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RESPIRATORY ACTIVITY IN
MEDULLA OBLONGATA
and its modulation by
ADENOSINE AND OPIOIDS

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The brainstem and perinatal respiratory activity

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


IV. Eric Herlenius, Ulrika Åden, Lie-Qi Tang and Hugo Lagercrantz. Adenosine in the immature rat brain and effects of maternal caffeine intake: development of respiratory control. *Manuscript*

V. Eric Herlenius and Hugo Lagercrantz. Adenosinergic modulation of respiratory neurones in the *in vitro* neonatal rat brainstem. *Submitted*
ABBREVIATIONS

aCSF artificial cerebrospinal fluid
ATP Adenosine-5’-triphosphate
Biphasic E Biphasic expiratory neurone (also called Pre-inspiratory)
C4 Cervical ventral root 4
CNS Central nervous system
CPG Central pattern generator
DAGO Try-D-Ala-Gly-[NMephe]-Gly-ol
DPCPX 8-cyclopentyl-1,3,dipropylxanthine
DPDPE [D-pen²⁵]-enkephalin
Exp Expiratory related neurone
EPSP excitatory postsynaptic potential
Insp Inspiratory related neurone
Naloxone naloxone hydrochloride
Naloxanazine naloxanazine dihydrochloride
Nor-BNI nor-Binaltorphimine hydrochloride
NTS Nucleus tractus solitarius
P a O 2 Partial pressure of oxygen in arterial blood
R-PIA adenosine A₁-receptor agonist
(N6-(2-phenylisopropyl) adenosine, R(-) isomer)
Theophylline 1,3,-di-methylxanthine
TTX Tetrodotoxin
U50,488 488 ( trans - (±) - 3,4 - dichloro - N - methyl - N - ( 2 - [ 1 - pyrrolidinyl] cyclohexyl) benzeneacetamide methanesulfonate)
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INTRODUCTION

Breathing is a fundamental physiological process produced and controlled by the nervous system which must be properly set into action from the moment of birth. Currently it is not exactly known how breathing is initiated and basic mechanisms, involved in the complex and fine-tuned regulation of respiration are not clearly delineated yet. A better understanding of normal development of respiratory control and its disturbances may lead to improved treatment and outcome of clinical conditions such as repeated episodes of apnoea in preterm babies, sudden infant death syndrome and postoperative hypoventilation. Hence, the study of mechanisms for central pattern generation of breathing is of great concern, not least in the perinatal period, which is the main theme in the present thesis focused on respiratory control and its modulation by adenosine and opioids.

GENERAL BACKGROUND

It was not until the French revolution that it was understood, during the use of the guillotine, that mammals do not need their heads to breathe. The French physician Le Gallois (1770-1814) established that the respiratory centre is localised in the medulla oblongata in a series of animal experiments (Le Gallois, 1812). This was the first time that an area within a major subdivision of the brain had been defined accurately by experimentation as having a specific function (Morton and Garrison, 1991). 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 to the organ and the medulla oblongata.

Since the discoveries of Le Gallois our understanding of the body, the brain and the control of autonomic functions has increased enormously, for reviews see (von Euler, 1991; Speck et al., 1993; Bianchi et al., 1995).

In order to investigate different levels of neuronal networks or single neurones, animal models have to be used. The anaesthetised or decerebrated cat was the model of choice for the study of respiratory control from 1920 up to about 1985. These in vivo studies localised the neuronal populations that contain the basic circuitry for respiratory rhythm in the brainstem as well as the firing pattern, interconnection and projections of respiratory neurones found in this region (Feldman, 1987; Bianchi et al.,
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Based on such studies it was determined that the generation of breathing rhythmogenesis does not critically depend on extrinsic feedback loops or reflexes, provided the excitability of these mechanisms is kept sufficiently high by adequate biasing inputs (von Euler, 1980).

During the last decade, thanks to the availability of novel techniques, especially *in vitro* en bloc and slice preparations, our understanding has increased concerning cellular and synaptic physiology of brainstem respiratory neurones. These *in vitro* preparations make it feasible to examine cellular and sub-cellular levels of respiratory control. Nevertheless, we still do not know how the neuronal networks in the medulla oblongata generate respiratory rhythm even though several hypotheses have been proposed. (Richter *et al*., 1992; Bianchi *et al*., 1995; Onimaru *et al*., 1997; Rekling and Feldman, 1998). The classical concept of a reciprocal inhibition between two symmetrical populations of inspiratory and expiratory premotor neurones, the “half-centre model”, has been abandoned. Instead, the prevailing theories divide the respiratory cycle into three phases (inspiratory, passive and active expiratory (Richter, 1982). Today, two main hypotheses exist: 1) Site hypothesis: the preBötzinger complex is the site for respiratory rhythm generation (Smith *et al*., 1991) and 2) rhythmogenesis hypothesis: pacemaker or group pacemaker neurones are the cellular kernel for respiratory rhythm (Rekling *et al*., 1996) (Onimaru *et al*., 1988). The preBötzinger complex in the rostral ventrolateral medulla oblongata seem to have an obligatory role in respiratory rhythmogenesis, whereas more caudal and dorsal medullary structures do not. Reciprocal synaptic inhibition between groups of respiratory neurones is not the cellular basis for respiratory rhythm. This has led to that neurones intrinsically capable of generating cyclic discharges, i.e., pacemaker neurones have been proposed as candidates for rhythm generation (Onimaru, 1987) for reviews see (Rekling and Feldman, 1998) and (Bianchi *et al*., 1995).

Thus, it has been established that a highly sophisticated central neuronal network is responsible for the central control of breathing. This central network is able to continuously adjust breathing to the requirements of the internal and external environment. Central control implies that the central nervous system is intrinsically capable of providing the proper timing of muscle activation. Fine-tuning of the central output is attained by a wide range of vagal afferents from the airways and lungs relaying sensory information to the nucleus tractus solitarius (NTS) in the dorsal medulla oblongata. In addition, the arterial baro- and chemoreceptors, both those relaying via the vagus from the aortic arch and those relaying via the IXth cranial...
nerve from the carotid bifurcation, send their fibres to the NTS. In addition, descending inputs from cortex, diencephalon and the cerebellum also influence the medullary network (Euler and Lagercrantz, 1987). Although suprapontine and sensory input modulates respiratory rhythm and adapts breathing, these influences only modulates the ongoing central respiratory rhythm generation. It is important, therefore, to understand the processes underlying the central respiratory rhythm generation and its intrinsic control. This thesis focuses on the intrinsic respiratory control system in the brainstem.
DEVELOPMENT OF RESPIRATORY CONTROL

The ability of central networks to produce rhythmic respiratory motor behaviour is a well conserved property of the brainstem reticular formation between species (Borday et al., 1997). Since 1991 an area in the ventral group of respiration related neurones, the pre-Bötzinger complex, has been considered by most researchers to be the crucial site of this rhythmogenesis (Smith et al., 1991). This area was identified by a series of transection studies in the in vitro brainstem spinal cord preparation from neonatal rats that is the main tool in this thesis. During recent years this area has been reported to also be important in vivo and in other species (Paton 1997, (Pierrefiche et al., 1998; Ramirez et al., 1998c). The ventral group of respiratory neurones (VRG) extends through the whole length of the ventrolateral medulla, partly along the nucleus ambiguous (Ezure, 1990) but it is mainly in the rostral VRG where bulbospinal neurones recide (Fig 1). The nucleus tractus solitarius in the dorsal group of respiratory neurones (DRG) receive afferent input from the lungs and also receive input from peripheral baro and chemoreceptor. It is thus an important relay for integrating afferent input that can influence respiration. However, in the rat the DRG makes little or no contribution to the respiratory drive transmitted from medulla to spinal motoneurones (Bianchi 1995).

Respiratory rhythm generation is established long before birth, and in the human foetus respiratory movements already occur at the 11th week of gestation (de Vries et al., 1982). These movements are necessary for normal lung development (Kitterman, 1996). Foetal breathing movements are episodic and are progressively more inhibited towards the end of pregnancy when periods without respiratory movements (apnoea) dominate (Maloney, 1975 However, from the dramatic moment of birth, these respiratory neuronal networks need to function and continuously generate a respiratory rhythm to sustain oxygenation and metabolism. The mechanisms of this transition from episodic to continuous breathing is still unclear. Respiration must also have the capacity to respond to changes in the external and internal environments to maintain body homeostasis. The rhythmic respiratory movements in foetuses, as well as the breathing in neonates and adults, are governed from neuronal networks in the brainstem which generate the respiratory rhythm. Thus, during the last decades researchers have tried to understand how this noed vital develops and functions...
through different experimental approaches, for review see (Mitchell, 1997) and (Speck et al., 1993).

**Respiratory depression during hypoxia**

Breathing movements before birth are inhibited by hypoxia (Boddy et al., 1974; Johnston, 1991). This is functional in foetal life when processes consuming oxygen have to be turned off if they are not vital for survival (Johnston, 1991; Hanson, 1996). However, this apnoeic response to hypoxia may be less appropriate after birth. During the early postnatal period respiratory control is still different than in adults. Both adult and new-born animals respond to hypoxia with a biphasic change of respiration (Schwieler, 1968; Lawson and Long, 1983; Runold et al. Fredholm, 1989). There is an initial increase of respiration due to stimulation of the peripheral chemoreceptors (Schwieler, 1968), activation of nervous structures localised rostral to the brainstem (Eldridge et al., 1981) and structures within the brainstem (Ramirez et al. 1998b). However, in newborn animals the response to hypoxia is similar to that of the foetus and the secondary depression of respiration is pronounced and ventilation falls below normoxic levels within minutes after initiation of hypoxic exposure. During severe hypoxia or anoxia there are progressive changes in the ventilatory pattern, from hyperpnoea to a sustained pause of respiration, which is then, after a period of expiratory apnoea, followed by gasping. Respiratory gasping is characterised by a series of brief bursts of phrenic activity with sudden onsets followed by a rapid decline and absence of expiratory activity (Guntheroth and Kawabori, 1975).

What are the brain structure(s) and neuromodulators that are involved in the hypoxia-induced respiratory depression? Several possibilities have been considered as mechanisms behind the secondary depression of respiration, for review see (Lawson and Long, 1983; Neubauer et al., 1990; England, 1993). These hypotheses can be divided into those stressing inhibitory supramedullary input to the brainstem as vital for the depression i.e. (Johnston, 1991; Waites et al., 1996) (Hanson, 1996; Okada et al., 1998) and those emphasising local neurochemical processes in the brainstem (Moss et al., 1986). Recent findings indicate that even in the reduced in vitro preparation the hypoxic response is biphasic both at the motor output level and within the respiratory network (Brockhaus et al., 1993; Ramirez et al., 1998a; Ramirez et al., 1998b). Both afferent and supramedullary input as well as intrinsic brainstem processes are thus probably involved in this complex behaviour. Diverse structures at
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several CNS levels are thus involved in the response to hypoxia but the basic mechanism(s) behind the central inhibition of breathing are still unclear. A proposed intrinsic brainstem mechanism is a depression of central respiratory neurones mediated by the release of several neurotransmitters/modulators. These include GABA (Neubauer et al., 1990) and prostaglandins $PGE_2$ (REF). Naloxone and theophylline partially abolish the respiratory inhibition caused by asphyxia, suggesting that opioids and adenosine are involved (Grunstein et al., 1981; Chernick and Craig, 1982; Hedner et al., 1984; Runold et al., 1989)

**Figure 2** Depicts the biphasic ventilatory response to hypoxia (10-15 % $O_2$ in $N_2$). The inhibitory phase is more pronounced in immature animals. Naloxone or theophylline can attenuate the inhibition, suggesting that opioids and adenosine are involved in the secondary inhibition. Administration of dipyridamole, which augments the endogenous levels of adenosine induces a more pronounced hypoxia-induced inhibition. (Modified after Lagercrantz, 1987, Grunstein, 1981, Darnall 1985).
OPIOIDS AND RESPIRATORY DEPRESSION

Opioid-induced respiratory depression is well known from clinical situations as well as from experimental studies (Yeadon and Kitchen, 1989). Administration of exogenous opioids is associated with depression of central respiratory activity in mammals.

Naloxone reverses neonatal depression caused by foetal asphyxia (Chernick and Craig, 1982). Thus, opioids are thought to be involved in hypoxia-induced respiratory depression (Moss et al., 1987) Nevertheless, the role of endogenous opioids in basal respiratory control has not yet been completely established. Some studies using naloxone have demonstrated that endogenous opioids do not have any significant influence on the basal regulation of breathing in rats (Steinbrook et al., 1984; Olson, 1987). Others have come to the opposite conclusion (Isom and Elshowihy, 1982). Recent findings by Greer et al. (Greer et al., 1995) indicate that naloxone has no effect on medullary respiratory control during in vivo or in vitro experimental conditions in newborn rats.

Most studies thus indicate that endogenous opioids are not involved during eupneic breathing but have an important role during hypoxia and postoperative hypoventilation. Opioids have also been proposed to be involved in the pathogenesis of sudden infant death syndrome (Orlowski, 1986; Morin et al., 1992).

Three major classes of opioid receptors μ, δ, and κ have currently, been identified, characterised and cloned, all with putative receptor subtypes. All are seven-transmembrane proteins and members of the G-protein coupled receptor superfamily. Endogenous opioid peptides with distinctive selectivity profiles exist namely the enkephalin (μ), endorphin (δ) and dynorphin (κ) groups. μ-receptor binding sites are present during mid-foetal time and have a high density in cardiorespiratory-related brainstem nuclei, whereas the δ-opioid receptors primarily appear during the postnatal period in rats (Xia and Haddad, 1991).

Opioid-induced respiratory depression has been suggested to be caused by direct actions on the brainstem (Flórez and Hurlé, 1993). Most previous in vivo studies have suggested that μ- and δ-opioid receptors participate in opioid-induced respiratory depression, while κ-opioid receptors are not involved (Shook et al., 1990; Flórez and Hurlé, 1993). However, κ- and μ-opioid receptors have a similar distribution in the brainstem (Mansour et al., 1988) and due to the somewhat unselective receptor agonists used, it is still debated if only μ-receptors are involved in the respiratory depression caused by opioids. Based on a subdivision into μ-1 and μ-2 isoreceptors...
(Pasternak et al., 1980), some authors have claimed that opioid-induced respiratory depression is mediated by μ-2 receptors (Ling et al., 1985; Ling et al., 1986). However, the exact actions of opioids on respiratory activity via activation of μ-, δ- and κ-opioid receptors in the medulla oblongata remain to be elucidated.

ADENOSINE AND RESPIRATORY DEPRESSION

As early as 1929, Drury and Szent-Györgyi reported that adenosine can inhibit respiratory and intestinal movements as well as decrease heart (Drury and Szent-Györgyi, 1929). Adenosine is a constituent of all body fluids, including the extracellular space of the central nervous system. It has multiple effects on organs and cells of the body (Berne, 1986). Thus, its levels are tightly regulated by a series of enzymatic steps (Fredholm, 1995). Adenosine can be regarded more as a neuromodulator in that it does not seem to be stored in vesicles with a regulated release from nerve terminals. Adenosine is produced by dephosphorylation of adenosine monophosphate (AMP) by 5’ nucleotidase, an enzyme occurring in both membrane-bound and cytosolic forms (Brundege and Dunwiddie, 1997). Degradation of intra- and extracellular ATP is the main source of extracellular adenosine (Dunwiddie and Fredholm, 1997). Specific bi-directional transporters maintain intra- and extracellular concentrations of adenosine at similar levels. During basal conditions adenosine levels are 30-300 nM and can rise following stimuli that cause an imbalance between ATP synthesis and ATP breakdown. Thus, the levels during ischemia or hypoxia can rise 100-fold (Winn et al., 1981; Fredholm, 1995).

\[
\begin{align*}
[\text{ATP}]_i & \downarrow \\
[\text{AMP}] & \Leftrightarrow [\text{ATP}]_e \\
\downarrow & \\
\text{Inosine} & \Leftrightarrow [\text{Adenosine}]_i & \Leftrightarrow [\text{Adenosine}]_e \\
\text{bidirectional transportor} & \\
\end{align*}
\]

**Figure 3.** Schematic representation of the degradation of ATP to adenosine
Hypoxia can trigger apnoea in the human neonate (Rigatto et al., 1972; Miller and Martin, 1992), whereas the adenosine antagonist theophylline blocks hypoxia-induced depression of breathing in rats (Neylon and Marshall, 1991), rabbits (Runold et al., 1989) and piglets (Moss et al., 1987; Lopes et al., 1994). The therapeutic effect of theophylline on neonatal apnoea has thus been suggested to be due to its antagonistic action on adenosine receptors (Lagercrantz Y et al., 1984; Darnall, 1985; Hedner et al., 1985). Several lines of evidence indicate that adenosine can inhibit respiration-related neurones in the brainstem (Eldridge et al., 1983; Thomas et al., 1994). However, adenosine also decreases metabolism and oxygen consumption through inhibiting lipolysis and non-shivering thermogenesis (Ball et al., 1996). Neonatal animals respond to hypoxia with decreased body temperature. Thus, it has been suggested that the main effect of adenosine in depressing breathing is indirect by a decrease of oxygen consumption (Lagercrantz et al., 1986).

To date four distinct receptor subtypes have been identified by cloning, denoted A1, A2a, A2b and A3-adenosine receptors. All subtypes belong to the family of rhodopsin G-protein-coupled receptors (Fredholm, 1995). Basal levels of adenosine can act on A1- and A2a-receptors whereas A2b-receptors only are activated at pathological adenosine levels. A2a receptors are coupled to GTP-binding (G) proteins classified as Gs, because of their stimulatory effect on adenylyl cyclas, and mainly expressed in dopamine-rich regions such as the striatum. Adenosine A1-receptors are coupled to Gαi(1-3) (inhibitory to adenylyl cyclas) or G0 (no effect on adenylyl cyclas) proteins and are ubiquitously expressed in the CNS with higher expression in regions such as the cortex, hippocampus and cerebellum (Fredholm, 1995). A1-receptors have been characterised by behavioural, biochemical and anatomical studies, for a review see (Dunwiddie and Fredholm, 1997). A1-receptors can modulate synaptic transmission in the CNS through several different mechanisms. Among these are decreased neurotransmitter release (Mynlieff and Beam, 1994) and reduction of neuronal excitability through hyperpolarisation of neurones (Thompson et al., 1992). Adenosine have been demonstrated to modulate cardiovascular control through its action in the rostral ventrolateral medulla (Thomas and Soyer, 1996). Responses to adenosine are different even in neuronal populations using the same transmitter, which makes it difficult to directly extrapolate data obtained from one group of neurones to another. Thus, a modulation shown for parts of a neuronal network may not be applicable when all its components are examined.

Adenosine is also an important neuromodulator in foetal life (Bissonette et al., 1990; Koos and Matsuda, 1990) and at birth (Irestedt et al., 1989). Changes of
Adenosinergic tonic activity due to caffeine, an adenosine antagonist, may interfere in the communication between developing neurones leading to long term sequela. Caffeine, is the most widely consumed neuroactive substance worldwide. Negative effects on fertility, birthweight, risk for prematurity and congenital malformations of high doses of caffeine have been demonstrated in animals, for review see (Nehlig and Debry, 1994). There is however little evidence that normal human caffeine consumption has any of these consequences.

Does maternal intake of adenosine antagonists, such as caffeine affect the development of respiratory rhythm generation and control during foetal and neonatal life? High doses of caffeine intake during gestation or the early postnatal period affect postnatal behaviour in rats (Holloway and Thor, 1982; Guillet, 1990; Devoe et al., 1993; Guillet and Dunham, 1995). Effects of more moderate prenatal caffeine intake on postnatal behaviour have also been studied, and several investigators have described behavioural hyperactivity in developing rodents (Sobotka, 1979; Sinton, 1981; Holloway Jr, 1982; Holloway Jr and Thor, 1982). If low doses of caffeine during gestation can induce long-term changes in adenosine receptor expression and if the development of respiratory control is affected this may have important clinical implications.

THE BRAINSTEM SPINAL CORD PREPARATION

Studies on respiratory control and the highly sophisticated neuronal networks that generate this complex behaviour can be performed at different levels: whole-body, organ, neuronal networks, cellular, synaptic and molecular. During the last 15 years in vitro experimental preparations have proved invaluable in unravelling the mechanisms of central respiratory control. Among the powerful advantages of in vitro preparations are: 1) direct access to neurones of interest; 2) mechanical stability, allowing intracellular recordings with whole-cell patch techniques; and 3) improved control of the environment surrounding the neurones.

A brainstem-spinal cord preparation from neonatal rats was first used by Suzue and co-workers (Suzue, 1984). This en bloc brainstem spinal cord preparation generates respiration-related rhythmic activity for several hours when perfused with oxygenated artificial cerebrospinal fluid (aCSF). Compared to the in vivo situation, the respiratory frequency is low. Inspiratory-phase discharge in vitro is rapidly peaking-slowly decrementing instead of a short duration, augmenting discharge envelope (Smith et al., 1990). The transformation of the respiratory motor pattern in vivo to that
of the isolated and reduced brainstem spinal cord preparation can be explained by several facts: 1) it does not have suprapontine inputs and can be further reduced by removing the pons; 2) removal of vagal mechanosensory afferent input; 3) low temperature (25-32 °C) (Smith et al., 1990; Onimar, 1995a).

Respiratory rhythm generation in neonatal in vitro models differs from that of adult in vivo models. In this neonatal model Chloride-mediated inhibition is not necessary for the respiratory rhythm whereas it probably is in the adult (OnimaruArata and Homma, 1990; Feldman Smith and L et al.iu, 1991; Onimaru et al., 1997; Ramirez et al., 1997). However, the isolated respiratory network in the brainstem is sufficient to generate the essential features of respiratory activity at the cellular and system levels that have previously been described for the intact respiratory system.

The relevance of neuronal mechanisms studied in this reduced preparation to those operating in the intact neonatal or adult rat has been determined by several studies during the last decade, for reviews see (Smith et al., 1990; Onimaru et al., 1997).

Synaptic events during the respiratory cycle have not been thoroughly analysed in any neonatal mammalian species, so the correspondence between the synaptic events producing the cycle phasing in the neonatal and adult system remains to be established (Smith et al., 1990) The studies that have been performed reveal remarkable similarities in the synaptic interactions between respiratory neurones although recordings from in vivo cat and in vitro rodents were compared. (Ramirez et al., 1997).

Neonatal rats, as all mammals, are born with a functional central pattern generator for respiration. However, the CNS and its connections with the internal and external environments develops before and after birth. As described in the previous pages it is known that both adenosine and opioids have different effects on respiration in the neonate compared to the adult mammal. Are these changes in modulatory effects initiated at the moment of birth, during the first postnatal days or are they dependent on progressive maturation of neuronal networks, receptor expression and affinity? We have in the following studies used the brainstem spinal cord preparation, complemented with in vivo and in situ techniques, to address some of these questions.
THE PRESENT INVESTIGATION

The overall aim of the present study was to further elucidate the perinatal development of central respiratory activity and its control. Specifically, the aims where to:

- investigate if there is a transition at birth of the properties of the isolated neuronal network that generate respiratory rhythm. (IV)

- determine if adenosine and opioids inhibit respiration by acting directly on the central neuronal networks generating respiratory activity. (I-V)

- determine if the adenosinergic and opioidergic modulation of respiratory activity in the medulla oblongata changes during the first days after birth. (I, III-V)

- investigate if chronic maternal intake of caffeine (a typical adenosine antagonist), during gestation and early postnatal life, alters the development of respiratory control. (IV)

- investigate the mechanisms behind and at which level adenosinergic modulation of central respiratory activity occurs, i.e. pre- vs. postsynaptically.(I and V)
MATERIALS AND METHODS

ANIMALS

Rats were bred by certified animal supplier and stored at the animal facilities according to local regulations. Neonatal rats were kept with their mothers until the experiments started. Pups were examined in vitro at postnatal age 0-4 days and at embryonic (E) day 18-21. Rat brains were examined at E14, E18, E 21, exactly 2 hours and 24 hours after vaginal delivery and postnatal day (P)3 and P7.

In the experiments performed in paper IV, examining the development of respiratory control, timed pregnant rats were used. The day when a vaginal plug was found was designed as embryonic day 0. The regional animal ethics committee approved the experiments, which followed the European Community regulations.

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An extended description of the blind whole-cell patch clamp technique, the accompanying histological method as well as the in vivo experiments follows below. The other methods used are described in the papers included.
BRAINSTEM-SPINAL CORD PREPARATION

The in vitro experiments were performed on the brainstems and spinal cords of newborn (0- to 4-day-old) Bkl:S-D or Wistar rats. Under deep ether anaesthesia, the brainstem-spinal cord was dissected and isolated as described previously (Suzue, 1984). The brainstem was then rostrally decerebrated between the VIth cranial nerve roots and the lower border of the trapezoid body. This preparation was subsequently transferred to a 2-ml chamber, where it was continuously perfused at a rate of 3.0-3.5 ml min\(^{-1}\) with artificial cerebrospinal fluid (aCSF) equilibrated with 95% O\(_2\) and 5% CO\(_2\) at 26-28 °C to give a pH of 7.4. Foetal rats were obtained from plugged dams by caesarean section. Respiratory activity was recorded employing suction electrodes applied to the proximal ends of cut C4 or C5 ventral roots containing respiratory motoneurone axons that innervate the intercostal muscles and the ribcage in vivo. (Fig. 2). The phasic C4/C5 activity corresponds to phrenic nerve discharges (Suzue, 1984; Smith et al., 1990). The regularity of respiratory activity was quantified as coefficient of variation (CV) of the interval between C4 burst discharges.

For further details see paper I-V

Figure 4 Brainstem-spinal cord preparation
BLIND WHOLE CELL PATCH CLAMP

Whole cell recordings from respiratory neurones were obtained using the modified blind patch clamp technique (Blanton et al., 1989; Onimaru and Homma, 1992). Briefly, patch pipettes were pulled in one step from borosilicate glass (GC100TF-10, outer diameter 1.0 mm, with a filament; Clark Electromed, Reading, UK) using a vertical puller (PE-2; Narishige, Tokyo, Japan). The electrode tips had inner diameters of 1.2-2.0 µm and a DC resistance of 4-8 MΩ. The electrodes were filled with a solution consisting of (mM): potassium gluconate 120, ethylenediaminetetraacetate (EGTA) 10, 4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid (HEPES) 10, CaCl₂ 1, MgCl₂ 1 and Na₂-ATP 1. KOH was used to adjust the pH to 7.3. In a number of experiments this electrode solution also contained 0.2 % Lucifer Yellow (LY; Sigma, St. Louis, Mo, USA). The solution was routinely filtered through a Millipore filter (pore size 0.45 µm) immediately before its introduction into the electrodes.

A small area of the pia mater was removed using a glass needle and the electrodes were inserted through this area into the ventral medulla. A slight positive pressure (5-15 cm H₂O) was applied during advancement of the electrode to keep the electrode tip clean. Extracellular signals and intracellular membrane potentials were measured with a voltage clamp amplifier (Nihon Koden, CEZ-311). Respiration-related neurones were sought in RVL (Arata et al., 1990) in the region of the ventral respiratory group (Richter et al., 1992), while monitoring extracellular signals amplified with a loudspeaker (Onimaru, 1995b). In whole cell experiments, when such a respiration-related neurone was found, a slight negative pressure was applied. The resulting formation of a gigaohm seal (>1GΩ) was monitored and confirmed by applying a hyperpolarising current pulse (0.1 nA; duration, 30 ms 2 Hz). When this seal had been established, the pressure was returned to zero. Rupture of the cell membrane was achieved by applying negative pressure (50-80 cm H₂O) often together with a single hyperpolarising current pulse (0.5-0.8 nA; duration 30 ms). When whole cell recording had been established the pressure was rapidly returned to zero ±2-3 cm H₂O. Series resistance (20 - 50 MΩ) was compensated by a bridge balance circuit. The voltage deflections induced by 1 Hz 30 ms hyperpolarising pulses were monitored and compensated for by eye.
Neurones were identified and classified on the basis of their characteristic firing patterns and the temporal correlation of this activity to the respiratory cycle of C4/C5 activity. Inspiratory neurones (Insp) receive excitatory synaptic input and discharge action potentials during inspiratory phrenic (C4/C5) activity. Expiratory neurones (Exp) discharge action potentials between the inspiratory phases and are inhibited during the inspiratory phase (Shao and Feldman, 1997). Biphasic expiratory neurones (also classified as Preinspiratory by Onimaru et al.) are characterised by pre- and postinspiratory excitation and inspiratory-related inhibition (Onimaru et al., 1990; Smith et al., 1990).

Inspiratory neurones were further classified into three subtypes, according to previous classifications performed with this preparation (Onimaru et al., 1996; Onimaru et al., 1997). Type I neurones (Insp I) receive excitatory postsynaptic potentials (EPSPs) prior to the onset, as well as after the termination of C4 activity, whereas type III neurones (Insp III) are hyperpolarised by synchronised inhibitory postsynaptic potentials (IPSPs) during the pre- and postinspiratory phases. Insp neurones, which only exhibited EPSPs during the inspiratory phase and no hyperpolarisation during the pre- or postinspiratory phase, were classified as type II neurones (Insp II).

<table>
<thead>
<tr>
<th>Neuronal type</th>
<th>Inspiratory</th>
<th>Peri-inspiratory</th>
<th>Expiratory-I</th>
<th>Expiratory-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insp type-I</td>
<td>++</td>
<td>(+)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Insp type-II</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>(+)</td>
</tr>
<tr>
<td>Insp type-III</td>
<td>++</td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Expiratory-I</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>Biphasic E</td>
<td>-</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 1 Respiration-related neurons in the brainstem spinal cord preparation, here categorised in relation to the C4 inspiratory activity according to Onimaru et al. (Onimaru 1997) * = hyperpolarised, Inspiratory (Insp), Biphasic expiratory (Biphasic E), tonic Expiratory (Expiratory-t), late Expiratory (Expiratory-l)

The current-voltage (I-V) relationship was determined by injection of an inward current (0.02-0.08 nA; duration 100 ms) during silent phases between bursts or during
negative holding potentials (-50 mV) in the case of certain expiratory neurones. Input resistance ($R_m$) was calculated from the slope of a least-squares regression line fitted to the data. In Exp neurones, the frequency (action potentials / min) of expiratory neuronal activity was examined. During the experiments, signals were displayed on a chart recorder, monitored using an oscilloscope, digitalised (Digidata 1200B, Axon Instr., Foster, CA, U.S.) and stored on DAT tape (RD-120TE, TEAC, Tokyo, Japan) or a hard disc for off-line analysis.

**HISTOLOGY**

After completing the *in vitro* recordings, those preparations containing neurones filled with Lucifer Yellow were removed from the recording chamber and placed in a solution of cold (4 °C) Lillie solution (10% formalin in phosphate buffer, pH 7.0) for at least 48 hours. The preparation was then rinsed and placed serially for 10 min each in 5, 10 and 15% sucrose in 0.1 M phosphate buffer (PB, Sorensen-Gomori, pH 7.2). The sample was subsequently stored in the 15% solution at 4 °C for at least 12 hours before 70-µm sections were cut on a cryostat. Sections were mounted on glass slides, coated with PBS and propriogallate. The intracellularly marked Lucifer Yellow-neurones were photographed and reconstructed with the aid of a camera lucida attached to a fluorescence microscope (Olympus, Tokyo, Japan). Counterstaining was performed with 2% neutral red. After dehydration in a series of solutions containing increasing ethanol concentrations, sections were clarified with xylene and a cover was placed on them.

**BAROMETRIC PLETHYSMOGRAPH**

*In vivo* ventilation was monitored with a barometric plethysmograph, a procedure which allows non-invasive recording of respiration in unanesthetized animals (Drorbaugh and Fenn, 1955). This method is based on the fact that warming a gas maintained at a constant volume increases its pressure. Thus, during inspiration, pressure inside the chamber increases and during expiration it decreases. The animals were placed in a plexiglass chamber (160 ml in volume) which, together with an identical reference chamber, was connected to a highly sensitive differential pressure transducer (Validyne DP103). Pressurised gas from gas tubes was continuously fed to the recording chamber and removed with a vacuum pump. The resistance of the inlet and outlet tubing was adjusted with needle valves in order to maintain approximately atmospheric pressure in the recording chamber. The rate of gas flow through the recording chamber was about 1.2 l min$^{-1}$. The time constant of the recording system
was 0.2 seconds. The pressure signal was amplified and recorded by an on-line computer. The measurements of breathing were only semiquantitative because core body temperature was not measured and because of the short time constant of the recording system. Animal and chamber temperatures were recorded using digital thermometers with an accuracy of 0.1°C. At the onset of the experiment the chamber temperature was kept at 29.5 ± 0.5°C via a thermoradiator and a heating pad.

**DRUGS**

The pH was assessed and adjusted to 7.4 with 95% O$_2$ and 5% CO$_2$ prior to bath application of drugs. Sufficient time for achievement of the steady-state drug response, determined for each drug used was allowed to elapse before determination of the drug response. In paper IV dams were given caffeine (0 or 0.3g/L) in the drinking water from embryonic day 2 (E2) throughout gestation and postnatal life. The daily intake of water was measured in all litters. Blood from five litters were collected in heparinized plastic tubes and centrifuged. Plasma concentrations of caffeine and its metabolites theophylline, theobromine and paraxanthine were analysed using HPLC. In paper II 17 animals received a subcutaneous injection (s.c.) of naloxanazine 35 mg/kg (=5 ml naloxanazine solution/kg) and 17 animals received saline 5 ml/kg s.c. 22-26 hours after these injections in vitro studies were performed. See Table 3 for drugs used.
## Table 3. Major pharmacological tools used in this thesis

<table>
<thead>
<tr>
<th>Compound</th>
<th>Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adenosinergic</strong></td>
<td></td>
</tr>
<tr>
<td>R-PIA</td>
<td>$A_1$-adenosine receptor agonist</td>
</tr>
<tr>
<td>Dipyridamol</td>
<td>nucleoside transport inhibitor</td>
</tr>
<tr>
<td>DPCPX</td>
<td>$A_1$-adenosine receptor antagonist</td>
</tr>
<tr>
<td>Theophylline</td>
<td>Adenosine receptor antagonist</td>
</tr>
<tr>
<td>Caffeine</td>
<td>Adenosine receptor antagonist</td>
</tr>
<tr>
<td><strong>Opioidergic</strong></td>
<td></td>
</tr>
<tr>
<td>Morphine</td>
<td>opioid receptor agonist</td>
</tr>
<tr>
<td>DAGO</td>
<td>$\mu$-opioid receptor agonist</td>
</tr>
<tr>
<td>DPDPE</td>
<td>$\delta$-opioid receptor agonist</td>
</tr>
<tr>
<td>U50,488</td>
<td>$\kappa$-opioid receptor agonist</td>
</tr>
<tr>
<td>Naloxone</td>
<td>opioid receptor antagonist</td>
</tr>
<tr>
<td>Nor-BNI</td>
<td>$\kappa$-opioid receptor antagonist</td>
</tr>
<tr>
<td>Naloxanazine</td>
<td>$\mu$-1 opioid receptor agonist</td>
</tr>
</tbody>
</table>

## DATA ANALYSIS AND STATISTICS

Off-line analysis was performed using a personal computer and the commercially available programs Axoscope (Axon Inc., Foster, CA, USA), Origin (Microcal Software Inc., Northampton, MA, USA) and JMP (SAS Inst. Inc., Cary, NC, USA). The results are presented as means ± standard deviations or standard errors of the mean when small or non-normally distributed data are presented. After analysis of the variance by the $F$-test, statistical analysis was performed using the multivariate analysis of variance (MANOVA) repeated measure design, two-tailed paired Student’s $t$-test or Wilcoxon's signed-rank test (when variances were unequal). The differences between several independent means were analysed by comparing all pairs using the Tukey-Kramer HSD test. Spearman rank nonparametric correlation was performed on measured variables with respect to postnatal age, in order to evaluate a possible dependency of the results on age. A $P$ value of $<0.05$ was considered to be statistically significant.
RESULTS AND DISCUSSION

ASPECTS ON METHODOLOGY

The region where respiration-related neurones in the neonatal rat are located is in the ventrolateral reticular formation at depths of 50-500 μm. In the brainstem spinal cord preparation there are substantial gradients of $\text{PO}_2$, $\text{PCO}_2$, $K^+$ and pH from the surface (Brockhaus et al., 1993; Okada et al., 1993; Voipio and Ballanyi, 1997) to the respiratory network but the oxygenation permits the neurones to operate under conditions of aerobic metabolism (Brockhaus et al., 1993). Furthermore, the brainstem spinal cord preparation is sensitive to changes in pH and oxygenation and responds in a similar manner at the neuronal and motor output levels, as do animals in vivo (Ramirez et al., 1998b). The preparation is thus used for studying cellular mechanisms and the localisation of central chemosensitivity as well as the biphasic respiratory response to hypoxia (Kawai et al., 1996; Ramirez et al., 1998b). The preparation is also suited for detailed pharmacological studies of the respiratory centre in anaesthesia-free conditions. Drugs can be applied at defined concentrations to the regions of interest and simultaneous recordings of respiratory output and from individual neurones made.

Although it has several advantages, the in vitro system is a reduced preparation with altered parameters of neuronal function including CNS temperature (25-30 °C). Several parameters of neuronal function, including the kinetics of membrane currents (Hille, 1984) are temperature dependent (Smith et al., 1990). Hypothermia can attenuate or enhance the action of neuromodulators/drugs affecting respiration (Vitez et al., 1974; Puig et al., 1987). The temperature therefore has to be considered when performing experiments using this preparation.

The size of the in vitro preparation is a serious limitation since substantial gradients for oxygen, carbon dioxide and pH may alter the condition of the tissue and its viability (Mitchell, 1993). The in vitro preparation is highly reduced by the removal of afferents and suprapontine inputs. This is desirable in that it excludes several compensatory mechanisms, i.e., cardiovascular changes, that may complicate data interpretation. However, this also alters the state of the central pattern generator by removing tonic inhibitory and excitatory input. Likewise, because of the artificial
environment of the neurones, variables such as pH, oxygenation and steady state have to be taken into consideration when determining effects of drugs.

Thus, data have to be cautiously interpreted and compared with data from in vivo models to determine the relationship between the nervous system behaviour in vitro with that of in vivo systems. We demonstrate below how results depend on time, temperature as well as perinatal age when performing experiments in this model using mammals still in a developmental phase.

**Time dependency of opioid and adenosinergic drugs**

The drug effect has been evaluated after 5 minutes in several of the previous studies. We demonstrate in the present studies (I-III and V) that steady state effect of both adenosinergic and opioidergic drugs can take as long as 10-20 minutes to establish. There are several possible explanations for this: 

- **Drug kinetics**: R-PIA is highly lipophilic, and thus it is possible that R-PIA binds to fat-rich glia before a gradient and slow diffusion establishes steady state concentrations within the region of the respiratory neurones, 50-600 μm below the ventral surface;

- **Receptor recruitment**: recent studies indicate that some G-protein linked receptors increase at the cell surface when stimulated. A1-receptors are recruited from the cytoplasm to the surface of neurones after stimulation (Ciruela et al., 1997). In addition, it has recently been reported that activation of dopamine D1-receptor, also G-protein coupled, induces a time- and dose-dependent recruitment of D1-receptors to the cell surface (Brismar et al., 1998). Thus, an alternative explanation for the time dependency could be that the full effect of ligands first is reached when ligand-induced aggregation of receptors to the cell surface is completed. However, our studies cannot determine the mechanisms for this time dependency, but nevertheless underline the importance of taking time into account when determining drug effects in this preparation.

**Perinatal age and experiments**

The inhibitory effect of morphine on the respiratory rhythmic activity increased with postnatal age (P0-P4) (III) while the depressant effect of adenosine on respiration decreased during both foetal (E18-E21) and postnatal life (P0-P4) (I, IV and V). Age has a significant effect on both control respiratory activity (I and IV) and the effects of
drugs (I-V) during the first postnatal days. This fact has been neglected in several studies using the in vitro brainstem spinal cord preparation. (Murakoshi, 1985; Greer et al., 1995). These findings will be discussed in the context of development of respiratory control later on.

**OPIOIDS AND RESPIRATORY DEPRESSION**

**Opioids inhibit respiration by μ- including μ-1 and κ-opioid receptors**

To study the effects of opioids on brainstem opioid receptors effects of the μ-receptor agonist DAGO, the δ-receptor agonist DPDPE and the κ-receptor agonist U50,488 were evaluated in the brainstem spinal cord preparation (II and III). The opioid receptor antagonists naloxone (μ) and naloxanazine (μ-1) were also used. DAGO reduced respiratory frequency and inspiratory time in a concentration-dependent manner and at high concentrations (10 μM) caused a reduction of peak integrated C4 amplitude. The μ-1 receptor antagonist naloxanazine shifted the concentration-respiratory frequency response curve for DAGO to the right (p<0.05). Thus, higher concentrations of DAGO were needed to give an equivalent respiratory depression as that in animals not pre-treated with the μ-1 antagonist. DPDPE had no effect on respiratory activities whereas U50,488, like DAGO, reduced respiratory frequency and amplitude of integrated C4 in a concentration-dependent manner. Thus, μ-opioid receptors, including the μ-1 receptors are involved in respiratory frequency reduction whereas κ-opioid receptors are involved in reduction of both respiratory frequency and respiratory amplitude. δ-opioid receptors do not seem to participate in respiratory modulation in the neonatal rat.

The finding that a κ-opioid receptor agonist depresses respiration is in conflict with the findings of Greer et al. (Greer et al., 1995). However, it should be noted that these authors only conducted their measurements 5 min after drug application (Greer et al., 1995) while our evaluations were performed after 20 min. This methodological difference most certainly explains the discrepancy between the studies.

Our results are in agreement with previous findings in adult rats in which microinjection of κ-opioid receptor agonists into ventral medullary structures reduced $V_t$ and respiratory frequency (Hassen, 1984). Nevertheless, most of the previous in vivo studies (Leighton et al., 1987; Yeadon and Kitchen, 1990; Dosaka-Akita et al.,
Results and Discussion

1993) have indicated that κ-opioid receptors do not participate in opioid-induced respiratory depression. This discrepancy between the results of these in vivo studies and the present in vitro study suggest that suprabulbar structures may modify medullary κ-receptors-mediated respiratory depressant effects. If κ-receptors are involved in the respiratory depression of opioids this is important knowledge in the development of future opioiergic analgesia without respiratory depressant effects.

Temperature and respiratory control

The temperature normally used in the different mammalian in vitro preparations is 25-26 °C. The low temperature compared to in vivo physiological conditions is used to decrease oxygen consumption and to sustain regular respiratory activity for long periods of time. Reduced temperature may have consequences beyond the desired effects of reduced metabolic rate and prolonging the viability of the preparation. Low temperatures may alter membrane fluidity, thereby altering membrane bound proteins including receptors and ion channels. (Mitchell, 1993). In paper III we examined the possible influences of temperature on respiratory depression induced by opioids. We demonstrate that lower temperature markedly reduced the ability of morphine (Fig. 6) and DAGO to decrease respiratory frequency. Changes in temperature did not affect the dose-response curve for κ-receptor activation by U50,488. We conclude that the respiratory effect of morphine and DAGO via activation of medullary μ-opioid receptors is temperature-dependent.
Figure 5 Temperature dependent respiratory depression by morphine

The influence of change in temperature on the C4 activity (upper trace) integrated C4 (lower trace) and their response to morphine 50μM. A) the respiratory activities recorded from the in vitro preparation in standard solution at 25.5 °C. B) 20 minutes after increasing temperature to 28.5 °C) 30 min after perfusion with morphine 50μM D) 30 min after decreasing chamber temperature to 25.5 °C.
The reduced respiratory frequency, caused by morphine in our study, is in agreement with what has been previously reported (Murakoshi, 1985). Hitherto, not much has been described about effects of temperature on opioid receptor mechanisms. It has been proposed that the affinity of the μ-receptor for opioids can be modified at different temperatures (Garaulet et al., 1992). Since receptor proteins can undergo conformational changes due to temperature changes and, hence, alter their affinities for the opioid molecule, this could explain our findings. However, the affinity of naloxone for opioid receptors is not temperature dependent (Puig et al., 1987; Kuemmerle and Makhlouf, 1992). This concords with previous studies of intestinal muscle preparations, in which the potency of DAGO (μ-agonist), DPDPE (δ-agonist) and morphine decreased with a lower temperature while in the range from 37-30 °C (Puig et al., 1987; Garaulet et al., 1992).

The opioid receptor is a classic seven transmembrane receptor with three extracellular loops deciding ligand specificity (Befort, 1997). μ- and δ-agonists require the third extracellular loop for binding. κ-selectivity lies primarily within the 1st and 2nd extracellular loop which may gain structural stability from the cysteine-cysteine bridge connecting these extracellular receptor domains (Traynor, 1996; Befort, 1997). A speculative explanation of the selective μ-receptor temperature dependency could be that the third extracellular loop, which is not stabilised with a disulphide bridge, is more susceptible to changes in the temperature. Thus, instability of the third extracellular loop of the opioid receptor may lead to conformational changes with temperature and alter the affinity for μ- and δ-agonists.

Our observations of temperature dependent opioid receptor function are of clinical interest since opioids are frequently used in patients, not least during hypothermia for cardiopulmonary bypass surgery. In the clinical setting analgesia and not respiratory depression is the goal. With large doses of opioids administered during hypothermia, significant side effects will appear at normothermia. Ling et al. (Ling et al., 1985) suggested that opioid-induced analgesia and respiratory depression are mediated via activation of different subtypes of μ-opioid receptors. According to their in vivo study, opioid-induced analgesia was linked to μ, opioid receptor activation and respiratory depression was linked to μ. However, using this brainstem spinal cord
Brainstem perinatal respiratory activity

preparation we demonstrate that the medullary $\mu_1$ opioid receptors also participate in respiratory frequency reduction (III).

This observation links analgesia with respiratory depression. Clinically it is well known that the respiratory depressant effect of opioids does not appear until significant pain relief is achieved. Such a clinical effect is straightforward and logical if the two modalities, analgesia and respiratory depression, are mediated via the same receptor. Our results are not necessarily in disagreement with the observation of Ling et al., who reported that $\mu_1$-receptors have an impact on respiratory timing. However, it challenges the assumption that pain relief via $\mu$-receptors can, one way or another, be separated from respiratory depression (Ling et al., 1985).

Do opioids and adenosine interact in respiratory depression?

Some of the pharmacological effects of morphine seem to be due to the presynaptic modulation of transmitter release of substances such as acetylcholine, substance P and adenosine (Jessell and Inversen, 1977; Sawynok et al., 1989; Taguchi et al., 1993). In the spinal cord a component of the antinociceptive action of morphine is due to the release of adenosine (Sollevi, 1997; Sawynok, 1998). Thus to test the hypothesis that part of the effect of morphine on respiration is mediated by indirect effect of other transmitters / modulators, we tried to reverse the morphine-induced respiratory depression by adding adenosine antagonists, substance P and acetylcholine. Acetylcholine (10 $\mu$M) and substance P (50 nM) could partly, but not fully, reverse the morphine-induced reduction of respiratory frequency via respiration-related structures in the rostral medulla (Takita, Herlenius et al. unpublished observations). However, the adenosine receptor antagonist theophylline (100 $\mu$M) does not affect morphine-induced respiratory depression at all. This would suggest that acetylcholine, substance P and adenosine are not crucially involved in morphine-induced respiratory depression.

ADENOSINE AND RESPIRATORY DEPRESSION

Adenosine levels increase during hypoxia and have been proposed to be involved in hypoxia-induced depression of breathing due to its inhibitory action on the central
nervous system. The following results describe where and how adenosine can modulate respiration through respiration-related neurones in the brainstem.

**Adenosine modulates respiratory activity by acting directly on brainstem respiration-related neuronal networks**

In paper I we demonstrate that R-PIA and dipyridamole decrease the activity of inspiratory neurones (I-neurones) and the C4 respiratory burst rate. Theophylline or DPCPX reverse the effects of both R-PIA and dipyridamole on C4 respiratory rate and intra-burst fq of I-neurones. Thus, adenosine depresses both the I-neurones in the RVL and the respiratory motor output. This depression of I-neurones and respiratory rate can be abolished by theophylline primarily through a blockade of medullary adenosine $A_1$ receptors. These results were confirmed and also shown to be valid in the foetal period in papers IV and V.

The studies presented in this thesis, demonstrate that endogenous adenosine modulates respiration by acting directly on the neuronal network for breathing rhythmogenesis (I, IV and V). This does not exclude the possibility that adenosine also may inhibit respiration via an indirect effect on body temperature and metabolism (Lagercrantz et al., 1986). However, as indicated in a recent study, the decrease in metabolism that occurs during hypoxia does not seem to be responsible for the decrease of respiration induced by hypoxia (Rehan et al., 1996). Our findings confirm and verify previous suggestions that adenosine modulates respiration through an action on medullary respiratory networks (Eldridge et al., 1985; Thomas et al., 1994).

Theophylline and caffeine are adenosine antagonists that cause ventilation to increase when given systemically (Aranda and Turmen, 1979; Eldridge et al., 1983). Furthermore, theophylline can attenuate both the hypoxia-induced depression of respiration *in vivo* (Darnall, 1985; Runold et al., 1989) and the depression of C4 motoneuronal discharge *in vitro* (Kawai et al., 1995). This indicates that part of the respiratory decrease induced by hypoxia (Cross and Warner, 1951; Haddad and Mellins, 1984) can be explained through increased adenosine levels and $A_1$-R mediated inhibition of respiration-related neurones in the RVL. This finding is important since two of the most widely used drugs to treat apnoea of prematurity are theophylline and caffeine (Aranda and Turmen, 1979). This also implies that the decreased incidence of neonatal apnoeas during theophylline treatment is mainly due to the antagonistic effect of theophylline on $A_1$-receptors in the medulla oblongata.
Adenosine modulates the synaptic activity in brainstem respiratory neurones

What are the mechanism(s) behind adenosinergic depression of respiration? After having found that adenosine acts on respiratory neurones in the rostral ventrolateral medulla to depress respiratory output we wanted to clarify the mechanisms behind this depression. To be able to determine synaptic events and changes in membrane properties of individual neurones intracellular recordings were necessary. To achieve this goal, the modified blind whole cell patch-clamp technique was used on respiratory neurones identified by their characteristic firing patterns and correlated in time to the respiratory cycle of C4/C5-activity. This allowed us to investigate adenosinergic effects on membrane potential and resistance of RVL neurones, simultaneously recording changes in C4 respiratory output. Lucifer Yellow labelled neurones were located 50-500 μm below the closest ventral surface and ventral of the ambiguous nucleus, in agreement with previous findings (Arata et al., 1990). Camera Lucida reconstruction of the major respiratory neurone subtypes found in vitro are illustrated in Fig. 6.

Figure 6. Camera Lucida reconstruction of respiratory neurones: biphasic expiratory (Pre-I) and expiratory neurone in proximity of an inspiratory neurone.

In paper V, we demonstrate that R-PIA consistently causes a reduction of spontaneous synaptic activities during the expiratory phase in all RVL neurones examined. Figure 7 (modified from paper V) depicts the characteristics of major
groups of respiration-related neurones found. The A₁-R agonist and antagonist induced modulation of excitatory postsynaptic potentials did not correlate with changes in membrane potential ($E_m$) or input resistance ($R_m$). This indicates that adenosine depresses respiration through presynaptic adenosine A₁-receptor mediated inhibition of synaptic transmission in the medulla oblongata. This is in agreement with the now well established role of adenosine as a regulator of CNS synaptic transmission (Snyder, 1985; Brundege and Dunwiddie, 1997; Dunwiddie and Fredholm, 1997). Several possible mechanisms exist for by which presynaptic A₁-receptor can decrease transmitter release (Brundege and Dunwiddie, 1997). One of these is mediated via a G-Protein dependent inhibition of N-type Ca$^{++}$ channels (Mynlieff and Beam, 1994). This will decrease Ca$^{++}$-induced transmitter release. Other mechanisms not involving Ca$^{++}$ entry from the extracellular space exist (Brundege and Dunwiddie, 1997). However, it has not been within the scope of the present studies to determine the exact mechanisms following presynaptic A₁-R activation. Nevertheless, we indicate here that the decreased synaptic activity by adenosine observed in other parts of the brain (Brundege and Dunwiddie, 1997) is also valid for the medullary central pattern generator for breathing.
Inspiratory (Insp) neurones receive excitatory synaptic input (EPSPs) and discharge action potentials during inspiratory C4 ventral root activity (C4). Insp-I neurones receive EPSPs prior to and after termination of C4 activity whereas Insp-III neurones are inhibited during both these phases. Biphasic expiratory (Biphasic E) are characterised by pre- and postinspiratory excitation and inspiratory related inhibition. Expiratory (Exp) neurones are inhibited by hyperpolarising IPSPs during the inspiratory phase and either discharge tonically in the expiratory phase (Exp-i) or receive continued inhibition and discharge primarily in the late expiratory phase.

Figure 7 Respiration related neurones in the neonatal rat brainstem
Figure 8    Irregular C4 respiratory activity (bottom) and discharges of a biphasic expiratory neurone, discharging before and after C4 and hyperpolarised during C4 inspiratory activity, preparation from new-born pup (2 hours after birth). Note the regular distance between neuronal discharge burst and how the long postinspiratory discharges
Adenosine and postsynaptic depression of respiration-related neurones

Except for reduced synaptic noise, evident in all neurones, the effects of adenosinergic agents were different between the major groups of respiration-related neurones. Expiratory neurones demonstrated a reversible decrease in input resistance, a depression of action potential discharges and a hyperpolarisation of the membrane potential during application of R-PIA. Similar responses of input resistance and $E_m$ to R-PIA were apparent after synaptic activity had been blocked by 0.5 μM tetrodotoxin (TTX). As a group, biphasic expiratory (biphasic E) and inspiratory neurones demonstrated no changes in input resistance or membrane potential during R-PIA application with or without TTX present. However, whereas the response to adenosinergic agents was uniform among inspiratory neurones, the response of biphasic E neurones varied. In some biphasic E neurones the synchronisation between neurone burst discharges and C4/C5 activity was reversibly disturbed by R-PIA. R-PIA induced a reversible hyperpolarisation in a third of Biphasic E neurones examined. Some of these neurones are characterised by a high level of excitatory (subthreshold) synaptic input and a reduction of ongoing excitatory postsynaptic potentials could lead to an apparent hyperpolarisation of the membrane potential. The fact that only one out of 11 Biphasic E neurones demonstrated a change in input resistance supports this hypothesis. The effects of R-PIA on respiratory activity, input resistance and membrane potential could be reversed by the $A_1$-receptor antagonist DPCPX (200 nM).

Our data suggest that the modulation of respiratory output induced by adenosinergic agents can be explained by: 1) a general decrease in synaptic transmission between medullary respiration-related neurones mediated by presynaptic $A_1$-receptors; and 2) an inactivation, via membrane hyperpolarisation, of medullary expiratory neurones mediated by postsynaptic $A_1$-receptors.

Expiratory neurones are hyperpolarised and inactivated through postsynaptic $A_1$-receptors. This inactivation of expiratory neurones is similar to what has been observed in vivo with intracellularly injected adenosine inducing a postsynaptic membrane hyperpolarisation and inactivation of expiratory neurones (Schmidt et al., 1995). Also in vitro, during hypoxic conditions, the expiratory neurones are reversibly
inactivated as demonstrated in the isolated neonatal mice respiratory network (Ramirez et al., 1998b). Thus, it is plausible that our in vitro findings could partially explain in vivo response to hypoxia. Adenosine has been proposed as a link between cellular energy metabolism and the excitability of neurones (Lloyd et al., 1993), since breakdown of ATP increases substantially during hypoxia and more adenosine is formed. Adenosine is thus considered as a retaliatory metabolite by which cells communicate their energy status to surrounding tissues (Newby, 1984).

**Inhibition of expiratory neurones does not abolish the respiratory rhythm**

As stated above, adenosine causes: 1) a general decrease in synaptic transmission between medullary respiration-related neurones mediated by presynaptic A$_1$-receptors; and 2) an inactivation, via membrane hyperpolarisation, of medullary expiratory neurones mediated by postsynaptic A$_1$-receptors. Inactivation of tonic and late expiratory neurones only modulates but does not abolish the generation of respiratory rhythm in vitro. These findings suggests that these neurones per se are not necessary for respiratory rhythm generation. This is consistent with the view that respiratory rhythm is generated primarily by inspiratory-related neurones (Bradley et al., 1975; Wyman, 1977; Richter, 1982) and that medullary expiratory neurones are less important for the rhythmogenesis of respiration. It is contradictory to network models for the generation of respiratory rhythm in adult mammals, which have phasic and reciprocal inhibitory interactions between inspiratory and expiratory neurones as a key feature (Ezure, 1990; Bianchi et al., 1995). However, the respiratory generation matures during the first two postnatal weeks (Paton and Richter, 1995) and the neurones in the reduced neonatal in vitro preparation may respond differently than in adult in vivo systems. Nevertheless, as demonstrated by Richter et al. (Richter et al., 1993), most expiratory type 2 neurones in the adult cat are inactivated by membrane hyperpolarisation and stop discharging actionpotentials during hypoxia. As adenosine levels increase during hypoxia (Winn et al., 1981) a possible explanation would be that expiratory neurones in vivo as well as in vitro are not essential for respiratory rhythm and can be inactivated by hypoxia-induced increased adenosine levels.
Figure 9  Proposed model for the actions of adenosine A₁-receptors (A₁-R) in the respiratory network of neonatal rats. Respiratory neurones are categorised into three subgroups: inspiratory (INSPI), peri-inspiratory (PERI-I) and expiratory (EXP).

1. Presynaptic A₁-R decrease neurotransmitter release through inhibiting Ca²⁺-channels (N-type) (Mynlieff and Beam, 1994). 2. Postsynaptic A₁-R hyperpolarises and inactivates expiratory neurones via a decreased K⁺-channel conductance (Thompson et al., 1992). This decreases the overall synaptic transmission in the network without stopping the respiratory rhythm generation. Identified components of the CPG for respiration in neonatal rats in vitro and their connectivity. (Inspiratory neurone (Insp), late expiratory neurone (Exp), tonic expiratory neurone (Exp), biphasic expiratory (Biphasic E). The Biphasic E neurone is also characterised as Pre-inspiratory neurone (Onimaru et al., 1987). (Modified, with kind permission of the authors, after (Onimaru et al., 1997).
DEVELOPMENT OF RESPIRATORY CONTROL

The neuronal network generating respiratory rhythm does not undergo major changes in the early perinatal period.

In paper IV we show that an irregular rhythmic respiratory activity is established in the foetal rat at embryonic day 18. The regularity of respiratory activity, measured as a decrease in the coefficient of variation (CV) of the interval between C4 burst discharges, increased with postnatal age (P<0.01). Also time of inspiratory activity in each C4 burst (T_i) increased with postnatal age (ANOVA P<0.05). On E21 a respiratory pattern resembling that after birth had been established with regard to pontine inhibition, control frequency and T_i. Mean frequency of respiratory activity did not differ between age groups when pons remained, but was significantly slower on E18 after ponto-medullary transection. Removal of pons resulted in a significant increase in frequency (from 2.3±1.4 to 9.1±3.1 burst/min, P<0.0001). The central pattern generator for respiratory rhythm in medulla oblongata and its inhibition from pontine structures is thus active on E18. Respiratory rhythmic activity in medulla oblongata matures pre- and postnatally with regard to both frequency and regularity of respiratory activity. Pontine inhibition of this respiratory rhythm do not seem to change from E21 to P3. Our results confirm previous results concerning the development of respiratory rhythm generation in the foetal and neonatal rat (Di Pasquale et al., 1992; Greer, 1992). Previously the inhibition by pons on respiratory activity in the medulla oblongata has been demonstrated to exist on E20 (Di Pasquale et al., 1992) but we find it already on E18.

In addition, the depressant effects of an adenosine A_1-receptor agonist decrease between E18 to P3. This parallels the increased regularity of respiratory rhythm and a possibility could be that a weaker adenosinergic influence on respiration increase its regularity. However, the increased maturation of dendrites and synaptic connections in the developing neuronal networks is a more plausible explanation (Bryan and Bryan, 1978). Thus, the decreased role of adenosine in modulating respiration could either be due to changes in receptor affinity with development (Runold et al., 1986), or that more mature neuronal network are less susceptible to the influences of an inhibitory modulator such as adenosine.
The development of respiratory activity and chronic maternal intake of caffeine.

The caffeine treated group differed significantly from control in their basal respiratory frequency, which was higher than control. Furthermore, the pontine inhibition of brainstem generated C4 activity was more pronounced in the caffeine group (MANOVA, repeated measures design; P<0.05) (Paper IV Fig. 2C). Furthermore, chronic exposure to a low dose of caffeine increased the pontine inhibition of breathing activity and changed the response to an anoxic challenge without detectable changes in A1-receptor ontogeny. Furthermore, this pontine inhibition is dependent on age and is influenced by chronic caffeine intake.

The *in vitro* findings are strengthened by the *in vivo* findings of a reduced gasping frequency during anoxia in the P7 caffeine group. Pons is involved in the respiratory depression during hypoxia (Okada *et al.*, 1998). Thus, it is possible that this reduced gasping frequency reflects an increased hypoxic respiratory depression mediated by structures in the pons (Okada *et al.*, 1998).

Even if the levels of adenosine A1-receptors increase with age and high levels are encountered in pons-medulla in postnatal life (Fig 8) the effects of adenosine on respiration decrease. It is difficult to quantify the effects of adenosine. The modulation of synaptic transmission and neuronal membrane properties involve a complex cascade of mechanisms mediated by several neuromodulators (Lagercrantz, 1987; Feldman, 1990). Changes may be direct by A receptors or indirect effects on other transmitter systems. Our data indicate that receptor expression is not equivalent to functional importance. This may be due to that the expression of receptors with increasing postnatal age are relatively lower in areas and neurones involved in respiratory regulation compared to surrounding brainstem regions. Post-translatory changes or changes in network properties may also be involved.
GENERAL DISCUSSION

Before birth the isolated respiration-related neuronal networks in the brainstem generate a similar output as that during the first postnatal days (IV). Thus, changing respiratory activity at birth from intermittent to continuous breathing does not seem to depend on major changes in the network.

Birth brings the foetus into contact with a totally different environment than that \textit{in utero}. New tactile stimuli, light, lower temperature, removal of the umbilical circulation, arousal, gas with oxygen and carbon dioxide in the upper and lower airways, increased pulmonary blood flow, increased oxygen consumption, vagal input from mechanoreceptors are all involved in the initiation and maintenance of breathing (Blanco, 1991). The decreased inhibition from neuromodulators such as adenosine and prostaglandin and increased excitatory afferent input after birth has given rise to the expression “the inhibited state of the foetus”.

As is apparent from the present studies, the modulatory effects of adenosine on central respiratory rhythm generation decrease within the first two postnatal days (I, IV and V). A decreased sensitivity to adenosinergic agents in the neonatal rat after birth could explain our observations. This concords with earlier findings in neonatal rabbits (Runold \textit{et al.}, 1986) and piglets (Elnazir \textit{et al.}, 1996), in which a considerably more pronounced effect of adenosine agonists on respiration was evident in newborns (1-3 days) compared with older animals (8 days and 3 weeks). Furthermore, the present studies indicate that the decreasing sensitivity for adenosine may contribute to the maintenance of continuous breathing during the first postnatal days. This can be concluded based on the facts that: 1) adenosine levels ought to decrease as partial pressure of oxygen in arterial blood rapidly increases after birth; 2), as presented in our studies (I, IV and V), the effect of A1-R activation on central respiratory activity decreases during the first two postnatal days.

The instability of breathing in the new-born period has been attributed to the immaturity of neuronal networks (Bryan and Bryan, 1978). Thus, according to this view the increased regularity of the respiratory rhythm can be partly explained by the increased organisation of central neurones as well as the afferent input. The increased regularity of control respiratory rhythm activities during the perinatal period investigated in our studies (E18-P7) may be explained by a maturation of the central neuronal networks. This maturation seems in part to be independent of birth since the
increased regularity of central respiratory output in postnatal life has already started in foetal life (IV). The CV

During pre- and postnatal brain development the brain becomes bigger because neurones grow in size, and the number of axons and dendrites as well as the extent of their connections increases (Shatz, 1992). To achieve the precision of the adult pattern, neuronal function is necessary.

There is a general requirement for neuronal activity to transform immature neuronal circuits into the organised connections that subserve adequate brain function (Katz and Shatz, 1996). A key function for neurones is to communicate with other neurones. “Neurones that fire together wire together while the others are left in the sink”. Changes in the tonic adenosinergic modulation of synaptic transmission by caffeine may interfere in the normal development of neuronal circuits. Rat pups whose mothers drank caffeine had a slightly changed development of respiratory activity and its control. A speculative explanation to these findings may thus be that the changes are not due to changes in receptor development but in the development of neuronal networks.

It may be of functional significance that vital brainstem neurones and their resulting respiratory outputs remain active, while overall synaptic transmission in the network is decreased. This will attenuate local neuronal damage induced by hypoxia and at the same time increase the time for respiration to possibly restore oxygen saturation to normal levels. Overall, adenosine decreases oxygen consumption and has neuroprotective effects (Fredholm, 1997) without itself stopping respiratory rhythm generation. The extensive use of xanthines may thus have unwanted side-effects and should, may be first be used as a secondary choice of treatment when equivalent alternatives exists.
CONCLUSIONS

• The central respiratory rhythm generation does not undergo major changes in the perinatal period around birth.

• Adenosine and opioids depress respiration by acting directly on the neuronal networks in Medulla Oblongata generating respiratory activity. Opioids depress respiration through μ- and κ-opioid receptors in the ventral Medulla Oblongata. The potency of μ-, but not κ-opioid receptors to depress respiration is temperature dependent and increases between 22.5-30.5 °C.

• The modulatory effect of adenosine and opioids on respiration changes during the first postnatal days. Whereas adenosinergic depression of respiratory activity is inversely correlated to perinatal age, the effects of morphine increases with postnatal age.

• Chronic maternal intake of caffeine during gestation does not affect A₁-receptor development but the development of central respiratory activity and its modulation by pontine structures in vitro is altered. Most likely this applies also in vivo since caffeine treated pups, at a postnatal age of 7 days, have a reduced anoxia-induced-gasping. This indicates that changes in pontine inhibition of respiratory neurones may have long-term functional consequences. This raises new questions of the extensive use of caffeine during pregnancy and in treatment of neonates.

• Adenosine depresses respiration through presynaptic adenosine A₁-receptor-mediated inhibition of synaptic transmission in the medulla oblongata. Furthermore, postsynaptic A₁-receptors hyperpolarises and inactivates tonic and late expiratory neurones but only modulates the generation of respiratory rhythm in vitro, thus indicating that these neurones per se are not necessary for respiratory rhythm generation.
Brainstem perinatal respiratory activity

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“NO BREATHE, NO LIVE” – The KARATE KID II
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