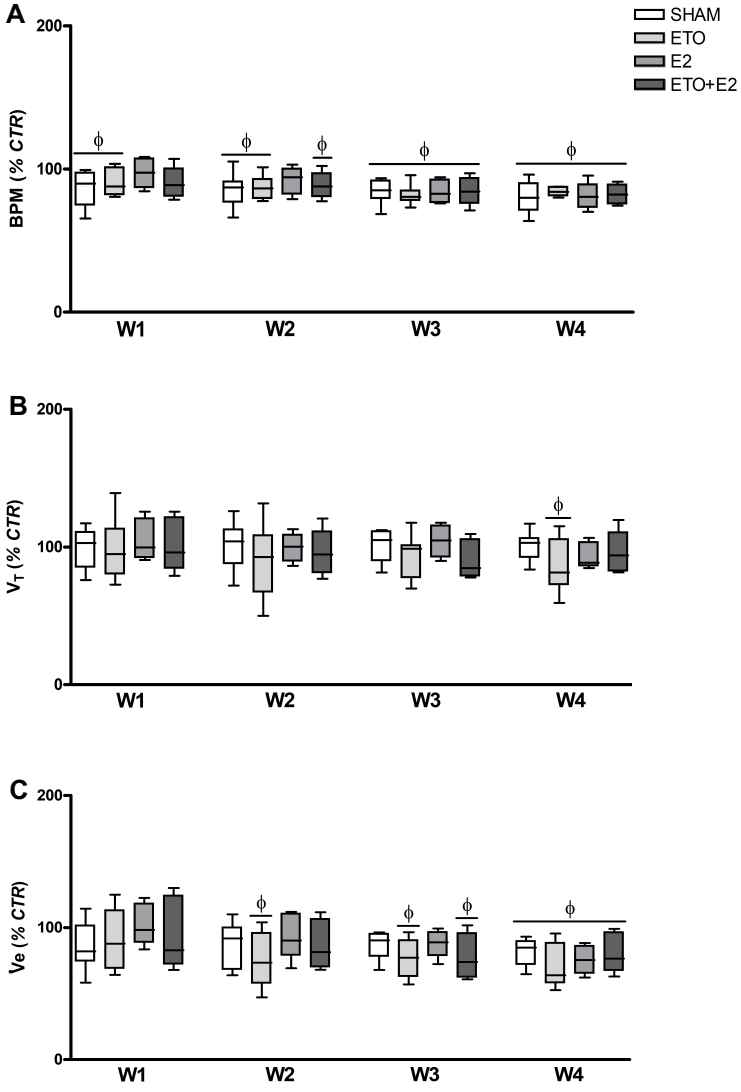

Figure 4.1. **Normoxic baseline breathing during hormonal treatment in healthy adult female rats**. Box and whisker plots illustrating the percent change from control (pre-treatment values) in normoxia of breathing frequency (breath per minutes, BPM, A), Relative Tidal Volume (V_T, B) and Minute Ventilation (Ve, C) in SHAM (n=9; white), ETO (n=10; light grey), E2 (n=6; grey) and ETO+E2 (n=6; dark grey) rats during week (W) 1 to 4 of hormonal treatment. A) BPM decreases in SHAM and ETO at W1-2 relative to control and it decreases at week 3 and 4 in all four groups relative to their own control recordings. B) No significant changes in V_T are observed between group and across time. C) Ve decreased in ETO rats in W2 to 4. On W4, all treatment groups display a reduced Ve relative to their control recordings. Repeated measures two-way ANOVA and post-hoc tests assessed significant changes ($P<0.05$). Theta symbols (Φ) indicate changes within groups relative to control.

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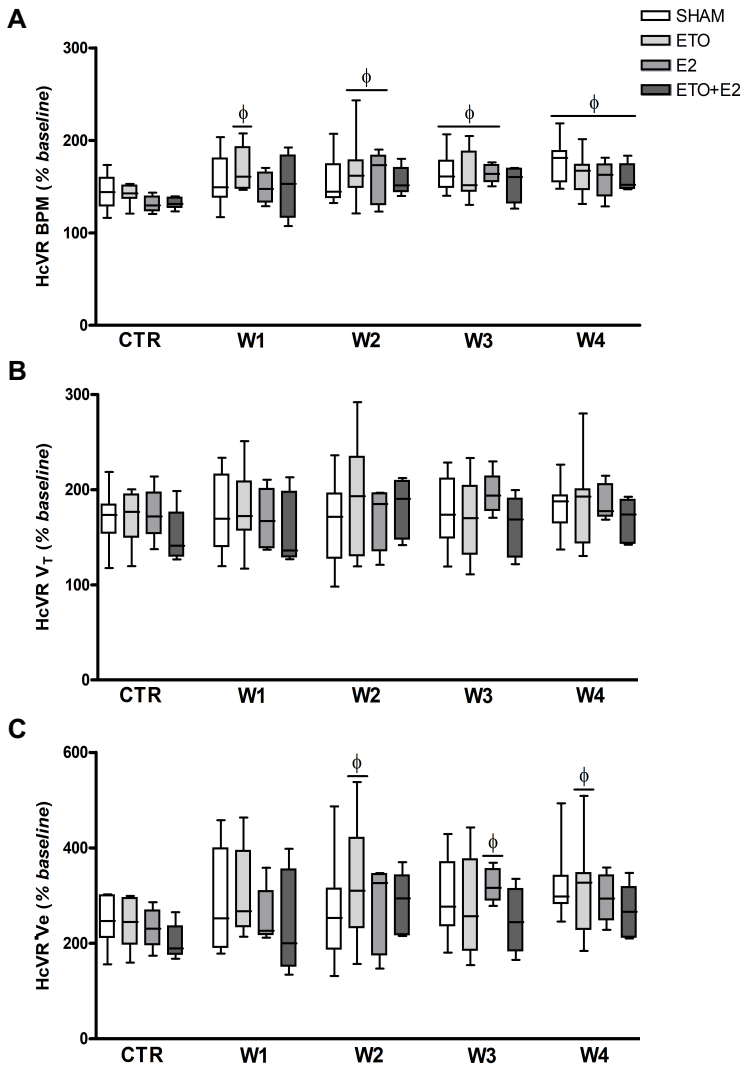

Figure 4.2. **Hypercapnic ventilatory response in control condition and during hormonal treatment in healthy female rats**. Box and whisker plots illustrate the percent of change from baseline (normoxia) values during the hypercapnic ventilatory response (HcVR). Response in breathing frequency (BPM, A), relative Tidal Volume (V_T, B) and Minute Ventilation (Ve, C) in SHAM (n=9; white), ETO (n=10; light grey), E2 (n=6; grey) and ETO+E2 (n=6; dark grey) rats in control and during weeks (W) 1 to 4 of hormonal treatment. A) HcVR BPM response increased in ETO treated rats from week 1 through 4 of treatment compared to CTR. At week 4, all treatment groups display a significantly increased HcVR BPM response compared to their CTR response. B) No changes in HcVR V_T response are in any treatment group in W1-4. C) Ve response is significantly increased in W2 and W4 of hormonal treatment in ETO rats compared to CTR. Repeated measures two-way ANOVA and post-hoc tests assessed significant changes where significance is indicated by $P<0.05$. Theta symbols (Φ) indicate changes within groups relative to control ($P<0.05$). No significant difference across treatment groups is observed.

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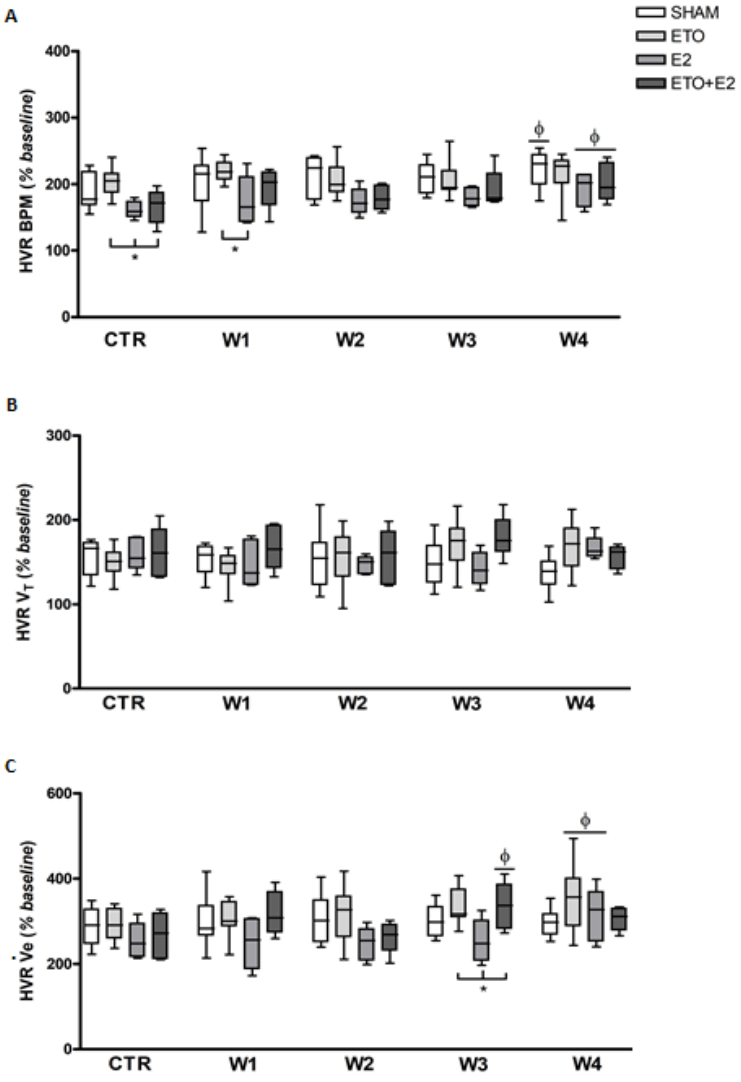

Figure 4.3. **Hypoxic ventilatory response in control condition and during hormonal treatment in healthy female rats**. Box and whisker plots illustrate the percent of change from baseline (normoxia) values during the hypoxic ventilatory response (HVR). Response in breathing frequency (BPM, A), relative Tidal Volume (V_T, B) and Minute Ventilation (Ve, C) in SHAM (n=9; white), ETO (n=10; light grey), E2 (n=6; grey) and ETO+E2 (n=6; dark grey) rats in control and weeks (W) 1 to 4 of hormonal treatment. A) The HVR BPM response significantly increased in SHAM, E2 and ETO+E2 rats compared to their own CTR response at W4. HVR BPM response in ETO is higher compared to E2 (CTR, W1) and ETO+E2 (CTR) rats. B) No changes in HVR V_T response are in any treatment group in W1-4. C) The Ve response significantly increased at week 4 in ETO and E2 compared to control. Repeated measures two-way ANOVA and post-hoc tests assessed significant changes ($P<0.05$). Theta symbols (Φ) indicate changes within groups relative to control. Asterisks (*) indicate changes across groups.

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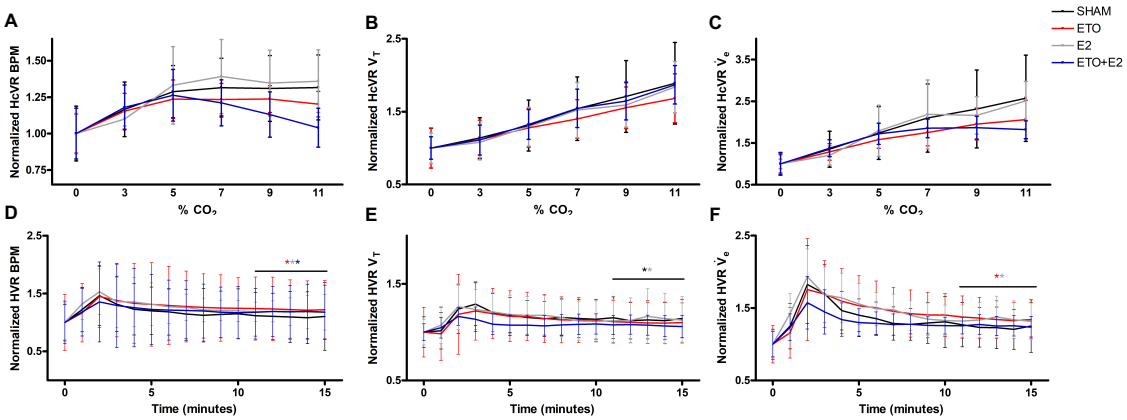

Figure 4.4. **Hypercapnic and hypoxic ventilatory responses after 4-weeks hormonal treatment in isofluorane anesthetized female rats**. Normalized change in breathing frequency (BPM), tidal volume (VT) and minute ventilation (Ve) during the hypercapnic ventilatory response (HcVR, A-C) and the hypoxic ventilatory response (HVR, D-F) in SHAM (n=6; black), ETO (n=6; red), E2 (n=4; green) and ETO+E2 (n=5; blue) female rats. Hypercapnia was delivered following a step-wise, 3minutes intervals increase in $\%CO_2$ (0-11%). No differences in the response were observed across groups. In HVR, no difference was observed in the initial peak of the hypoxic response, whereas during the secondary phase of the hypoxic response (i.e, in the last 5 minutes of hypoxia) the BPM response increased in ETO, E2 and ETO+E2 compared to SHAM rats. The HVR V_T response was reduced in ETO+E2 rats relative to SHAM and E2 rats, and the HVR Ve response was greater in ETO compared to SHAM and ETO+E2 rats and was greater in E2 compared to SHAM rats. Repeated measures two-way ANOVA and post-hoc tests assessed significant changes in HcVR and one-way ANOVA assessed HVR ($P<0.05$). Asterisks (*) indicate significant difference between groups.

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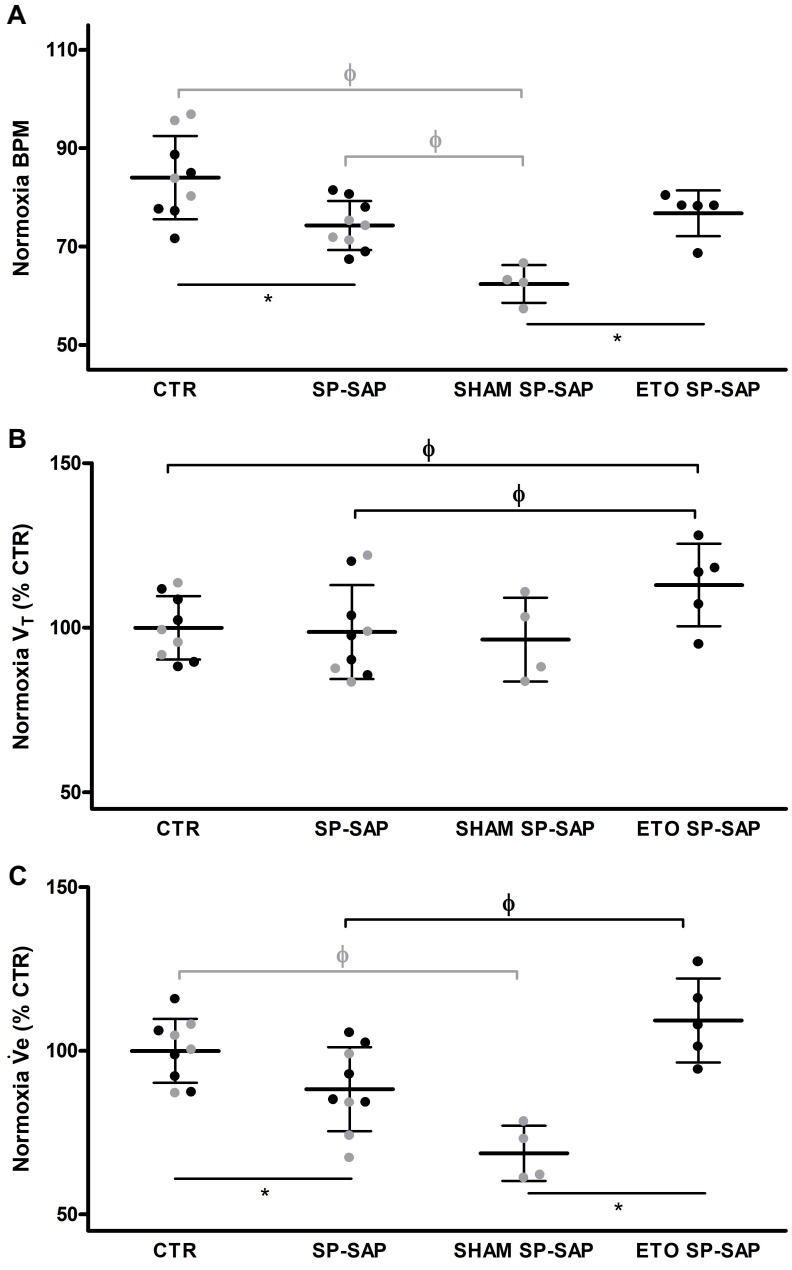

Figure 4.5. Normoxic baseline breathing in SHAM and ETO-treated RTN-lesioned rats. Absolute value of breathing frequency (BPM, A) and percent change from control conditions of tidal volume (V_T, B) and minute ventilation (Ve, C) prior to SP-SAP injection into the retrotrapezoid nucleus (RTN; CTR; n=9), 1-4 weeks following SP-SAP induced RTN lesion (SP-SAP; n=9), and 1-4 weeks following sham surgery (SHAM SP-SAP; n=4) or Nexplanon instrumentation (ETO SP-SAP; n=5) in normoxia. A) Normoxic BPM is significantly reduced in SP-SAP rats compared to their own control recordings. BPM is also reduced in SHAM SP-SAP compared to its own CTR and SP-SAP conditions (grey). BPM was greater in ETO SP-SAP compared to SHAM SP-SAP and was not different from values prior to RTN lesion (CTR). B) V_T was significantly increased in ETO SP-SAP rats compared to their own CTR and SP-SAP conditions (black). C) Ve was significantly reduced in SP-SAP rats compared to CTR. Ve was also significantly reduced in SHAM SP-SAP rats compared to their own CTR (grey), whereas, Ve in ETO SP-SAP rats increased compared to both SP-SAP (black)

and to SHAM SP-SAP rats. Repeated measures two-way ANOVA and post-hoc tests assessed significant changes within groups (Φ, P<0.05). Paired t-tests assessed significant differences between CTR and SP-SAP conditions (n=9, P<0.05), whereas unpaired t-tests assessed significant changes between SHAM SP-SAP (n=4) and ETO SP-SAP (n=5) rats. Asterisks (*) indicate changes across groups at each given time point (P<0.05).

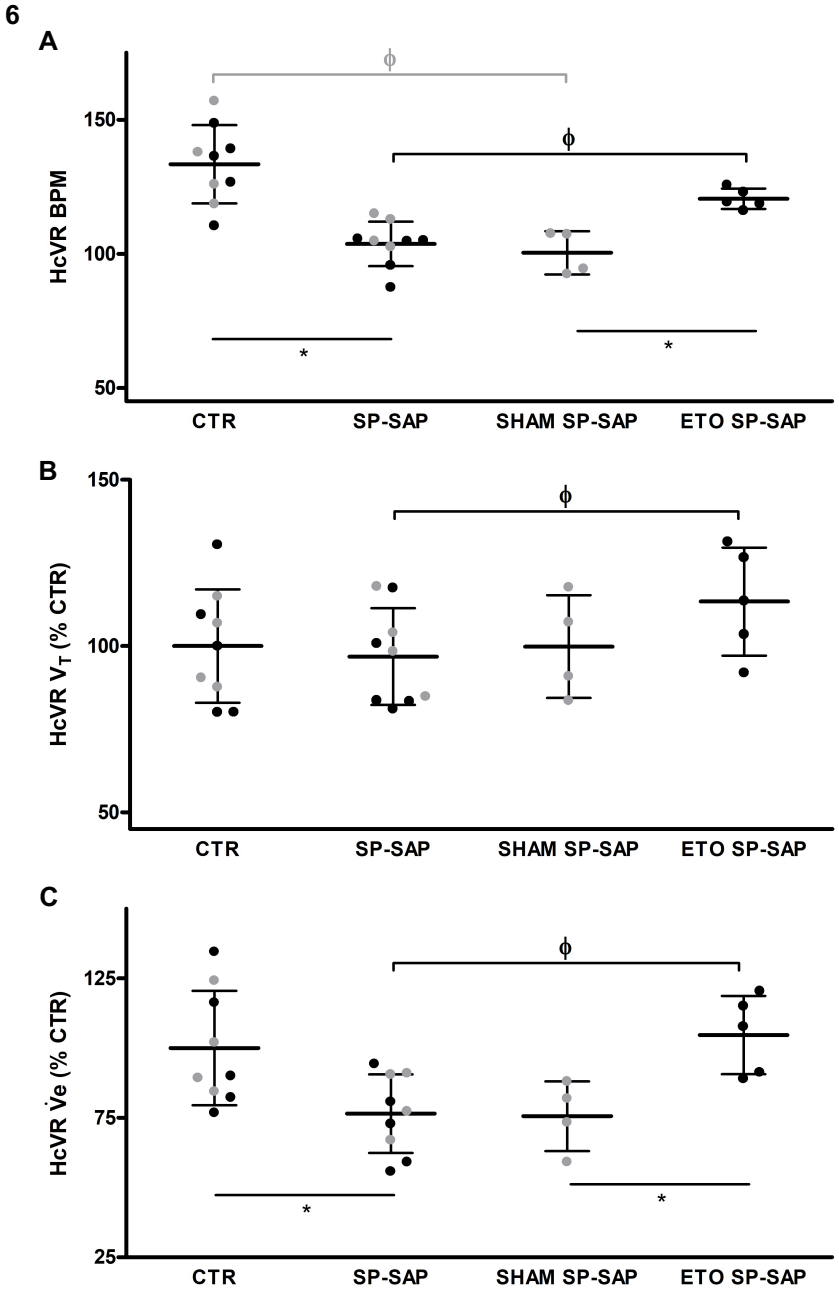

Figure 4.6. Hypercapnic ventilatory response is increased in ETO treated, RTN lesioned rats. Breathing frequency (BPM, A) and percent change from control of tidal volume ($V_{T,}$ B) and minute ventilation (Ve, C) prior to SP-SAP injection into the RTN (CTR; n=9), 1-4 weeks following SP-SAP induced RTN lesion (SP-SAP; n=9), and 1-4 weeks following sham surgery (SHAM SP-SAP; n=4) or Nexplanon instrumentation (ETO SP-SAP; n=5) in hypercapnia (HcVR. A) HcVR BPM response was significantly reduced in SP-SAP rats compared to CTR. BPM also decreased in SHAM SP-SAP compared to their own CTR (grey) conditions, whereas HcVR

BPM response in ETO SP-SAP significantly increased compared to both SP-SAP conditions (black) and SHAM SP-SAP. B) V_T increased in ETO SP-SAP rats compared to their SP-SAP conditions (black), although it was not different from SHAM SP-SAP rats. C) HcVR Ve response decreased in SP-SAP compared to CTR rats. Furthermore, HcVR Ve response increased in ETO SP-SAP rats compared to both their SP-SAP conditions (black) and to SHAM SP-SAP rats. Repeated measures two-way ANOVA and post-hoc tests assessed significant changes within groups (Φ, $P<0.05$). Paired t-tests assessed significant differences between CTR and SP-SAP conditions ($n=9$, $P<0.05$), whereas unpaired t-tests assessed significant changes between SHAM SP-SAP ($n=4$) and ETO SP-SAP ($n=5$) rats. Asterisks (*) indicate changes across groups at each given time point ($P<0.05$).

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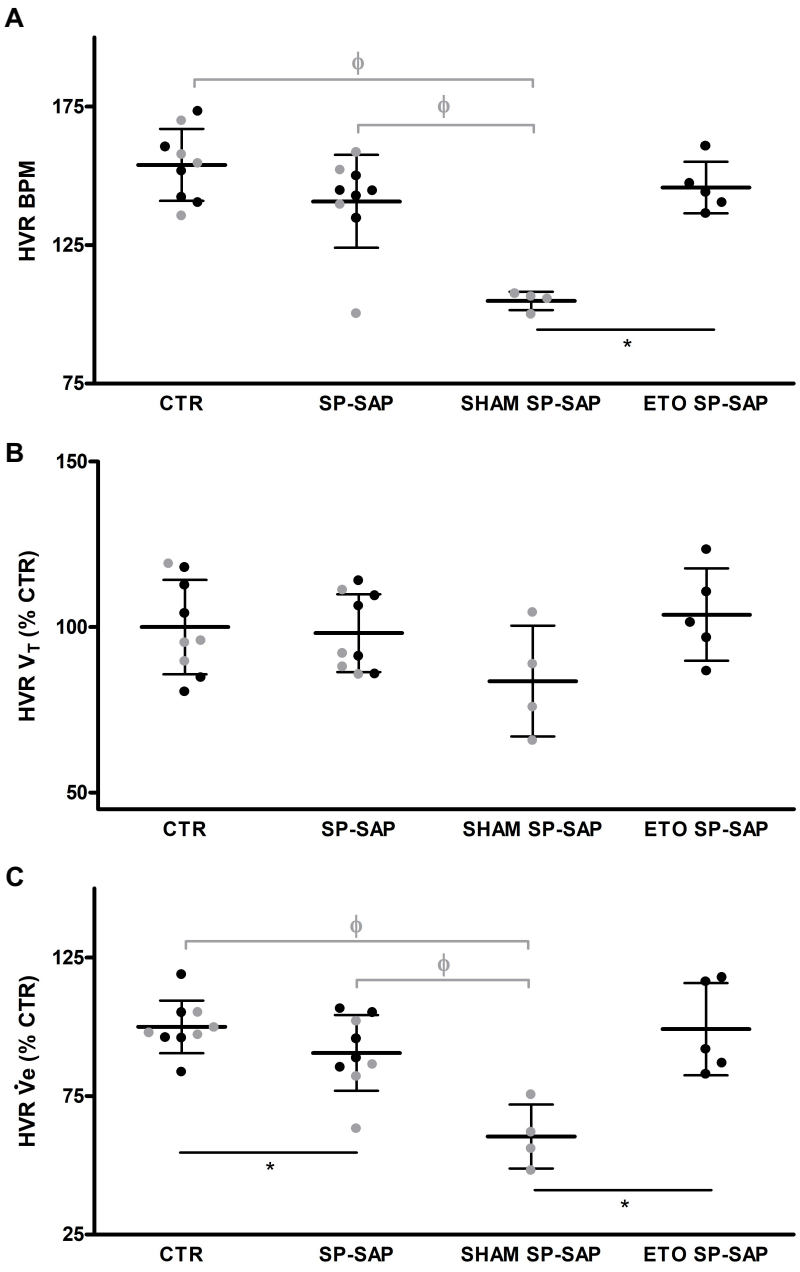

Figure 4.7. Hypoxic ventilatory response is enhanced in ETO treated, RTN lesioned rats. Breathing frequency (BPM, A) and percent change from control of tidal volume (V_T, B) and minute ventilation (Ve, C) prior to SP-SAP injection into the RTN (CTR; n=9), 1-4 weeks following SP-SAP induced RTN lesion (SP-SAP; n=9), and 1-4weeks following sham surgery (SHAM SP-SAP; n=4) or Nexplanon rod instrumentation (ETO SP-SAP; n=5) in hypoxia (HVR). A) HVR BPM response is significantly reduced in SHAM SP-SAP rats compared to both CTR and SP-SAP (grey) conditions. BPM response increased in ETO SP-SAP rats compared to SHAM SP-SAP. C) The response in Ve during HVR was reduced in SP-SAP rats compared to CTR condition. Furthermore, Ve response in SHAM SP-SAP was reduced compared to both CTR and SP-SAP conditions (grey) in addition to ETO SP-SAP rats. Repeated measures two-way ANOVA and post-hoc tests assessed significant changes within groups (Φ, $P<0.05$). Paired t-tests assessed significant differences between CTR and SP-SAP conditions (n=9, $P<0.05$), whereas unpaired t-

tests assessed significant changes between SHAM SP-SAP (n=4) and ETO SP-SAP (n=5) rats. Asterisks (*) indicate changes across groups at each given time point (P<0.05).

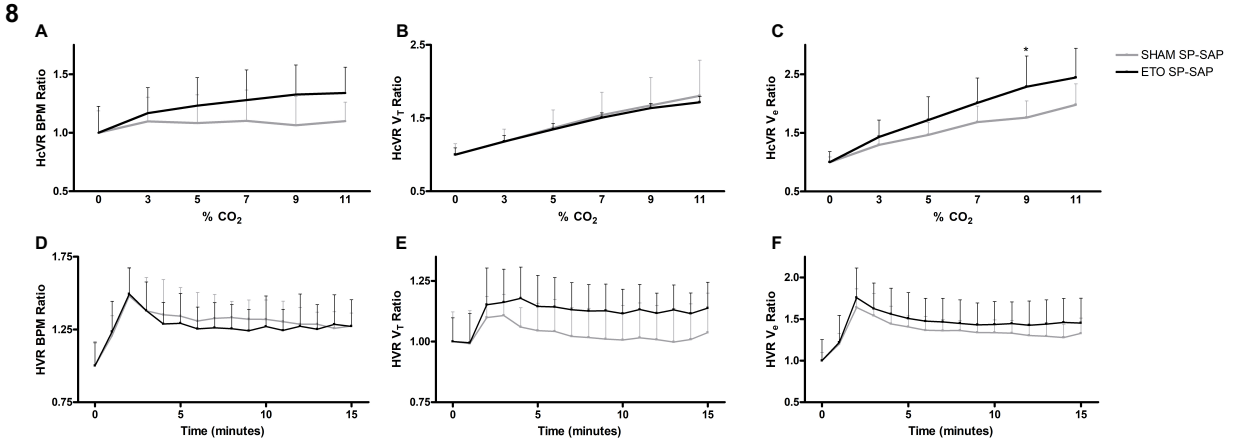

Figure 4.8. **Hypercapnic and hypoxic ventilatory responses following RTN lesion and 4 weeks ETO treatment in isofluorane anesthetized female rats.** Normalized respiratory changes during the hypercapnic (HcVR, A-C) and hypoxic (HVR, D-F) ventilatory responses in SHAM SP-SAP (n=4; grey) and ETO SP-SAP (n=5; black) rats. Breathing frequency (BPM, A,D), tidal volume (V_T, B,E) and minute ventilation (Ve, C,F) during the 3-minute step-wise increase in % of inspired CO_2 (0-11%) and during hypoxia (10% O_2) under isoflurane anesthesia). Although a consistent trend for an increased HcVR BPM and Ve responses in ETO SP-SAP rats compared to SHAM SP-SAP rats was observed, HcVR Ve response was statistically increased only at 9% CO_2 compared to SHAM SP-SAP rats (A-C Repeated measures two-way ANOVA and post-hoc tests assessed significant changes in HcVR and one-way ANOVA assessed HVR D-F. Asterisks (*) indicate significant difference between groups (P<0.05).

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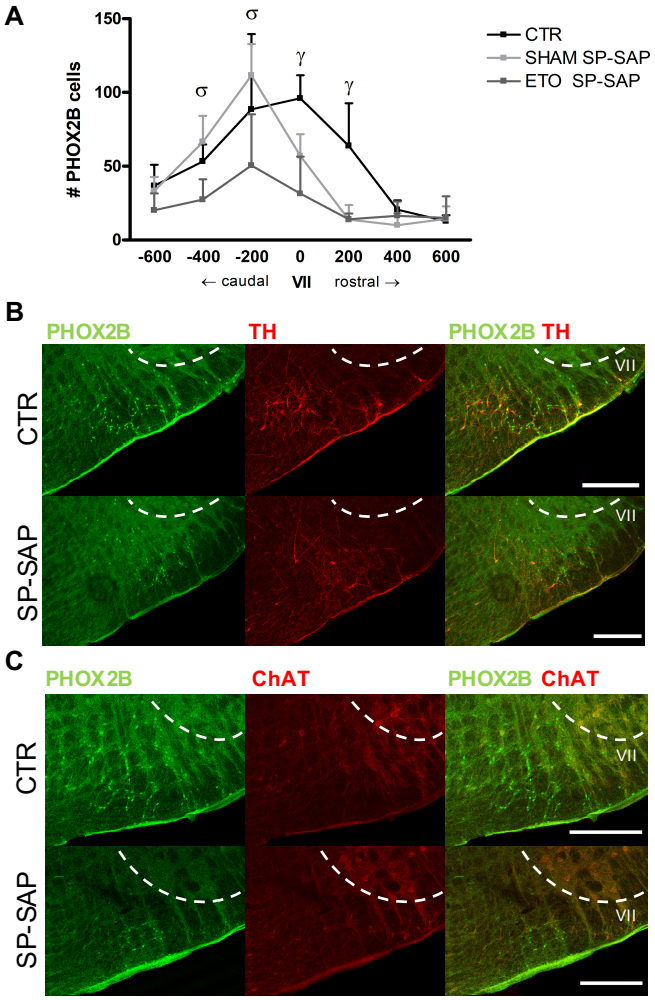

Figure 4.9. Rostro-caudal distribution of PHOX2B cells in control and RTN lesioned rats. A) Rostro-caudal distribution of PHOX2B immunopositive cells of control/naÏve (CTR; n=4), SHAM SP-SAP (n=4) and ETO SP-SAP (n=5) rats in the medulla ventromedial to the facial nucleus (VII). The number of PHOX2B immunopositive cells was decreased in SHAM SP-SAP and ETO SP-SAP rats compared to CTR rostral to the caudal tip of VII (region indicated by the dotted line), indicating localized lesion of Phox2b/NK1 chemosensitive RTN cells. B) Expression of PHOX2B (green), TH (red) in CTR (top) and ETO-SP-SAP (bottom) rat brains, 200 microns caudal to the caudal tip of VII. C) Expression of PHOX2B (green) and ChAT (red) at the VII (region indicated by the dotted line) caudal tip in CTR (top) and ETO SP-SAP (bottom) rats. Calibration bars: 300µm. Gamma (Colten, Altevogt, & Institute of Medicine (U.S.). Committee on Sleep Medicine and Research.) symbol indicates reduced number of PHOX2B nuclei in SHAM SP-SAP and ETO SP-SAP rats compared to CTR, and delta (Colten, Altevogt, & Institute of Medicine (U.S.). Committee on Sleep Medicine and Research.) indicates reduced number of PHOX2B nuclei in ETO SP-SAP rats relative to both CTR and SHAM SP-SAP.

Chapter 5: General Discussion

5.1 Overview

Respiratory research has progressed significantly over the last several decades. Developments in the field have facilitated a greater understanding of the microcircuits involved in rhythm generation and the neural populations responsible for modulating ventilation (Del Negro et al., 2018; Huckstepp et al., 2016). The application of advanced technologies like optogenetics, chemogenetics and the use of adeno-associated viral vectors in neuroscience has enabled the integration of molecular, cellular, and developmental systems knowledge, which in turn contributed to the development of novel therapeutics for respiratory disorders (Cui et al., 2016; Huckstepp et al., 2016; Pagliardini et al., 2011; Tan et al., 2008). Although major advancement have occurred in the past 30 years, several questions remain on the sites and mechanisms of respiratory rhythm generation and how different neurotransmitters and neuromodulators affect respiration. The objectives of my research projects aimed to understand the contribution of expiratory abdominal activation in neonatal rodents and human infants during sleep; I also investigated the potential therapeutic application of a potent progestin drug as a respiratory stimulant in conditions of central hypoventilation in rodents.

The central pattern generator comprises microcircuits that are critical for rhythm and pattern generation (Del Negro et al., 2018; Feldman et al., 2013; Smith et al., 1991). Together, the spinal and cranial motor nuclei, which innervate the pump and airway muscles, enable the movement of air into and out of the lungs (Del Negro et al., 2018). Under resting condition in mammals, inspiration is an active process and expiration is passive, relying on the lungs elastic recoil to expel air. Typically, active expiration is only observed during conditions of high respiratory drive, like exercise or hypercapnia (Iscoe, 1998). In vivo investigations in experimental animals identified a conditional rhythmic oscillator responsible for the generation of active expiratory activity in the parafacial region (Abdala et al., 2009; Janczewski & Feldman, 2006a; Pagliardini et al., 2011; Zoccal et al., 2018a).

Prior studies investigating muscle activity in healthy adult rats indicate that expiratory abdominal activation is present not only during conditions of high respiratory drive but also during sleep, both in nREM and, more surprisingly, in REM sleep, despite REM-induced muscle atonia (Andrews & Pagliardini, 2015; Iscoe, 1998; Sherrey et al., 1988a). Our laboratory previously observed a pattern of abdominal muscle activation in sleeping rats when respiration was irregular, and its recruitment was associated with an increase in respiratory stability and

minute ventilation, suggesting that the expiratory generating neural networks are active during sleep (Andrews & Pagliardini, 2015).

Sleep and breathing are two homeostatically regulated processes that undergo substantial developmental changes in the postnatal period. These changes introduce critical time points of vulnerability for breathing during sleep. Sleep-disordered breathing (SDB) is described as an irregular breathing pattern, which includes respiratory cessations and depressions in ventilation. Amongst SDB disorders is Congenital central hypoventilation syndrome, a rare genetic disorder characterized by a mutation in the transcription factor PHOX2B (Amiel et al., 2009). CCHS is associated with the inability to generate a chemoreflex response with changes in blood gases (in particular increase in arterial CO_2) (Dauger et al., 2003; Trang et al., 2005). It has been long established that progesterone serves as a respiratory stimulant, evidenced by hyperventilation periods experienced by women in the luteal phase of their menstrual cycle and during pregnancy (Behan et al., 2003; England & Farhi, 1976). A serendipitous study reported a recovery in the CO_2 response of two patients with CCHS, after implementation of the oral contraceptive desogestrel (Straus et al., 2010).

Treatment options for CCHS and most SDB syndromes are limited and not always effective. The projects outlined in this thesis were designed to investigate the occurrence and contribution of expiratory abdominal muscle activation to respiration in the neonatal period during healthy and pathological sleep, and how the administration of a synthetic progesterone agonist as potential alternative, non-invasive therapeutic option for patients with hypoventilation.

5.2 Abdominal muscle activation in infant rodents and humans

Infants are subject to greater vulnerability to respiratory disturbances during sleep, due to their immature nervous and respiratory systems (Colten, Altevogt, Institute of, et al., 2006; Ivanhoe et al., 2007; MacLean et al., 2015; Peirano et al., 2003). Given previous investigations in the Pagliardini lab, which indicate that abdominal activity is present during sleep and is associated with improved respiratory period regularity and increased minute ventilation (Andrews & Pagliardini, 2015), I investigated respiratory activity during sleep in infant rodents (Saini & Pagliardini, 2017b) and human (Saini et al., 2022) to analyze the occurrence of abdominal muscle activation during sleep and how it may be contribute to respiration and respiratory events. I hypothesize that both infant rodents and humans, who are more susceptible

to experience respiratory instabilities compared to adults, will exhibit a pattern of abdominal activation pattern during sleep, in particular during active sleep, and that during the time of active expiration there will be enhanced ventilation and more regular breathing.

In my initial project with infant rodents, I provided a comprehensive developmental (P0-P15) analysis of ventilatory parameters across sleep states. By combining plethysmographic recordings with recordings of nuchal EMG and overt behavior I demonstrated that infant rats, like infant humans, spend over 50% of their time sleeping and the larger proportion of this time was in AS during the first post-natal week. With development, the amount of time spent in sleep gradually decreased and rats spent more time in QS compared to AS, indicating that the neuronal network regulating sleep/wake cycles mature within the first two post-natal weeks. These results corroborated previous studies which showed that sleep and wakefulness bout length increased into the second postnatal week (Blumberg et al., 2005) (Jouvet-Mounier et al., 1970; Seelke & Blumberg, 2008). In these recordings, although we did not observe a significant amount of apneas at birth, breathing variability and apnea occurrence were greater during active sleep compared to quiet sleep at all ages observed.

Prior investigations in rodents and humans showed that at rest, expiratory abdominal activity is absent (Iizuka & Fregosi, 2007; Iscoe, 1998), however, through disinhibition or activation of pF_L neurons or hypercapnia, active expiration may be recruited (Pagliardini et al., 2011); (Leirao et al., 2018; Pisanski et al., 2020). Our data also showed that expiratory abdominal muscle activity was present across postnatal development, and during both QS and AS. Similar to what was observed in adult rats, expiratory abdominal muscle activation was associated with increased ventilation and respiratory regularity, suggesting a functional relationship between abdominal muscle activation and increased minute ventilation in infant rodents. We propose that this increase in ventilation has two potential means: 1. A mechanical effect, advancing the movement of air out of the lungs by expiratory abdominal activation and decreasing upper airway resistance and 2. A central effect, restructuring the dynamics of the respiratory network and stimulating the preBötC. This study lays the groundwork for evaluating breathing during sleep in the perinatal period in pathological conditions (e.g., SDB and apnea of prematurity.

In my second project, I performed a similar investigation in human infants, with similar and exciting results. I characterized abdominal muscle activation from the PSG records of

infants aged 0-2 years, suspected of OSA with no other risk factors (DeHaan et al., 2015). Our data showed that infants experienced expiratory abdominal activation during sleep, with greater occurrence in nREM and REM sleep compared to SWS. Interestingly, abdominal activation events occurred both during regular breathing and during episodes with multiple respiratory events (i.e., apneas, O_2 desaturation). In the significant majority of the latter episodes, the respiratory events occurred before the abdominal activation episodes, suggesting that respiratory events may trigger the occurrence of ABD activity. Furthermore, if abdominal recruitment occurred in association with O_2 desaturation, the recruitment of abdominal muscle activity ended with a recovery in O_2 saturation. Interestingly, a large number of abdominal events were not associated with any visible respiratory events.

Although abdominal activity has been rarely seen in infant recordings during sleep (Praud 1989), our study investigated and identified abdominal activity in PSG recordings during a longer period (minutes vs hours) and analyzed it in relation to sleep states and respiratory events. We found these expiratory activation occurrences particularly intriguing as they could suggest that active expiration may be part of the strategies that the respiratory network has to improve ventilation when obstructions of the airways (as in OSA) or sleep-related depression of the respiratory network (as in central sleep apnea) occur. The triggering factors need to be further investigated since hypercapnia/high end tidal CO_2 (a commons trigger of active expiration in animal studies) was not present in a large portion of abdominal events. Interestingly, I observed some abdominal activity during O_2 desaturation and its resolution, which may indicate that O_2 desaturation, either through activation of carotid bodies or central chemoreception, may be involved in triggering active expiratory networks (Abdala et al., 2009; Dempsey et al., 2006; Iizuka, 2011; Prabhakar et al., 2005; Zoccal & Machado, 2010; Zoccal et al., 2008). In support of this hypothesis, rodents treated with chronic intermittent hypoxia experienced heightened expiratory abdominal activity (Zoccal & Machado, 2010).

Results from these studies and from our laboratory indicate that during sleep abdominal activity is present, despite inactivity or muscular atonia (especially during REM sleep). The physiological mechansims responsible for this muscle activation are currently unknown. I hypothesize that abdominal recruitment is driven by activation of the expiratory oscillator in the medulla, either through release of inhibition (similar to hypercapnic challenges) or through direct activation of these oscillatory neurons. While the source of potential inhibitory inputs has been

attributed to gabaergic and glycinergic neurons in the preBotC and Botzinger area. The source of excitatory inputs is still under investigation but potential candidates may be the chemosensitive RTN neurons, or other respiratory neurons in the medulla, although glutamatergic neurons in sleep related structures are also plausible candidates (Biancardi et al., 2021)

Mechanistically, release of inhibition could occur also at the level of abdominal premotor and motoneurons to activate abdominal muscles, although this may not be the case during active/REM sleep as stronger inhibition is present at the level of spinal motoneurons to support REM-induce motor atonia (Arrigoni et al., 2016).

We propose that the abdominal muscles are activated as a protective mechanism to support ventilation and recovery from respiratory events that occur or avoid them altogether. Previously, an important role of expiratory muscle activity involves constraining the rise in end expiratory lung volume, which serves a protective role for diaphragm functionality (De Troyer & Estenne, 1984; Iscoe, 1998) and increases respiration (Leirao et al., 2018). Our observational study in infant rodents does not demonstrate that abdominal activity or the respective central expiratory microcircuitry is the reason for stabilized and increased ventilation. However, we observed stabilization in ventilation upon activation of expiratory muscles, most often occurring when breathing was irregular. Previous research in rodents has shown that vagatomized neonates under anesthesia (Iizuka, 2009) and those that experienced a quantal slowing in breathing induced by opioid administration (Janczewski et al., 2002) experience expiratory abdominal activation prior to an apnea. Interestingly, *in vitro* application of mu opioid receptor agonist in the brainstem spinal cord preparation suppresses phrenic nerve activity supplying the diaphragm but not the rhythmic activity that governs the abdominal muscles (Janczewski et al., 2002). Exposure to opioids in the neonatal period elicits differing effects on the two oscillators, reconfiguring the respiratory network (Mellen et al., 2003). The inspiratory oscillator is supressed by opioids whereas, the expiratory oscillator activity is unaffected and takes over to provide a persistent pace to the inspiratory rhythm, thereby facilitating respiratory rhythm (Janczewski et al., 2002; Mellen et al., 2003). A recent study investigating inspiratory and expiratory motor patterns in cheyne-stokes breathing, also demonstrated that cyclic increases in CO_2 activate the expiratory oscillator to serve as an ‘anti-apneic’ centre, further supporting the potential protective role of abdominal activation (Casarrubios et al., 2023).

One limitation in our study was the inability of obtaining inspiratory (intercostal or diaphragmatic) EMG activity in rodent younger than P4, preventing us to perform a direct analysis of EMG activity across the first 2 postnatal weeks. A second limitation in our study was intrinsic to our recording system set-up that prevented us from providing absolute values of tidal volume, minute ventilation and air convection requirements. Even though we did not provide these values, the relative measures of respiratory frequency, tidal volume and minute ventilation were sufficient to provide measurements of relative changes (before/after recruitment) and supported my hypothesis, showing that in presence of abdominal recruitment intercostal EMG activity (>P4) and relative tidal volume and ventilation increased with occurrence of abdominal activity in the first two post-natal weeks.

The study in human infants (Chapter 3) investigated occurrence of abdominal activity in sleeping infants and provided details regarding the temporal relationship between the respiratory events and onset of abdominal activation episodes. We observed that nearly half of the abdominal activation episodes that occurred in infant humans were correlated with a respiratory or desaturation events (or some combination of the two). A few constraints in these investigations included the limited number of PSG recordings to capture this data. Also, there were significant disruptions in the surface EMG signals in both the diaphragm and abdominal muscles which further reduced the pool of recordings available for additional analysis. Lastly, this study was solely observational and limited to infants that were suspected of OSA, but otherwise healthy.

In this studies, I primarily focused on healthy neonatal rodents and human infants with potential OSA. To further advance the research in this field, our laboratory proposes to replicate and extend this analysis to rodent models of SDB. Two notable models that would be worth exploring include the chronic intermittent hypoxia rodent model (O'Halloran, 2016; Reeves & Gozal, 2006; Zoccal et al., 2008), or a model with focal lesions of the respiratory network, that lead to respiratory impairments indicative of SDB (Amiel et al., 2009; McKay & Feldman, 2008). The utilization of rodent SDB models is particularly beneficial as they would allow detailed insights into the physiological changes that occur in pathological conditions.

In human infants that are undergoing PSG's for suspected SDB, it would be of significant value to collect surface EMG data in healthy infants and at all severity levels of SDB syndromes. This comprehensive dataset would enhance our ability to make comparative assessments

between healthy and SDB patients in age-, weight- and gender-matched infants. Investigations in at-risk populations could provide an important insight into the pattern of abdominal activation in neonates that are more prone to respiratory disturbances.

Future investigations focusing on evaluating this pattern of activity in pathological animal models and in human infants that have respiratory disease can help us comprehend and validate whether expiratory abdominal activation is a respiratory compensation or recovery mechanism when breathing is unstable. These findings would opens an array of opportunities to further explore how abdominal activation can be use to improve condition of SDB.

5.3 Sex steroid hormones and breathing

Hypoventilation syndromes encompass a broad range of conditions characterized by inadequate ventilation, leading to hypercapnia and, typically hypoxemia. These disorders, which include CCHS, obesity hypoventilation syndrome, and COPD among others, are often underpinned by issues related to the central respiratory centers in the brainstem, or structural abnormalities in the chest wall or lungs. In CCHS specifically, the severity is determined by the extent of the polyalanine mutation expansion in the PHOX2B transcription factor (Weese-Mayer et al., 2010).

Current therapeutic approaches for CCHS, are quite restricted, mainly involving invasive and non-invasive mechanical ventilation, which often disrupt sleep patterns (Weese-Mayer et al., 2010) and reduce quality of life (Giberson et al., 2023; Weese-Mayer et al., 2010; Weese-Mayer et al., 1992). Given the limited efficacy of existing treatment strategies, our research emphasizes the dire need for alternative, non-invasive options for individuals suffering from respiratory disorders such as CCHS.

For decades, researchers have been exploring the potential of female sex steroid hormones, like progesterone, as respiratory stimulants (Bayliss & Millhorn, 1992; Brodeur et al., 1986; Griffith, 1929; Hasselbalch & Gammeltoft, 1915; Hosenpud et al., 1983). The use of respiratory stimulants in CCHS patients has had unsatisfactory results until recently, when two female CCHS patients displayed a 2-3 fold increase in ventilation with the onset of treatment with desogestrel, a potent progestin contraceptive (Straus et al., 2010). Further work in neonatal rodents demonstrated that acute delivery of ETO increased respiratory frequency in both neonatal rats (Joubert et al., 2016) and in a model of CCHS (Casciato et al., 2022). This

encouraged our investigations to deliver chronic administrations of the potent progestin in adult female rats.

I hypothesized that ETO interacts with progesterone receptors (PR) and stimulate PR expressing neurons in the respiratory network and thus potentiate respiration. In order to investigate this hypothesis I selected the same chronic administration approach that women use for long-term contraception, Nexplanon rods. Nexplanon is a 68mg radiopaque rod that slowly and constantly releases etonogestrel for up to 3 years. The initial and crucial step of this study was to evaluate the appropriate thickness of the Nexplanon rods to administer consistent levels of ETO in rodents. We determined that a size adjusted rod of 1mm in thickness was able to provide similar ETO plasma level concentration observed in women. As shown in Chapter 4, this dose provided very limited effect on basal breathing and ventilator responses in adult female rats.

These results were further investigated in our laboratory, where a further comprehensive investigation on ETO effects on respiratory function and transcriptional activity was performed in healthy female rats (Cardani et al., 2022). The results of that study indicate that ETO does not significantly change breathing parameters in baseline, hypoxic or hypercapnic conditions but metabolic activity (i.e, O_2 consumption and CO_2 production) was reduced in ETO treated rats compared to SHAM rats, therefore affecting CO_2 responses and causing an increase in the air convection requirement and consequent alveolar hyperventilation in hypercapnia (Cardani et al., 2022).

Furthermore, genomic analysis of brain regions expressing PGR demonstrated that PHOX2B mRNA and its downstream genes decreased selectively in the NTS (Cardani et al., 2022). These findings make the NTS an area of significant interest when coupled with earlier research indicating that local microinjections of progesterone into the NTS were associated with an increase in respiratory frequency (Bayliss et al., 1987). The collective outcome of these studies suggests that the NTS could be responsible for ventilatory, and metabolic changes induced by ETO and requires further studies.

I also tested whether ETO affected respiratory function in a model of central chemoreflex impairment (Chapter 4) and demonstrated that lesioning the RTN in female rats reproduced the respiratory dysfunction (i.e., impaired CO_2 chemoreflex) previously observed in male rats (Souza et al., 2018). Interestingly, I observed that ETO administration in these chemoreflex-impaired

rats increased normoxic, hypercapnic and hypoxic ventilatory responses to restore respiratory function.

One limitation of the study presented in chapter 4 was the small sample size of both healthy and chemosensitively impaired rodents analyzed in the study and the use of an open whole-body plethysmograph that was setup to measure relative changes in ventilatory parameters and not a closed barometric approach that would have allowed me to measure absolute values of tidal volume and minute ventilation for a more in depth analysis (as described in (Drorbaugh & Fenn, 1955; Mortola & Frappell, 2013).

Given these limitation and my time constraint, these experiments were carried on further in the Pagliardini laboratory by Drs. Janes and Cardani . The results of this study are resported in a different manuscript and are currently under submission in peer reviewed journals. Janes and colleagues demonstrated that the CO2 chemoreflex impairment was dependent on the magnitude of the RTN lesion, and that ETO significantly recovered respiratory function in rats that showed up to 80% reduction in chemosensitive cells, while rats with lesion >80% did not show significant improvement. The fact that ETO successfully recovered respiratory function with medium impairment, but it was not sufficient to recover function with more substantial impairment may explain the controversial ETO results with CCHS patients (Joubert et al., 2016; Li et al., 2013; Straus et al., 2010). The results from Janes et al (submitted) also support my original preliminary findings and point to potential mechanisms of action of ETO in respiratory control, which could entail: 1) direct stimulation of surviving RTN neurons; 2) activation of other chemosensitive neurons that express PGR and enhance the chemoreflex response in this pathological condition.

Cardani and colleagues have demonstrated an intriguing relationship between ETO administration and its effect on PHOX2B at the molecular level and its downstream target genes, which opens up an array of additional considerations as a possible targets for CCHS treatment (Cardani et al., 2018; Cardani et al., 2022). Further studies in our laboratory will thus focus on determining the mechanism of action of ETO in SP-SAP RTN lesion rats by investigating areas that are potentially affected by ETO. Given the molecular changes observed in the NTS and its role in respiratory control and metabolism, it will be important to determine whether ETO similarly affect transcriptional activity of PHOX2B dependent genes, as in Cardani 2022. Furthermore, it will be key to determine whether local application of ETO on either RTN

surviving neurons or other potential sites of interest (e.g., NTS) will be sufficient to promote recovery in SP-SAP treated rats.

Recent work by Casciato (2023) in a mouse model of CCHS show improvement of respiratory function with acute ETO administration (Casciato et al., 2022). In our laboratory, acute ETO administration did not change respiratory function (Chapter 4, (Cardani et al., 2022). Although we can't explain differential responses with acute injections, our data indicate that ETO is effective after 3 weeks from chronic treatment onset, suggesting that genomic changes may be critical for ETO long term effects on respiration.

The results of this work demonstrate that ETO acts as respiratory stimulant in models of central hypoventilation, supporting further investigations towards the use of sex hormones as a safe and suitable treatment option in conditions of hypoventilation, for example CCHS. Although hormonal treatment has had contradictory clinical outcomes so far, our studies contribute to the support of the use of specific progestins in enhancing respiratory function.

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