Karolinska Institutet

Thesis for doctoral degree (Ph.D.) 2008

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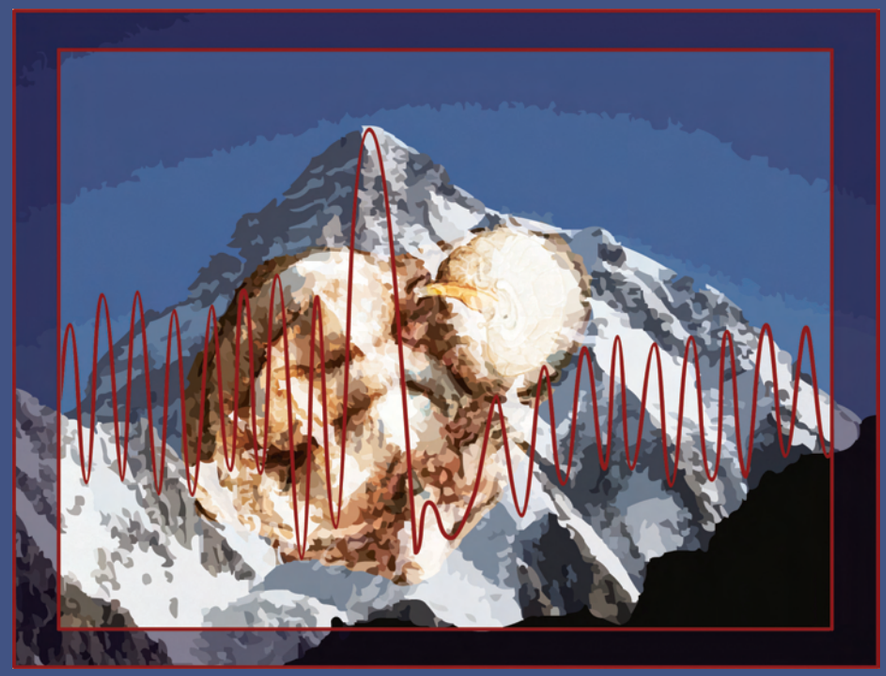

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"you've got to understand
that a seagull is an unlimited idea of freedom, an image of the
Great Gull, and your whole body, from wingtip to wingtip, is
nothing more than your thought itself."

from "Jonathan Livingstone Seagull"
by Richard Bach

To my wife, Maria and

my children Nicole, Livia and Gabriel

ABSTRACT

We breathe in order to maintain oxygen, carbon dioxide and pH levels within the physiological range in response to the metabolic demands of the body. To achieve this, the respiratory control system is dependent on input from peripheral and/ central chemosensitive areas and on participation of different neuromodulator systems. This thesis focuses on the role of one of the neurotransmitters, substance P, involved in the complex and finely tuned control of respiration. It also explores how prenatal nicotine exposure affects the substance P-ergic system and the effects of morphine when this system is non-functional.

To investigate if endogenously released substance P is necessary for the hypoxic ventilatory response (HVR) in the intact newborn rat pup (postnatal day five, P5) we used a neurokinin 1-receptor antagonist (RP67580) injected intracerebroventricularly. We could demonstrate that RP67580-treated animals displayed an altered HVR but normal respiration during normal conditions, indicating that endogenously released substance P is necessary for an adequate response to hypoxic stress. Furthermore, *in situ* hybridisation demonstrated that c-fos mRNA expression, used as a marker for neuronal activation, was decreased in respiration related areas in the brainstem in RP67580-treated animals, indicating structures involved in the perturbed HVR.

We also used a transgenic mouse model (Tac1 $^{-/-}$), lacking substance P and neurokinin A (NKA), to investigate the respiratory response to intermittent hypoxia and hypercapnic stress at P2-3 and at P8-10 to identify developmental changes. *In vivo* experiments, using flow-plethysmography, displayed an attenuated increase in tidal volume during intermittent hypoxia in transgenic mice, P8-10, whereas the younger animals did not differ from controls except from an altered breathing pattern with fewer apneas and more augmented inspiratory breaths with a pause during intermittent hypoxia. Brain-stem spinal cord preparations of P2-mice revealed that intermittent hypoxia did not induce an increase in burst frequency, reflecting long-term facilitation, in Tac1 $^{-/-}$ mice as displayed in controls. This was also manifested *in vivo* as an impaired augmentation of ventilation during post-hypoxic periods. Furthermore, transgenic mice displayed a more prominent posthypoxic frequency decline *in vivo* and posthypoxic neuronal arrests appeared more often *in vitro*. In line with previous studies the hypercapnic response did not differ between strains, confirming that substance P is not involved. Thus, our results show that a functional substance P/NKA system is essential to generate an adequate respiratory response and that it is also involved in the plasticity of respiratory network during early development.

Human sudden infant death victims have elevated levels of substance P-like immunoreactivity (-LI) in the brainstem and nicotine increases the risk for sudden infant death syndrome (SIDS) by up to four-fold. We could demonstrate elevated substance P-LI levels in the brainstem and alterations of the substance P-precursor, preprotachykinin A mRNA expression in carotid body and petrosal/ jugular ganglia following prenatal nicotine exposure in newborn rat (P1). This may offer a biochemical link between nicotine exposure and SIDS.

We also show an increase in morphine analgesia and reduced main (respiratory depression) and other side-effects in Tac1 $^{-/-}$ mice. Since morphine is a widely used analgetic drug, also in neonates, with a narrow therapeutic window, our result offers the possibility to decrease the activity of substance P/NK- receptor signalling and thereby improve the pharmacological potential of morphine.

In conclusion, this thesis demonstrates the involvement of substance P in the HVR and plasticity of the respiratory network. Prenatal nicotine exposure severely affects the substance P-ergic system, a possible underlying mechanism for SIDS. Furthermore, it offers a correlation between the functionality of the substance P-ergic system and the breathing disturbances seen in Rett syndrome.

LIST OF PUBLICATIONS

I. Wickström HR, **Berner J**, Holgert H, Hökfelt T, Lagercrantz H, "Hypoxic response in newborn rat is attenuated by neurokinin-1 receptor blockade", Respir Physiol Neurobiol. 2004 Apr 20;140(1):19-31

II. **Berner J**, Shvarev Y, Lagercrantz H, Bilkei-Gorzo A, Hökfelt T, Wickström R. " Altered respiratory pattern and hypoxic response in transgenic newborn mice lacking the tachykinin-1 gene", J Appl Physiol. 2007 Aug;103(2):552-9. Epub 2007 May 24

III. **Berner J**, Ringstedt T, Brodin E, Hökfelt T, Lagercrantz H, Wickström R " Prenatal exposure to nicotine affects substance P and preprotachykinin-A mRNA levels in newborn rat", In Press, Pediatr Res

IV. **Berner J***, Zimmermann J*, Wickström R, Racz I, Zimmer A, Bilkei-Gorzo A "Increased morphine analgesia and reduced side-effect in mice lacking the tac1-gene", *authors have contributed equally. Submitted manuscript.

CONTENTS

LIST OF ABBREVIATIONS

ACh	Acetylcholine
BötC	Bötzinger complex
CNS	Central nervous system
CSF	Cerebrospinal fluid
DA	Dopamine
DRG	Dorsal respiratory group
DRGs	Dorsal root ganglia
f	Respiratory frequency
HVR	Hypoxic ventilatory response
ICV	Intracerebroventricular
KF	Kölliker-Fuse nucleus
LC	Locus coeruleus
LTF	Long-term facilitation
MORs	Mu-opioid receptors
mRNA	Messenger ribonucleic acid
NE	Norepinephrine
NK-1,2,3	Neurokinin receptors 1,2,3
NKA	Neurokinin A
NKB	Neurokinin B
NTS	Nucleus tractus solitarii
PBC	Pre-Bötzinger complex
pFRG	Parafascial respiratory group
PPT-A	Preprotachykinin A
PRG	Pontine respiratory group
RTN	Retrotrapezoid nucleus
RT-PCR	Reversed transcriptase-polymerase chain reaction
RVLM	Rostroventrolateral medulla
SIDS	Sudden infant death syndrome
V_E	Ventilation
VLM	Ventrolateral medulla
VRG	Ventral respiratory group
V_T	Tidal volume

1 INTRODUCTION

1.1 RESPIRATION HISTORICAL BACKGROUND

The understanding of respiratory physiology was of interest already for the ancient Greeks such as Hippocrates (about 460-377 B.C.) that considered air to be "the instrument of the body". Breathing was for a long time believed to serve to cool the innate burning flames and combustion of the heart (the Galenic doctrine, 2nd century A.D.). This view lasted until Robert Boyle in 1660 discovered that air was vitally necessary for life. During the French Revolution (1789-1799) it was further understood that there must be a kernel of respiratory control somewhere in the spinal cord because when the guillotine divided the head and kept a larger portion of the spinal cord, the body retained breathing movements. These observations were later corroborated by animal experiments performed by Legallois in the beginning of the 19th century (Legallois 1812), concluding that respiratory movements did not originate from the spinal cord but from the brainstem. Today, we still do not have full insight in central respiratory control. The functionality of this control is of course of great importance and is the main theme of this thesis with special reference to the role of substance P (SP) and effects of morphine and nicotine.

1.2 RESPIRATORY CONTROL - AN OVERVIEW

The respiratory system must be stable from the very first breath and yet respond to different stimuli such as emotions, exercise, disease and vocalization. To achieve this balance all neuronal afferent input is integrated in the respiratory network situated in the brainstem.

1.2.1 Central respiratory control

In the brainstem respiration related afferent input is incorporated to produce a respiratory effort responding to the organism specific demands on a moment-to-moment basis by integrating chemical and mechanical information. The central respiratory control in the brainstem involves at least three regions, the pontine respiratory group (PRG), the ventral respiratory group (VRG) and the dorsal respiratory group (DRG). The parabrachial and the Köllicker-Fuse nuclei constitute the pontine

group, involved in the modulation of respiratory rhythm (von Euler 1983), control of wake and sleep state of airway muscles and airway protection including the diving reflex (Alheid, Milsom et al. 2004). The border of the PRG is not clear cut but is considered caudally to be just rostral to the parafacial nucleus.

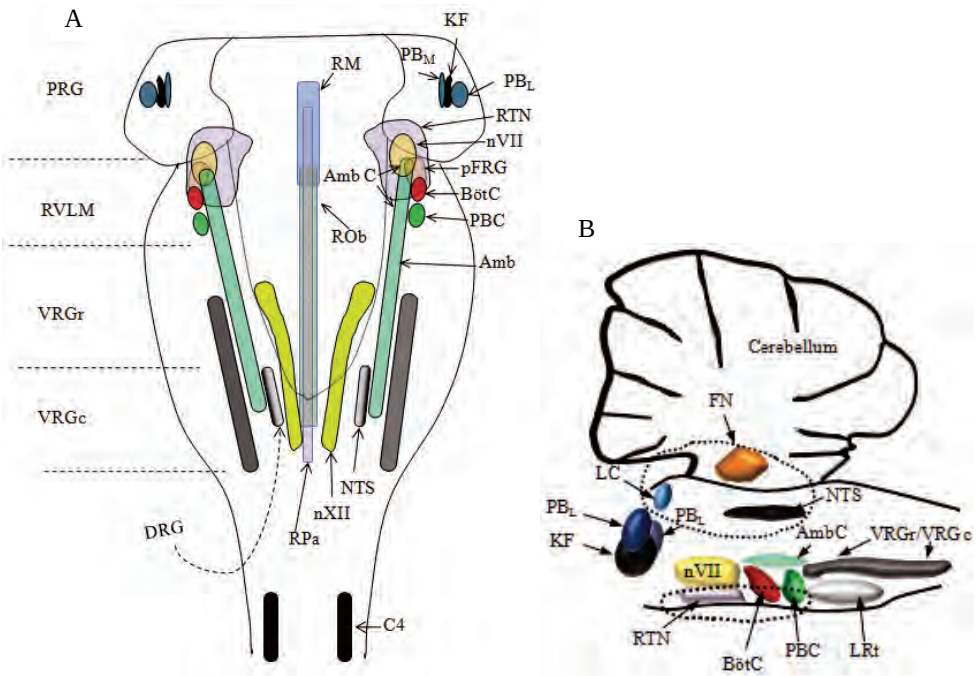

Figure 1. A. *Schematic overview of respiration related nuclei in the brainstem, ventral view longitudinal axis.***B.** *Sagittal view. Dotted lines represent central chemosensitive regions. Abbrevations: Amb, nucleus ambiguus; AmbC, nucleus ambiguus pars compacta; BötC, Bötzinger complex; DRG, dorsal respiratory group; FN, fastigial nucleus; KF, Kölliker-Fuse nucleus; LC, locus coeruleus;LRt, lateral reticular nucleus; nVII, facial nucleus; nXII, hypoglossal nucleus; NTS, nucleus tractus solitarii; PBC, preBötzinger Complex; PB$_L$, lateral nucleus parabrachialis; PB$_M$, medial nucleus parabrachialis; pFRG, parafascial respiratory group; RM, raphé magnus; ROb, raphé obscurus; RPa, raphé pallidus; RTN, retro trapezoid nucleus RVLM, rostroventrolateral medulla; VRGc, caudal ventral respiratory group; VRGr, rostral ventrolateral respiratory group, C4, cervical root four.*

The VRG is situated in the ventrolateral region of the medulla oblongata and is functionally divided in a caudal and rostral part (VRGc and VRGr, respectively). The VRGc comprises the nucleus retroambiguus with premotoneurons of mainly expiratory function (Shen and Duffin 2002). The VRGr includes the Bötzinger complex (BötC), the preBötzinger Complex (PBC) and the parafacial respiratory group (pFRG, hereafter also including the retrotrapeziod nucleus, RTN). Furthermore, the rostral part of the VRGr is known as the rostral ventrolateral medulla (RVLM) with its caudal limit at the PBC-level. This region has been of special interest in the last decade and the current knowledge is based on *in vivo* and *in vitro* experiments mainly on neonatal rodents and is considered to contain respiratory rhythm generating and chemosensitive properties (Feldman, Mitchell et al. 2003). The BötC, situated near the rostral end of the nucleus ambiguous, has mainly expiratory neurons and maintain the expiratory phase by inhibiting inspiratory neurons (Bryant, Yoshida et al. 1993). The pFRG and PBC are regarded as two regions with intrinsic rhythm generating or pacemaker-like properties that work either together or independently (Onimaru and Homma 2003; Janczewski and Feldman 2006; Onimaru, Kumagawa et al. 2006). Finally, the DRG which lies in the dorsal medulla in the region of the nucleus tractus solitarii (NTS) receives information from peripheral chemo- and mechanoreflexes (Hering-Breuer reflex) and contain efferent connections to premotoneurons in the medulla and to spinal motoneurons. NTS is also involved in the cardiovascular reflexes and has an important integrative function of sensory afferent input (Lawrence and Jarrott 1996). A schematic overview of the above described respiratory regions is summarized in figure 1A and B.

1.2.2 Respiratory rhythm generation

The respiratory research field has long searched for the kernel of rhythm generation and in the last decades several studies have pointed to two regions with intrinsic rhythm generating or oscillating properties. The timing and activation of respiratory related neurons are determined by the respiratory network, i.e. the central pattern generator, integrating autonomic and behavioural input (von Euler 1983). In the last decade, progress has been made in our understanding of the rhythmicity of respiration. *In vitro* studies first located an area in the brainstem, rostrally in the ventrolateral medulla, necessary for respiration-related motor nerve output with pacemaker-like activity (Smith, Ellenberger et al. 1991), designated the preBötzinger

Complex (PBC). Subsequent anatomical studies defined the PBC as neurons in this area expressing neurokinin 1 (NK1) receptors (Gray, Rekling et al. 1999). Using a treatment strategy based on saporine-labeled substance P, it was possible to selectively lesion the NK1-expressing neurons in the PBC and thereby profoundly affect the normal respiratory pattern (Gray, Janczewski et al. 2001; Wang, Germanson et al. 2002; Wenninger, Pan et al. 2004). This indicates a vital role for these substance P-sensitive neurons for generation of a normal respiratory pattern. Interestingly, the PBC is not only a rhythm generator but might also have intrinsic properties that gives rise to different breathing patterns such as eupnea (normal respiration), sighing and gasping (Lieske, Thoby-Brisson et al. 2000; Shvarev, Lagercrantz et al. 2003). The second region is a cluster of pre-inspiratory neurons within the pFRG (Onimaru and Homma 2003). These neurons rhythmically discharge before *and* after inspiration, thereby making the term pre-inspiratory somewhat misleading. One hypothesis is that the pFRG and PBC interact and form a coupled oscillatory system regulating respiratory rhythm in mammals (Janczewski and Feldman 2006). Pre-inspiratory neurons are opiate-insensitive which distinguishes them from inspiratory neurons within the PBC that, consequently are opiate-sensitive, implicating that PBC neurons also have opioid receptors (Takeda, Eriksson et al. 2001). This discovery deepened the understanding of rhythm generation and *in vitro* experiments revealed the so called quantal, or step-like, slowing of respiratory frequency. This is also seen *in vivo*, but is more distinct in vagotomised animals than in intact animals with their retained sensory input (Janczewski, Onimaru et al. 2002; Mellen, Janczewski et al. 2003; Janczewski and Feldman 2006). This issue is at present unresolved, and there is an ongoing discussion on how closely coupled these two oscillatory networks are and if the pFRG is pre-inspiratory or solely expiratory.

1.2.3 Chemosensitivity

In order to keep the pCO_2, pO_2 and pH in a physiological range the respiratory system needs instant information about the metabolic status of the organism. The theory of a central and peripheral chemosensitivity was postulated by Winterstein in 1956 (Winterstein 1956) stating that O_2 was only sensed peripherally whereas CO_2/H^+ -ions were also sensed in the brain. In 1960s Loeschcke et al reported the localisation of chemosensitive neurons on the ventral surface of the medulla (see review (Loeschcke 1982)) and further studies have confirmed this (Feldman, Mitchell et al. 2003; Guyenet,

Stornetta et al. 2008). Since the reaction $CO_2 + H_2O \leftrightarrow HCO_3 + H^+$ is rapid and exists in all five compartments (the red blood cell, plasma, endothelium (blood-brain barrier), extracellular fluid and intracellular glia cells or neurons), it is somewhat unclear whether it is in fact CO_2 and/or the hydrogen ion that is sensed. Central chemosensitive neurons regulate ventilation following changes in CO_2/pH in a physiological range, since all cells will react in a severely acidotic milieu. The functional chemosensitive areas are believed to be located at the surface of RVLM, including pFRG/ retrotrapezoid nucleus (RTN) and PBC, NTS, locus coeruleus (LC), fastigial nucleus and the midline medullary raphe (Fig 1B). Furthermore, the RTN and NTS sensitivity to CO_2 changes with sleep/ wake state (Feldman, Mitchell et al. 2003).

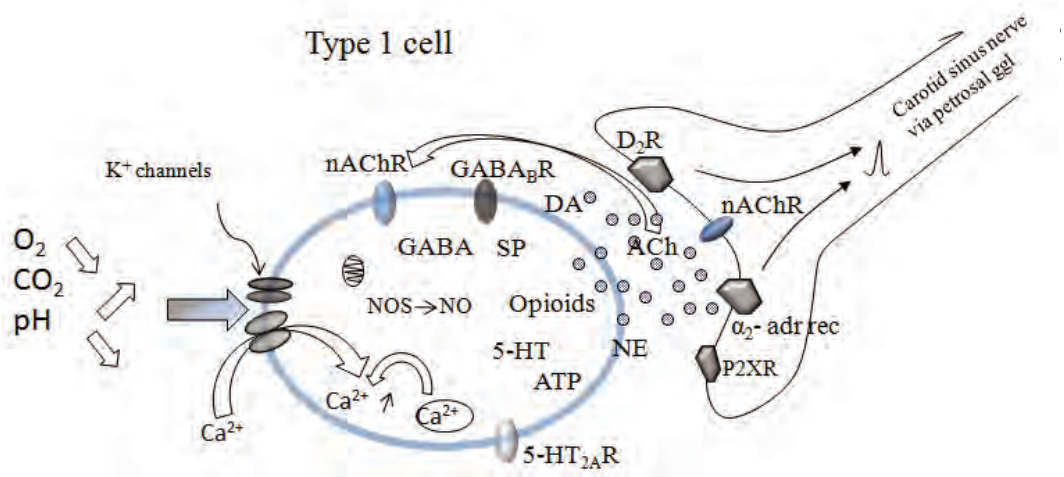

Figure 2. *Schematic overview of chemosensitivity and proposed transduction pathways in carotid body (CB) type I cell, in synapse with a petrosal ganglion cell projecting through carotid sinus nerve to nucleus tractus solitarii (NTS). Chemical stimulation provoke the release of conventional neurotransmitters i.e. dopamine (DA), serotonin (5-HT), acetylcholine (ACh), norepinephrine (NE), substance P (SP), adenosine-5´-triphosphate (ATP), gamma-aminobutyric acid (GABA) and opioids (enkephalins) whereas nitric oxide (NO) is considered an unconventional transmitter. If oxygen (O_2) decreases several possible transduction pathways are postulated; activation of ionchannels (voltage-gated Ca^{2+}-channels leading to an influx of Ca^{2+} and/or inhibition of K^+-channels); inhibition of NO-synthase (NOS); inhibition of mitochondrial respiratory chain, all of which are resulting in transmitter release. Excitatory transmitters include ACh, SP and ATP, whereas DA and opioids are considered inhibitory. GABA and 5-HT acts through feedback loops on presynaptic receptors which inhibit respectively enhance CB activity. Other used abbreviations; carbon dioxid (CO_2) and purinergic receptors (P2XR).*

The central chemosensitivity is thus a rapidly responding system, whereas the sensitivity to oxygen is activated later, e.g. in case of prolonged apnea.

Peripheral chemosensitivity is mainly mediated by a cluster of cells, the carotid bodies, located at the bifurcation of the internal and external carotid arteries. These chemoreceptors consists of type I cells, or glomus cells, of neural crest origin and of type II cells or sustentacular cells. This small organ possesses an impressive array of responses on stimulation, such as cardiovascular, endocrine, renal and respiratory responses (Fitzgerald 1997). The theme of this thesis is on the respiratory response to O_2, CO_2 and pH, although it also senses glucose levels, potassium levels, osmolarity and temperature (Kumar and Bin-Jaliah 2007). The ability to detect chemicals in the blood-stream was originally postulated by De Castro in 1928 (De Castro 1928). Later on Heymans was awarded the Nobel Prize in 1938 for "the discovery of the role played by the sinus and aortic mechanisms in the regulation of respiration". However, the mechanisms involved in O_2-sensing in the type I cell is not fully understood, it seems as if an essential step, at least for CO_2/H^+-ion is the inhibition of K^+ -channels leading to influx of Ca^{2+}-ions and a membrane depolarisation and a subsequent transmitter release (Nurse 2005). There are several hypotheses about how O_2 actually is sensed by the glomus cells. Heam-containing enzymes in the cell (mitochondrial and non-mitochondrial enzymes), sensitive ion-channels or a combination of both these so-called metabolic and membrane hypotheses involved in the O_2 transduction process, have been suggested (Prabhakar 2006). The strategic localisation of carotid bodies, just before the arterial blood reaches the brain, make them ideal for mediating a rapid response to changes in chemical composition. The glomus cells are synaptically connected to carotid sinus nerve endings (a branch of the glossopharyngeal nerve), whose cell bodies reside in the petrosal ganglion. In case of hypoxia, hypercapnia or change in pH, chemoafferent activity is conveyed into the central nervous system (CNS), primarily to the NTS and then further directed to the central pattern generator leading to adjustment of ventilation (see Fig 2). Several transmitters are released in response to chemoactivation of glomus cells. Some of these have primarily excitatory effects, such as acetylcholine (ACh) acting via nicotinic acetylcholinergic (nACh) receptors, adenosine triphosphate via P2X-receptors, noradrenaline (α_2-adrenoreceptors) whereas substance P (presumably through NK-1 receptors (Cragg, Runold et al. 1994)), serotonin (5-HT), opioids and dopamine (DA) are considered to have a more modulatory effect on hypoxic/ hypercapnic response (Prabhakar 2000;

Iturriaga and Alcayaga 2004; Nurse 2005). The knowledge about which putative neurotransmitters are released and/ or co-released and acting on which pre- and postsynaptic receptors in the carotid body is increasing but partly because of species and age specific differences (Fitzgerald, Shirahata et al. 2007; Milsom and Burleson 2007) the chemotransduction is not yet fully understood. A schematic overview is presented in figure 2.

1.3 RESPIRATORY CONTROL BEFORE AND AT BIRTH

Fetal development may be viewed from an evolutionary perspective where the relatively hypoxic (pO_2 of 5 kPa) fetal environment would correspond to the Ordovician period (ca 480-440 million years ago) when all organisms still lived in water. The fetus has an impaired chemosensitivity and hypercapnia, at higher levels than in adults, causes an increase in regularity and depth of breathing movements whereas, in contrast, hypoxia inhibits breathing efforts (Boddy, Dawes et al. 1974). Birth is therefore an "evolutionary" step from amniotic fluid out into the air where the neonate has to acclimatize to a normoxic environment. Thus, the stress of being born is a crucial challenge requiring immediate transition from passive gas-transport via the placenta to active breathing, reflected by the huge surge of catecholamine (Lagercrantz and Slotkin 1986). The hypercapnic response is intact in the newborn, although the CO_2-sensitivity weakens in rat pups at postnatal day seven to ten (P7-10) (Wickstrom, Hokfelt et al. 2002) and reaches adult levels at two weeks of age, displaying a three-phasic pattern (Putnam, Conrad et al. 2005).The relative hyperoxia (air) initiates a resetting of the O_2 sensitive arterial chemoreceptors resulting in a decreased sensitivity during the first days of life (Blanco, Hanson et al. 1988; Holgert, Hertzberg et al. 1993; Hanson and Kumar 1994; Holgert, Hokfelt et al. 1995; Carroll, Sterni et al. 1996; Cohen and Katz-Salamon 2005). The main effect of hypoxia is instead a reduced metabolic rate and a lowered body temperature, a safety mechanism promoting hypoxic survival not seen in adults (Mortola 2004). Prolonged hypoxia subsequently results in apneas, gasping and finally a terminal apnea (Martin and Abu-Shaweesh 2005). During fetal life, episodic breathing movements occurs predominantly during rapid eye-movement-sleep (Dawes 1984). Later, in gestation, fetal breathing becomes irregular and is interrupted by apneas, probably due to supra-pontine inhibition. Neurotransmitters and neuromodulators such as neuropeptide Y, endogenous opioids

and adenosine, are involved in suppressing fetal activity like breathing movements, locomotion and arousal (Herlenius and Lagercrantz 2001). Several of those neurotransmitters are abundant during the perinatal period (glutamate, catecholamine and substance P) and for instance γ-aminobutyric acid (GABA) transcends from being excitatory prenatally into inhibitory postnatally. Immediately after birth, respiration must provide the organism with oxygen, eliminate carbon dioxide and keep the pH in a physiological range. Furthermore, the liquid-filled lungs must be cleared by the initial first breaths effectuating a negative intrathoracic pressure of – 50 cm H_2O (P. Karlberg 1962; Mortola 1987). The newborn is initially aroused and breaths continuously probably due to the cooling effect, sensory input (e. g. cutaneous stimulation), loss of inhibition and activation of catecholaminergic systems. Respiratory system activity is balanced by the participation of different neurotransmitters and neuromodulator systems that regulate the central rhythm generation and finally, respiratory output.

1.4 HYPOXIA AND RESPIRATION

The ability to adequately control ventilation with an appropriate response to hypoxia is vital for survival. Acute hypoxia leads to an increase in respiration within seconds depending on peripheral chemoreceptors in the carotid body (see above). Prolonged hypoxia will ultimately result in brain damage within minutes in humans.

1.4.1 Hypoxic ventilatory response

The biphasic hypoxic ventilatory response (HVR) is characterized by an initial increase in ventilation followed by a later suppression of ventilation termed hypoxic ventilatory depression (HVD) (Lawson and Long 1983; Powell, Milsom et al. 1998). The augmentation phase is inversely related to how low the fractional inspired oxygen level is and the HVD is mainly due to a decline in tidal volume (Bureau, Zinman et al. 1984; Eden and Hanson 1987). This first phase is probably due to an activation of excitatory neurotransmitters (ACh and substance P) via the carotid body-NTS pathway, whereas the HVD might depend on several processes such as release of inhibitory neurotransmitters (DA and GABA) and/or a hypometabolic reaction in response to hypoxia (Mortola 1999; Liu, Lowry et al. 2006; Prabhakar 2006). The hypoxic hypometabolic response is similar to the fetal reaction and is probably more dominant in the newborn than later during development. The respiratory system gradually

matures and undergoes developmental changes during the neonatal period displayed as differences in HVD (Liu, Lowry et al. 2006). In the neonate the HVD reaches levels near or below baseline (Mortola and Rezzonico 1988; Cohen, Malcolm et al. 1997), whereas in adult mammals ventilation slowly decreases remaining at levels above baseline (Maxova and Vizek 2001). At least in rat, the HVR undergoes dynamic developmental changes during first two postnatal weeks, leaving a vulnerable window of imbalance between inhibitory and excitatory systems (Wickstrom, Holgert et al. 1999; Viemari, Burnet et al. 2003; Wong-Riley and Liu 2005; Liu, Lowry et al. 2006).

1.4.2 Plasticity of respiratory control

Many neural systems undergo structural and functional development due to genetic and environmental factors. Furthermore, the same systems are capable of changing their performance and response as a result of experience as for example in learning, memory, vision and pain (Wiesel 1982; Kato and Yoshimura 1993; Liu and Sandkuhler 1997; Afrah, Fiska et al. 2002; Bruel-Jungerman, Davis et al. 2007). The respiratory system is no exception and there has been a growing interest in this phenomenon in recent years. Indeed, the same stimulus presented continuously or repeatedly will result in different responses also depending on when during development it occurs. Thus, there is a period when developing neural networks undergo synaptogenesis, modulation of neurotransmitters and their receptors and apoptotic mechanisms, which are highly susceptible to stimulus or stress such as hypoxia (Huttenlocher 1984; Rabinowicz, de Courten-Myers et al. 1996; Herlenius and Lagercrantz 2001; Carroll 2003). Not only *when* during development the stimuli occurs but also *how* matters, in this case the biphasic HVR to sustained hypoxia differs from the long-term facilitation (LTF) in response to intermittent hypoxia (repeated hypoxic episodes separated with normoxia) (Millhorn, Eldridge et al. 1980). The interest in LTF and intermittent hypoxia has increased the last years since intermittent hypoxia resembles several physiological relevant conditions such as recurrent apneas in the neonate and obstructive sleep apneas in the adult. LTF was initially observed in anesthetized, vagotomised and paralyzed cats on a mechanical ventilator where increased phrenic nerve activity was recorded long after repeated carotid body stimulation had ceased (Millhorn, Eldridge et al. 1980; Ling, Olson et al. 1997). Since LTF is attenuated by serotonin receptor antagonists, LTF is considered to be serotonin-dependent (Millhorn, Eldridge et al. 1980; Bach and Mitchell 1996). This is also

interesting as multiple serotonergic brainstem abnormalities have been found in SIDS-victims (Paterson, Trachtenberg et al. 2006). There is evidence that the neuronal plasticity and LTF occurs in the carotid bodies as well as in the central respiratory rhythm generating network (Blitz and Ramirez 2002; Peng, Rennison et al. 2004; Prabhakar, Dick et al. 2007). The underlying mechanisms are still poorly understood and in addition to serotonin the involvement of reactive oxygen species-mediated signalling and activation of hypoxia-inducible factor-1 have been proposed in recent studies (Peng, Yuan et al. 2006; Prabhakar, Peng et al. 2007).

1.5 SUBSTANCE P

Substance P is considered to be one of the neuromodulators involved in respiratory control. It is an undecapetide belonging to the tachykinin family and was the first neuropeptide discovered by von Euler and Gaddum (von Euler 1931). Other members of the tachykinin family are neurokinin A (NKA), neurokinin B (NKB), neuropeptide K and neuropeptide γ. All tachykinins are encoded by the Tac1 gene in mouse, except for neurokinin B that is encoded by the Tac 2 gene. From the precursor peptide preprotachykinin-A (PPT-A) substance P is converted by convertases and stored in vesicles which are transported to nerve terminals and released when the neurons are activated (Moss and Laferriere 2002). Once released, the neuropeptide is metabolized by endopeptidases and/ or angiotensin-converting enzyme and cannot, like classical neurotransmitters be re-used by the neurons or synthesized by nerve-terminals. Thus, it has to be synthesized *de novo* followed by axonal transportation (Hokfelt, Broberger et al. 2000). This also implicates that the sites of action could also be on adjacently located synapses, i.e. paracrine signalling. Substance P acts preferentially via neurokinin 1 (NK-1) receptors, whereas NKA and NKB interact with NK-2 and NK-3 receptors, respectively, although substance P has affinity for all three receptors (Regoli, Boudon et al. 1994; Severini, Improta et al. 2002; Rigby, O'Donnell et al. 2005). The NK-receptors are seven-transmembrane G protein coupled receptors and activate phospholipas C with an elevation of intracellular calcium as a result (Guard, McKnight et al. 1991). Elegant studies have demonstrated how the neurokinin receptor is internalized within minutes after activation and returned to the cell-membrane after half an hour, thereby being an indicator of endogenously released substance P (Bowden, Garland et al. 1994; Mantyh, DeMaster et al. 1995). Substance P is one of the most

abundant and widespread neurokinin peptide in the central and peripheral nervous system and is involved in several physiological processes including cardiovascular, respiratory, gastrointestinal, nociceptive, and also modulates the immune response and mood (Pernow 1983; Lagercrantz, Srinivasan et al. 1991; Otsuka and Yoshioka 1993; Hokfelt, Pernow et al. 2001). Except for its main neuronal localization substance P has also been found in peripheral blood and thereby resembles paracrine or endocrine action probably secreted from gastrointestinal mucosa (Pernow 1983). Regarding substance P´s neuronal distribution it is present in sensory afferents from arterial baroreceptors and chemoreceptors and is also found in areas in the brainstem thought to be involved in respiratory regulation (Ljungdahl, Hokfelt et al. 1978; Gillis, Helke et al. 1980; Kalia, Fuxe et al. 1984; Holtman 1988; Mazzone, Hinrichsen et al. 1997). Other localizations where substance P is found are the enteric neurons in the gut (Pearse and Polak 1975), the lung and respiratory tract (vagal and thoracic spinal origin) (Saria, Martling et al. 1985; Manzini, Conti et al. 1989), the spinal cord (Hokfelt, Kellerth et al. 1975), the skin (Hökfelt 1977) and in the urinary tract (Maggi, Geppetti et al. 1988). This widespread distribution confirms the variety of physiological actions of substance P. Furthermore, there is a growing body of data for the involvement of substance P in the respiratory control and the hypoxic response (Yamamoto and Lagercrantz 1985; Chen, Hedner et al. 1996; Kumar and Prabhakar 2003; Morgado-Valle and Feldman 2004). The respiratory system undergoes crucial changes during the perinatal period and substance P may play an important role during this maturation, since high concentrations are present perinatally in the medulla reaching maximum levels postnatal day 5-15 (Yamamoto and Lagercrantz 1985; Quirion and Dam 1986; Pagliardini, Ren et al. 2003; Viemari, Burnet et al. 2003). A corresponding localisation and timeframe is seen also for the NK1-receptor (Moss and Laferriere 2002). An interesting aspect on tachykinins and especially substance P, is the neuroimmuno communication, i.e. the link between the central nervous and the immune system. NK-1 receptors and substance P are found in immune cells such as monocytes, lymphocytes, macrophages and mastcells and substance P is considered to modulate the peripheral immune response (Bost 2004; Siemion, Kluczyk et al. 2005). Interestingly, substance P can stimulate glial cells to produce cytokines thereby amplify the inflammatory cascade in the CNS (Marriott 2004).

Taken together, substance P and its receptor NK1 appear to play an important role in respiratory control when the organism is challenged by hypoxia and undergoes developmental changes with special vulnerability during the postnatal period.

1.6 NICOTINIC EFFECTS ON THE SUBSTANCE P-ERGIC SYSTEM

Nicotine acts as a stimulant in humans and is the main factor in tobacco smoke responsible for developing dependency. Approximately 20% of fertile women in Sweden smoke three months before pregnancy and about 10% used nicotine in some way during the first trimester (Socialstyrelsen 2006), despite convincing evidence for multiple adverse effects. These effects include growth retardation, increased risk for fetal mortality, impaired cognitive development, four-fold increase in risk for sudden infant death syndrome (SIDS) and later in life a decreased lung function and asthma (Wickstrom 2007; Slotkin 2008). In relation to respiratory control, animal studies have revealed that acute nicotine exposure activates carotid body chemoreceptor response involved in HVR (Jonsson, Kim et al. 2002; Jonsson, Wyon et al. 2004), whereas prenatal exposure to nicotine impair the ability in newborn rats to autoresuscitate during hypoxia (Fewell and Smith 1998) and display an abnormal ventilatory response to hypoxia (Bamford and Carroll 1999). Recent studies on transgenic mice lacking the nicotinic acetylcholinergic (nACh) receptor display an unstable breathing and a perturbed arousal capacity (Dauger, Durand et al. 2004; Cohen, Roux et al. 2005). Furthermore, prenatal nicotine-exposure also effects several neuromodulatory systems involved in respiratory control (Robinson, Peebles et al. 2002; Wickstrom, Mas et al. 2002; Paterson, Trachtenberg et al. 2006) and in peripheral chemoreception (Holgert, Hokfelt et al. 1995; Gauda, Cooper et al. 2001). The interaction between the acetylcholinergic neurotransmitter system and substance P is not fully understood but substance P probably modulates the acetylcholinergic effect through a) direct receptor interaction on neuronal nACh-receptors (Stafford, Oswald et al. 1998) and/ or b) desensitizing the nACh-receptors via second messengers (Andoh, Itoh et al. 2001). Furthermore, presynaptic nACh-receptors causes substance P release shown in myenteric neurons (Schneider and Galligan 2000).

1.7 MORPHINE

Morphine, along with codein, heroin and naloxone, is an opiate which refers to opioids derived from Papaver Somniferum. Opioid receptors and their subtypes include $\mu_{1\text{-}3}$ (mu), $\kappa_{1\text{-}3}$ (kappa) and $\delta_{1\text{-}2}$ (delta) and are G-protein (guanine-nucleotide-binding protein) coupled and act as both positive and negative modulators. Morphine mainly acts on the μ-opioid receptor of which the μ_1-opioid receptor mediates analgesia in the CNS and the μ_2–opioid receptor in the peripheral nervous system (PNS). Morphine is still a widely used drug, neonates included, for acute and chronic pain relief, for preoperative sedation and as a supplement to anaesthesia. In spite of its narrow therapeutic index morphine also has a high interindividual variability (Aubrun, Langeron et al. 2003). Other clinical effects are cough-suppression, sedation, hypotension and nausea/ vomiting. The μ–opioid receptors (MORs) also mediate a wide range of both acute and chronic side effects of different severity, such as euphoria, miosis, decreased gastrointestinal motility, respiratory depression and physical dependence including withdrawal syndrome (Benyamin, Trescot et al. 2008).

From a respiratory point of view the fatal adverse effect of morphine, respiratory depression, is known to be mediated through the MORs in the brainstem respiratory areas (Takita, Herlenius et al. 1997; Feldman, Mitchell et al. 2003; Mellen, Janczewski et al. 2003; Janczewski and Feldman 2006). Furthermore, studies on MOR deficient transgenic mice have shown that morphine has no analgesic or respiratory depressant effect (Matthes, Maldonado et al. 1996). In addition, endogenous opioids (endorphins, enkephalins and endomorphines), as well as endogenous substance P, are released during acute hypoxia (for review Moss and Laferriere 2002). Both these endogenous peptide systems are activated during hypoxia and attention to their respective receptors and following activation also their internalization has been made. Recent research in this perspective has shown an imbalance in the time-course of NK-1Rs and MORs due to repeated hypoxia in the brainstem, resulting in a higher degree of NK-1R internalisation (Laferriere, Liu et al. 2003). This could explain the reduced respiratory drive as the residual dominating effect is of endo- or contingently exogenous opioids.

1.8 CLINICAL IMPLICATIONS

Sudden infant death syndrome (SIDS) is one of the leading causes of postnatal death among infants below one year of age in developed countries. Since the “Back to

sleep" campaign (advocating a non-prone sleep position) started in 1992, the incidence has been reduced by more than 70%. In Sweden, the SIDS incidence was 0.23 per 1000 live births in 2005, whereas the figure in the United States is 0.54 per 1000 live births, there corresponding to 22% of all deaths below one year of age (Hauck and Tanabe 2008). SIDS is defined as a sudden and unexpected death in an infant below one year of age that remains unexplained after thorough investigation and complete autopsy, examination of death scene and review of the clinical history (Willinger, James et al. 1991). SIDS is generally regarded to be multi-factorial in origin, displayed by the wide range of risk-factors. It is a well known fact, yet unexplained, that the natural variation of SIDS incidence coincides with that of infectious diseases (Highet 2008), also defined as seasonality in the Nordic countries with an increased incidence during winter (Alm, Norvenius et al. 2001). However, a functional autonomic control of the cardiorespiratory system as well as arousal responsiveness from sleep is considered to be of vital importance. Several studies have reported alterations in neurotransmitter systems associated with cardiorespiratory control and arousal, including elevated levels of substance P found in medulla oblongata in SIDS-victims (Bergstrom, Lagercrantz et al. 1984; Obonai, Takashima et al. 1996; Ozawa and Takashima 2002; Dauger, Durand et al. 2004), abnormalities of serotonergic and cholinergic systems in the brainstem (Kinney, Filiano et al. 1995; Paterson, Trachtenberg et al. 2006) as well as alterations in catecholamines (Obonai, Yasuhara et al. 1998). Due to the complexity of this syndrome a triple-risk hypothesis has been proposed including 1) a vulnerable infant 2) a critical developmental period in homeostatic control and finally 3) an exogenous stressor. A demanding challenge for research still remains in order to identify vulnerable children and to better understand the underlying mechanisms for autonomic respiratory control during this critical period.

Rett syndrome was first described as brain atrophy in girls by Andreas Rett in 1966 (Rett 1966). It is a severe genetic neurodevelopmental disorder that becomes evident at 6-18 months of age with regression of achieved motor skills, communication, a dysfunctional autonomic system and a characteristic stereotype hand wringing eventually develops as well as an autistic like condition (Hagberg, Aicardi et al. 1983; Williamson and Christodoulou 2006). Rett syndrome only affects females since male fetuses with this disorder do not survive until birth due to the X-linked methyl-CpG binding protein 2 (MECP2) gene mutation (Amir, Van den Veyver et al. 1999). Affected girls develop an irregular breathing pattern that alters depending on sleep-

wake state indicating the influence of suprapontine structures (Kerr 1992; Julu, Kerr et al. 2001; Rohdin, Fernell et al. 2007). The breathing pattern is characterized by either intermittent forceful breathing causing hypocapnia and alkalosis or weak breathing with or without recurrent apneas resulting in hypercapnia and acidosis. The dysfunctional autonomic system in the brainstem causing these breathing abnormalities is suggested to finally result in sudden death in one fourth of Rett patients (Julu, Kerr et al. 1997; Julu, Kerr et al. 2001). Several neurotransmitter systems involved in cardiorespiratory and autonomic control are considered to be altered in Rett syndrome such as catecholamine, endorphins and glutamate (Dunn 2001). A transgenic mouse model, lacking the MECP2 gene, has revealed the involvement of NE and also serotonin in the respiratory irregularities during development (Viemari, Roux et al. 2005). Furthermore, decreased levels of substance P was detected in cerebrospinal fluid (CSF) and substance P-like immunoreactivity (-LI) was also decreased in several brainstem areas involved in respiratory control (Matsuishi, Nagamitsu et al. 1997; Deguchi, Antalffy et al. 2000). As shown in figure 3, substance P has multiple sites of action and Rett syndrome is characterized by dysfunction of many of these sites.

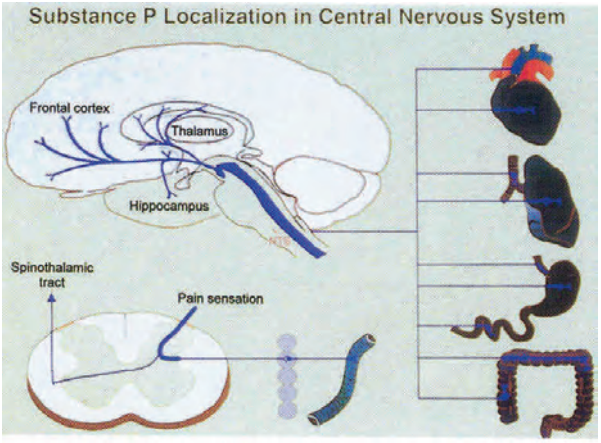

Figure 3. *Substance P localization in CNS and in the periphery, resembling several organ and system dysfunctions found in Rett syndrome. (Illustrated by Kimiko Deguchi, reproduced with permission from* Oxford University Press).

In addition, during early development, brain-derived neurotrophic factor (BDNF) is essential for the survival of sensory afferents in the petrosal and nodose ganglia comprising chemoafferent neurons and baroreceptors as well as for the

modulation of the central respiratory network (for review see (Ogier and Katz 2008)). Levels of BDNF are low in Rett syndrome (as BDNF is a transcriptional target for the MECP2-gene) and recent findings show that BDNF, presynaptically stimulates the release of substance P, at least in the spinal cord (Merighi, Bardoni et al. 2008). Even if there are, at present, only a few studies confirming a perturbed substance P-ergic system in Rett patients, it is of great concern to further evaluate the involvement of substance P and endorphins in the irregular breathing pattern displayed in Rett patients.

Recurrent apneas in the neonatal period, especially in the preterm infant, might need intervention by the clinician such as medical treatment or even invasive or non-invasive respiratory support. Apneas are categorized as obstructive, central or of mixed origin (Martin and Abu-Shaweesh 2005). The obstructive apneas are caused by a compromised airway, whereas central apneas originate from the respiratory network and mixed apneas are a combination of the two. There are several underlying causes for this to occur. It might be physiological for the organism to breathhold in order to obtain normalization of CO_2, to prevent both atelectasis and for lung compliance (Martin and Abu-Shaweesh 2005). Apneas have also been shown to represent reconfigurative properties of the respiratory network indicating immaturity of respiratory control (Lieske, Thoby-Brisson et al. 2000). Deficits in sigh activity have previously been shown to correlate to SIDS (Khan 1988). Infections are well known to cause recurrent apneas and interleukin-1β has been demonstrated to mediate respiratory depression and apneas via the prostaglandin E_2 pathway (Hofstetter, Saha et al. 2007).

2 AIMS OF THE STUDY

The main objective of this thesis was to evaluate the involvement of substance P on respiratory control in the newborn with special reference to interactions of morphine and nicotine.

Specific aims of the studies:

- To clarify the role of endogenously released substance P and of the NK-1 receptor in the hypoxic response of the newborn rat. *(Paper I)*

- To determine the intermittent hypoxic and hypercapnic respiratory response and breathing pattern during development and whether substance P/ NKA is involved in the plasticity of the respiratory network and finally alterations in central chemosensitivity in newborn mice lacking the Tac1-gene. *(Paper II)*

- To investigate the effect of prenatal exposure to nicotine on the substance P-like immunoreactivity in brainstem regions and on changes in mRNA of the precursor (PPT-A) to substance P and NKA in respiratory related versus sensory ganglia and in brainstem regions. *(Paper III)*

- To characterize the influence of substance P/NKA on respiratory control, pain and on side-effects of morphine in mice lacking the Tac1-gene. *(Paper IV)*

3 ASPECTS OF METHODOLOGY

In this section aspects of methodology will be discussed and detailed information about specific methods used can be found in papers as listed below.

Methods used:

- Flow plethysmography — *Papers I, II, IV*
  - -sustained hypoxia — *Paper I*
  - -intermittent hypoxia — *Paper II, IV*
  - -hypercapnia — *Paper II*
- *In situ* hybridization — *Paper I*
- Intracerebroventricular injection — *Paper I*
- Brainstem-spinal cord preparations — *Paper II*
- Administration via mini-osmotic pumps — *Paper III*
- Real-time reverse transcription PCR — *Paper III*
- Radioimmunoassay — *Paper III*
- Intra-peritoneal injection — *Paper IV*
- Analgesic activity; hot plate test — *Paper IV*
- Locomotor activity — *Paper IV*
- Conditioned place preference and aversion — *Paper IV*

3.1 ANIMALS

Rats and mice have become suitable models for studies on respiration both *in vivo* and *in vitro*, in particular during the neonatal period (Feldman and Del Negro 2006). The maturation of the brain in the newborn rodent is comparable to a preterm human infant (Bayer, Altman et al. 1993). In *paper I-II* and *IV* we used electrophysiological or immunohistochemical as well as physiological approaches in order to correlate these findings with those in the intact animal. In *paper I* and *III*, Sprague-Dawley rat pups (B&K Universal, Sollentuna, Sweden) were studied on postnatal day five (P5) and postnatal day one (P1), respectively, of both sexes. At P5 injection through the skull was still possible and the ventricular system including the cerebral aqueduct was large enough to locate.

Transgenic mouse models have offered the possibility to define the role for specific neurotransmitters, tachykinins included and we studied a transgenic mice lacking the tachykinin 1 gene ($Tac1^{-/-}$). This gene encodes for preprotachykinin A (PPT-A), the precursor for substance P and NKA and the selective deletion is described previously (Zimmer, Zimmer et al. 1998). The mouse line was engineered from C57/BL6J mice, which therefore were used as controls (B&K Universal, Sollentuna, Sweden). There is a variation in HVR and the response to hypercapnia not only between species but also in different mouse strains which is important to bear in mind when comparing with other studies (Tankersley, Elston et al. 2000). All animals were born naturally and were kept on a regular 12-h light-dark cycle under standardized conditions with food and water provided *ad libitum* in accordance to the European Community guidelines approved by the local ethics committee (N28/01, N88/02, 116/03, N321/98, N40/04, N69/07, 50.203.2 BN 34 19/05).

3.2 PLETHYSMOGRAPHY

It is complicated to study respiration in small infants and intact animals. The alternative has, for a number of years, been to anesthetize and intubate newborn mammals in order to obtain respiratory physiological data. In 1955, the barometric method was introduced and is still the principle upon which the plethysmographic method rests, although debated (Drorbaugh and Fenn 1955). In *paper I –II* we used the whole body flow plethysmography method with the advantage of being un-restrained, but with the disadvantage that tidal volume is not possible to accurately measure and only qualitative and not quantitative assessments were made to calculate relative changes (Enhorning, van Schaik et al. 1998). This set-up allows us to control the composition of gases exposed to the animal during an indefinite period of time. In *paper II* we wanted to evaluate the effect of intermittent hypoxic periods as well as posthypoxic periods which would not have been feasible with a traditional closed chamber system. Also in *paper II*, neonatal pups at two different ages, P2-3 and P8-10, were studied *in vivo*, in order to evaluate developmental maturation of respiratory control, known to undergo crucial changes during the perinatal period and the substance P-ergic system may play an important role during this developmental window (Yamamoto and Lagercrantz 1985; Quirion and Dam 1986; Ptak, Di Pasquale et al. 1999; Wickstrom, Holgert et al. 1999).

A combination of closed chamber- and flow-through system has recently been developed and was used in paper IV. This system allows us to control the composition of gases given but also continuously measure O_2 consumption and production of CO_2. Furthermore, since it is a so-called nose-out method the tidal volume can be exactly calculated after calibration with a known volume and also inspiratory and expiratory time was obtained. These last parameters are of interest since opioids affect respiratory timing (Janczewski and Feldman 2006; Onimaru, Kumagawa et al. 2006). The chamber and ambient temperature is important to maintain in accordance with the thermo-neutral range for rat or mice at similar age (Mortola, Rezzonico et al. 1989). Since the newborn mammal changes metabolic rate and temperature as well as ventilation in response to hypoxia (Mortola 1999). In *paper I* the thermo-neutral zone for the animal was kept using a heating blanket and in *paper II* and *IV* the chamber was immersed in a thermostat-controlled water bath. A schematic illustration of the principle of flow plethysmography is shown in figure 4. Protocols for plethysmography experiments are displayed in figure 5.

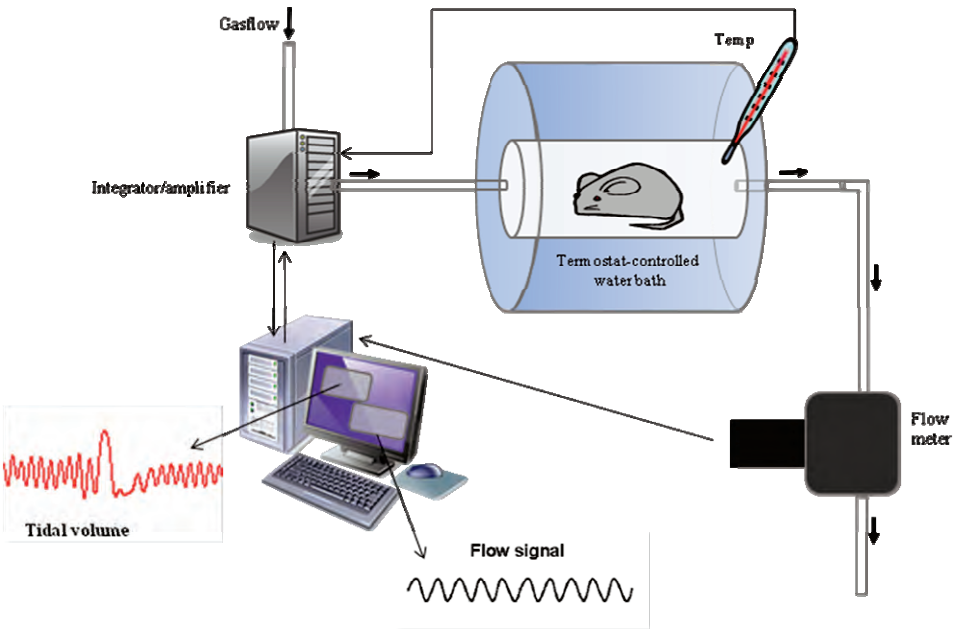

Figure 4. *Flow plethysmography, a schematic illustration. The raw data is further processed in an analysing program before calculations of breathing frequency and relative tidalvolume are made.*

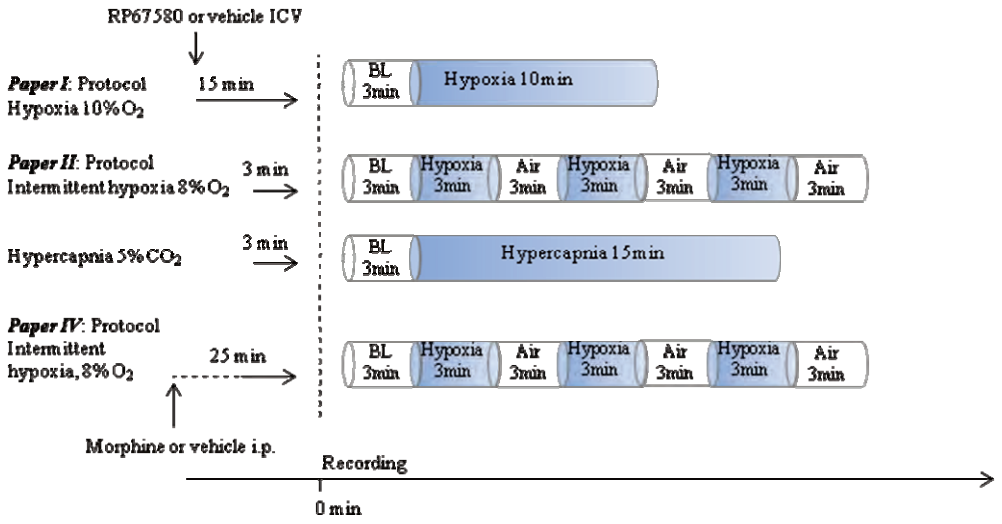

Figure 5. *Protocols for plethysmography experiments. Paper I contained two groups (treated and untreated rat pups, P5), paper II contained four groups (Tac1 $^{-/-}$ or wild type at two ages, P2-3 and P8-10) and paper IV also included four groups (Tac1 $^{-/-}$ or wild type, treated or untreated). Abbreviations; baseline (BL) and Intracerebroventricular (ICV).*

3.3 REAL-TIME RT-PCR

To detect small changes or small amounts of messenger ribonucleic acid (mRNA), the transcript from the deoxyribonucleic acid (DNA) template and the result from gene-expression, RT-PCR is the most sensitive technique for detection and quantification. The advantage with real-time RT-PCR is that it automates data analysis, standard curve generation and is especially useful to detect small amounts of mRNA. In *paper III* we wanted to analyze PPT-A mRNA, the precursor to substance P and NKA, in respiratory related ganglia (carotid bodies and petrosal/jugular ggl) as well as in sensory ganglia (trigeminal ggl and dorsal root ganglia at cervical and lumbar level). Also, RNA was isolated from three levels in the brainstem using transversal cryostat sections, i.e. 1) at the locus coeruleus 2) at the rostral end of the lateral reticular nucleus 3) and finally at the NTS (figure 6). The tissue homogenization and RNA extraction were performed according to the protocol supplied by the manufacturer (RNAas Mini kit, Qiagen, Stanford, Calif, USA) and the relative quantification of PPT-A mRNA was performed by a real-time RT-PCR machine (iQ5 Real-Time Detection System, Bio-Rad Lab, Sundbyberg, Sweden) . We used SYBR green, to detect PCR products, that

binds to double-stranded DNA and emits light when excited. A disadvantage is that it might bind to non-specific reaction products including primer-dimers. In negative controls no reverse transcriptase was added to check that no primer-pairs were yielded. Furthermore, the real-time RT-PCR machine also determines melting curves that are used to check that all PCR products of interest have the same melting curve and if not, indicating that there has been a contamination. The PCR amplification reactions were run on 1.5% agarose gels to confirm the analyzed product. As internal standard we used Quantum RNA classical 18s (Ambion, Austin, TX, USA) in order to finally perform a relative quantification of PPT-A gene expression by using the delta delta Ct method (Livak and Schmittgen 2001). The delta delta Ct method is only an approximation method and to check the accuracy for the PCR we performed dilution curves.

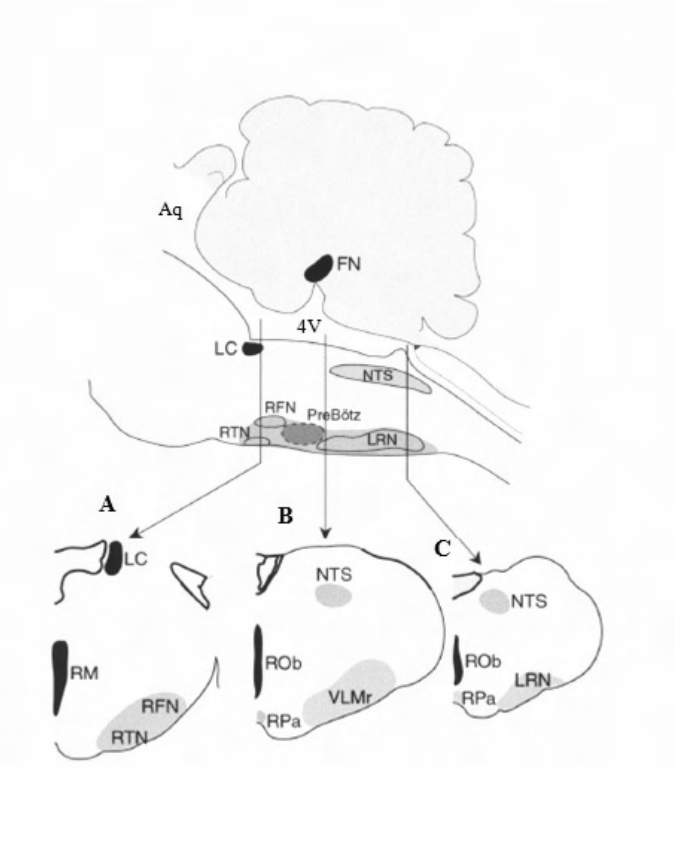

Figure 6. *Transversal cryostat sections at three levels; A) locus coeruleus (LC) B) rostral end of lateral reticular nucleus (LRN) and C) nucleus tractus solitarii (NTS). Other abbreviations: cerebral aqueduct (Aq) and fourth ventricle (4V).*

3.4 BRAINSTEM-SPINAL CORD PREPARATIONS

The *in vitro* technique offers a unique possibility to investigate the respiratory network on a cellular level and has contributed to the understanding of the mechanisms involved in rhythm generation (Suzue 1984; Smith, Ellenberger et al. 1991; Rekling and Feldman 1998; Gray, Janczewski et al. 2001). The set-up also allows us to stress the network to hypoxia/anoxia, hypo-/ hypercapnia, different pH-levels and also to several drugs. There are, however, limitations since peripheral and descending afferent input is absent and normally a non-physiological low temperature of the set-up (room-temperature) resulting in a different breathing pattern and a slower bursting frequency than in the intact animal (Feldman and Del Negro 2006). In *paper II* we used P2 pups with Tac1 $^{-/-}$ and Tac $^{+/+}$ (C57/BL6J) genotypes and the isolated brainstem-cervical spinal cord preparation were obtained as described previously (Nsegbe, Wallen-Mackenzie et al. 2004). The isolated preparation was continuously perfused with artificial cerebrospinal fluid at a pH of 7.4. Inspiratory discharges of respiratory motoneurons were monitored with glass suction electrodes applied to the proximal cut end of the C4 ventral roots of the spinal nerves.

3.5 NICOTINE ADMINISTRATION

Mini-osmotic pumps are widely used to investigate teratological and toxicological effects of different drugs. They offer a safe administration route of for instance nicotine and are well tolerated, especially important in pregnant animals where stress has to be avoided (Murrin, Ferrer et al. 1987; Slotkin, McCook et al. 1997). In *paper III* mini-osmotic pumps (Model 2ML4, Alzet, Palo Alto, CA, USA) were inserted subcutaneously on the dam, lightly anesthetized with ether, on the fifth day of gestation. The pumps delivered nicotine bitartrate solved in sodium chloride corresponding to a dose of 3 mg nicotine free base per kg bodyweight (of the dams' initial weight) per day. Thus, the dose per kg bodyweight declined during pregnancy. This dose is comparable to moderate smoking in man (Nasrat, Al-Hachim et al. 1986; Lichtensteiger, Ribary et al. 1988). However, the continuous way of releasing nicotine gives a steady state concentration of nicotine, in contrast to peak nicotine levels achieved by intermittent smoking, but on the other hand it resembles that of transdermal nicotine replacement.

3.6 OTHER DRUGS AND ADMINISTRATION ROUTS

To investigate the role of endogenously released substance P and its preferred receptor in the HVR, RP67580, a potent nonpeptide NK-1 receptor antagonist was used, kindly provided by Dr. C. Garret (Aventis Pharma, Vitry-sur-Seine, France). The non-peptide RP67580 is potent, specific, competitively acting, with high affinity for the NK-1 receptor, as previously described (Garret, Carruette et al. 1991; Culman, Wiegand et al. 1995). This potency is not seen after systemic administration, indicating a poor blood-brain barrier penetration (Holzer-Petsche and Rordorf-Nikolic 1995). Intracerebroventricular injection in neonatal animals with a surgically implanted cannula is not an option because of the softness of the skull and the rapid growth. To solve this, we used in *paper I*, a drawn glass pipette and during light anaesthesia with isoflurane, and injected into the cerebral aqueduct and the fourth ventricle, schematically shown in figure 6. We did not use a stereotactic device because the placing and positioning process was time consuming and prolonged the time under anaesthesia; instead we manually held the pup with a light bulb beneath the skull and the ventricular system appeared clearly. The landmarks used were the midline, 2 mm caudal to the bregma and 1.5 mm deep, vertical to the surface. To ensure that the injected fluid landed in the intended place we used pontamine blue sky, a tissue dye, which ink coloured the ventricular surface in 5/5 tested animals. The dose injected was 200 pmol (RP67580) per animal, corresponding to equivalent effective dose in adult animals as previously described (Unger, Chung et al. 1996), whereas control animals received an equal amount of artificial CSF, both fluids were equilibrated to pH 7.4. In order to permit passive penetration, i.e. diffusion into the brain tissue, 15 min appeared enough to block the NK-1 receptor. This was preceded of a small pilot study with 25 min post injection and no respiratory changes were observed.

In *paper IV*, morphine hydrochloride (Meda AB, Solna, Sweden and Merk Darmstad, Germany) and naloxone hydrochloride (Sigma-Aldrich Deisenhofen, Germany) were used. Both drugs were dissolved in sterile 0.9% saline and administered in the same volume as vehicle (saline 0.9%), 10 ml/kg bodyweight. Morphine was administered either subcutaneously or intraperitoneally with different doses (1-50mg/kg bodyweight) or vehicle, depending on testing protocol. When testing acute effects of morphine the allowed time-span was 25-30 min from injection until intended testing started which corresponds to peak effect of morphine (Kalvass, Olson et al. 2007). Upper intestinal tract motility was tested 30 min after vehicle or

morphine was injected , with Carmine, a nonabsorbable red dye, given intragastrically through an orogastric tube and after 30 min the animals were sacrificed and the length of Carmine stained gut was measured.

3.7 DATA ANALYSIS

Ventilatory measurements: whole-body flow plethysmography was used in *paper I* and *II* where the raw signal from the highly sensitive gas flow sensor was amplified and integrated with a four-channel amplifier. The signal was then digitally converted using DasyLab software (Datalog, Mönchengladbach, Germany). Analysis of the respiratory signal was made on at least 30-s periods without movement artefacts, which were chosen subjectively. Respiratory frequency (*f*, breaths/min), tidal volume (V_T, µl) and ventilation (V_E, ml/min) were calculated by the software and normalized to facilitate comparison. In addition, number of apneas (defined as a ventilator pause twice the duration of the preceding breaths), sighs or inspiratory augmented breaths (AIB, defined as a biphasic inspiration, twice the V_T of the preceding breaths (Thach and Taeusch 1976; Cherniack, von Euler et al. 1981)) as well as *f* (to exclude any movement artefact recorded as a breath by the analyzing program) were calculated manually. V_T and V_E were divided by bodyweight (g) and expressed as µl/g and ml/g/min, respectively. In addition to the obtained values stated above, we used an alternative form of plethysmography in *paper IV*, that also measured inspiratory and expiratory time and more accurately calculated V_T (PowerLab, Chart v5.5.4, ADInstruments; Colorado Springs, CO, US).

In the *in vitro* brainstem-spinal cord preparation in *paper II* the burst frequency (calculated as number of C4 bursts/min) and amplitude in C4 activity were analyzed. Burst amplitude was quantified, in arbitrary units, as the distance from the baseline to the peak of the integrated burst slope. The electrophysiological data was collected by Axoscope software and Digidata 1200B interface (Axon Instruments, Foster, CA, US). The preparations were superfused with control artificial CSF for 40 min to reach steady state and baseline values were then obtained. Thereafter testing solutions were applied, i.e. anoxia (sustained or intermittent) or hypo-/hypercapnia.

Comparison of c-fos mRNA levels using *in situ* hybridisation (ISH) was made after exposure under identical conditions and a computerised quantification, using a free download NIH Image program (http://.rsb.info.nih.gov/nih-image/) was performed in *paper I*. ISH is a reliable method to detect and determine the mRNA distribution in

tissue. We used either a main intensity or a minimum and maximum threshold approach, when applicable, in order to quantify all labelled neurons in a certain area and to eliminate background staining and artefacts. The activation of immediate early genes, such as c-fos, is an often used marker for neuronal activation (Kovacs 2008). However, there are limitations such as unspecific activation, the need of a strong stimulation and the fact that inhibited neuronal pathways are not detected (Chan and Sawchenko 1994). With this in consideration it is important to have a standardized experimental milieu to reduce confounding factors and to interpret the results judiciously.

3.8 STATISTICS

All statistical analyses were made on absolute values before normalization for graphical clarity. In *paper I* the statistical analysis was performed using a two-way analysis of variance (ANOVA, Statistica software). Where ANOVA indicated a significant interaction a planned comparison was made to detect differences within and between groups. In *paper II* Student´s *t*-test was used in paired comparisons and one- and two-way ANOVA for multiple comparisons. Post-hoc analysis was conducted by the Tukey´s test. Concerning statistical analysis of breathing pattern (apneas, sighs and AIBs) we used the non-parametric Mann-Whitney´s *U*-test since these parameters were not normally distributed. In *paper III* we used a one-way ANOVA followed, when indicated, by a planned comparison test. In paper IV we used two- and three-way ANOVA, followed by a post-hoc test when indicated. Data are shown as means ± SD and ± SE in figures for graphical clarity. A confidence level of $P<0.05$ was considered significant.

4 RESULTS AND DISCUSSION

4.1 NORMOXIC CONDITIONS

During normal conditions substance P and the activation of NK-1 receptors is not essential for eupnea *(papers I and II)*. This is in accordance with previous studies (Yamamoto and Lagercrantz 1985; Ptak, Hunt et al. 2000; Ptak, Burnet et al. 2002; Telgkamp, Cao et al. 2002; Grasemann, Gerard et al. 2007). However, we could demonstrate differences in breathing pattern in Tac1 $^{-/-}$ mice at P2-3 during normoxic conditions. Transgenic mice displayed almost a 50% reduction in the number of apneas/min as compared to wild-type animals (WT) at this age. This difference disappeared at P8-10, probably due to maturation of the respiratory network. Also, we did not see any differences in breathing pattern at P5 in *paper I*, although only NK-1 receptors in the brainstem were blocked leaving the functionality of afferent input related to NK-1 receptors intact. The interest in breathing pattern recognition has emerged in recent years (Feldman and Gray 2000; Lieske, Thoby-Brisson et al. 2000) and is considered to relate to reconfigurative properties of the respiratory network. Our finding that the apneic pattern was not visible *in vitro* displays the need of integration of peripheral input and influence from higher centra. Hence, these findings indicate involvement of the substance P-system in neuronal networks acting to stabilize eupneic respiration during early development.

4.2 HYPERCAPNIA

Our *in vivo* and *in vitro* studies of the hypercapnic response, with an anticipated increase in ventilation, did not reveal any differences following alterations of the substance P-ergic system (*paper II)*.This corroborates findings by others (Prabhakar, Mitra et al. 1987; De Sanctis, Green et al. 1991) and indicates that substance P is not of importance for this response. Rather, serotonin has been proposed to be involved in the hypercapnic response (Richerson 2004; Taylor, Li et al. 2005; Li and Nattie 2008). Although, these two neurotransmitters are co-localized in respiratory related neurons (including the carotid body), they are activated by different stimuli (Sasek, Wessendorf et al. 1990; Wang, Stensaas et al. 1992) and a differential effect may be expected.

4.3 HYPOXIA

4.3.1 Sustained hypoxia and NK-1 receptor activation

In order to investigate involvement of NK-1 receptor activation in the brainstem in the HVR, the antagonist RP67580 was injected ICV. Thereby, we could examine if substance P was endogenously released upon hypoxia. We demonstrate, in *paper I*, that activation of the NK-1 receptor in the brainstem is essential for an adequate HVR. The normal increase in *f* and V_T resulting in augmented ventilation was initially seen in both groups, but after 10 min of sustained isocapnic hypoxia (10% O_2 and 3% CO_2 in N_2) RP67580-treated animals were incapable of maintaining *f*. However, V_T did not differ and in contrast displayed a tendency to increase which counterbalanced the decreased *f* and maintained ventilation. This finding appeared late and probably reflects the major contribution to HVR by peripheral pathways, i.e. the carotid body projecting to NTS and that the blocked receptors in the brainstem only contribute after prolonged hypoxia. However, it cannot be ruled out that the effect of substance P is mediated by some other receptor, most likely NK-2 and/or NK-3 receptors. The differentiated effect with a decreased *f* and an unchanged V_T reflects a differential regulation of the respiratory network. This is supported by previous findings demonstrating that a local injection in NTS of substance P only increases V_T (Mazzone and Geraghty 2000) and that both NK-1 *and* NK-3 receptors are involved (Mazzone and Geraghty 2000).

4.3.2 Sustained hypoxia and c-fos expression

Several brainstem areas known to be involved in respiratory control displayed an increase in c-fos mRNA expression after 60 min of hypoxic (10% O_2 and 3% CO_2 in N_2) exposure (*paper I*). These areas comprise caudal reticular nucleus and deep thereof towards the nucleus ambiguous, an area referred to as caudal VLM, RTN, the caudal NTS and rostral VLM. In animals treated with RP67580 before hypoxic exposure this increase was attenuated in rostral VLM and RTN. Other areas displayed the same increase as in controls, and were thus not affected by the NK-1 receptor antagonist. The attenuated response to RP67580 in the caudal NTS known to be involved in hypoxic activation of peripheral chemosensitive pathways and release of substance P (Lindefors, Yamamoto et al. 1986) may be explained by other neurotransmitters and/ or receptors involved, e. g. NKA and/or NK-3 receptor. More interesting is the attenuation by RP67580 in the rostral VLM, containing parts of the VRG and the PBC with rhythm

generating properties which may be correlated to the inability to maintain *f* in HVR *in vivo*.

4.3.3 Intermittent hypoxia

Intermittent hypoxia resembles a more physiological way to investigate HVR and the plasticity of the respiratory network and early-life experiences such as episodic hypoxia may cause a potentially maladaptive development of respiratory control. These changes are also long-lasting when occurring during this vulnerable window, effects not seen in the adult animal (Carroll 2003; Feldman, Mitchell et al. 2003). In *paper II*, we demonstrate that substance P is involved in long-term facilitation (LTF) of respiration, previously described for serotonin (Fregosi and Mitchell 1994; Bach and Mitchell 1996; Fuller, Zabka et al. 2001). LTF is considered to occur in the carotid body but in our study we show that this plasticity at least also occurs in the brainstem (figure 7). This figure also illustrates the different effect between continuous and intermittent hypoxia.

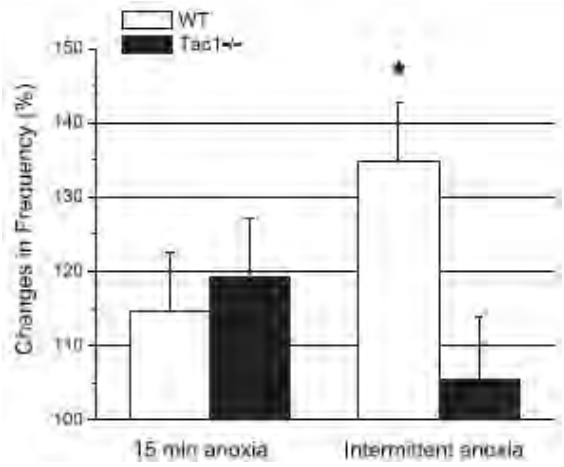

Figure 7. *Showing in vitro changes in burst frequency (±SE) 40 min after completed continuous or intermittent anoxia. * indicates both a significant increase in f from baseline (P<0.001) and between WT and Tac1 -/- animals.*

The newborn pups at age P2-3 did not display any LTF or HVR in response to intermittent hypoxia in either experimental group. This may be due to immaturity of the respiratory network, insensitivity of the plethysmography setup or rather reflect that the hypometabolic response is more prominent in newborn mammals (Mortola, Rezzonico

et al. 1989; Mortola 2004). It is notable that the central respiratory network contains this plasticity underlying LTF since the *in vitro* experiments were performed at P2. Thus, the immature properties may be of peripheral chemosensitive origin and the hypometabolic response may override the ventilatory response at this age. *In vitro*, the hypoxic (anoxic) response was affected in Tac1 $^{-/-}$ preparations by 1) less amplitude reductions, 2) the recovery period was shorter when the burst amplitude was restored, and 3) posthypoxic neuronal arrest (PHNA) appearing more frequently following resumption of oxygenation (Figure 8). In line with previous studies, compensatory mechanisms may have developed during early ontogeny to manage normal conditions but not during stress such as, in this case, anoxia (Zimmer, Zimmer et al. 1998; Telgkamp, Cao et al. 2002). Other neurotransmitters and neuromodulators (e. g. glycine, opioids, adenosine and serotonin) have been demonstrated to affect PHNA duration (Kinkead and Mitchell 1999; Kato, Hayashi et al. 2000) and could be possible candidates exerting compensatory respiratory output mechanisms in Tac1 $^{-/-}$ mice. The physiological implications of PHNA is not clear, but may be related to a decreasing metabolism during resumption of oxygen (Dick, Dutschmann et al. 2001) when the respiratory related neurons in the VLM recover from anoxia (Ballanyi, Volker et al. 1994). These findings may contribute to the understanding of the clinically related disorders such as SIDS and Rett syndrome with features such as abnormal breathing pattern during early development.

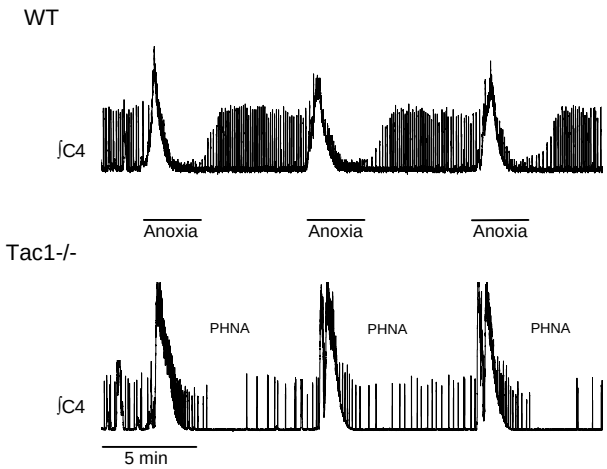

Figure 8. *Showing registrations of C4 burst activity on intermittent anoxia. Posthypoxic neuronal arrest (PHNA) was observed in 100% of Tac1 $^{-/-}$ preparations which also displays less amplitude reduction and shorter recovery period as compared to wild-type (WT).*

4.4 MORPHINE EFFECT ON RESPIRATORY CONTROL

One of the main side-effects of morphine is respiratory depression. How morphine effects the HVR has been poorly described in rodents. In our study (*paper IV*), we demonstrate a blunted HVR that attenuates with repeated hypoxic challenges in WT. In contrast, transgenic Tac1 $^{-/-}$ mice displayed a normal HVR, even when subjected to intermittent hypoxia. However, the sensitivity to morphine was equal in both experimental groups during normoxia, i.e a significant reduction of *f* and V_T to the same level, summarized in figure 9. Furthermore, in *paper IV*, young Tac1 $^{-/-}$ mice display elevated ventilation during basal conditions, whereas newborn pups have a similar respiration as controls during normal conditions (*paper II*). This may be due to compensatory mechanisms such as the serotonergic system (co-localized with substance P) and/ or the dopaminergic system known to be activated during hypoxia. The interpretation of these findings is difficult and further studies are needed to understand how substance P and/ or NKA is involved.

4.5 MORPHINE AND OTHER SIDE-EFFECTS

To evaluate to what extent substance P and NKA influence other side-effects of morphine than respiration we studied analgesic activity, motility of the intestines, rewarding effect, locomotion and conditioned place preference and aversion. Our results demonstrate that morphine had a higher analgesic effect in transgenic mice lacking substance P/ NKA, which is supported by previous data (Guan, Xu et al. 2005). This may be explained by the long-term reduction of NK-1 receptor activity at a spinal and supraspinal level, leading to a reduction of opioid peptide release and consequently, as a compensatory change to the lowered transmitter level, leading to the sensitization of opioid receptors in areas related to pain sensation. Furthermore, Tac1 $^{-/-}$ mice had fewer side-effects, except for intestinal motility where no interaction was found. Likewise, the rewarding effect was similar in Tac1 $^{-/-}$ mice and controls. On the other hand, transgenic mice were less sensitive to morphine withdrawal, suggesting that substance P-NK1-receptor signalling is involved in the development of opioid withdrawal symptoms. This has also been shown in pharmacological studies (Maldonado, Girdlestone et al. 1993; Michaud and Couture 2003) and substance P has a modulatory effect on dopamine pathways during opioid withdrawal (Zhou, Kindlundh et al. 2004).

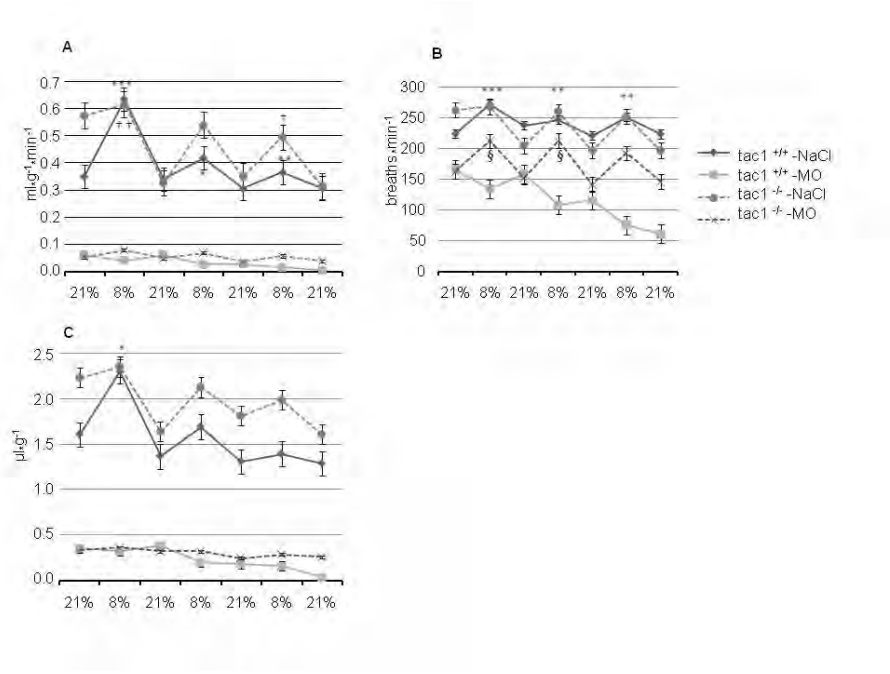

Figure 9. *Effects of morphine during normoxia and repeated hypoxia.* ***A)*** *ventilation/body weight* ***B)*** *frequency and* ***C)*** *tidal volume/body weight). Ventilation was depressed in both tac1$^{+/+}$ and tac1$^{-/-}$ mice. Transgenic animals displayed a significantly stronger hypoxic ventilatory response resulting from an increase in f. *p < 0.05; ** p < 0.01; *** p < 0.001 displays significant difference between saline and morphine treated tac1$^{+/+}$,† p <0.05; †† p < 0.01 displays significant difference between saline and morphine treated tac1$^{-/-}$ mice. § p < 0.05 display significant difference between morphine treated tac1$^{+/+}$ and tac1$^{-/-}$ animals.*

4.6 PRENATAL NICOTINE EXPOSURE

Prenatal nicotine exposure influences neuronal development including effects on several neurotransmitter systems (for review, see (Wickström 2007; Slotkin 2008)). It also attenuates the ventilatory response to hypoxia (Fewell and Smith 1998; Bamford and Carroll 1999), known to require a functional substance P-ergic system. Previous studies have shown that nicotine increases the risk for sudden infant death syndrome (SIDS) by four-fold (Haglund and Cnattingius 1990) and that SIDS-victims have elevated brainstem levels of substance P (Bergstrom, Lagercrantz et al. 1984). Thus, in *paper III* we studied the effect of prenatal nicotine exposure on the levels of substance

P-like immunoreactivity (-LI) by radioimmunoassay in the brain and adrenals as well as the PPT-A mRNA expression by real-time RT-PCR in respiratory related pathway; carotid bodies and petrosal/jugular ganglia as well as in sensory ganglia (trigeminal ggl and DRGs at cervical and lumbar level), in newborn rat pups at P1.

We could demonstrate elevated substance P-LI in the brainstem, a five-fold increase of PPT-A mRNA in the carotid body and a 40-fold increase in petrosal/jugular ganglia. In contrast, cervical DRGs display a significant decrease and a tendency towards a decrease in trigeminal ganglia. One possible explanation is a differential effect of prenatal nicotine exposure on sensory neurons (giving rise to most substance P in the brainstem) in general versus the respiratory carotid body-petrosal/jugular ganglia-NTS pathway. Furthermore, nicotine did not affect PPT-A mRNA levels in lumbar DRGs or in the brainstem at any of the three different levels studied (fig. 6).

Different effects of nicotine depending on site of action have been shown previously (Damaj, Meyer et al. 2000), and are probably due to the heterogeneous distribution of nicotinic receptor subunits (Albuquerque, Alkondon et al. 1997). We found PPT-A mRNA in carotid bodies in control newborn animals, whereas previous studies could only detect PPT-A mRNA in petrosal ganglia in adult animals but not in carotid bodies. This further emphasizes the importance of tachykinins during early development. The results of elevated levels of substance P-LI in the brainstem and affected PPT-A mRNA expression in respiratory related structures following prenatal exposure may contribute to the understanding of possible underlying mechanisms for SIDS.

5 FUTURE PERSPECTIVES

The coincidence of the seasonality of the incidence of SIDS and the infectious period, at least in Nordic countries, where a connection to interleukins, apneas and SIDS have been proposed (Hofstetter 2006) is interesting in the light of substance P as a neuro-immuno communicating peptide (Bost 2004; Siemion, Kluczyk et al. 2005), severely affected by prenatal nicotine exposure. Future studies on the interaction between tachykinins and the immune response, in particular with interleukins, is of great importance.

In Rett syndrome promising treatment trials are made with norepinephrine re-uptake inhibitors. Several characteristics of Rett syndrome resemble that of a dysfunctional substance P-ergic system, in particular the breathing disturbances. The onset of symptoms also coincides in time when substance P in CNS returns to adult levels. Whether this is due to an uncontrolled decrease *per se* or due to a perturbed neurotransmitter balance due to low levels of BDNF remains to be revealed.

Another interesting result of the present thesis is the findings of a nearly intact hypoxic ventilator y response following morphine injections in animals lacking a functional substance P system, i.e. a constitutional knock-out. Whether this is true also in a situation with an acute decrease in substance P-ergic transmission such as receptor inhibition remains to be elucidated. At the moment, the only NK1-receptor antagonist registered drug is Emend ®, an anti-emetic drug used during chemo-therapy. If the same ventilatory response is seen using such an antagonist, it may be of use as a possible adjuvant to morphine in order to reduce side-effects, including fatal respiratory depression, and to enhance the analgetic potential.

6 CONCLUSIONS

This thesis describes the role of substance P in the finely tuned respiratory control system in the newborn and the effects of morphine and perinatal exposure to nicotine.

- Central endogenous tachykinin release is important for an adequate hypoxic ventilatory response but NK-1 receptor blockade does not affect respiration during basal conditions.
- NK-1 receptor blockade inhibits activation in the rostral ventrolateral medulla (VLM) and the retrotrapezoid nucleus (RTN) known as respiratory related areas in the brainstem.
- *In vitro* and *in vivo* experiments demonstrate that Tac1 $^{-/-}$ mice exhibit an abnormal respiratory response and breathing pattern when exposed to hypoxic but not to hypercapnic stress. Furthermore, our results show that substance P is involved in long-term facilitation and that it occurs also at a central level. This indicates that substance P is a required neuromodulator for an adequate hypoxic ventilatory response. Thus, a dysfunctional substance P-ergic system may contribute to the fatal outcome of SIDS and to the respiratory disturbances seen in Rett patients.
- We offer a biochemical link between SIDS and prenatal nicotine exposure, a relation previously shown in epidemiological data, with increased levels of substance P-LI in the brainstem and decreased levels of PPT-A mRNA expression in respiratory related structures (carotid bodies and petrosal/jugular ggl). We also show that PPT-A mRNA expression is detected in the carotid bodies, which is absent in the adult mice, further stressing the importance of tachykinins during early development.
- Morphine depresses ventilation both during normal conditions and intermittent hypoxia in control animals. The sensitivity to morphine in Tac1 $^{-/-}$ animals was, likewise, a depressed ventilation during basal conditions but displayed an enhanced ventilatory response to intermittent hypoxia. In addition, the analgesic potential of morphine was increased in Tac1 $^{-/-}$ animals, whereas the addictive potential was reduced. Thus, a reduced activity in the tachykinin system could be a strategy to improve the pharmacological potential of morphine and minimize the main side-effect.

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