From the Neonatal Unit,
Department of Women’s and Children’s health
Karolinska Institutet and Astrid Lindgren Children’s Hospital, Stockholm, Sweden

NEONATAL RESPIRATORY CONTROL - INSPIRATION, INFLAMMATION AND THE PROSTAGLANDIN E$_2$ PATHWAY

Veronica Siljehav

Stockholm 2014
For Sebastian – a result of paediatric research
Immature or deficient autonomic control is a common problem in infants born at a premature age, and is of central importance in apneas. The pre- and perinatal development of the brainstem neural circuits that control autonomic functions is vital for survival after birth and for the regulation of breathing movements. Children with immature brainstem respiratory control have periods of irregular breathing with potential detrimental apneas that are increased during sleep and infection. This thesis investigates the pathophysiology behind apneas and its correlation to infection, hypercapnia, and hypoxia. The focus is particularly on the mediatory role of prostaglandin E\(_2\) (PGE\(_2\)) in modulating central respiratory activity and breathing, as well as in evaluating its role as a potential biomarker of apneic infants.

To elucidate the association between infection and apnea, respiration was examined in neonatal mice using whole-body plethysmography after administration of the cytokine interleukin-1\(\beta\) (IL-1\(\beta\)) or PGE\(_2\). Neonatal mice given IL-1\(\beta\) or PGE\(_2\) exhibited a lower respiratory frequency, depressed hypoxic gasping, and a reduced ability to autoresuscitate following hypoxic apnea compared to control animals. IL-1\(\beta\) and PGE\(_2\) also reduced the respiratory response to hypercapnia.

Cardiorespiratory activity was evaluated in extremely preterm infants and term infants using impedance pneumography, electrocardiography, and pulse oximetry. Lumbar puncture was also performed and PGE\(_2\) and prostaglandin metabolite (PGEM) levels were measured. The incidence of apnea, bradycardia, and desaturations was associated with prostaglandin levels in cerebrospinal fluid (CSF). Infants with sepsis and meningitis had high levels of PGEM.

PGE\(_2\) reversibly inhibited brainstem respiratory activity \textit{ex vivo} in brainstem-spinal cord en bloc preparations and provoked apnea and irregular breathing patterns in neonatal mice. IL-1\(\beta\) rapidly induced brainstem microsomal prostaglandin E synthase-1 (mPGES-1), an enzyme crucial for PGE\(_2\) biosynthesis. Attenuation of the mPGES-1-pathway decreased respiratory depression during hypoxia and it increased survival in the neonatal mice. Moreover, mice lacking the EP3 receptor (EP3R) for PGE\(_2\) had no PGE\(_2\) induced apneas \textit{in vivo} and no PGE\(_2\)-induced inhibition of respiratory activity \textit{ex vivo} compared to wild type mice. In addition, the EP3R turned out to have a pivotal role in the hypercapnic response. In newborn infants PGE\(_2\) concentrations in CSF could predict cardiorespiratory disturbances and the highest levels of PGEM in CSF were found during meningitis and sepsis. This thesis provides evidence that PGE\(_2\) release, induced by hypoxia or inflammation, in the vicinity of the brainstem areas related to central respiratory pattern generation and control, adversely affects breathing and its control by binding to EP3 receptors in the rostral ventrolateral medulla. These findings have impact on our ability to screen, detect and protect against neonatal apnea associated with infections, hypercapnia and hypoxia.
LIST OF PUBLICATIONS

This thesis is based on the following publications. The papers will be referred to in the text by their Roman numerals.


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<td>Vₑ</td>
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- "Nog finns det mål och mening i vår färd - men det är vägen, som är mödan värd"

Karin Boye, 1900-1941
1 BACKGROUND

This thesis explores the pathophysiology of apnea and cardiorespiratory disturbances in the neonate. It examines the association between infection, hypoxia, hypercapnia and apnea, specifically the role of prostaglandin E\textsubscript{2} in respiratory regulation.

RESPIRATORY CONTROL

Breathing is a rhythmic motor behavior generated and regulated by brainstem neuronal networks. The functions of breathing are clearly definable, where its most important role is to regulate blood O\textsubscript{2}, CO\textsubscript{2} and pH in the body. The French physician Le Gallois (1770-1814) was first to establish that the respiratory rhythm center is localized in the medulla oblongata, after a series of animal experiments [1]. Le Gallois declared that life in an animal or in any of its organs depends on two obligatory conditions. One is the integrity of the medulla oblongata and its nervous output. The second is the circulation of arterial blood of the organ and the medulla oblongata. During the 20\textsuperscript{th} century the research on respiratory control continued, and it was found that breathing rhythmogenesis does not critically depend on extrinsic feedback loops or reflexes, but rather on central pattern generators (CPG) [2]. The gathered data since has pointed out two distinct, but functionally interacting rhytmogenic networks as the CPGs in the rostral ventrolateral medulla oblongata: the pre-Bötzinger complex (pre-BötC) and the retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG) (Figure 1) [3]. It is postulated that the former serves as the dominant inspiratory group and the latter functions as the main expiratory rhythm generator in adults [4]. However, there are studies showing that if one of the two is non functioning, this can at least partially be compensated by the other, stating that they can perhaps counterweigh for the others dysfunction [5]. But at birth, they seem to be dependent on each other, since null-mutants have revealed that the function of both oscillators is required for survival, concluding that the interaction of the two networks starts already at the embryonic stage [6-9].

![Figure 1. Anatomical overview of the rostral ventrolateral medulla of the brainstem.](image)

Pre-Bötzinger complex (Pre-BötC) is located next to Nucleus Ambigus (nA). Retrotrapezoid nucleus (RTN) and parafacial respiratory group (pFRG) are located in the vicinity of the facial motor nucleus (VII\textsub{In}). They interact through reciprocal connections. Modified from Mellen 2012 [10].

There are three main respiratory rhythms generated by the CPG: eupnea (\textit{e.g.}, normal resting respiration); sighing (\textit{e.g.}, large inspiratory efforts overlying and interspersed
within eupnea); and gasping (*e.g.* short inspiratory efforts of high amplitude preceding long expiratory pauses).

The control of the rhythm generation is further regulated primarily by central chemoreceptors. Apart from being CPGs the RTN/pFRG and the Pre-BötC also function as central chemoreceptors. However, many areas in the central nervous system have been pointed out as potential chemoreceptors. The medullary raphe [11], locus coeruleus [12], hypothalamus [13], dorsolateral pons [14] as well as the cortex and cerebellum in general [15] are all potential modulators of respiration. Current evidence is insufficient to assert if chemoreceptive distinct areas take care of central respiratory chemoreception or if it is a property widely distributed throughout the brain. In addition peripheral chemoreceptor and pulmonary sensory receptors [16-19], as well as temperature [20] and sensory stimuli from the airways [21] affect respiratory regulation. Finally, there are the voluntary and the emotional parts of respiratory rhythm regulation, reared by the limbic system as well as by the hypothalamus [22].

In summary, the organization of a fine-tuned respiratory rhythm regulation makes up an orchestra, and the Pre-BötC and the RTN/pFRG is the kernel. An overview is provided in Figure 2.

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**Figure 2. Functional organization of respiratory control in the brainstem.** Many systems regulate the respiratory rhythm which is generated within the central pattern generators (CPG) *i.e.* pre-Bötzing complex (Pre-BötC); retrotrapezoid nucleus (RTN) and parafacial respiratory group (pFRG). Nucleus of tractus solitarius (NTS) acts as a processor of respiratory afferents, which integrates the information from both peripheral chemoreceptors and mechanoreceptors and transmits it to the CPG.
FETAL BREATHING, TRANSITION AT BIRTH AND NEONATAL RESPIRATION

Fetal breathing

The fetus relies on the placenta for respiratory gas exchange rather than its own lungs. Although of no importance when regulating pH, CO₂ and O₂ in the fetus, fetal breathing movements have important roles in lung growth and in the development of respiratory muscles and neural regulation [23, 24]. Changes in pCO₂ and pO₂ does however regulate fetal breathing movements, and as hypercapnia stimulates ventilatory efforts hypoxia, it on the other hand inhibits them [25].

In human infants, respiratory rhythm generation is established about the 11th week of gestation [26]. A two-step developmental process that establishes fetal breathing has been described in mouse. The researchers show how the pFRG connects with the Pre-BötC and initiate fetal breathing [8].

In the early gestation fetal breathing movements are nearly continuous, but throughout the course of pregnancy they become interrupted by prolonged apnea [27]. Interestingly, as the presence of apneas increases in the fetus during the last trimester, so does PGE₂ levels in utero [28]. In the fetus prostaglandin inhibits breathing movements and maintains patency of the ductus arteriosus to facilitate fetal circulation [29]. Functional arterial chemoreceptors exist in utero, and their activity is stimulated by low pO₂ and pCO₂ [30]. Their threshold for sensing hypoxia is lower than in the neonate, probably reflecting the lower fetal pO₂. If the fetus is challenged by asphyxia, it is not excited, but rather becomes immobilised, stops breathing and becomes bradycardic [31]. This paralytic state of the fetus can be due to inhibition of chemical neurotransmission. PGE₂ as well as adenosine are postulated as suppressive neuromodulators in the fetal brain that decrease oxygen consumption and have neuroprotective effects [32]. Of note is that high doses of caffeine during gestation, altering adenosine receptor development, increase the incidence of apnea in rats postnatally [33, 34]. In addition, maternal smoking as well as the use of snuff during pregnancy may increase the presence of apnea after birth [35]. Taken together, these results implicate that events prenatally have fatal consequences on respiratory regulation in the perinatal period. This is further supported in animal models where changes in the prenatal genetic programs operating at very early stages in the embryo caused devastating abnormalities after birth [36].

Transition at birth

Extra-uterine life puts high demands on infant respiratory control which must develop from sporadic fetal into independent continuous breathing [37]. The most essential adaptation to birth is the initiation of breathing, but also the clearance of fetal lung fluid from the lungs [38]. From the dramatic moment of birth the respiratory neuronal networks need to function and continuously generate a respiratory rhythm to sustain oxygenation and metabolism. It has been shown that neuronal networks generating respiratory rhythm do not undergo major changes in the early perinatal period [39]. Interestingly, PGE₂ levels in utero-ovarian vein plasma [40] and uterine tissues [41] as well as in fetal plasma reach their peaks just
before delivery [42]. What exactly initiates the first breath of life is still a mystery. The CO\textsubscript{2} drive, which is an important driving mechanism of respiration, seems to be strongly up-regulated after birth and its sensitivity appears to be driven by a cholinergic mechanism [43]. Several genes encoding for excitatory neurotransmitters are switched on during the perinatal period, and a surge in these transmitters may play a crucial role in the respiratory transition and general arousal at birth [44-48]. At the same time, circulating levels of adenosine decrease, which perhaps also is true for PGE\textsubscript{2}, and the decrease in adenosinergic inhibition may contribute to the establishment of postnatal breathing [49].

**Neonatal respiration**

Development of the intrinsic properties and functional organization of the central respiratory network continues after birth in order to stabilize respiratory activity. There is a maturation of dendritic morphology and increase in synaptic connections and myelination after birth [50], as well as a change in the motor pattern and neurotransmitter sensitivity of respiration-related neurons with advancing postnatal age [51, 52]. The carotid body is the primary site for detection of hypoxia in the older infant [53]. When oxygen tension decrease, the carotid body activity increase and stimulate the CPG, which results in increased ventilation. The maturation of the carotid body is well studied, and in human infants the estimated range for full influence of the carotid body is from a few days pup to 10 weeks postnatal age [54, 55]. This maturation concurs with the decrease of periodic breathing present after birth [56], which could be explained by the decreased release of dopamine, an inhibitory modulator in the carotid body [57].

The ventilatory response to CO\textsubscript{2} in healthy term neonates resembles that of adults [58]. This would suggest that the central chemoreceptors are functional immediately after birth, which is further supported by evidence of c-fos mRNA expression in the central chemoreceptor area at the ventral medullary surface at the same time-point, and which is enhanced by hypercapnia [59].

Control of neonatal respiration is also dependent on reflex responses from the lungs, respiratory muscles and the airways. The Hering-Breuer reflex and laryngeal chemoreflex are more profound in the neonates compared to adults [60-62]. During the immediate postnatal period the neonate also experiences a hypotonic upper airway, increased chest wall compliance, lower functional residual capacity and decreased coordination between respiratory muscles [63]. Taken together the postnatal regulation of respiration differs from that in adult life, however, apneas are still considered a failure in the neonatal breathing control.
NEONATAL APNEA

Definition

Apnea is defined as cessation of breathing that lasts for more than 20 seconds or if accompanied by hypoxia or bradycardia [64]. Apnea is classified into subtypes depending on its origin, *e.g.*, central, obstructive, or mixed events (for review, see [65]). Central apnea is the total cessation of inspiratory effort, with no apparent airway obstruction. Obstructive apnea is the absence of airflow associated with respiratory efforts against an obstructed upper airway [66]. During mixed apnea there is an initial disruption of central respiratory drive during the central component, followed by a delayed upper airway dilator response to airway occlusion [67]. In preterm infants the most common type of apneas is the mixed type, typically accounting for 50% of long apneic episodes, followed by central apnea (40%) [68].

Pathophysiology

The main reason for central and mixed apnea of prematurity is attributed to immaturity of respiratory control. For the preterm infants, this immaturity could be explained by the simple fact that their breathing is made for the intrauterine life, where the infant is not depending on its own ventilation and experiences frequent respiratory pauses. This could also explain why apnea of prematurity most often ceases by term gestation [69]. Whilst single apneas without significant desaturations may not be of great concern, chronic intermittent hypoxia (CIH) as a result of repetitive desaturations, have shown to give consequences such as retinopathy of prematurity [70] and adverse neurological outcome [71]. It is thought that the concurrent inflammation of the lung in preterm infants contributes to the chronic intermittent hypoxia [72].

Altered peripheral chemoreceptor activity has also been addressed as underlying the pathophysiology of apneas and especially responsiveness to O₂ is different in preterm infants with apnea. Apneic infants demonstrate enhanced peripheral chemoreceptor activity as evidence of greater immediate increase in ventilation during hypoxic challenge, as well as respiratory depression in response to hyperoxia [73, 74]. Infants with apneas have a more pronounced hypoxic ventilatory depression, which is explained by immaturity in central respiratory networks as well as significant suprapontine inhibition, which are thought to be important in the fetal response to hypoxia [75-77].

In addition, disrupted response to CO₂ has been shown to generate more apneas in premature infants as they exhibit impaired ventilatory response to hypercapnia [78]. A higher threshold for sensing CO₂ changes further potentiates the response [79] as well as an impaired upper airway muscle tone when subjected to increased CO₂ [80]. In conclusion, dysfunction in both central respiratory regulation and chemoreceptor input may increase the frequency of apneas in the neonate.

The immaturity in the respiratory control makes the infants more susceptible to apneas during postnatal events such as infection, thermal instability, necrotising
enterocolitis, particularly if they occur during critical periods during the maturation of respiratory control [81]. Infections are among the most usual complications in the neonatal period of preterm infants and they are frequently inducing apneas [82]. The role of infection in regulating neonatal respiration is one of the major questions of this thesis and will be further explored.

**Incidence**

The incidence of apneas is 25% in neonates who weigh <2.500 g at birth, and it approaches 84% for those under 1.000 g at birth [83]. Since the publishing of this study the panorama of preterm infants has changed, with greater survival of patients born at a younger gestational age, making apneas of prematurity an increasing feature in the neonatal intensive care unit. Their immature cardiorespiratory function puts them at greater risks for apnea, bradycardia and desaturation events, which seem to persist beyond term gestation [84].

**Treatment**

Since the 1970s, the first-line treatment for apneas in the premature infant consists of methylxanthines, such as aminophylline, theophylline, and caffeine [85, 86]. Their efficacy in preventing apneas is well documented [87]. They stimulate respiration and reduce hypoxia-induced respiratory depression by inhibiting brainstem adenosine receptors [88]. Although, deleterious effects of caffeine have been described in animal models [89-91], the CAP (Caffeine for Prematurity Study) a RCT study in human neonates, has proven it to be harmless [86]. Some neonates though are unresponsive to these drugs, why another respiratory stimulant, Doxapram, is used for treatment of apneas [90, 92]. However, its use is limited and the mechanism not fully understood [93]. In addition to medical treatment, ventilator support or therapies targeting secondary causes of apnea are also in use.

**INFECTION, APNEA AND SIDS**

Apnea and cardiorespiratory disturbances are common presenting signs of infection in neonates [94]. Infection and inflammation may play a crucial role in the pathogenesis of neonatal apnea and Sudden Infant Death Syndrome (SIDS). SIDS is the leading cause of death in infancy following the early postnatal period. The triple risk model has gained most attention in explaining the pathogenesis behind SIDS, involving 1) a vulnerable infant, 2) a critical developmental period in homeostatic control, and 3) an outside stressor(s) [95]. Infections are postulated to be outside stressors, and mild viral or bacterial infections often precede death in SIDS victims, Figure 3 [96].

Although the instability of breathing and its sensitivity to infections are well known facts among preterm infants, and also which other risk factors that add to its presence (e.g. viral upper respiratory tract infections and maternal smoking [35, 97], the exact mechanisms have not been fully understood. Cytokines, such as interleukin-1β (IL-1β), have been proposed to act as a critical mediators of infection, apnea and SIDS.
IL-1β is produced during an acute phase immune response to infection or inflammation and has shown to alter hypoxic gasping and autoresuscitation that have been implicated in the pathogenesis of SIDS [99]. Recently, it was found that the majority of unexpected infant deaths was associated with an up-regulation of inflammatory markers [100]. In addition immaturity in CO₂ regulation is also thought to contribute to apnea of prematurity and SIDS [101, 102].

**Figure 3. The triple risk model for SIDS**

**EARLY AND LATE ONSET SEPSIS IN THE NEONATE**

Neonates, and especially preterm infants are more prone to contract infections due to their immaturity of the immune system, as well as decreased placental passage of maternal antibodies [103]. Sepsis in the neonate is divided into early (within the first 48-72 h of life) and late onset sepsis (usually from 72 h of age) and the two carry different risk factors as well as different predominant pathogens [104, 105]. Even though the incidence of documented early onset sepsis is low (0.5-1% of all deliveries [106] the incidence of late onset sepsis is as much as 25% in very low birth weight infants treated in neonatal intensive care units [107]. Due to the high mortality and morbidity, inversely related to gestational age, it is not surprising that sepsis in the neonate is a feared complication and antibiotics are frequently prescribed[108, 109]. Unfortunately the use of antibiotics is not harmless. There are both short-term and long-term consequences, where on the short-term the nephrotoxicity is most prevalent [110] but also the risk of Necrotising Enterocolitis (NEC) [111] and invasive candidiasis are associated with antibiotic therapy [112]. On the long term, obesity and disrupted gut flora [113] have been linked to neonatal antibiotic exposure [114]. Currently, blood culture is the golden standard for diagnosing sepsis in the neonate, but usually takes days before the result arrives. In addition CRP and WBC are useful [115]. However, both CRP and WBC are proven to be quite unreliable biomarkers in the neonatal population [116, 117]. The need for other ways to predict sepsis has been underlined [118]. Cardiovascular monitoring has arisen as an alternative to biomarkers [119].
PROSTAGLANDIN E₂ AND ITS METABOLITES

PGE₂

Prostaglandin E₂ (PGE₂) is a critical component of the immune response to infection. IL-1β, a proinflammatory cytokine, induces the microsomal prostaglandin E synthase-1 (mPGES-1) activity in the blood-brain barrier leading to PGE₂ release in the brainstem [120]. High levels of PGE₂ are detected in the fetus as well as in all term and preterm infants, in comparison with older infants [28, 121]. Its actions in the fetus as well as in the neonate are diverse. In the fetus prostaglandins exert inhibition of the tal movements, maintain patency of the ductus arteriosus and decrease metabolic rate and energy turnover and thus may protect the brain when oxygen and energy resources are scarce [32]. In the neonate PGE₂ has actions on kidney development [122] as well as on synaptic plasticity [123]. PGE₂ has also turned out to be a potent regulator of respiration in the neonate. In vivo animal studies demonstrate that PGE₂ depresses fetal and neonatal respiration by decreasing respiratory frequency, tidal volume, and central CO₂ sensitivity [124-126]. Frequency and duration of apneas is also affected by PGE₂ [124]. In vitro, PGE₂ inhibits Pre-BötC neurons involved in both eupnea and gasping [127]. A recent study showed how the central actions of PGE₂ are mainly governed by synthesis of PGE₂ in brain endothelial cells [128]. Other novel findings have shown how astrocytes in the ventrolateral medulla are involved in respiratory control through pH-dependent release of ATP [129]. Their action is regulated by PGE₂ through induction of glutamate release in the subventricular zone [130].

Apnea is a common side effect of PGE₂ treatment in the neonatal intensive care unit, and in human neonates, PGE₂ has been correlated to both higher apnea frequency and duration of apneas, especially in those infants weighing under 2000g [131-133].

PGE metabo-lite{s}

PGE₂ is primarily catabolized enzymatically by stepwise oxidation and reduction into a metabolite 13,14-dihydro-15-keto PGE₂, which is further metabolized non-enzymatically into 13,14-dihydro-15-keto PGA₂. 13,14-dihydro-15-keto accumulates in biological fluids and its stability and detectability makes it an interesting biomarker [134]. However, the most stable of all metabolites is the final urine metabolite 9,15-dioxo-11α-hydroxy-13, 14-dihydro-2,3,4,5-tetranor-prostan-1,20-dioic acid (tetranor-PGEM) which is considered the best reflector of PGE₂ production [135]. Recently, tetranor-PGEM has proven to be a reliable biomarker of inflammation, in ulcerative colitis [136] as well as in infants with fever due to viral infections [137].

EP₃R

PGE₂ exerts its actions via E prostanoid-receptors (EPRs). They belong to the group of rhodopsin-type G-protein-coupled membrane receptors (GPCRs). There are to date

EP3R is found in respiratory related regions of the brainstem (e.g., the nucleus of tractus solitarius (NTS) and rostral ventrolateral medulla) [139]. In sheep brainstem, the peak expression of EP3R is found just after birth [140], which implicates the importance of EP3R in brain development and perhaps also on transition of respiration at birth.

Activation of human EP3R causes a decrease of [cAMP], and a modest increase in [Ca^{2+}] [141]. Reduction of cAMP decreases the firing amplitude and rate in respiration-related brainstem neurons and duly breathing activity [127].

To date, little is known on the EP3R and its involvement in respiratory regulation in the neonate. This thesis aims to explore the possible mediating role of EP3R in PGE$_2$ induced cardiorespiratory disturbances.
2 HYPOTHESIS AND AIMS

The general aim of this thesis was to examine the prostaglandin pathway and its implications on the pathophysiology of neonatal apnea. This can be divided into the following goals:

• To investigate a potential mechanism by which inflammation can exert respiratory depression, i.e., via a prostaglandin E\textsubscript{2}-mediated pathway (Papers I, II and III).

• To investigate the association between the prostaglandin pathway, its metabolites, infection and autonomic dysfunction in human neonates (Papers I and IV).

• To investigate how alterations in the prostaglandin pathway would change the outcome during respiratory challenge (Papers I, II and III).
3 SUBJECTS AND METHODS

The methods used in the present investigations are described below. Detailed descriptions of the methods can be found in the original papers. Subjects and methods are summarized in Table 1 below.

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Table 1. Summary of number of subjects and methods used in Papers I-IV.

ANIMAL MODELS

Transgenic mouse models play an important role in the investigation of respiratory control mechanisms [142, 143]. In Papers I and II, male and female inbred DBA/1lacJ mice at postnatal age of 9 (P9) days were used. The microsomal prostaglandin E synthase 1 (mPGES-1) gene was selectively deleted in knockout mice as described before [144]. Heterozygote mice were used as well. There is a large variability in the development and sensitivity to hypoxia and hypercapnia between mouse strains [145]. The strain DBA/1lacJ was chosen since it has proven to be highly sensitive to hypoxia [146] and has also been used to study inflammatory processes [144, 147]. Thus, the strain seemed particularly appropriate for examining the effects of immune system mediators on the hypoxic ventilator response. In Papers I and III, male and female inbred C57BL/6J mice at postnatal age of 9 and 2 days were also used (P9 and P2), and the EP3 receptor (EP3R) gene was selectively deleted in knockout mice as described previously [148]. These mice enable us to
further elucidate the particular mechanism by which PGE$_2$ may alter respiration-related neurons within the brainstem, i.e., via the EP3R.

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 (N117/04; N126/03; N305/03; N354/03; N87/08; N533/11).

HUMAN SUBJECTS

In Papers I and IV, we investigated the correlation between central PGE$_2$ and PGE$_2$-metabolite, infectious marker C-reactive protein concentrations and cardiorespiratory disturbances in human neonates. Infants were eligible for inclusion if they underwent a lumbar puncture for routine clinical indications such as suspected infection, CRP elevation, cardiopulmonary changes 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, invasive material i.e. tube or central lines 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).

CARDIORESPIRATORY MONITORING

Impedance pneumography detects chest wall movements were used as an indicator of respiratory volume changes, and a three-lead ECG detects heart rate variability. Mean heart rate and respiratory frequency during the 15 and 30 sec immediately prior to an apnea or bradycardia, respectively, were recorded using the KIDS monitor. The 60 second periods both before and after the event were stored in the monitor’s memory. When possible, the patient was also connected to the Cardas monitoring device (Maternal and Infant Telemonitoring Centre, Oxford, England). In Paper I and IV cerebrospinal fluid (CSF) was collected in infants with a clinical indication for lumbar puncture (i.e. suspected 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.

DRUGS

IL-1β or saline were administered by an intraperitoneal (i.p.) injection in newborn rodents in order to induce an immune response resembling that which occurs during
an infectious or inflammatory process [149]. PGE₂ was administered by an intracerebroventricular (i.c.v.) injection in order to examine its effects on respiration in vivo in wild type mice lacking parts of or all of mPGES-1 and EP3R. Recombinant mouse IL-1β and PGE₂ 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. Concentrations of IL-1β and PGE₂ were chosen based on concentrations used in similar rodent studies [146, 147, 150-152].

WHOLE BODY PLETHYSMOGRAPHY

Evaluation of respiration in vivo can be carried out using several techniques. Non-invasive methods are preferable in the newborn animal since they reflect both the autonomic and behavioral components of the ventilator response in hypoxia [153]. The only non-invasive method available for ventilatory measurements to date is whole-body plethysmography, which is implemented in our studies. 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 [154]. Tidal volume can then be calculated from the temperature, humidity and pressure values [155]. Flow plethysmography is based upon the principle that fluctuations in airflow superimposed upon the baseline flow through the plethysmograph chamber are the results 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 VT measurements [156]. 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 VT should be examined qualitatively, not quantitatively [155]. Similarly, as VE depends on VT it may also be important to emphasize relative changes in VE rather than to focus on absolute measurements.

Papers I and II Protocol: Each mouse received an intraperitoneal injection (0.01 ml/g) of IL-1β (10 µg/kg) or vehicle. Baseline skin temperature was recorded immediately before injection. At 70 min, skin temperature was remeasured, and the mouse was placed unrestrained into the plethysmograph chamber. Respiration was assessed during 4 min of normoxia (21% O₂) followed by a 1 min hyperoxic challenge (100% O₂). After a 5 min recovery period in normoxia, the chamber was flushed with 100% N₂ for 5 min, and the anoxic response was examined. Finally, 100% O₂ was administered for 8 min, and the ability to autoresuscitate was evaluated. Skin temperature was recorded at the end of each experiment.

Paper II Protocol: Each mouse was exposed to the same protocol as described above with the exception that anoxic exposure (100% N₂) continued until 1 min after the animal’s last gasp.

Paper III Protocol: Each mouse received an i.p. injection of IL-1β or vehicle. Baseline skin temperature was recorded immediately prior to injection. After 70
min skin temperature was measured again, and the mouse placed within the dual-chamber plethysmograph. Respiration was assessed during 10 min of normoxia (21% O₂) followed by 5 min of hypoxia and hypercapnia, 7 min of normoxia and then 5 min of hypercapnia. A recovery period of 5 min in normoxia was followed by a hyperoxic challenge during 1 min and after a final recovery period of 4 min normoxia the animals were subjected to anoxic exposure during 5 minutes. A subgroup was subjected to different anoxic periods ranging between 6-20 min. Finally, 100% O₂ was administered for 8 min and the ability to autoresuscitate was evaluated. Skin temperature was recorded at the end of each experiment.

**Papers I-III Protocol:** The respiratory response to central PGE₂ was also investigated in neonatal mice using flow plethysmography. Immediately after anesthesia administration and i.c.v. injection of PGE₂ 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 as described in *Papers I-II* above. Plethysmography recordings are summarized in Figure 4.

![Figure 4. Plethysmography recordings from Papers I, II and III.](image)

**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 [157, 158]. Thus, in all plethysmography experiments, chamber temperature was maintained at approximately 30°C in accordance with the documented thermo neutral range for mice of similar age [159]. 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 and PGE₂ alter basal respiration. The mice were exposed to a brief hyperoxic challenge in order to blunt peripheral chemoreceptor activity and unmask central respiratory drive [160]. This enabled us to better assess whether the ventilatory effects of IL-1β and PGE₂ 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. IL-1β has been shown to increase COX-2 mRNA expression at 1 h after i.p. injection [120, 161]. Thus, respiratory recordings were performed between 60 – 95 min after i.p. administration of IL-1β in order to allow sufficient time for respiratory effects to occur while attempting to minimize confounding systemic effects.

IL-1β and PGE₂ 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 [162, 163], and an increased metabolism has been associated with shorter gasping duration [164]. 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. These temperature measurements corresponded to previously reported values for mice of similar age [165]. Previous studies in rodents indicate that IL-1β does not induce significant temperature increase until at least 90 min after i.p. injection [150, 152, 161, 166] and that PGE₂ does not induce maximum fever until 20 – 25 min after i.c.v. administration [167]. Consequently, respiratory recordings were performed within these time frames. Moreover, fever induced by IL-1β does not affect the duration of hypoxic gasping nor does it hinder autoresuscitation following repeated hypoxic exposure in newborn rats [168].

**BRAINSTEM-SPINAL CORD EN BLOC PREPARATION**

Whereas *in vivo* measurements also reflect the influence of peripheral factors, experiments on isolated central structures offer insight into central regulations. Experiments were therefore performed on the brainstem-spinal cord preparation of 2 day old C57BL/6J mice with EP3R⁺/⁺ and EP3R⁻/⁻ genotypes. The brainstem and spinal cord of newborn rodents were dissected and isolated as described previously [169, 170].

*Papers I and III Protocols*: Inspiratory discharges of respiratory motor neurons were monitored by extracellular recording using glass suction electrodes applied to the proximal cut end of C4 and C3 ventral roots of spinal nerves (Figure 5). Burst activity of respiratory related motor neurons was analyzed and calculated as the number of C4 bursts per minute. After the preparation they are superfused with control artificial CSF (aCSF) for 40 min and when C4 activity reached a steady state, the control perfusate was replaced by testing solutions. Every preparation was exposed only to one testing protocol.

In *Paper I*, the preparations were initially perfused with control aCSF. This was followed by perfusion with aCSF containing PGE₂ for 20 min. There was a final washout period with control a CSF.
In Paper III, 3 protocols were used: In Protocol 1, the intermittent anoxia consisting of three 3-minute intervals of anoxia separated by 5 minutes of normoxia was applied to the preparation. The last anoxic episode was followed by a 30-minute interval of washing out with control aCSF.

In protocol 2, the anoxic solution was applied for 15 minutes followed by control aCSF.

In protocol 3, control a CSF was replaced for 20 min by hypercapnic solution followed by control aCSF.

The control values of the inspiratory burst frequency were calculated during application of normoxic aCSF as the mean of the last 5 minutes before testing anoxic aCSF application.

ENZYMATIC ASSAY

In Paper I, microsomal prostaglandin E synthase-1 (mPGES-1) activity was assessed in the cortex and brainstem of neonatal wild type mice as well as mGPES-1 knockout mice using a quantitative enzymatic assay first described by Thorén and Jakobsson in 2000 [172]. This assay has been shown to recover 85 ± 11% of PGE_2 [172]. 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 highest 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 1 mM-reduced glutathione. This was followed by sonication. The 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.

**ENZYME IMMUNOASSAY**

In *Paper I* and *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 [173]. EIA allows enzyme detection using small sample volumes, which is crucial given the small CSF volumes 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$_2$, was 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.

**DATA ANALYSIS**

*In vivo plethysmography experiment:* Since the animals were placed unrestrained in the plethysmograph 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 artifact). Respiratory frequency ($F_R$, breaths/min) was calculated manually. Tidal volume ($V_T$) 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 autoresuscitation 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. In *Papers I, II* and *III*, we attempted to perform all recordings at age P9 since there is a variable response to anoxia based upon age [174]; 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, however they were also compared with their littermates.
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, PGE_{2}, hypercapnia and anoxia were assessed.

Infant cardiorespiratory data analyses: The monitor software was used to calculate baseline respiratory rate, heart rate, pulse rate, and SpO₂ 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 = numbers of (n) 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 seconds and expressed as bradycardia index (BI = n bradycardias/hour recording), duration, and HR nadir. Oxygen desaturation was defined as a SpO₂ value ≤ 90% and characterized by hypoxemia index (HI = n hypoxemias/hour recording), duration, and nadir SpO₂ values. Periodic breathing was defined as an episode of three or more successive apnea pauses of > 3 breathe durations separated by < 20 seconds of normal respiration. The occurrence of periodic breathing in the 60 seconds following an apnea event was examined. Mean RR, HR, and SpO₂ immediately prior to an event were recorded. All movement artifacts as well as recordings < 2 h duration were excluded from analysis.

STATISTICS

Statistical analysis of paired comparisons was performed by Student’s t-test. ANOVA compared those parameters with normal distribution and equal variance. Two-way ANOVA was performed when there were more than one independent variable or multiple observations, and multiple comparisons were accounted using the Tukey-Kramer (T-K) method. Unequal variance was tested using Levene’s test and if positive parametric data was tested using Welch’s test and nonparametric data using Wilcoxon χ² test. A generalized linear model was used to determine which variables are associated with PGE_{2} levels, including univariant analysis to compare cytokine concentrations to each independent variable and multivariant analysis to determine which variables independently contribute to the prediction of PGE_{2} concentrations. P values refer to two-way ANOVA and data are presented as mean ± SEM unless stated otherwise. A value of P < 0.05 was considered statistically significant.
4 RESULTS AND DISCUSSION

IL-1B ATTENUATES RESPIRATION VIA CENTRAL ACTIONS

In Papers I-III, the ventilatory effects of IL-1β were investigated in two different strains of wild type (WT) neonatal mice. We hypothesize that IL-1β affects the central respiratory network rather than peripheral chemosensitivity. Mice were subjected to hyperoxic challenge to unmask central respiratory drive, by inducing a physiological denervation of peripheral chemoreceptors, “Dejours test” [160]. During normoxia IL-1β did not alter basal frequency in either DBA/1lacJ or C57BL/6J. All mice exhibited an appropriate peripheral chemoreceptor response with a lowered respiratory frequency, and IL-1β induced a more pronounced respiratory depression during hyperoxia in DBA/1lacJ mice. This finding implicates a compensatory activation of peripheral chemoreceptors during normoxia in IL-1β-treated mice in order to balance the IL-1β-induced depression of central respiration-related neurons.

In Paper III we reveal that IL-1β depresses respiration in C57BL/6J mice during hypercapnia. This is a new and interesting finding showing how inflammation also affects respiratory response to CO₂.

Papers I-III demonstrate how IL-1β depresses hypoxic ventilatory response by decreasing gasping duration and amount; this is evident in both DBA/1lacJ as well as in C57BL/6J. This inability to sustain respiratory effort during anoxia is correlated to worsened survival in mice [175]. IL-1β also reduced the ability to autoresuscitate after a hypoxic event in DBA/1lacJ mice resulting in death.

However, due to its lipophilic properties, peripheral IL-1β can not exert its respiratory modulating actions within the brainstem without a mediator. We provide evidence of its actions in the brainstem via the induced prostaglandin pathway.

PGE₂ MEDIATES THE RESPIRATORY EFFECTS OF IL-1B

In Papers I-III we investigate how IL-1β induces PGE₂ release in the brainstem as a mediator of its respiratory effects. IL-1β is used as our proinflammatory cytokine in all of the experiments when inducing peripheral inflammation. IL-1β effected respiration in WT mice during hypoxia, hyperoxia and hypercapnia. However, it was unable to exert its effects in mice lacking microsomal prostaglandin E synthase 1 (mPGES-1) as well as the eicosanoid receptor 3 (EP3R) during hypoxia. IL-1β effected survival rate in DBA/1lacJ mice Papers I-II, this was not seen in C57BL/6J mice Paper III. C57BL/6J turned out to be more sturdy against hypoxic challenge, they were for instance more able to gasp all the way through the 5 min period of severe hypoxia, as well as when challenged for longer periods when they could continue for up to 20 minutes in an hypoxic environment and this would still not affect their survival rate. It would however effect time to autoresuscitation,
where longer anoxic period would require longer time to resuscitation. This made conclusions on survival difficult. Previous studies have shown that the hypoxic ventilatory depression on postnatal day 1-3 (P1-P3) in mice of the strain C57BL/6J is nonexistent, and signs of this response is not apparent until P7 [176]. DBA/1lacJ on the other hand were very sensitive to hypoxic challenges, and pretreatment with IL-1β drastically changed their ability to survive. The more pronounced response to hypoxia in DBA/1lacJ is not an unknown feature and has been experienced before [146].

On the contrary during a hypercapnic challenge, IL-1β did not alter respiratory response in DBA/1lacJ, whereas it did in C57BL/6J wild type mice. These results suggest that DBA/1lacJ are more sensitive to hypoxia, whilst C57BL/6J are more sensitive to hypercapnic changes. This is important to keep in mind when comparing results between these two strains.

**PGE₂ INHIBITS RESPIRATORY ACTIVITY VIA EP3R**

It’s a well established fact that PGE₂ treatment in infants induces apneas [132]. In Papers IV and I we find a correlation between PGE₂ levels in cerebrospinal fluid and apneas. In Paper I we investigated the co-localization of EP3R with neurokinin-1 receptors in respiratory related regions, and co-localization was found in the RVLM ventral to nucleus ambiguous and including the Pre-BötC. Previous studies have also found EP3R expressed within the NTS and RVLM [139].

Papers I and III challenge the hypothesis of EP3R being the main receptor for the depressive effects of PGE₂ on respiration. PGE₂ is used for both in vivo and in vitro experiments and its effects on EP3R in both systems during hypercapnia are coherent. In the in vivo system PGE₂ affects the hypercapnic response by attenuating both FR and expiratory time via EP3R. In the in vitro system the central pattern generator for respiration did not respond to hypercapnia when EP3R was lacking. In Paper I we show that PGE₂ injected in the vicinity of the brainstem induces apnea an irregular breathing, but not in EP3R⁻/⁻ mice. This would suggest that PGE₂ induces a hypoventilation during hypercapnia via EP3R in respiratory related regions in the brainstem. In Paper III we demonstrate that EP3R deficient mice are more responsive to CO₂ changes. Infants with apneas among term children have a depressed and delayed response to CO₂ [78]. Preterm infants have a blunted response to CO₂ which is associated with apneas of prematurity [101]. Our results suggest that the attenuated response to CO₂ in apneic term and preterm infants could be due to PGE₂ activity in the brainstem.

During hypoxia, a EP3R’s action as a mediator in respiratory control is also evident. Mice without EP3R were able to sustain longer and more active respiration throughout the hypoxic challenge. The fact that EP3R insufficient mice were coping better during an anoxic event compared to control mice would also suggest that hypoxia per se induces PGE₂ which effects the ability to handle the challenge. However, also IL-1β worsened the response to hypoxia in wild type mice, stating the more direct effects of PGE₂.
In conclusion, our studies provide evidence of EP3Rs existence in respiratory related regions and its direct involvement in respiratory regulation as the receptor mediating the respiratory depressive effects of PGE₂, both in vivo and in vitro.

**IL-1β AND HYPOXIA ACTIVATE MPGES-1**

*Paper I* shows how brief anoxic exposure increases mPGES-1 activity in the mouse cortex but proportionally more in mice brainstem. Also IL-1β induced the mPGES-1 activity in a time-dependent manner, and the effects of anoxia and IL-1β were additive in the in vitro system. In *Papers I and II*, mice with different expressions of mPGES-1 are investigated (i.e. wild type, heterozygotic and knock-out mice) in an in vivo system. IL-1β adversely effects respiration both under hyperoxic conditions but mostly under anoxic conditions, also in the in vivo system showing an additive effect of IL-1β and hypoxia. Of note is that we in *Paper II* show that a partial but not complete depletion of mPGES-1 during anoxia and inflammation turned out to be most beneficial in terms of overcoming the challenge. Anoxia has previously been proposed to induce mPGES-1 and PGE₂ release [177]. The exact mechanism on how anoxia induces mPGES-1 is to our knowledge not unraveled. Induced gene expression and mPGES-1 activation would be to slow processes to explain the release within the brief 5 min of our experimental hypoxia. Potential mechanisms include post-transcriptional regulation or stabilization of mPGES-1 mRNA, an etiology previously shown in neonatal mouse cardiocytes [178]. Of interest is that PGE₂ released at birth is thought to have acute neuroprotective effects during perinatal hypoxia [179]. But another study has found that PGE₂ might exacerbate hypoxia neuronal injury [180]. This poses important questions on PGE₂ effects on brain during hypoxia, but they are far from the scope of the present thesis and still to explore. However, our studies do implicate that an attenuation of the mPGES-1 is beneficial, and this would open doors to new treatment regimes. Specific prostaglandin inhibitors have been developed [181], and the blocking of endogenous prostaglandin production increases breathing movements and central respiration during early postnatal life [125, 182].

Taken together, our results from the in vivo and in vitro systems show how mPGES-1, induced by either hypoxia or inflammation depresses central respiratory mechanisms via its release of PGE₂ in the vicinity of respiratory related regions and binding to EP3R in this area (Figure 6).
Figure 6. This model shows how inflammation and hypoxia induce the microsomal prostaglandin E synthase-1 (mPGES-1)-mediated pathway to respiratory depression and autoresuscitation failure, described in the present thesis. During a systemic immune response, the proinflammatory cytokine interleukin-1β (IL-1β) is released into the peripheral bloodstream. It binds to its receptor (IL-1R) located on endothelial cells of the blood-brain barrier (BBB). Activation of IL-1R induces the synthesis of PGH₂ from arachidonic acid (AA) via COX-2 and the synthesis of prostaglandin E₂ (PGE₂) via the rate-limiting enzyme mPGES-1. Moreover, hypoxia itself activates mPGES-1 and the activation is additive to that of IL-1R. PGE₂ is released into the brain parenchyma and binds to its EP3R located in the respiratory control regions of the brainstem, e.g. nucleus of tractus solitarius (NTS) and the rostral ventrolateral medulla (RVLM). This results in depression of central respiration-related neurons and breathing, which may fatally decrease the ability to gasp and autoresuscitate during hypoxic events. Figure modified from Siljehav et al., Ped Res 2012 [183].
PGE₂ AND PGEM ARE ASSOCIATED WITH INFECTION AND POSSIBLY MEDIATE CARDIO-RESPIRATORY DISTURBANCES

In *Paper IV* we find that infants investigated due to cardiorespiratory disturbances have higher levels of PGEM, also infants with infections such as sepsis and meningitis exhibit the highest levels of PGEM. This suits well with the fact that one of the predominant first presenting sign of infection is cardiorespiratory disturbances [184]. In a mouse sepsis model, LPS induced cytokines present at the start of infection and inflammation depressed heart rate variability (HRV) [185]. Previous studies have shown that IL-1β depresses respiration in neonatal rats, and the effects can be ameliorated using indomethacin [146]. The results from *Paper IV* where infants were investigated due to susceptibility to infection, indicate that PGE₂, as one of the presenting cytokines of infection, causes cardiorespiratory disturbances. Apnea index in infants with high level of PGE₂ is also high, which we show in *Papers I and IV*. These findings suggest that PGE₂ and its metabolite are interesting novel biomarkers for both infection and cardio-respiratory disturbances. Since neonatal infections carry a high mortality and morbidity it is of utmost interest to diagnose and treat these neonatal infections early [96]. Fear of sepsis and meningitis makes neonatologists frequently prescribe antibiotic treatment for the neonates [186]. Antibiotic treatment in neonates is not without adverse effects, the number needed to harm has been found to be high [187]. The overuse of antibiotics also leads to the development of multi-resistant bacteria in the neonatal intensive care units (NICUs) [188]. Establishment of an appropriate microbiota is essential for gut and brain development and antibiotics may disrupt an evolving ecosystem, leading to both direct and long-term consequences for health [113]. This is why novel strategies for diagnosing sepsis and meningitis must emerge, in order to more precisely predict infectious status at an early stage. In this thesis we demonstrate that PGE₂ and PGEM are good candidates as future biomarkers for infection and cardiorespiratory disturbances.

INCREASED LEVELS OF PGEM IN PRETERM INFANTS

In *Paper IV* we demonstrate that the levels of PGEM in neonatal cerebrospinal fluid varies depending on the gestational age of the infant. In the neonate PGE₂ has multiple effects, especially on the kidney, and particularly on hemodynamics and water/electrolyte balance, but also on the development [122]. Preterm infants have higher levels of urinary PGE₂ [189] and these levels also increase proportionally more during the first days of life compared to in term infants [190]. The renal biosynthesis as a mechanism of compensatory response is thought to be the reason, this in order to prevent decrements in renal plasma flow, since prostanoids play an important role in protecting the immature kidney form high levels of angiotensin II. In addition, premature infants are often exposed to infections and inflammatory agents prior to birth and thereafter. 78% of infants born at weeks 26-28 and 31% born at weeks 23-25 are exposed to chorioamnionitis [191]. This inflammatory process has effects on lung development that can be both detrimental and beneficial [192, 193]. PGE₂ is thought to play a role in the development of inflammation in the fetal lung.
[194], hence elevated levels in the preterm infants could be seen due to the fact that they are more often subjected to inflammation. However, since we in Papers I and IV show how PGE$_2$ levels in CSF correlate with apneas and cardiorespiratory events in the neonate, and these are more present in preterm infants [84], the increased levels of PGEM could also reflect the immaturity in respiratory regulation leading to apneas of prematurity.
“Ingen dröm är för stor!”
Vinnie, 2014
Breathing is a continuous and vital behavior from birth. This thesis explores the vulnerability of the neonatal respiratory regulation to hypoxia, hypercapnia and inflammation by the induced prostaglandin E$_2$ pathway. We explore the prostaglandin pathway from its synthesis by the mPGES-1, to its actions via the EP3R. We find that PGE$_2$ is involved in the physiological as well as pathophysiological regulation of respiration via EP3R present in respiratory centers in the brainstem. PGE$_2$ adversely affects breathing and its control, resulting in increased apnea frequency, decreased hypercapnic response and hypoxia-induced mortality.

In human infants we find that PGE$_2$ as well as PGEM are correlated to degree of both infection and respiratory disturbances. Moreover, infants investigated due to suspected infection more often than non-infected infants presented cardiorespiratory disturbances prior to diagnosis. These findings will have implications on possible ways to predict infants at risk of both invasive infections as well as detrimental apneas. However, repetitive lumbar puncture is not a feasible method to investigate infants at risk. Instead, Tetranor-PGEM, a stable urinary metabolite of PGE$_2$ has evolved as a potential biomarker for inflammation ensuing cardiorespiratory dysregulation and disturbances.

This thesis will improve the current knowledge of normal and dysfunctional respiratory control in the neonate, and especially show how it is changed by inflammation, hypoxia and hypercapnia. The conceptual change introduced by our data, that endogenous prostaglandins are central pathogenic factors in respiratory disorders and the hypoxic response, open new diagnostic and therapeutic avenues that should significantly improve the diagnostics and treatment of newborn patients.
6 FUTURE PERSPECTIVES

The group of extremely preterm infants, “new survivors”, continues to grow. The reported morbidity in this group is high, which challenges current treatment and surveillance. These infants are susceptible to retinopathy of prematurity, neurodevelopmental disorders, as well as infections, the latter that holds true for neonates in general. Both infection and neurodevelopmental disorders will affect, and be affected, by ventilation. To predict and prevent future impairment in respiratory control is the foremost aim for current and future studies within the field of developmental respiratory regulation. The directions to take are many, through deeper knowledge of pathophysiology and development of biomarkers, as well as interpreting changes in cardiorespiratory patterns.

- “The measure of greatness in a scientific idea is the extent to which it stimulates thought and opens up new lines of research.”
  
  Paul A.M. Dirac, 1902-1984

Several projects are still ongoing:

- mPGES-1 expression in the brainstem and its change in expression by inflammation and hypoxia. The brainstems of lambs, that have been subjected to preterm delivery, are currently being investigated using immunohistochemistry.

- Tetrnor-PGEM levels in the neonate, and the possible differences in term and preterm birth. Urine is collected from the NICU and will be investigated for Tetrnor-PGEM levels.

- Development of Tetrnor-PGEM as a biomarker for autonomic dysfunction and infection. A prospective pilot study has started at the NICU, enrolling preterm infants who will provide consecutive urine samples. These are investigated for Tetrnor-PGEM levels, inflammatory and also cardiorespiratory parameters.

Våra studier baseras på försök i möss och människa. I avhandling ingår fyra delarbeten:

**Delarbete I** kartlade att PGE$_2$ är involverat i andningsregleringen, och hur PGE$_2$ kan försämra förmågan att klara av syrebrist och infektion hos nyfödda möss. Vi undersökte även om en av prostaglandinreceptorerna, EP3 receptor (EP3R), fanns i de centra i hjärnstammen som reglerar andningen. **Delarbete I** visade också att även hos spädbarn tycks PGE$_2$ påverka antalet andningsuppehåll, där höga nivåer i ryggmärgsvätska leder till ökad mängd andningsuppehåll. Denna mekanism för hur infektion, genom frisättning av PGE$_2$ nära hjärnstammens andningscentrum, aktiverar EP3R, vilket i sin tur leder till andningsstörning och andningsuppehåll samt försämrad förmåga att överleva akut syrebrist, öppnar nya möjligheter för diagnostik och behandling.

Syftet med **Delarbete II** var att se om minskad förmåga hos möss att producera PGE$_2$ påverkade deras andningsreglering, något som skulle motsvara en farmakologisk behandling av prostaglandins signaleringsväg. Vi fann att under tillstånd som syrebrist och infektion, klarade sig möss med minskad förmåga att producera PGE$_2$ sig bättre än de som hade full produktion eller ingen produktion alls.


**Delarbete IV** försöker pröva om kunskapen som vi erhållit från försöken på möss gäller även för spädbarn. För tidigt födda och fullgångna barn från den neonatala intensiv-vårdavdelningen och från BB på Karolinska sjukhuset undersöktes. Andningsstörningar registrerades och jämfördes med nivåer av PGE$_2$, och dess metabolit PGEM, i ryggmärgsvätska. Vi fann att prostaglandin-nivåer korrelerade med både andningsstörningar och invasiva infektioner såsom hjärnhinne-inflammation och blodförgiftning och dessutom med störningar i reglering av andning och hjärtfrekvens.

**7 POPULÄRVETENSKAPLIG SAMMANFATTNING**
Sammanfattningsvis har vi i denna avhandling kartlagt att den av inflammation, syrebrist och koldioxid framkallade PGE$_2$ signaleringsvägen i allra högsta grad påverkar andningsregleringen hos spädbarn. PGE$_2$ bidrar till att försämra andningen vid infektion och syrebrist. Denna vetskap är av vikt både för att bättre kunna förutspå och identifiera spädbarn med risk för andningsuppehåll, och för att kunna förbättra behandlingen och övervakningen av dessa barn.
8 ACKNOWLEDGMENTS

Eric Herlenius, my main supervisor, for his inevitable enthusiasm for research and his kindness, introducing me and supporting me in the field of respiratory physiology. He has also encouraged me to see the world outside the laboratory and taught me one of life’s greatest lessons – Enivrez-vous!

Per-Johan Jaboksson, my co-supervisor, for his ability to make the impossible happening, in providing stringent supervision and for always looking at bright side of life.

Mireille Vanpée, my co-supervisor, for being an excellent role model in combining good clinical skills and research and inspiring me to continue within the field of neonatology.

Annika Hofstetter, with whom I took my first steps into the lab, performing plethysmography patiently by my side, and continuing to help out with providing excellent English corrections.

Lars Björk, who hasn’t just taught me everything I know about immunohistochemistry, but also on wines and food.

Jennifer Frithiof, Eva Lundberg, Ann-Christine Eklöf and Astrid Häggblad for their patience and kindness helping me with logistics and administration.

Hugo Lagercrantz, a true source of inspiration in his never-ending struggle for children’s health.

Kristina Broliden, my mentor, who showed up just in time, and whose wisdom and experience has guided me through to the end.

Britta Wahren, I’m grateful for your contribution to the thesis, taking your time and giving me impeccable corrections.

During the finalization of this thesis I also give my warmest thanks to Eric Thelin for reading through and commenting on the thesis and Stefan Persson for all the help with the figures.

I would like to thank all of my lab-mates and research colleagues and especially Johan Hamrin, the ultimate colleague both in the clinic and in the field of research, Zachi Horn, the nicest person alive and an inspiration on moving to “the other side”, I hope you come back soon, Eric Thelin, for infusing enthusiasm and encouragement, David Forsberg, who keeps on growing on me, the sky is the limit!, Anna Gunnerbeck, thanks for all wisdom and humour, Ruth Detlofsson, for your patience and warmth, Linus Olsson, the IT catcher in the rye, Jenny Turesson, the...
sunshine, Anna-Karin Edstedt-Bonamy, for your wise comments and for the role model you are, Yuri Shvarev, for thorough comments and help to improvement, Jonas Berner, for all the help with the plethysmography, where would I have been without you? and that goes for Gary Cohen as well.

Miriam Katz-Salamon, Anna Nilsson, Ronny Wickström, Thomas Ringstedt, Eli Gunnarson, Ulrika Åden, Malin Rohdin, Panos Papachristou, Georgios Alexandrou, Béatrice Sköld, Pierre Kuhn, Jakob Frie Carlsson, Hanna Ingelman-Sundberg, Sofia Ygberg, Emiliija Wilson, Lena Swartling Schlingiz, Lena Legnevall, Ajaya Ravella, Mónica Pérez-Manso, Evangelina Tserga, Suzanne Witteveen Pronk, Cici Dyberg, Marika Strindberg, Johan Jäderstad, Linda Jäderstad, and last but not least Marco Bartocci – thank you all for creating an inspiring lab environment!

I would like to aim special thanks to my Australian mates, Graeme Polglase, Stuart Hooper, David Tingay, Mary Tolcos, Nadia Hale and Amy Shields, who gave me an amazing time at Monash Institute and Royal Children’s in the most stimulating research climate!

My former colleague in Uppsala, Fredrik Hedborg, with whom I shared my tears of happiness seeing my first manuscript in press. I wish you could have joined me until the finish line.

Oili and Lars-Olof Tiderman, thank you for your constant support and encouragement. You have been an enormous safety since coming to Stockholm 19 years old on a cold winters day, becoming my “Stockholm family”, the kind that everyone should have.

Mom and Dad for always being there. Sebastian, my real source of inspiration and my adorable big baby brother. Marin, Måns, Jessica, Patrik, Christian, Sonja, and Solvieg. My beloved grandfather, Bror, who hopefully gets to see it all from above although I would have preferred it if you were actually here. Maria and Johan, for making my time in Stockholm artsy and jazzy.

The Family Petersson, without you this would not have been possible. Göran, Marie, Helge, Ragnar and last but not least Anna Malou, my dear friend throughout the years, thanks!

My wonderful friends, who have brought light to my time as a PhD-student and stood by, Manuela, Christian, Angelica, Agnieszka, Emilia, Anna, Anna and Ellinor.

Enskede Ridskola, who has taught me everything I know about my own respiratory regulation, or at least the voluntary part.

Stefan – the love of my life and my best friend. I can’t thank you enough!
Finally, thanks to all my colleagues at the Astrid Lindgren Children’s Hospital, the wonderful people that works hard for the best of the children and brings about a friendly working environment. I would also like to thank my paediatric residency program at Astrid Lindgrens Children’s Hospital as well as Stockholm County Council for their support in completing my doctoral education.

This thesis was generously supported by: Stockholm County Council (Forskar ST), Karolinska Institutet (KID), Axel Tielmans Memory, Queen Silvia’s jubilee fund, Freemasons Children’s House and Swedish National Heart and Lung foundations.
9 REFERENCES

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