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Apnea and infection in neonates:
Mediatory role of interleukin-1β and prostaglandin E_2

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Dedicated to Christoph and my parents

ABSTRACT

The breathing pattern of infants, particularly preterm infants, is often irregular or periodic and is frequently interrupted by apnea. The latter represents a major concern in neonatology, yet much remains unknown about its incidence, appearance, and pathophysiology. This thesis further characterizes cardiorespiratory activity in preterm infants during postnatal development and investigates the association between infection and apnea in neonates, focusing on the mediatory role of interleukin-1β and prostaglandin E_2 in depressing central respiration.

Cardiorespiratory activity was evaluated in extremely preterm infants between birth and term-equivalent age using impedance pneumography, electrocardiography, and pulse oximetry. The incidence of apnea, bradycardia, and hypoxemia diminished with advancing age, although these events often persisted at term-equivalent age and after hospital discharge. Infection was clearly associated with an increased apnea and hypoxemia incidence.

To further elucidate the association between infection and apnea, respiration was examined in neonatal rodents using whole-body plethysmography after administration of the cytokine interleukin-1β (IL-1β) or the bacterial endotoxin lipopolysaccharide (LPS). Animals given IL-1β or LPS exhibited a lower basal respiratory frequency, depressed anoxic gasping, and a reduced ability to autoresuscitate following hypoxic apnea compared to control animals. Hyperoxic challenge revealed functioning peripheral chemoreceptors in all animals, suggesting a central mechanism underlying the ventilatory effects of these immunomodulators. However, IL-1β did not affect the respiration-related activity in neonatal rat brainstem-spinal cord preparations, indicating that it may communicate indirectly with this central respiratory network.

Prostaglandin E_2 (PGE_2) may serve as a critical mediator of ventilatory changes induced by IL-1β. In newborn infants, the infectious marker C-reactive protein was correlated with an elevated PGE_2 concentration, which in turn was associated with an increased apnea frequency. In newborn rodents, PGE_2 reversibly inhibited respiratory neurons *in vitro* and induced apnea and irregular breathing patterns *in vivo*. Moreover, IL-1β rapidly induced brainstem microsomal prostaglandin E synthase-1 (mPGES-1), an enzyme crucial for PGE_2 biosynthesis. Pretreatment with indomethacin, a prostaglandin synthesis inhibitor, clearly attenuated the adverse effects of IL-1β and LPS on basal respiration and anoxic ventilatory response in neonatal rats. Additionally, mPGES-1 knockout mice did not exhibit IL-1β-induced respiratory depression during hyperoxia and anoxia. Similarly, mice lacking the EP3 receptor for PGE_2 had fewer PGE_2-induced apneas *in vivo* and less PGE_2-induced inhibition of brainstem respiratory activity *in vitro* compared to wildtype mice. These findings strongly suggest that IL-1β alters breathing and hypoxic defense via central mPGES-1 activation and subsequent PGE_2 synthesis and binding to brainstem EP3 receptors. These studies have important clinical implications for the diagnosis, surveillance, and treatment of neonatal apnea associated with infection.

LIST OF ORIGINAL PAPERS

This thesis is based upon the following papers, which will be referred to by their Roman numerals:

I. Hofstetter AO, Legnevall L, Herlenius E, Katz-Salamon M. Cardiorespiratory function in extremely preterm infants during early postnatal development. *Manuscript*.

II. Hofstetter AO, Herlenius E. Interleukin-1β depresses hypoxic gasping and autoresuscitation in neonatal DBA/1lacJ mice. *Respiratory Physiology and Neurobiology, 146 (2-3): 135-146, 2005*.

III. Olsson A, Kayhan G, Lagercrantz H, Herlenius E. Interleukin-1β depresses respiration and anoxic survival via a prostaglandin-dependent pathway in neonatal rats. *Pediatric Research, 54 (3): 326-331, 2003.*

IV. Hofstetter AO, Saha S, Siljehav V, Jakobsson PJ, Herlenius E. The induced prostaglandin E_2 pathway – a key regulator of the respiratory response to infection and hypoxia in neonates. *Manuscript submitted*.

ABBREVIATIONS

AI	apnea/hypopnea index
BI	bradycardia index
COX-2	cyclooxygenase-2
CRP	C-reactive protein
CSF	cerebrospinal fluid
EP3R	EP3 receptor
f_R	respiratory frequency
GA	gestational age
HI	hypoxemia index
HR	heart rate
IL-1β	interleukin-1β
LPS	lipopolysaccharide
mPGES-1	microsomal prostaglandin E synthase-1
NTS	nucleus tractus solitarius
PCA	post-conceptional age
PGE_2	prostaglandin E_2
PNA	postnatal age
preBötC	pre-Bötzinger complex
RR	respiratory rate
RVLM	rostral ventrolateral medulla
SIDS	Sudden Infant Death Syndrome
V_T	tidal volume
V_E	minute ventilation

CONTENTS

1. Introduction

This thesis explores the incidence, appearance, and pathophysiology of apnea, or the cessation of breathing, in newborns. It examines the association between infection and apnea, specifically the role of two immunomodulators, interleukin-1β and prostaglandin E_2, in altering respiration.

1.1. Respiratory rhythm generation

Respiratory efforts are generated and regulated via a complex integrative system consisting of a central respiratory network and multiple feedback mechanisms (Figure 1). The Greek physician Galen (ca 130 – 201 A.D.) was one of the first to purpose that the brainstem was crucial for respiratory rhythmogenesis based upon his observations of injured animals in the arena and criminals on the scaffold (81). This hypothesis has been confirmed and refined since ancient times (76, 128, 216), and recent investigations indicate that the central respiratory network is formed by neurons in three distinct regions of the brainstem: 1) the ventral respiratory group (VRG) in the ventrolateral medulla (VLM); 2) the dorsal respiratory group (DRG) in the nucleus tractus solitarius (NTS); and 3) the pontine respiratory group (PRG) in the dorsolateral pons (for review, see (143)). The exact site and mechanism of rhythm generation have been much debated. The pre-Bötzinger complex (pre-BötC) as well as the retrotrapezoid nucleus (RTN) and parafacial respiratory group (pFRG) have been proposed as crucial centers for respiratory rhythmogenesis (71, 155, 190, 191). Recently, Feldman *et al* hypothesized that the pre-BötC and RTN/pFRG create a coupled oscillator system, whereby the former serves as the dominant inspiratory rhythm generator and the latter functions as the main expiratory rhythm generator (71). Three main respiratory rhythms are generated: eupnea (*e.g.*, normal resting respiration); sighing (*e.g.*, large inspiratory efforts overlying and interspersed within eupnea); and gasping (*e.g.*, short inspiratory efforts of high amplitude preceding long expiratory pauses). These ventilatory patterns may be modulated by input from suprapontine structures within the central nervous system (CNS), chemoreceptors, mechanoreceptors, and other sensory afferents. Ultimately, this complex respiratory system regulates oxygenation, CO_2 removal, and acid-base homeostasis in the body.

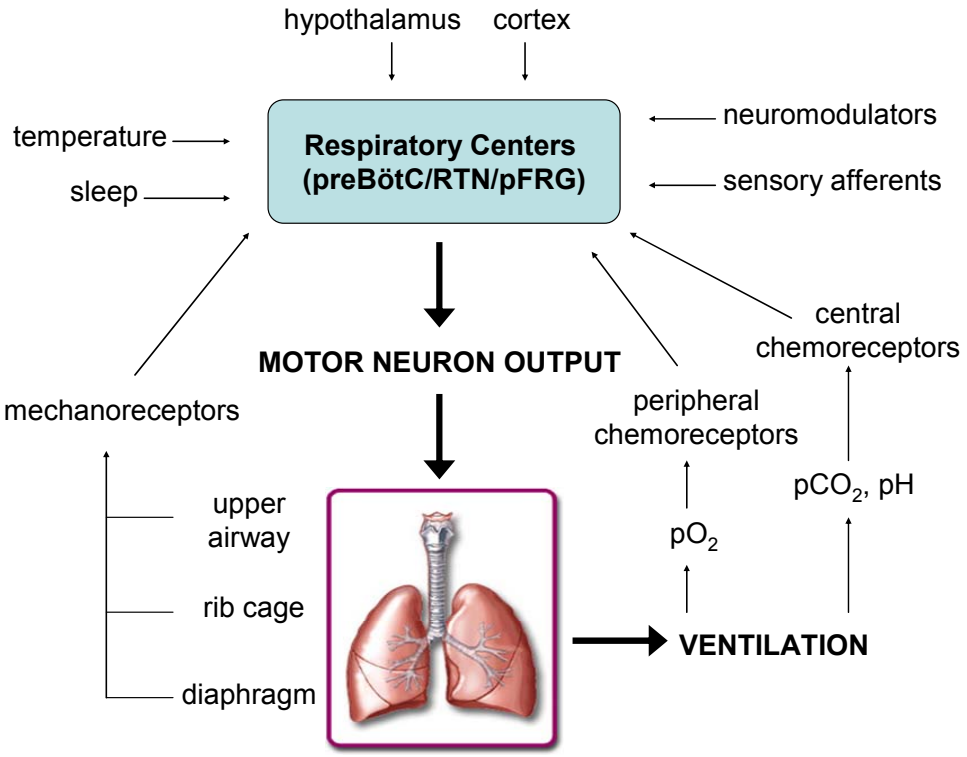


Figure 1. Control mechanisms of central respiratory rhythm generation. preBötC = pre-Bötzinger complex; RTN = retrotrapezoid nucleus; pFRG = parafacial respiratory group.

1.2. Fetal breathing and transition at birth

The central respiratory network initiates respiratory rhythmogenesis *in utero* (for review, see (88)), and consequently the fetus exhibits irregular breathing movements beginning in early pregnancy (24, 47). Fetal breathing movements are nearly continuous during early gestation, whereas they are interrupted by prolonged apnea later during fetal development (for review, see (143)). This change in respiratory pattern may be explained by brain maturation, sleep state development, and respiratory control mechanisms existing *in utero.*

Fetal breathing movements are closely regulated by changes in pCO_2 and pO_2 (for review, see (173)). Specifically, hypercarbia stimulates ventilatory efforts, whereas hypoxia inhibits fetal breathing (25). The latter may be explained by suprapontine inhibition *in utero* since midcollicular brainstem transection as well as lateral pontine lesion result in respiratory stimulation in response to hypoxia (48, 110). Removal of this central inhibition unmasks peripheral chemoreceptor activity. Functional arterial chemoreceptors exist *in utero*, and

their activity is increased in response to low pO_2 and high pCO_2 (19). However, their sensitivity is set at a lower pO_2 than after birth, probably due to the lower fetal pO_2.

Neuromodulators such as adenosine, prostaglandin, and endorphins also play an active role in the regulation of fetal breathing. Adenosine inhibits central respiration-related neurons in fetal rats (99), and its release during hypoxia may make it more abundant *in utero* due to the lower pO_2 (for review, see (101)). Similarly, prostaglandin and endorphins inhibit fetal breathing movements (118, 141, 181). Conversely, fetal breathing is stimulated by administration of their antagonists, *e.g.*, methylxanthines, indomethacin, and naloxone, respectively (39, 99, 117, 119, 148, 182).

At birth, there is a transition from irregular breathing movements to continuous respiration. The exact mechanism underlying this transition remains unclear. Central cooling of the newborn infant is an important trigger of continuous breathing at birth (22, 121, 122). Moreover, several genes encoding for respiratory-stimulating neurotransmitters are switched on during the perinatal period, and a surge in these excitatory neurotransmitters may play a key role in the respiratory transition and general arousal after birth (123-126, 176, 192, 223). Perinatal changes in inhibitory neurotransmitter expression and activity have also been implicated in the maintenance of continuous respiration, *e.g.* lower adenosine concentrations and less adenosine A_1-receptor inhibition (99) and removal of placental inhibitory factors such as prostaglandin (5, 6). Changes in suprapontine stimuli, chemosensitivity, and other reflex responses during the early postnatal period play a crucial role in the stabilization of neonatal respiration and will be discussed in greater detail below.

1.3. Neonatal respiration

Development of the intrinsic properties and functional organization of the central respiratory network continues after birth. Not only is there a change in the motor pattern and neurotransmitter sensitivity of respiration-related neurons with advancing postnatal age (99, 160, 169), but there is a maturation of dendritic morphology and increase in synaptic connections and myelination after birth (167). Collectively, these processes help stabilize respiratory activity during the postnatal period.

Central chemosensitivity plays an important role in modulating neonatal respiration. The ventilatory response to CO_2 in healthy term neonates is similar to that in adults (8), indicating that the central chemoreceptors are functional immediately after birth. This is supported by evidence that the primary central chemoreceptor area at the ventral medullary

surface exhibits *c-fos* mRNA expression directly after birth, and this expression is further enhanced by hypercarbia at one day after birth (221).

While arterial chemoreceptors are functional *in utero*, they become quiescent immediately after birth. However, within the first few days of postnatal life, peripheral chemoreceptors increase their responsiveness towards adult levels (19, 103, 104). This resetting process most likely results from a rise in pO_2 concentrations at birth (21). Peripheral chemosensitivity continues to develop during the postnatal period and plays an important role in respiratory regulation (29, 41, 174). Differential expression of neuromodulators within the carotid body may alter chemosensitivity. For example, a decreased release of dopamine, an inhibitory neuromodulator in the carotid body, coincides with the enhanced chemosensitivity after birth (103).

The ventilatory response to hypoxia also changes after birth. In newborn mammals, hypoxia induces a biphasic respiratory response that is comprised of an increase in ventilation followed by a decrease in respiratory efforts (for review, see (137)). This biphasic ventilatory response persists until at least 8 weeks postnatal age in preterm infants (138). The initial hyperventilation, lasting 1 – 2 min, results from activation of peripheral chemoreceptors. The hypoxic ventilatory depression, characterized by primary apnea, gasping, and secondary or terminal apnea (32), may result from the persistence of descending inhibitory tracts involved in the fetal response to hypoxia (20, 48, 58, 203). Additionally, modulation of central respiration-related neurons may contribute to the hypoxic ventilatory depression (159). Inhibitory neurotransmitters such as adenosine (100, 127, 180), endorphins, and GABA have also been implicated (for review, see (188)).

Control of neonatal respiration is also influenced by a variety of reflex responses from the lungs, respiratory muscles, and airways. The Hering-Breuer reflex and laryngeal chemoreflex are more profound in neonates than adults (44, 154, 204). Additionally, a hypotonic upper airway, increased chest wall compliance, lower functional residual capacity, and decreased coordination between respiratory muscles contribute to instability of ventilation during the immediate postnatal period (for review, see (143)). Development of these reflex responses and respiratory mechanics is a key factor involved in the maintenance of adequate respiration after birth.

1.4. Pathophysiology of neonatal apnea

Apnea, or the cessation of breathing, occurs frequently in the neonatal population, and immaturity of the central neuronal network plays a crucial role in its pathogenesis. Preterm

infants with frequent apnea exhibit prolonged auditory evoked responses, indicating that they may have decreased neuronal diameter, less myelination, or slower synapse transmission time (97). This immaturity increases their vulnerability to postnatal events such as infection, intracranial hemorrhage, and thermal instability, particularly if they occur during critical periods of respiratory plasticity (37, 142).

Immature chemosensitivity to CO_2 may also contribute to apnea. Preterm infants in general and those with apnea in particular exhibit an impaired ventilatory response to hypercarbia (77, 84, 115, 174). This is further potentiated by a narrow window between baseline CO_2 levels and the apneic CO_2 threshold (116) as well as a higher CO_2 threshold for upper airway muscle tone (36). In these infants, CO_2 sensitivity increases with advancing postnatal age (77, 174), which may be due to activation of additional chemosensitive regions in the brainstem (222). It may also reflect maturation of respiratory mechanics.

Premature infants with apnea also exhibit abnormal O_2 responsiveness. They demonstrate enhanced peripheral chemoreceptor activity as evidenced by a greater immediate increase in ventilation in response to hypoxia and respiratory depression in response to hyperoxia (3, 153). They also have a more pronounced hypoxic ventilatory depression (4, 175) that may be due to immaturity of the central respiratory network as well as significant suprapontine inhibition, which plays a crucial role in the fetal response to hypoxia. Neuromodulator expression is also pronounced during early postnatal life, and developmental changes in expression may explain alterations in the hypoxic responsiveness (99, 101). Chemoreceptor dysfunction as well as central respiratory depression may impair the infant's ability to autoresuscitate following an apnea event (115, 138).

Apnea in preterm infants may also result from the marked excitability of pulmonary stretch receptors as well as mechano- and chemoreceptors in the laryngeal mucosa, particularly during hypoxia (220). Furthermore, apnea may be secondary to conditions such as infection, which is one of the most frequent problems encountered in preterm infants (170). The role of infection in altering neonatal respiration is the focus of the present thesis and will be discussed in greater detail below.

1.5. Apnea characteristics and treatment

Conventionally, apnea has been defined as a respiratory pause greater than 20 seconds or a pause of shorter duration accompanied by bradycardia or hypoxemia (1). Apnea has been classified into subtypes, *e.g*, central, obstructive, or mixed events (for review, see (137)). Central apnea occurs when there is a lack of inspiratory effort, but no apparent

airway obstruction. Obstructive apnea occurs when the infant initiates a respiratory effort against an obstructed upper airway. Mixed apnea occurs when there is a lack of respiratory effort in the setting of airway obstruction. The distribution of apnea subtypes has been frequently described (30, 55, 74, 129, 150). Findings are variable (*i.e.,* 40 – 93% of apnea in preterm infants is of central origin), which likely reflects different methodological approaches and infant populations (74, 129).

While apnea commonly occurs in preterm infants, its incidence in this population remains unclear. The largest investigation of apnea was conducted between 1974 and 1979, and it showed an increased frequency and prolonged duration of recurrent apnea in infants born at earlier gestational ages (GA) (95). However, this study included few very preterm infants (less than 8% infants were born before 28 weeks GA). Since it was published, infant demographics have changed dramatically with greater survival of patients born at a younger gestational age. Their immature cardiorespiratory function puts them at greater risk for apnea, bradycardia, and hypoxemia events, which seem to persist beyond term gestation (42, 59, 168). The appearance of cardiorespiratory events in preterm infants has been described in multiple cross-sectional studies (13, 35, 96, 165, 217). The majority of apnea events are not accompanied by clinically significant changes in heart rate (HR) or oxygen saturation (SpO_2) (168), although apnea may occasionally occur concomitantly with bradycardia and/or hypoxemia (35, 96, 165, 210, 217). Prolonged apnea in particular is associated with a greater incidence, duration, and severity of bradycardia and hypoxemia (35, 74, 96, 163, 165, 210).

Methylxanthine derivatives such as caffeine and theophylline are the preferred treatment worldwide for neonatal apnea. In newborn mammals, methylxanthines stimulate respiration and reduce hypoxia-induced respiratory depression by inhibiting brainstem adenosine receptors (78). They consequently reduce apnea frequency and mechanical ventilation use in infants (98). However, deleterious effects have been described in animal models (57, 89, 207), which may result from blocking the neuroprotective effects of adenosine during ischemia. A large multi-center investigation, including infants from the neonatal intensive care unit at Karolinska University Hospital, is underway to examine the long-term neurodevelopmental outcome of preterm infants who received caffeine to treat apnea (184). In addition to methylxanthines, ventilatory support or therapies targeting secondary causes of apnea may be utilized.

1.6. Infection, apnea, and SIDS

Apnea is a common presenting symptom in infants with infection. Approximately 20% of newborns hospitalized with a respiratory syncytial virus (RSV) infection and 55% of infants with late-onset sepsis suffer from apnea (27, 70). Infection and hypoxia have been linked to Sudden Infant Death Syndrome with the majority of SIDS victims exhibiting minor signs of infection (*e.g.*, intermittent cough, congestion) or evidence of hypoxia (*e.g.*, hypoxic gasping, elevated hypoxanthine and vascular endothelial growth factor concentrations) prior to death (18, 111, 156, 166, 170, 178, 205). The SIDS incidence is greatest between two and four months when infants exhibit reduced maternal antibodies and an immature immune system that make them more vulnerable to the effects of infection (170). Cytokines such as interleukin-1β (IL-1β) have been proposed to act as critical mediators of infection, apnea, and SIDS (92, 170) (Figure 2). The ability of immunomodulators to alter hypoxic gasping and autoresuscitation has been implicated in the pathogenesis of SIDS (79, 162).

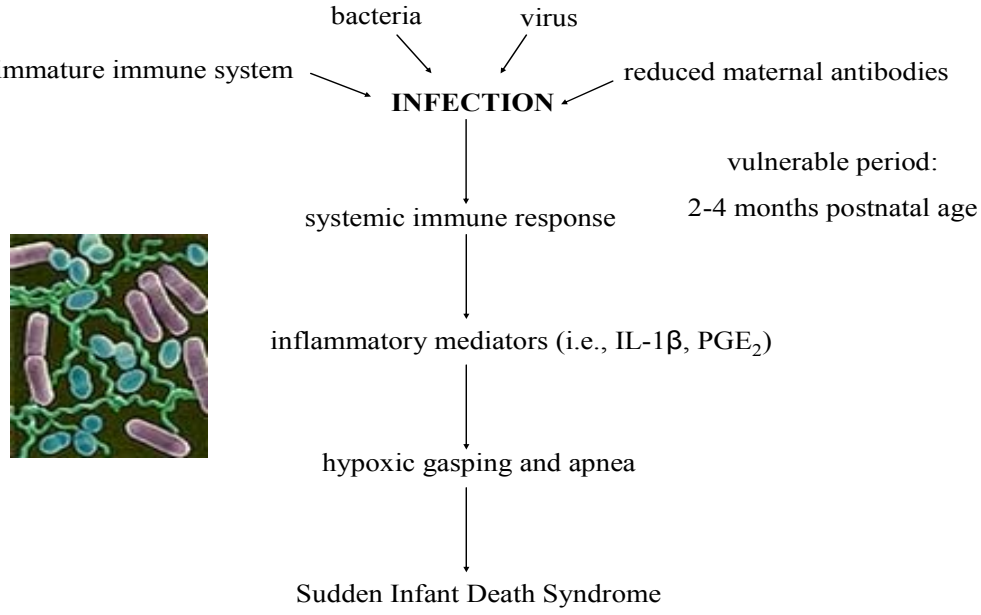


Figure 2. A proposed pathway by which infection in newborn infants may lead to apnea and Sudden Infant Death Syndrome via the mediatory actions of pro-inflammatory cytokines such as IL-1β. *Adapted from Raza 1999 (170).*

1.7. Interleukin-1β

Interleukin-1β (IL-1β) is a pro-inflammatory cytokine that is synthesized and released from activated monocytes and macrophages, neutrophils, and brain glial cells during an acute phase immune response to infection and inflammation (for review, see (46)). IL-1β induces a variety of sickness behaviors, including fever (54), hypersomnia (120), hypophagia (161), and neuroendocrine changes (15). These physiological changes are highly organized and strategically implemented during the body's fight against infectious pathogens. While these responses have distinct benefits, they can have adverse effects as well. In a newborn infant at a critical stage of development, the simultaneous occurrence of an infectious process and a hypoxic event may have a deleterious outcome. Infection with respiratory syncytial virus (RSV) in newborn lambs prolongs the duration of apnea induced by laryngeal stimulation (133). IL-1β may contribute to this finding as it similarly increases the duration of reflex apnea in piglets (80, 195). Additionally, IL-1β concentrations in pharyngeal secretions of human infants with RSV infection are positively correlated to the clinical severity of apnea (132). IL-1β may also have detrimental effects on autoresuscitation as it has been shown to reduce the respiratory frequency following apnea in piglets (80, 195). Furthermore, increased levels of IL-1β have been found in the cerebrospinal fluid of SIDS victims (215).

As IL-1β induces behavioral and physiological changes of central origin, many studies have investigated the presence and activation of IL-1 receptors (IL-1Rs) within the CNS. In the rat, Type 1 IL-1R (IL-1R1) mRNA has been localized primarily to elements associated with the blood-brain barrier such as the vascular endothelium, leptomeninges, ependyma, and choroid plexus (69). In the mouse, IL-1R1 expression has been observed predominantly upon endothelial cells, the choroid plexus, and the meninges as well as within the dentate gyrus and over the midline raphe system (11, 45). Previous studies illustrate that systemic administration of IL-1β and lipopolysaccharide (LPS), an endotoxin that increases IL-1 bioactivity, immunoreactivity, and mRNA expression (for review, see (152)), induces time- and dose-dependent expression of the immediate-early gene *c-fos* in respiratory regions of the brainstem such as the NTS and RVLM (26, 49, 63, 68, 69). Interestingly, these specific areas of IL-1β-induced Fos immunoreactivity do not appear to express IL-1R mRNA (69).

There are several routes by which systemic IL-1β may relay immune signals to autonomic regulatory centers in the brain. Although IL-1β is a large, lipophobic protein, it may enter the CNS via carrier-mediated transport across the blood-brain barrier or by passage

through circumventricular organs (12, 23, 113). However, active transport systems have a low capacity and are rapidly saturated (64). Moreover, barrier cells may prevent IL-1β diffusion through circumventricular organs (219). Thus, IL-1β may communicate with the central respiratory network via an indirect mechanism.

IL-1β may alter central behavior by inducing the synthesis and release of prostaglandin E_2 (PGE_2) at the blood-brain barrier. Circulating IL-1β has been shown to bind to and subsequently activate vascular endothelial cells expressing IL-1R mRNA at the blood-brain interface (69, 213, 224). Within an hour of intravenous administration of IL-1β, an increased expression of cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1) mRNA is observed at these endothelial cells (61). This results in enhanced prostaglandin immunoreactivity and a dose-dependent rise in PGE_2 production (213). Similarly, peripheral LPS induces COX-2 and mPGES-1 co-localized to brain endothelial cells (225), which in turn evokes a time-dependent increase in PGE immunoreactivity and production in the choroid plexus and brain microvasculature (16, 214). After synthesis, PGE_2 may diffuse throughout the brain parenchyma and bind to its receptors within the CNS. A high density of prostaglandin binding sites exist near respiratory-related regions in the brainstem, including the NTS, nucleus ambiguus, and nucleus parabrachialis (139, 197). Furthermore, systemic IL-1β administration results in colocalization of mRNA expression of the PGE_2 receptor subtypes EP3 and EP4 with *c-fos* activation in the NTS and VLM (60, 227).

1.8. Prostaglandin E_2

Prostaglandin E_2 (PGE_2) is a critical component of the immune response to infection and inflammation. Prostaglandin H_2 (PGH_2) is synthesized from arachidonic acid (AA) by cyclooxygenase-2 (COX-2). PGE_2 is then synthesized from PGH_2 by microsomal prostaglandin E synthase-1 (mPGES-1). Prostaglandins mediate many of the central effects of IL-1β, including the induction of fever (40), behavioral responses (43), and neuroendocrine changes (67, 114, 151). Prostaglandin also appears to play an important role in respiratory control. *In vivo* animal studies demonstrate that PGE_2 depresses fetal and neonatal respiration by decreasing respiratory frequency, tidal volume, and central CO_2 sensitivity (90, 118, 181, 198). PGE_2 also increases the frequency and duration of apneas in newborn animals (90) and has been correlated to a higher apnea frequency in human neonates, particularly those weighing less than 2000 g (105, 130, 189). *In vitro* studies reveal

that PGE_1 inhibits Pre-BötC neurons involved in both eupnea and gasping (10). Given these findings, we hypothesize that IL-1β alters respiratory mechanisms within the brainstem via a central PGE_2-dependent pathway.

2. Aims

The general aim of this thesis was to examine the incidence, appearance, and pathophysiology of neonatal apnea. This can be divided into the following goals:

- To characterize cardiorespiratory activity in neonates during the early postnatal period (*Studies I, II*)

- To explore the association between infection and cardiorespiratory events in human neonates (*Studies I, IV*)

- To examine the role of interleukin-1β in altering respiratory control in newborn rodents and humans (*Studies II – IV*)

- To investigate a potential mechanism by which interleukin-1β may exert such effects, i.e., via a prostaglandin E_2-mediated pathway (*Studies III, IV*)

3. Methodology

3.1. Human subjects

In *Study I*, the main objective was to examine cardiorespiratory maturation in extremely preterm infants with a focus on longitudinal changes in apnea incidence and characteristics. Infants were eligible for the study if they were born before 29 weeks gestational age (GA) and were not on mechanical ventilation at the time of recruitment. Infants suffering from certain conditions that cause secondary apnea such as intraventricular hemorrhage (grade > 2) and white matter disease were excluded from the study.

In *Study IV*, we investigated the correlation between the infectious marker C-reactive protein, central PGE_2 concentrations, and apnea events in human neonates. Infants were eligible for inclusion if they underwent a lumbar puncture for routine clinical indications such as suspected infection or neurological changes. While we were particularly interested in those infants with infection, infants with other medical conditions served as valuable controls.

For both studies, informed written consent was obtained from infant guardians. Pertinent medical information was documented for each infant, including neonatal delivery data, medical conditions, infection status, respiratory therapy, and medications. The studies were performed in accordance with European Community guidelines and approved by the regional hospital research ethics committee (*Dnr:* 00-328, 03-174).

3.2. Animal models

Transgenic mouse models play an important role in the investigation of respiratory control mechanisms (83, 199). In *Studies II* and *IV*, male and female inbred DBA/1lacJ mice at postnatal age 9 days were used. The microsomal prostaglandin E synthase 1 (mPGES-1) gene was selectively deleted in knockout mice as described previously (208). There is a great variability in the development and sensitivity to hypoxia and hypercarbia between mouse strains (200). The strain DBA/1lacJ was chosen since used previously to evaluate inflammatory processes (65, 208) and mice of a related strain (DBA/2J) are highly sensitive to hypoxia (201) suggesting that DBA/1lacJ mice might also respond strongly to pO_2 changes. Thus, this strain seemed particularly appropriate for examining the effects of immune system mediators on the hypoxic ventilatory response. In *Study IV*, male and female inbred C57BL/6 mice at postnatal age 9 days were also used, and the EP3 receptor (EP3R) gene was selectively deleted in knockout mice as described previously (75). These mice

enabled us to further eludicate the particular mechanism by which PGE_2 may alter respiration-related neurons within the brainstem, *i.e.*, via the EP3R.

In *Study III*, male and female rats of postnatal age 0-4 days (*in vitro*) and 7 days (*in vivo*) were used. This investigation was performed in rats for two primary reasons: 1) the rat is a well-established model system for the evaluation of respiration using *in vitro* and *in vivo* techniques (71); and 2) our hypothesis and protocol were based upon previous investigations using the rat as the model species (60, 68, 213, 214, 226).

All newborn rodents were born and reared by their mothers under standardized conditions with food and water provided *ad libitum*. The studies were performed in accordance with European Community guidelines and approved by the regional animal research ethics committee (N141/99; N126/03; N305/03; N354/03).

3.3. Drugs

IL-1β or LPS was administered (i.p.) in newborn rodents in order to induce an immune response resembling that which occurs during an infectious or inflammatory process (46). PGE_2 was administered (i.c.v.) in order to examine its effects on respiration *in vivo* in wildtype mice and mice lacking mPGES-1 and EP3R. Recombinant mouse IL-1β, recombinant rat IL-1β, and PGE_2 were also applied to the *en bloc* brainstem-spinal cord preparations of neonatal rodents in order to determine their direct effect on respiration-related neurons. Pretreatment with indomethacin crystalline, a nonspecific cyclooxygenase inhibitor, was performed in select rats to prevent the subsequent induction of prostaglandin synthesis by IL-1β or LPS. Concentrations of IL-1β, LPS, PGE_2 and indomethacin were chosen based upon concentrations used in similar rodent studies (38, 62, 65, 82, 94, 136, 179).

3.4. Cardiorespiratory monitoring

Impedance pneumography and electrocardiography (ECG) recorded baseline respiratory rate (RR) and heart rate (HR) as well as apnea/hypopnea and bradycardia events. Pulse oximetry continuously monitored changes in pulse rate and oxygen saturation. Impedance pneumography translates changes in alternating current between surface electrodes into a waveform corresponding to thoracic movements (53). While it is a well-established method for cardiorespiratory surveillance, there are potential disadvantages to its use. Since impedance pneumography monitors chest wall movements and is dependent on posture, it may be difficult to quantitatively assess respiratory tidal volume. Thus, qualitative comparisons must be employed. Additionally, cardiogenic artefacts may occur since this

technique detects electrical currents through other conducting materials (211). Furthermore, it may be difficult to ascertain apnea types (i.e., central, obstructive, or mixed) using this system. While respiratory inductance plethysmography is a more accurate system for monitoring cardiorespiratory activity, its current use is limited in the clinical setting and in home monitoring systems (9).

Study I Protocol: Overnight cardiorespiratory recordings were performed weekly between birth and term-equivalent age. Home recordings were done following hospital discharge and after parental training of monitor use.

Study IV Protocol: Cerebrospinal fluid (CSF) was collected in infants with a clinical indication for lumbar puncture (*i.e.*, infection), and a cardiorespiratory recording was performed as soon as possible thereafter. Early evaluation was crucial given that the infection-induced synthesis and central effects of PGE_2 are time-dependent and that common treatments (e.g., antibiotics, anti-pyretics, respiratory therapies) may alter the intrinsic immune response and cardiorespiratory function.

3.5. Whole-body plethysmography

Evaluation of respiration *in vivo* can be carried out using several techniques. Unrestrained whole-body plethysmography was implemented in our investigations as it is a non-invasive alternative to methods such as spirometry and pneumotachography in smaller animals (146). Barometric plethysmography, which was first described in 1955, is based upon the principle that warming and humidification of inspired air results in an increased pressure within the plethysmograph chamber (56). Tidal volume can then be calculated from the temperature, humidity, and pressure values (66). Flow plethysmography is based upon the principle that fluctuations in airflow superimposed upon the baseline flow through the plethysmograph chamber are the result of the animal's respiratory efforts. While there are advantages to using plethysmography for monitoring respiration, there are also disadvantages. With the barometric method, alterations in pressure and temperature within the chamber (*i.e.*, due to changes in gas composition or heat production) can profoundly influence V_T measurements (146). The introduction of an open flow plethysmography system reduces the effects of pressure and temperature gradients within the chamber. Nonetheless, it has been suggested that V_T should be examined qualitatively, not quantitatively (66). Similarly, as V_E depends upon V_T, it may also be important to emphasize relative changes in V_E rather than focus on absolute measurements.

Studies II and IV Protocol: At 70 min after intraperitoneal injection (i.p.) of IL-1β or NaCl, mice were placed in the plethysmograph chamber and their respiratory activity was measured using the following protocol: a) 4 min normoxia (21% O_2); b) 1 min hyperoxia (100% O_2); c) 5 min normoxia; d) anoxia (100% N_2) until 1 min after the animal's last gasp (*Study II*) or for 5 min (*Studies II* and *IV*); e) 8 min hyperoxia (Figure 3). In all animals, skin temperature was measured at baseline as well as immediately before and after experimentation.

Study III Protocol: Each rat received an initial i.p. injection of NaCl or indomethacin 30 min prior to a second i.p. injection of NaCl, IL-1β, or LPS. The animal was placed in the plethysmograph chamber at 60 min after the second injection and exposed to 7 min normoxia, anoxia until 1 min after its last gasp, and then 100% O_2 for 15 min or until autoresuscitation (Figure 3). In a sample population, skin temperature was measured during experimentation.

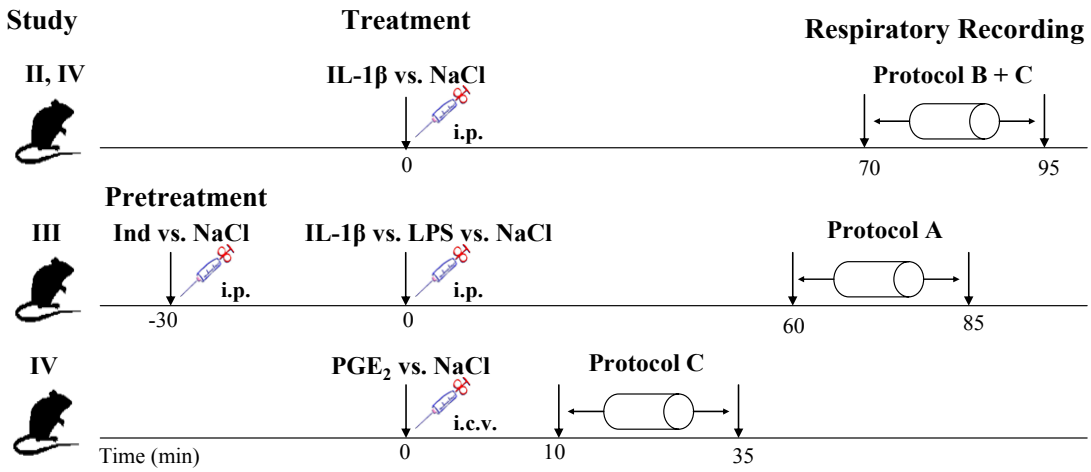
Supplemental Protocols: In order to characterize the respiratory behavior of neonatal DBA/1lacJ mice, the ventilatory response to varying concentrations of O_2 and CO_2 was examined in six neonatal DBA/1lacJ mice in *Study II*. Each animal was exposed sequentially to normoxia (21% O_2), mild hypercapnia (3% CO_2 in synthetic air), moderate hypoxia with mild hypercapnia (10%O2, 3% CO_2), severe hypercapnia (8%CO_2, 21%O_2), severe hypoxia with mild hypercapnia (5% O_2, 3% CO_2), and hyperoxia (100% O_2). Normoxia was administered between periods. In *Study IV*, the respiratory response to central PGE_2 was also investigated in neonatal mice using flow plethysmography. Immediately after anesthesia administration and i.c.v. injection of PGE_2 or vehicle, the mouse was placed into the plethysmograph chamber. After a 10 min recovery period in normoxia, the mouse was exposed to the same gas protocol described in *Study IV* above (Figure 3).

General considerations: Several important factors were considered in the design and implementation of these studies. First, ambient temperature can strongly influence the respiratory response to anoxia (31, 186). Thus, in all plethysmography experiments, chamber temperature was maintained at approximately 30°C in accordance with the documented thermoneutral range for rats and mice of similar age (109, 145). Second, gases were chosen with specific objectives. Normoxia was used to establish baseline respiratory characteristics within the control population and to determine how IL-1β, LPS, and PGE_2 alter basal respiration. The mice were exposed to a brief hyperoxic challenge in order to blunt peripheral chemoreceptor activity and unmask central respiratory drive (51). This enabled us to better assess whether the ventilatory effects of IL-1β and PGE_2 occur via peripheral or

central actions. Anoxia was used to induce hypoxic gasping, while chamber reoxygenation permitted the examination of autoresuscitation.

Drug administration protocols were based on careful evaluation of previous investigations using these drugs. Indomethacin was given 30 min prior to IL-1β or LPS in order to allow sufficient time to block cyclooxygenase before these immunomodulators could induce enzymatic activity (28, 86). IL-1β and LPS have been shown to increase COX-2 mRNA expression at 1 hr after intraperitoneal (i.p.) injection (34, 61). Thus, respiratory recordings were performed between 60 – 95 min after i.p. administration of IL-1β or LPS in order to allow sufficient time for respiratory effects to occur while attempting to minimize confounding systemic effects.

IL-1β, LPS, and PGE_2 evoke a broad array of centrally mediated adaptive responses, which themselves may contribute to alterations in respiratory control. For example, IL-1β has been shown to increase metabolic rate (14, 209), and an increased metabolism has been associated with a shorter gasping duration (108). However, animals exhibited similar skin temperatures at baseline, post-anesthesia in the i.c.v. experiments, and 60 – 70 min after i.p. injection of IL-1β or LPS. These temperature measurements corresponded to previously reported values for mice and rats of similar age (85, 185). Previous studies in rodents indicate that IL-1β and LPS do not induce significant temperature increases until at least 90 min after i.p. injection (33, 34, 38, 73, 82, 179) and that PGE_2 does not induce maximum fever until 20 – 25 min after i.c.v. administration (212). Consequently, respiratory recordings were performed within these time frames. Moreover, fever induced by IL-1β does not affect the duration of hypoxia gasping nor does it hinder autoresuscitation following repeated hypoxic exposure in newborn rats (73). Lastly, gross motor activity was similar between animals. This is consistent with previous studies demonstrating that IL-1β does not evoke sleep or hyperalgesia in rats until 2 or 4 hours after peripheral administration, respectively (144, 157).



Ind (indomethacin): 10 mg/kg
IL-1ß: 10 µg/kg
LPS: 100 µg/kg
PGE_2: 4 nM

Plethysmography Protocols:

A) 7 min normoxia → anoxia until 1 min after last gasp → 15 min hyperoxia

B) 4 min normoxia → 1 min hyperoxia → 5 min normoxia → anoxia until 1 min after last gasp → 8 min hyperoxia

C) 4 min normoxia → 1 min hyperoxia → 5 min normoxia → 5 min anoxia → 8 min hyperoxia

Figure 3. Plethysmography protocols from *Studies II, III,* and *IV.*

3.6. Brainstem-spinal cord preparation

The brainstem and spinal cord of newborn rodents were dissected and isolated as described previously (102, 196). This *in vitro* technique is valuable as it permits the evaluation of specific cellular processes involved in the generation and control of respiration (196). It also enables us to assess how respiration is affected by changes in pH, pO_2, or pCO_2 as well as by exposure to various chemicals or drugs. However, there are potential limitations of the brainstem-spinal cord preparation, including the absence of afferent input and the generation of a slower respiratory rhythm and a unique bursting pattern compared to eupnea in an intact animal (172, 193).

Studies III and IV Protocols: Brainstem–spinal cord preparations were rapidly isolated from 0 – 4 d-old rats (*Study III*) and 2 d-old C57BL/6 mice with $EP3R^{+/+}$ and $EP3R^{-/-}$ genotypes (*Study IV*). The preparations were initially perfused with control artificial CSF (aCSF). In *Study III*, this was followed by perfusion with aCSF containing either IL-1β or PGE_2 for 30-60 min. In *Study IV*, this was followed by perfusion with aCSF containing PGE_2 for 20 min. In both studies, there was a final washout period with control aCSF. Respiratory activity

corresponding to the inspiratory rhythm was recorded using glass suction electrodes applied to the proximal end of the cut C4 ventral root.

3.7. Enzymatic assay

In *Study IV*, microsomal prostaglandin E synthase-1 (mPGES-1) activity was assessed in the cortex and brainstem of neonatal wildtype mice as well as mPGES-1 knockout mice using a quantitative enzymatic assay first described by Thorén and Jakobsson in 2000 (206). This assay has been shown to recover 85 ± 11% of PGE_2 (206). Our study objective was to evaluate endogenous PGE_2 production as well as the ability of IL-1β and hypoxia to induce mPGES-1 activity. It also enabled us to determine the location of greatest mPGES-1 activity, *i.e.*, cortex *vs*. brainstem.

Protocol: Newborn mouse brains were homogenized in 0.1M KPi buffer containing 0.25M sucrose, 1X complete protease inhibitor, and 1mM reduced glutathione. This was followed by sonication. Membrane fraction was isolated by sub-cellular fractionation. Protein concentration was determined by the Bradford method. mPGES-1 activity was assayed by incubating the membrane fraction with 10uM PGH_2 followed by termination of the reaction using an acidified $FeCl_2$ solution. Solid phase extraction of the reaction product was then performed using C18 chromabond columns. PGE_2 was eluted with acetone, evaporated under nitrogen flow, and dissolved in 33% acetonitrile. An aliquot was analyzed by RP-HPLC combined with UV detection at 195 nm. Enzymatic formation of PGE_2 was calculated after subtracting the non-enzymatic PGE_2 formation in the buffer.

3.8. Enzyme immunoassay

In *Study IV*, PGE_2 concentrations in infant cerebrospinal fluid (CSF) were measured using enzyme immunoassay (EIA). CSF bathes the central nervous system, and thus CSF concentrations may provide an estimate of levels within the brain parenchyma (52). EIA allows enzyme detection using small sample volumes, which is crucial given the small CSF volume in neonates. Since PGE_2 is rapidly metabolized to 13,14-dihydro-5-keto PGE_2 *in vivo*, concentrations of 13,14-dihydro-15-keto PGA_2, a non-enzymatically formed stable metabolite of 13,14-dihydro-5 keto PGE_2, were also measured using EIA.

Protocol: In study patients, a small volume of cerebrospinal fluid (0.75 – 1.5 ml) was collected for research purposes. PGE_2 and PGE_2 metabolite concentrations were then determined using a standardized EIA protocol. In order to maximize sample integrity as well

as increase compliance amongst study collaborators, all samples were immediately stored at –18°C and transferred as soon as possible to –80°C.

3.9. Data analysis

In vivo plethysmography experiments: Since the animals were placed unrestrained in the plethymograph chamber, we used visual observations during experimentation as well as two different analysis methods to select the best periods for analysis during normoxia, hyperoxia, hypercapnia, and hypoxia (*i.e.*, calm respiration without movement artefact). Respiratory frequency (f_R, breaths/min) was calculated manually. Tidal volume (V_T, μL/breath), minute ventilation (V_E, mL/min), time of inspiration (T_i, s), and time of expiration (T_e, s) were also measured for flow plethysmograph data. In response to severe hypoxia and anoxia, the duration of hyperpnea, primary apnea, gasping phases, and secondary apnea was determined. The f_R during hyperpnea was calculated manually, and the V_T was calculated in mice that were calm during the analysis period. The number, frequency, and appearance of gasps were determined. Survival was recorded for all animals. The duration of secondary apnea and time required to autoresuscitate following O_2 administration were calculated in survivors. The f_R following autoresuscitation was also calculated. Apnea was defined as cessation of breathing for ≥ three respiratory cycles. Regularity of breathing was quantified in some i.c.v. experiments using the coefficient of variation (C.V.) (i.e., SD of Δ f_R / mean of Δ f_R during 60 s period). In *Study III*, similar findings were obtained with both flow and barometric plethysmography; thus, data were normalized to facilitate statistical comparisons. In *Studies II* and *IV*, we attempted to perform all recordings at age P9 since there is a variable response to anoxia based upon age (72); however, some mice may have been evaluated at P9 ± 1 d. Thus, we attempted to minimize confounding age-related effects by using weight as a correlate of age and excluding those mice weighing > 1 SD of the mean population weight in the anoxia and survival analyses.

In vitro brainstem-spinal cord preparation experiments: Respiratory frequency (f_R, burst/min) was calculated from the mean C4 burst interval during consecutive 2-5 min periods. Baseline f_R and changes in f_R in response to IL-1β and PGE_2 were assessed.

Infant cardiorespiratory data analyses: The monitor software was used to calculate baseline respiratory rate, heart rate, pulse rate, and SpO_2 values and to visualize cardiorespiratory events for each recording. Apnea/hypopnea was defined as a ≥ 10 sec reduction of the impedance signal amplitude to < 16% of the mean amplitude. It was described by

apnea/hypopnea index (AI = # apneas/hypopneas per hour recording), duration, and morphology. The latter was characterized by a predominant reduction in either RR or V_t. Bradycardia was defined as a HR < 80 bpm for > 1 sec and expressed as bradycardia index (BI = # bradycardias/ hour recording), duration, and HR nadir. Oxygen desaturation was defined as a SpO_2 value ≤ 90% and characterized by hypoxemia index (HI = # hypoxemias/ hour recording), duration, and nadir SpO_2 values. Periodic breathing was defined as an episode of three or more successive apnea pauses of > 3 breath duration separated by < 20 sec of normal respiration. The occurrence of periodic breathing in the 60 sec following an apnea event was examined. Mean RR, HR, and SpO_2 immediately prior to an event were recorded. All movement artefacts as well as recordings < 2 h duration were excluded from analysis.

4. Results and discussion

4.1. Cardiorespiratory development in preterm infants

There is a paucity of longitudinal data describing maturational changes in baseline cardiorespiratory function in extremely preterm infants during early postnatal development. In *Study I*, we reveal that the resting respiratory frequency did not change significantly between birth and term-equivalent age in extremely preterm infants, whereas a reduction in baseline respiratory rate during postnatal development has been shown previously in older preterm infants (106, 112). The lack of age-dependent changes in respiratory rate may reflect delayed maturation of respiratory control mechanisms in this very preterm infant population. Conversely, we show a diminishing heart rate during the postnatal period, which may indicate comparatively earlier cardiovascular development. The lowering of HR may be a consequence of increased parasympathetic tone, which also influences baroreceptor reflex sensitivity and contributes to HR variability during the postnatal period (7, 135). Our infants, as well as those born at later gestation (163), also exhibited an improvement in oxygen saturation with advancing post-conceptional age. This finding may reflect complex mechanisms such as changes in ventilation to perfusion matching, chemosensitivity, and lung mechanics. Ventilatory management may also influence baseline saturation in premature infants, *e.g.*, a target saturation of 88 – 92% SpO_2 is frequently used in the neonatal unit.

Since our study was performed in a relatively small cohort of extremely preterm infants, it would be beneficial to expand this investigation to include more infants who may be further stratified according to pertinent demographic and clinical variables. Nonetheless, our study provides useful reference data for baseline cardiorespiratory function in this population.

4.2. Cardiorespiratory events during early postnatal life

Age-dependent changes in the incidence and appearance of cardiorespiratory events have not been thoroughly described in extremely preterm infants during early postnatal life. In *Study I*, we reveal that all infants born between 23 and 28 wk GA experienced recurrent apnea, and 67% of infants continued to exhibit apnea/hypopnea events beyond 36 wk PCA. Our findings confirm retrospective data describing an age-dependent reduction in apnea incidence during the hospitalization period in a similar patient population using nursing records (59), which is a less reliable method of apnea detection (150). Our findings differ from those shown in older preterm infants, where a more pronounced decline in apnea

incidence results in fewer apnea events beyond term-equivalent age (95, 168). In general, this discrepancy may reflect maturational changes in the central respiratory network, chemoreceptor activity, reflex responses, and respiratory mechanics, but it may also mirror differences in methodological approach (*e.g.*, apnea definition, monitoring technique, data collection).

In our study, the appearance of apnea/hypopnea changed during the postnatal period. We reveal a dynamic age-dependent alteration in apnea/hypopnea frequency, *i.e.,* an initial rise followed by gradual decline in frequency, resembling that shown in older preterm infants at a later PCA (129, 158, 194). Additionally, there was a predominant RR reduction during apnea/hypopnea events occurring at an earlier PCA. This change may mirror the development of hypoxic responsiveness in preterm infants since hypoxic ventilatory depression is initially characterized by a marked reduction in RR than V_t (171), but subsequently the neonate is better able to sustain RR during hypoxia (218). Unlike these postnatal changes, the duration of apnea/hypopnea events remained constant with advancing age. Prolonged apnea/hypopnea occurred beyond term-equivalent age and was associated with a prolonged HR depression. This finding emphasizes the importance of close surveillance of this infant population after discharge from the well-controlled hospital environment. Furthermore, the long-term consequences of such events in extremely preterm infants must be ascertained in future investigations.

A small percentage of apnea/hypopnea events occurred concomitantly with bradycardia and/or hypoxemia events. There was a strong correlation between AI and BI, which may be secondary to peripheral chemoreceptor activation following apnea-induced hypoxemia (96) as well as a reflex response to cessation of lung inflation or superior laryngeal and trigeminal nerve stimulation (142). It may also indicate that infants with more immature respiratory control frequently exhibit cardiovascular dysregulation. Bradycardia incidence and duration declined rapidly with maturation. This finding, along with age-dependent changes in baseline HR, suggests that the cardiovascular system may undergo developmental changes more rapidly than the respiratory system. The hypoxemia index also diminished during postnatal development, which has also been shown in older preterm infants (163). Cardiorespiratory events may cause hypoxemia via reduced alveolar ventilation or arterial perfusion, although transient hypoxemia may also occur without simultaneous apnea or bradycardia episodes (164). Age-dependent changes may also be due to improved chemoreceptor function, which enables infants to better maintain their O_2 saturation.

4.3. Infection increases cardiorespiratory events in infants

In *Study I*, infection was clearly correlated with a higher incidence of apnea/hypopnea and hypoxemia events in preterm infants less than 31 wk PCA, but not in infants greater than 31 wk PCA. This indicates that younger preterm infants are particularly vulnerable to postnatal insults such as infection. These findings are consistent with a large multi-center study demonstrating that an increased apnea frequency occurs in the majority of infants with sepsis (70) and in 20% of infants hospitalized with respiratory syncytial virus (RSV) (27). In human neonates with RSV, IL-1β has been positively correlated to the clinical severity of apnea (132). Thus, we propose that IL-1β plays a crucial mediatory role in this association between infection and apnea in the newborn population. This hypothesis was explored further in *Studies II – IV*.

4.4. Respiratory behavior in neonatal DBA/1lacJ mice

In *Study II*, we characterized the respiratory behavior of newborn DBA/1lacJ mice since it was crucial to determine baseline function in these mice, which had not been done previously, prior to examining the effect of immunomodulators on ventilation in wildtype mice as well as mPGES-1 knockout mice. We describe respiratory frequency (f_R), tidal volume (V_T), and minute ventilation (V_E) during normoxia in wildtype mice, and these values resembled those reported previously in Swiss CD-1 and C57BL/6 mice of similar age (17, 177). The DBA/1lacJ mice exhibited a characteristic reduction in f_R in response to hyperoxia, although f_R decreased to a greater extent than described in other newborn mice (147). The DBA/1lacJ mice also demonstrated a more pronounced response to hypoxia and anoxia compared to that shown previously in other mouse strains (85, 108). Conversely, mild hypercapnia did not alter ventilation compared to normoxia, and severe hypoxia without hypocapnia (3% CO_2) produced a hypoxic ventilatory depression similar to that observed during anoxia with hyperpnea-induced hypocapnia. These findings indicate that DBA/1lacJ mice have a heightened sensitivity to O_2 concentration, but are less sensitive to pCO_2 changes. This is important to consider when comparing our findings in DBA/1lacJ mice with those of C57BL/6 mice. For example, whereas all DBA/1lacJ mice in *Study II* and *IV* terminated their gasping response during the five minutes of anoxic exposure, all neonatal wildtype C57BL/6 mice were able to sustain their gasping response beyond the five-minute anoxic period. This suggests that the DBA/1lacJ mice have a greater sensitivity to pO_2 changes than the C57BL/6 mice. Interestingly, hypoxic ventilatory depression is absent in

P1-P3 mice of the C57BL/6 strain, and it does not appear until P7 (17). This finding may explain why our C57BL/6 mice demonstrate a less robust hypoxic ventilatory depression at age P9 compared to the DBA/1lacJ mice.

4.5. IL-1β depresses respiration via central actions

In *Studies II – IV*, the ventilatory effects of IL-1β were investigated in wildtype rodents. We show that IL-1β lowered f_R during normoxia in newborn rats and reduced basal V_T, V_E, and a weight-normalized f_R in neonatal wildtype mice. LPS also tended to depress basal respiration in newborn rats. These findings are in accordance with data showing that IL-1β, given together with TNF-α, decreases normoxic f_R in rabbits and that LPS reduces f_R and V_T during normoxia in BALB/c mice (107, 209).

We hypothesize that IL-1β alters the central respiratory network rather than peripheral chemosensitivity. In *Studies II* and *IV*, mice were subjected to hyperoxic challenge in order to induce a physiological denervation of peripheral chemoreceptors and consequently unmask central respiratory drive. All mice exhibited an appropriate peripheral chemoreceptor response, and IL-1β induced a more pronounced respiratory depression during hyperoxia. These findings suggest that a compensatory activation of peripheral chemoreceptors occurs during normoxia in IL-1β-treated mice in order to balance the IL-1β-induced depression of central respiration-related neurons.

In our studies, IL-1β depressed the anoxic ventilatory response by lowering the gasping frequency in newborn rats and by reducing the number of gasps and the ability to sustain respiratory efforts during anoxia in newborn DBA/1lacJ wildtype mice. IL-1β also markedly reduced the ability of neonatal rodents to autoresuscitate following hypoxic apnea. Prolonged anoxia causes a gradual loss of afferent inputs, central accumulation of inhibitory neurotransmitters, and reconfiguration of neurons in the brainstem responsible for respiratory rhythm and pattern generation (131, 218). IL-1β may selectively modulate these processes responsible for hypoxic ventilation depression. However, we reveal that IL-1β was unable to directly alter bursting activity of central respiratory neurons *in vitro*, and a previously published study shows that Type 1 IL-1 receptor mRNA are not localized to respiration-related regions of the brainstem (69). These findings indicate that IL-1β communicates with the central respiratory network via an indirect mechanism. We suggest that these actions occur via a prostaglandin-mediated pathway.

4.6. Endogenous PGE_2 exerts tonic respiratory effects

In *Study IV*, we clearly demonstrate an endogenous expression of mPGES-1 activity in wildtype mice, particularly in the brainstem. Additionally, respiratory depression was greater in wildtype mice than in mPGES-1 knockout mice when central respiratory drive was unmasked during hyperoxia. These findings indicate that endogenous PGE_2 has a tonic effect on respiratory rhythm generation during the perinatal period. These results are consistent with data showing that prostaglandin synthesis inhibitors increase fetal breathing movements as well as central respiratory activity the early neonatal period (91, 117, 134), although indomethacin failed to stimulate respiration beyond basal levels in the newborn rats of *Study III*. This may be explained in part by developmental changes in the modulatory effects of prostaglandin with an initial inhibition of ventilation (90, 118) followed by little or no alteration in central respiration beyond the perinatal period (198). These changes may be secondary to a reduction of brainstem PGE_2 receptor expression beyond the perinatal period (197). An investigation of the ontogenesis of EP3R in respiration-related regions of the brainstem would be valuable.

4.7. PGE_2 inhibits respiratory activity via EP3R

In *Study IV*, the infectious marker C-reactive protein was positively correlated with PGE_2 levels in the cerebrospinal fluid of human infants, and the latter was associated with a higher apnea frequency. Although our study cohort was small and relatively heterogeneous, these findings support our hypothesis that infection induces neonatal apnea via a PGE_2-mediated mechanism. They are in accordance with previous investigations showing an independent association between CRP levels and apnea/hypopnea index in children with sleep apnea (202) as well as a positive correlation between urine PGE metabolite and central apneas in newborn infants (105). Moreover, human neonates treated with prostaglandin often display an increased apnea frequency (130, 189). Further evaluation in a larger patient population is warranted.

We hypothesize that PGE_2 depresses respiration centrally by binding to EP3 receptors in the brainstem. In *Study IV*, all PGE_2-treated mice demonstrated an appropriate peripheral chemoreceptor response to hyperoxic challenge, indicating a central mechanism underlying the ventilatory effects of PGE_2. This corroborates data showing that PGE_2 inhibits fetal breathing movements in sheep after denervation of the carotid sinus and vagus nerve (149). Furthermore, in our investigation, central administration of PGE_2 induced apnea events and

irregular breathing patterns in neonatal wildtype mice, but not in EP3R knockout mice. PGE_2 also depressed central respiratory-related bursting activity *in vitro* in newborn rats and EP3R wildtype mice, but not in mice lacking the EP3 receptor. Thus, this study provides clear evidence that the central respiratory effects of PGE_2 occur via EP3R, which is consistent with evidence that EP3 receptors are located within the NTS and RVLM (60). We are currently investigating the co-localization of EP3R with neurokinin-1 receptors found specifically within the preBötC and pFRG (87) using double immunohistochemistry.

4.8. IL-1β and hypoxia activate mPGES-1

In *Study IV*, IL-1β and brief anoxic exposure increased mPGES-1 activity in the mouse brainstem. Previous investigations have shown that anoxia increases PGE_2 production in the mouse cortex *ex vivo* (187) and transient asphyxia increases PGE_2 concentrations in the newborn guinea pig brain (2). The exact mechanism of mPGES-1 upregulation in our study remains unclear. Induced gene expression and mPGES-1 activation are less likely to occur during such a brief hypoxic event. Potential etiologies include post-transcriptional regulation or stabilization of mPGES-1 mRNA, which has been previously shown in neonatal mouse cardiomyocytes (50).

4.9. PGE_2 mediates the respiratory effects of IL-1β

In *Studies III* and *IV*, we further explored the mediatory role of prostaglandin in IL-1β-induced respiratory changes *in vivo*. First, indomethacin pretreatment attenuated the basal respiratory depression induced by IL-1β in neonatal rats. Indomethacin also markedly improved the ability of IL-1β-treated rats to survive anoxic challenge. Similarly, IL-1β was unable to alter basal respiration or the ventilatory response to hyperoxia in mice lacking mPGES-1 or EP3R. Additionally, it had no effect on anoxic gasping or the ability to autoresucitate following hypoxic apnea in mPGES-1 and EP3R knockout mice. Collectively, these findings suggest that IL-1β inhibits central respiratory mechanisms indirectly via activation of mPGES-1 and PGE_2 binding to EP3 receptors (Figure 4).

We hypothesize that IL-1β- and hypoxia-induced PGE_2 selectively modulates respiration-related neurons in the preBötC. There is persuasive evidence that preBötC neurons are crucial for the neurogenesis of gasping and subsequent autoresuscitation from hypoxia (159). Lesions within the preBötC have been shown to disrupt anoxic gasping and evoke central apneas and ataxic breathing (71, 140). Other neuromodulators inhibit these

neurons and slow respiration-related rhythm (87, 100). Recurrent apnea may lead to a loss of preBötC neurons, which may worsen the hypoxic ventilatory depression and increase the threshold for autoresuscitation (71). Thus, we theorize that acute hypoxia and infection, via release of IL-1β and PGE_2, may result in a pronounced inhibition of preBötC neurons, thereby impairing anoxic gasping and autoresuscitation. These effects may worsen with recurrent hypoxia and infection, which frequently occur in preterm infants.

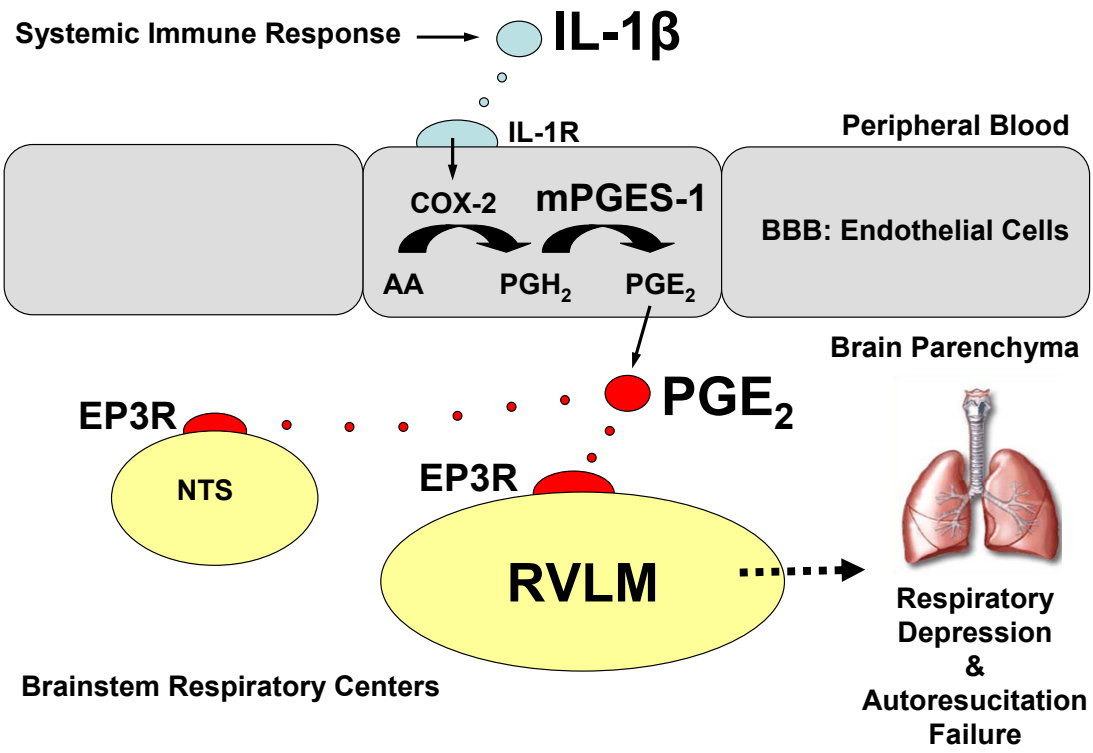


Figure 4. This schematic illustrates how, according to the present thesis, infection alters respiratory control via an interleukin-1β-induced, prostaglandin E_2-mediated pathway. During an acute phase response to infection, interleukin-1β (IL-1β) is released into the peripheral circulation. IL-1β then binds to Type I IL-1 receptors (IL-1R) located on vascular endothelial cells of the blood-brain-barrier (BBB), thereby activating cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1). COX-2 converts arachidonic acid (AA) to prostaglandin H_2 (PGH_2). Subsequently, mPGES-1 catalyzes the formation of prostaglandin E_2 (PGE_2) from PGH_2. PGE_2 then diffuses throughout the brain parenchyma, binding to its EP3 receptors (EP3R) located in the nucleus of the solitary tract (NTS) and the rostral ventrolateral medulla (RVLM) of the brainstem. This, in turn, results in respiratory depression and autoresuscitation failure.

5. Conclusions

This thesis describes age-dependent changes in the incidence and characteristics of cardiorespiratory events during the early postnatal period in extremely preterm infants. Importantly, we identified a high incidence of cardiorespiratory events beyond term-equivalent age and an increased vulnerability to postnatal insults such as infection in this population. These findings are of particular concern and emphasize the importance of careful surveillance and management outside of the hospital environment.

This thesis also identifies a novel mechanism linking systemic infectious response with respiratory control disturbances in neonates. We show that IL-1β alters basal respiration and hypoxic ventilation via central mPGES-1 activation and PGE_2 binding to brainstem EP3 receptors. Moreover, PGE_2 appears to play an important role in the respiratory response to anoxia. These findings have important implications for the clinical management of neonates. The rapid synthesis of PGE_2 in response to cytokine and transient anoxia may make it particularly useful in the diagnosis and monitoring of infants with increased apneas due to suspected infection or hypoxia. Our studies may also influence treatment strategies for neonatal apnea related to infection or hypoxia by selectively targeting mPGES-1 or EP3R. In conclusion, the cytokine-induced, PGE_2-dependent pathway described in the present thesis could potentially explain the association between infection, apnea, and Sudden Infant Death Syndrome.

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