Meeting abstracts
Neural control of breathing

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ORAL PRESENTATIONS – SESSION 1
Ontogeny and phylogeny of respiratory control

1.1

Early development of respiratory rhythm generation in mice and chicks

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Breathing in mammals starts in the foetus and acquires a vital importance at birth. The ability to produce rhythmic motor behaviours linked to respiratory function is a property of the brainstem reticular formation, which has been remarkably conserved during the evolution of vertebrates. Therefore, to understand the biological basis of the breathing behavior, we are investigating conservative developmental mechanisms orchestrating the organogenesis of the brainstem. In vertebrates, the hindbrain is one of the vesicles that appears at the anterior end of the neural tube of the embryo. Further morphogenetic subdivision ensues whereby the hindbrain neuroepithelium becomes partitioned into an iterated series of compartments called rhombomeres. The segmentation process is believed to determine neuronal fates by encoding positional information along the rostro-caudal axis. Before and at the onset of segmentation, genes encoding transcription factors such as *Hox*, *Krox-20*, *kreisler*, are expressed in domains corresponding to the limits of future rhombomeres. Inactivation of these genes specifically disturbs the rhombomeric pattern of the hindbrain. The presentation will address the problem of whether this primordial rhombomeric organisation influences later function of respiratory control networks in chicks and mice.

Experiments were performed in embryos and after birth in transgenic mice. They show that, although expression of developmental genes and hindbrain segmentation are transient events of early embryonic development, they are important for the process of respiratory rhythm generation by brainstem neuronal networks. We have found in chick that at the end of the period of segmentation, the hindbrain contains neuronal rhythm generators that conform to the rhombomeric anatomical pattern. We have also identified a minimal rhombomeric motif allowing the post-segmental maturation of a specific (GABAergic) rhythm-promoting circuit. Furthermore, *in vivo* and *in vitro* analysis of neurons in transgenic mice revealed postnatal respiratory phenotypes associated with defects of central pontine and/or afferent respiratory control in *Krox-20*, *Hoxa1* and *kreisler* mutants. Neonatal respiratory phenotypes are also induced in mice by treatment with low doses of retinoic acid that slightly change the early embryonic development of the Pons. Altogether, these experiments indicate that segmentation-related specifications of the hindbrain rhythmic neuronal network influences the respiratory patterns after birth. Therefore, early developmental processes have to be taken into account to understand normal and pathological diversity of the breathing behaviour in vertebrates.

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1.2

Development of gill and lung breathing in amphibia

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In the 25 morphological stages of larval bullfrog development there exists a gradual transition from gill to lung ventilation associated with a developmental decrease in the contribution of the skin in gas exchange. Bath application of GABA and/or glycine inhibited gill but not lung burst activities of cranial nerve (CN) VII in the premetamorphic (stages 16–19) *in vitro* tadpole brainstem preparation [1]. It was proposed that the neural basis of gill rhythmogenesis involved network inhibition, whereas lung rhythmicity was pacemaker driven [1]. Bath application of a bicuculline/strychnine solution abolished gill and enhanced lung bursting in stages 16–19 *in vitro* [1]. Bath application of the $GABA_B$ receptor antagonist 2-hydroxy-saclofen disinhibited the lung central pattern generator (CPG) resulting in precocious lung bursting patterns as early as developmental stage 6 [2].

We recorded efferent activity from CN VII and spinal nerve (SN) II in the *in vitro* tadpole brainstem preparation in three successive developmental groups (3–9; 10–15; 16–19) before and after bath application of a 10 μM bicuculline and 5 μM strychnine solution. We also exposed the brainstem to bath pH 7.4, 7.8, and 8.2 before and after bath application of bicuculline/strychnine. Bicuculline/strychnine produced lung ventilatory bursts in all developmental stages tested, indicating the presence of the lung CPG as well as excitatory synapses to respiratory motor neurons as early as stage 3.

We also designed an experiment to examine the importance of lung ventilation on the developmental shift from gill to lung bursting. Two groups of tadpoles were hatched from eggs. Control tad-

poles had free access to the air-water interface throughout development, whereas "barrier" tadpoles were denied access to the air-water interface *via* the placement of Plexiglas 2.5 cm below the surface of the water. CN VII and SN II efferent activities were recorded *in vitro* at bath pH 7.4, 7.8, and 8.2 before and after bath application of 10 μM bicuculline and 5 μM strychnine. Postmetamorphic barrier tadpoles exhibited different motor patterns than stage-matched controls. Tadpoles reared in the barrier condition to stages 20–25 possessed fictive gill and lung ventilatory activities of premetamorphic tadpoles. All barrier tadpole preparations exhibited robust, spontaneous lung burst activity following bath application of bicuculline/strychnine.

We propose that developmentally dependent GABA- and glycinergic mechanisms lead to disinhibition of the lung CPG such that early in development gill motor patterns are the dominant respiratory rhythm, whereas in late development the lung CPG is the dominant respiratory rhythm. Denying the tadpole the ability to "practice" lung breathing during metamorphosis produces a morphologically correct postmetamorphic tadpole with an immature, or premetamorphic respiratory rhythm. We propose that prevention of lung inflation in barrier tadpoles leads to premetamorphic levels of GABA- and glycinergic inhibition, and that this inhibition may be altered by pulmonary stretch receptor feedback in control tadpoles.

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1.3

Phrenic motoneuron and diaphragm development during the perinatal period

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Several key events in the development of the perinatal rat phrenic nerve and diaphragm have been determined, including the following: i) Fetal inspiratory motor discharge commences within the phrenic motoneuron (PMN) pool on embryonic day (E)17; gestation period is 21 days. ii) Phrenic axons grow to innervate the full extent of diaphragmatic musculature by E17–E18. iii) There is a radical maturation of PMN morphology during the period from E16–E20. We have subsequently gone on to examine functional changes by examining PMN electrophysiological and diaphragm contractile properties prior and subsequent to these pivotal developmental stages (E16–P0).

Summary data will be presented demonstrating the following: 1) As PMNs develop from E16 to P0, there are changes in passive membrane properties; resting membrane potential becomes hyperpolarized by ~10 mV, the input resistance decreases ~3-fold, and the mean rheobase increases by a factor of ~2.6. 2) There are significant changes in the amplitude, duration and the afterpotentials of action potentials from E16–P0 which places restrictions on the repetitive firing patterns of fetal PMNs. 3) The changes in PMN firing properties are primarily due to age-dependent changes in the expression of voltage-sensitive calcium and calcium-activated potassium currents. 4) Both dye and electrical coupling have been detected amongst subpopulations of PMNs between ages E16 and P0. 5) There are marked changes in diaphragm muscle contractile properties that develop in concert with PMN repetitive firing properties so that the full-range of diaphragm force recruitment can be utilized at each age and potential problems of diaphragm fatigue are minimized.

Data presented will be derived from the following references:

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1.4

Central neuromodulation and adaptations during respiratory development

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Consecutive daily hypoxia in developing swine results in a relatively lower hyperventilatory response than does a single exposure to the same hypoxic protocol [1]. We have hypothesized that this relative hypoventilation is associated with a decreased excitatory versus inhibitory neuromodulatory influence on central integrative pathways of breathing control.

Among the many central neuromodulatory systems shown to influence respiration, our laboratory has focused on the excitatory substance P/neurotachykinin and on the inhibitory opioid systems, the natural peptides and receptors of which are found in central respiratory-related regions. During hypoxia, neuropeptide release from neurons into the interstitial brain space is enhanced. Theoretically, therefore, the increased extracellular ligand concentrations should enhance receptor activation. On the other hand, however, the signal transduction pathways of ligand-activated G-protein receptors, including the neurotachykinin and opioid receptors, entail temporary internalization of the receptors from the membrane into the cytoplasm, rendering them temporarily unavailable for further activation. Thus, the functionality of each neuromodulator system depends on the ratio of extracellular peptide molecules to the number of available receptors at any moment. We have asked whether a differential degree of receptor internalization between neurotachykinin and opioid receptors might contribute to the relatively diminished hypoxic response observed in developing piglets.

To answer this question, we have first focused on the binding of neurotachykinin-1 receptors (NK-1R) and mu-opioid receptors (MOR) in the brainstem of the mature rat in response to recurrent or single episodic hypoxia as compared to normoxia. The hypoxia protocol was 6 episodes of 8% O_2 –92% N_2 for 5 min, each fol-

lowed by 21% O_2 –79% N_2 for 5 min, either on 6 consecutive days (recurrent episodic hypoxia), or on the 6th day only, following 5 daily normoxic exposures (single episodic hypoxia). Brains were collected either 5 min or 2 h after the last gaseous exposure. Brainstem sections underwent autoradiography with iodinated substance P for NK-1R or DAMGO for MOR. In nucleus tractus solitarius (NTS) from brains collected 2 h after the last exposure, the binding densities of NK-1R and MOR were unaltered by a single episodic hypoxia, and were greatly increased after recurrent episodic hypoxia, indicating an upregulation of both receptors by the recurrent episodic stimulus. In the NTS from the brains collected 5 min after the last hypoxic exposure, there was an important discrepancy in the binding response between the NK-1R and the MOR: The NK-1R displayed a significant decrease in ligand binding after both single and recurrent episodic hypoxia, implying enhanced receptor internalization. In contrast, the binding of MOR was not changed, implying that the rate of receptor internalization was not altered by the hypoxic exposure. This difference would result in relatively greater availability of functional MOR to opioid peptides during hypoxia.

At present, we are carrying out the same experiments in developing piglets, using the same hypoxia protocols. If the findings in piglets' brains (to be presented at the Satellite meeting) are the same as those in the adult rat brain, the greater availability of functional MOR as compared to NK-1R during hypoxia may explain the attenuated ventilatory response to repeated hypoxia during development in piglets.

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1.5

Phylogeny of central CO_2/pH chemoreception in vertebrates

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The traditional view has been that respiratory chemoreceptors responsive to changes in P_{CO_2}/pH first evolved in air-breathing vertebrates at both peripheral and central sites. Levels of arterial P_{CO_2} in water breathing fish are typically less than 2–3 torr and it has been assumed that the ventilatory responses of fish to changes in aquatic P_{CO_2}/pH were due to the effects of acidosis on haemoglobin oxygen transport. There is growing evidence, however, to suggest that fish also possess peripheral chemoreceptors responsive to changes in P_{CO_2}/pH *per se* that reside primarily in the gills, are innervated by the glassopharyngeal and vagus nerves, and respond primarily to changes in aquatic rather than arterial P_{CO_2}. Their distribution overlaps extensively with that of the gill O_2 chemoreceptors in fish and it is not yet clear whether both responses arise from the same sensory cells. To date, there is no convincing evidence that strictly water breathing fish possess central chemoreceptors.

There is, however, growing evidence to suggest that some species of air-breathing fish possess central CO_2 chemoreceptors. Central chemosensitivity has been reported in *in vitro* brainstem-spinal cord preparations from both a primitive (holostean) and a modern (teleost) actinopterygian (ray finned) fish. Stimulation of these putative receptors had no effect on fictive gill ventilation but stimulated fictive air breathing. Unfortunately, the fictive breathing rates of these preparations were more than 25 times the resting rates reported for intact animals raising questions about the physiological significance of changes in the fictive motor output identified as air breathing under these conditions. In the South American lungfish (a sarcopterygian fish belonging to the lineage giving rise to higher vertebrates), on the other hand, central perfusion with mock csf of differing pH stimulated air breathing at rates similar to those seen in control animals. While these data suggest that central chemoreceptors have arisen several times in evolutionary history, hand in hand with the evolution of air breathing, this issue is not yet totally resolved.

An intriguing and related finding is that central CO_2 chemosensitivity appears to develop slowly in amphibian tadpoles. It is not present in young tadpoles but develops over time. The receptors initially stimulate gill ventilation but transfer their influence to lung ventilation just prior to metamorphosis from the aquatic larval stage into the air breathing adult form. In association with this the primary location of the receptors in the brainstem shifts from a diffuse distribution to a rostral concentration. While central chemoreceptors have been demonstrated in reptiles and birds, not much is known of their properties or distribution. They have been well studied in mammals where there is growing evidence for multiple sites of central CO_2/pH chemoreception and evidence to suggest that the mechanism of sensory transduction may vary both within and between receptor populations. While there has been much recent interest in the membrane channels, receptors and electrical coupling of several chemosensitive sites, this work has largely been on cells with downstream respiratory rhythmicity in preparations whose responses are very different (in terms of changes in frequency and amplitude of phrenic output) from that which is seen in intact animals.

The phylogenetic trends that are emerging indicate that the use of CO_2 chemoreception for cardio-respiratory processes may have arisen much earlier than previously believed, that CO_2/pH chemoreception arose in the periphery before the evolution of central CO_2/pH chemoreceptors, that the sites of CO_2/pH chemoreception (both peripheral and central) have increased throughout the course of vertebrate evolution and that the mechanism of CO_2/pH chemotransduction may vary. The sum of the data suggests that CO_2/pH chemoreceptors have arisen multiple times, at multiple sites during vertebrate evolution.

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1.6

Transgenic approaches to the study of respiratory function

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Genetically engineered mice have proven an invaluable tool for establishing linkages between individual genes and the generation of complex behaviors, including respiration. This presentation will focus on the use of mice carrying targeted gene deletions (gene knockouts) to elucidate the role of neuronal growth factors in the development of the neural respiratory controller and breathing behavior. In particular, I will focus on the role of brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) in development of peripheral chemoafferent neurons and central respiratory output. Initial studies in my laboratory demonstrated that mice that are homozygous for a null mutation at the *bdnf* locus exhibit a severe developmental deficit in

control of breathing, characterized by depressed and irregular ventilation and central respiratory output and a lack of hypoxic ventilatory drive [1,2]. These deficits are due at least in part to loss of peripheral chemoafferent neurons that require BDNF for survival during fetal development [1,3,4]. Surprisingly, null mutations in the *gdnf* gene result in a similar phenotype, despite the fact that BDNF and GDNF are structurally unrelated and signal through wholly different classes of receptors. However, we recently found that BDNF and GDNF are both required for survival of the same population of chemoafferent neurons and that null mutations in either gene results in chemoafferent cell loss [5]. This dual requirement for BDNF and GDNF appears to be related to the fact that both molecules are expressed in the fetal carotid body and act as target-derived survival factors for chemoafferent neurons [5,6]. Loss of chemoafferent input at fetal stages is particularly deleterious for maturation of ventilatory function, as chemoafferent drive is required for stabilization of central respiratory output after birth. Potential implications of these findings for human developmental disorders of breathing will be discussed.

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1.7

Prenatal nicotine exposure up-regulates catecholaminergic traits in peripheral arterial chemoreceptors of newborn rat pups

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Infants born to smoking mothers have depressed hypoxic arousal responses, reduced respiratory drive, and blunted ventilatory responses to hypoxia. These abnormalities in respiratory control are believed to be features of infants at risk for Sudden Infant Death Syndrome (SIDS). The peripheral arterial chemoreceptors in the carotid body are extensively involved in modulating respiratory responses to hypoxia, and arousal responses during sleep. Similar to findings in infants exposed prenatally to tobacco smoke, prenatal exposure to nicotine, a major component of tobacco smoke, alters acute ventilatory responses to short exposures to hypoxia and hyperoxia (Dejours test), manipulations that are used as a test of peripheral arterial chemoreceptor in function in newborn rats. Nicotine, through binding to cholinergic receptors, causes depolarization and catecholamine and opioid release from cells and neurons. Chemosensory type 1 cells in the carotid body and a subset of chemoreceptor sensory neurons in the petrosal ganglion are rich in catecholamines and contain nicotinic receptors. Physiology and pharmacology data also suggest that the carotid body contains met-enkephalins. Release of dopamine and norepinephrine from chemosensory type 1 cells with subsequent binding to inhibitory dopaminergic and adrenergic receptors on carotid sinus nerve fibers results in diminished neuronal output from the carotid body leading to depressed hypoxic ventilatory responses. Similarly, the release of met-enkephalins from type 1 cells and binding to delta-opioid receptors on the carotid sinus nerve decreases hypoxic chemosensitivity.

We used *in situ* hybridization histochemistry to determine the effect of prenatal nicotine exposure on the change in the levels of mRNAs for the enzymes regulating dopamine and norepinephrine synthesis, tyrosine hydroxylase (TH) and dopamine-beta-hydroxylase (DβH), respectively and preproenkephalin (PPE) mRNA in the rat carotid body and petrosal ganglion during postnatal development. We also determined the change in the level of D_2-dopamine receptor mRNA expression in these tissues induced by prenatal exposure to nicotine. In the carotid body, prenatal nicotine exposure increased TH mRNA expression in animals at 0 and 3 postnatal days (both, $P<0.05$ vs control) without affecting TH mRNA levels at 6 and 15 days. However, at 15 postnatal days, DβH mRNA levels were increased in the carotid body of animals prenatally exposed to nicotine. D_2-dopamine receptor mRNA expression in the carotid body increased with postnatal age and was unaffected by nicotine exposure. In the petrosal ganglion, prenatal nicotine exposure increased the number of ganglion cells expressing TH mRNA in animals at 3 days ($P<0.01$ vs control). DβH mRNA expression was not induced nor was PPE mRNA expression up-regulated in the petrosal ganglion in treated animals. PPE was not expressed in the carotid body in control or prenatally treated animals. In conclusion, prenatal nicotine exposure up-regulates mRNAs for synthesizing enzymes for two inhibitory neuromodulators, dopamine and norepinephrine, in peripheral arterial chemoreceptors that might contribute to abnormalities in cardiorespiratory control in animals prenatally exposed to nicotine. A possible role for brain-derived neurotrophic factor (BDNF) in nicotine induced up-regulation of catecholaminergic traits in peripheral arterial chemoreceptors will be discussed.

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ORAL PRESENTATIONS – SESSION 2
Respiratory rhythm generation and its state-dependent modulation

2.1

Role of the pre-Bötzinger complex in respiratory rhythm generation *in vivo*: influence of respiratory network drive

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We have previously demonstrated that microinjection of DL-homocysteic acid (DLH), a glutamate analog, into the pre-Bötzinger

complex (pre-BötC) can produce either phasic or tonic excitation of phrenic nerve discharge. Our initial findings were obtained during hyperoxic normocapnia; thus, the influence of respiratory network drive, including intrinsic pre-BötC neuronal excitability, on the DLH-induced modulation of phrenic motor output requires clarification. We, therefore, examined the effects of chemical stimulation of the pre-BötC during increased respiratory network drive elicited by hypercapnia, hypoxia (ie, peripheral chemoreflex), or focal pre-BötC tissue acidosis in chloralose-anesthetized, vagotomized, mechanically ventilated cats. For these experiments, we selected sites in which unilateral microinjection of DLH into the pre-BötC during hyperoxic normocapnia ($PaCO_2$ = 37–43 mmHg) produced a non-rhythmic tonic excitation of phrenic nerve discharge. During hypercapnia ($PaCO_2 = 59.7 \pm 2.8$ mmHg; $n = 17$), similar microinjection produced excitation in which respiratory rhythmic oscillations were superimposed on varying levels of tonic discharge. A similar pattern of modulation was observed in response to microinjection of DLH into the pre-BötC during normocapnic hypoxia ($PaCO_2 = 38.5 \pm 3.7$; $PaO_2 = 38.4 \pm 4.4$; $n = 8$) and during focal pre-BötC tissue acidosis ($n = 7$). Further, during increased respiratory network drive, these DLH-induced respiratory rhythmic oscillations had an increased frequency compared to the preinjection baseline frequency of phrenic bursts ($P < 0.05$). These findings demonstrate that tonic inspiratory motor activity evoked by chemical stimulation of the pre-BötC is influenced by and integrates with modulation of respiratory network drive mediated by input from both central and peripheral chemoreceptors, as well as focal pre-BötC CO_2/H^+ chemosensitivity.

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2.2

Distribution of calcium binding proteins, sodium channels and persistent sodium current in the rat ventral respiratory group

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The ventral respiratory group (VRG) is important in generating both the breathing rhythm and motor pattern. The VRG is generally subdivided into 4 regions, the Bötzinger complex, preBötzinger Complex (pBc), rostral VRG, and caudal VRG based on neuron discharge patterns and axonal projections. Anatomical markers provide a potential bridge between the physiological analysis of the respiratory system and the neurochemical elements that are the ultimate building blocks of this system. An example of this approach is the coincidence of NK1 receptors with neurons in the pBc complex [1]. VRG labeling by antibodies to calcium binding proteins (parvalbumin, calbindin, and calretinin) in some respects complements the NK1 immunoreactivity. Parvalbumin has been suggested to label VRG neurons [2]. We have verified this by demonstrating that parvalbumin positive neurons project to the ipsilateral and contralateral VRG as well as to the phrenic nucleus. We have also found that a prominent gap in the column of VRG related parvalbumin cells [2] likely corresponds to the pBc since parvalbumin cells are rare in this zone and never co-localize with NK1 receptors. Calbindin and calretinin immunoreactive cells are scattered in the pBc and rostral VRG but rare in the Bötzinger complex. Calbindin neurons are intermingled, but not colocalized with pBc NK1 cells. Calretinin is not colocalized with NK1, except for a small population of cells at the caudal ventral edge of the pBc, which likely corresponds to NK1 bulbospinal neurons [3]. Finally, preliminary evidence indicates glycine immunoreactivity in some parvalbumin neurons within the Bötzinger complex region. In addition to this compartmentalized distribution of calcium binding proteins within the VRG, preliminary evidence is consistent with a differential distribution of Na channel alpha subunits. A slowly inactivating persistent Na current is postulated to underlie the pacemaker activity seen in a subset of pBc neurons [4]. Within the CNS at least 5 different Na channel alpha subunits have been identified, termed Nav1.1, 1.2, 1.3, 1.5 and 1.6. Of these Nav1.6 has been most strongly linked to persistent Na current. Immunohistochemical examination of Nav1.2, 1.3 and 1.6 demonstrated Nav1.2 is widely expressed in the VRG including some NK1 neurons. Nav1.3 was present in cranial motoneurons. In neurons acutely dissociated from the pBc of 1–15 day old rats, whole-cell voltage clamp recordings were used to analyze transient and persistent Na currents and single-cell RT-PCR was used to probe for Nav1.1, 1.2 and 1.6 mRNA. Whole-cell recordings were made using an external solution containing 50 mM NaCl. Slow ramp depolarization from −80 to +30 mV revealed a TTX-sensitive, persistent Na current. The current kinetics were dependent upon the ramp speed. A slow ramp (100 mV/s), elicited an inward non-inactivating current in 42% (10 of 24) of neurons sampled from the pBc. These results are consistent with a role for persistent Na current in regulation of the subthreshold behavior, including pacemaker activity. Moreover, single-cell RT-PCR revealed the presence of Nav1.6 in 40 of 72 cells (55%). The Nav1.1 subunit mRNA was present in 47 of 82 neurons (57%) and was co-expressed with Nav1.6 in 28% of cells. Preliminary findings on Nav1.2 mRNA are consistent with the immunohistochemistry with it present in 7 of 18 (38%) pBc neurons.

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2.3

Information processing at the nucleus tractus solitarii and respiratory rhythm generation

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The nucleus tractus solitarii (NTS) relays information from primary visceral receptors to the central nervous system and is critically involved in the reflex control of autonomic functions. In the respiratory system, it is a part of so-called dorsal respiratory group (DRG) and plays an important role in the regulation of respiration. Although people have noticed that the NTS in general is not a simple relay nucleus but a place of information processing, such concept has not necessarily been realized in the respiratory

system. Based on our recent data on NTS neurons, we now show some aspects of information processing taking place at the NTS.

Inflation or deflation of the lungs evokes various respiratory reflexes by activating mainly two types of pulmonary stretch receptors. Slowly adapting receptors (SARs) are activated solely by inflation of the lungs and firmly involved in the Hering-Breuer reflex; on the other hand, rapidly adapting receptors (RARs) are activated by both inflation and deflation of the lungs but their functional role is still controversial. We aimed to determine the input-output properties of their second-order relay neurons (P-cells and RAR-cells, respectively) in the NTS. P-cells are key neurons of SAR-induced reflexes and have long been regarded as simple relay neurons; however, our experiments in the rat clearly showed that firing of P-cells (and RAR-cells) was modulated with central (not *via* afferents) respiratory rhythm, indicating that these second-order relay neurons are under influence of the respiratory center. In other words, the respiratory center gates the afferent inputs at the NTS before such inputs act on respiratory neurons that underlie the elaboration of various respiratory reflexes. Now P-cells and RAR-cells have been revealed to receive complex synaptic inputs involving glycinergic and GABAergic inhibitions and non-NMDA and NMDA glutamate receptor-mediated excitations [1]. Therefore, these "relay neurons" are crucially organized into the medullary respiratory network and can exert a phasic influence on the central rhythm generating mechanisms even without receiving afferent inputs from SARs and RARs.

RAR-cells respond characteristically to lung deflation but do not necessarily respond to lung inflation. This is somewhat peculiar since RAR afferents respond to both inflation and deflation of the lungs. This suggests the possibility that lung inflation that activates RARs on one hand suppresses RAR-cell firing on the other hand. Recently we found evidence that this suppression is caused by synaptic inhibition of RAR-cells from P-cells (P-R linkage) [2]. This implies that some P-cells are inhibitory and that the SAR and RAR systems are not independent but work cooperatively to evoke respiratory reflexes. On the assumption that RAR pathways mediate inspiratory facilitation, this P-R linkage seems to explain the neuronal mechanisms underlying the Hering-Breuer inflation and deflation reflexes. That is, inflation of the lungs activates preferentially SAR pathways by inhibiting RAR pathways and deflation of the lungs activates RAR pathways.

In the NTS area, ie in the DRG, we identified a novel group of inspiratory neurons, which receive monosynaptic inputs from low-threshold vagal afferents and respond to lung deflation [3]. It is quite possible that RAR afferents innervate these deflation-sensitive inspiratory neurons (tentatively termed Iγ neurons). Until now we have no suitable answer to the question why two types of inspiratory (Iα and Iβ) neurons exist in the DRG. The fact that DRG inspiratory neurons are classified into at least three groups, Iα, Iβ and Iγ neurons, has made the situation more complex but provides new insight into the organization and role of the NTS, which integrates afferent inputs and the central respiratory rhythm.

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2.4

Central mechanisms controlling upper airway patency

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The neurones of the ponto-medullary respiratory network drive two functionally and anatomically distinct pools of motoneurones. One set is located within the spinal cord that innervates the diaphragm and intercostal muscles. A second group of motoneurones is located within the nucleus ambiguus imbedded in the ventral respiratory group that project *via* cranial motor outflows to co-ordinate the activity of laryngeal and bronchial muscles to control airway resistance and airflow. These spinal and cranial motor activities have to be precisely co-ordinated to ensure efficient ventilation. We recently included this behaviour as imperative for a definition of eupnoea. Thus, beside a rhythmic eupnoeic (ramp) discharge pattern of pump motoneurones, a phasic respiratory modulation of glottal resistance should be observed and expressed as glottal dilation during inspiration and transient glottal constriction during post-inspiration [1]. During the last decade mechanisms underlying respiratory rhythm generation were studied primarily in reduced *in vitro* preparations. The work concluded that the respiratory rhythm is generated by pacemaker neurones located in the Pre-Bötzinger complex and is independent of inhibitory glycinergic synaptic transmission (for review see [2,3]). However, a potential role for glycinergic transmission for the eupneic co-ordination of circuitry controlling the upper airway was largely disregarded.

To determine a role for glycinergic inhibition within the pontomedullary network, we used the arterially perfused working heart-brainstem preparation (WHBP) of neonatal and mature rat. This preparation allows both kinesiological and cellular studies of central and peripheral mechanisms controlling upper airway resistance [1]. Recording of the recurrent laryngeal nerve activity as an index of motor output to the glottis revealed post-inspiratory activity that shifted towards the inspiratory phase after strychnine antagonism of glycine receptors (0.5–1.5 μM). This shift of post-inspiratory activity was also obtained at the cellular level: intracellular recordings of post-inspiratory neurones revealed that the hyperpolarisation during the inspiratory phase was converted to a depolarisation with spike discharge after exposure to strychnine. This lead to a massive disturbance of the eupnoeic modulation of glottal resistance by converting the inspiratory glottic dilatation (seen during control) to a paradoxical constriction, as demonstrated by measuring changes in laryngeal resistance. Similar results were obtained in both neonatal and juvenile rats suggesting that glycinergic mechanisms co-ordinating ventilatory movements with upper airway resistance are functional at birth. The effects of glycinergic inhibition were mimicked during exposure of neonatal preparations to prolonged hypoxia. During the secondary hypoxic depression of respiration post-inspiratory activity was shifted towards inspiration causing a paradoxical glottic constriction during neural inspiration.

We conclude that integrity of glycinergic neurotransmission within the ponto-medullary respiratory network is essential for co-ordinating the neuronal activities which control upper airway resistance and ventilatory movements and consequently the eupnoeic breathing pattern in rats from birth.

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2.5

Mechanisms of respiratory rhythm generation *in vitro*. I. Pacemaker neurons and networks in the pre-Bötzinger complex (pre-BötC)

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We have proposed that the inspiratory rhythm-generating kernel in the pre-BötC consists of a heterogeneous network of glutamatergic neurons with voltage-dependent pacemaker-like bursting properties. This model is based on our optical imaging [1], electrophysiological [2], and mathematical modeling [3] studies of inspiratory (I) neurons within the pre-BötC of rhythmically active *in vitro* transverse slice preparations from neonatal rats. For electrophysiological and imaging studies, we have developed novel methods to optically identify I neurons with Ca^{2+}-sensitive dyes combined with IR-DIC imaging for whole-cell current camp (CC) and voltage clamp (VC) analyses of neuronal membrane conductance and synaptic mechanisms. In computational modeling studies we have developed models of single pacemaker neurons and synaptically-coupled networks of these neurons (50–500 cells) with heterogeneous distributions of cellular/network parameters. Model predictions have been tested experimentally from single-cell electrophysiological measurements and from macroscopic recordings of neuron population activity within the pre-BötC [2].

Electrophysiological and optical measurements demonstrate a subpopulation of pre-BötC I neurons that exhibit voltage-dependent rhythmic bursting under CC after blockade of non-NMDA glutamatergic synaptic transmission or after blocking synaptic transmission with Ca^{2+} channel blockers. The intrinsic bursting frequency of these pacemaker-type cells was a monotonic function of baseline membrane potential, spanning a frequency range of over an order of magnitude (~0.05–1 Hz); the voltage-dependence and frequency range varied for different cells indicating heterogeneity. Under VC with synaptic transmission intact, these pacemaker neurons exhibited glutamatergic synaptic drive currents. Optical imaging and cross-correlation of rhythmic Ca^{2+} activities of multiple cells demonstrate that the glutamatergic synaptic interactions synchronize bursting within the heterogeneous population, but with a temporal dispersion in neuronal spiking including pre-I spiking patterns [2,3]. Measurements of pre-BötC population activity show network frequency control by changing pre-BötC excitability, like predictions from our network models [3].

We have obtained evidence that a persistent Na^+ current (INaP) is the main subthreshold-activating cationic conductance underlying the voltage-dependent pacemaker bursting. We measured Na^+ currents in bursting pacemaker and non-bursting pre-BötC I cells. In all cells tested under VC, voltage ramp commands at rates of <100 mV/s inactivated the fast Na^+ current and yielded N-shaped IV curves with a negative slope region between −60 and −35 mV. TTX (1 μM) blocked this inward current. The slowest ramp speed tested (3.3 mV/s) failed to fully inactivate the negative slope generating current. The current is therefore a TTX-sensitive INaP. The peak amplitude of INaP ranged from −50 to −100 pA at Em = −20 mV (peak conductance = ~1–2 nS). Boltzmann plots gave half-maximal activation voltage of ~−40 mV and a slope factor of ~5, very similar to values used for INaP in our pacemaker neuron models [3]. In non-pacemaker I cells, the ratio of INaP/Ileak was insufficient to support intrinsic bursting as predicted by our models. Further tests of the role of INaP have been performed using the *dynamic clamp* to artificially add INaP to non-pacemaker neurons. INaP transformed these neurons into rhythmic bursters with voltage-dependent behavior quantitatively similar to our models and experimental observations.

These results generally support our conceptual and computational models for the rhythm-generation kernel as a heterogeneous pacemaker network.

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2.6

PreBötzinger complex (preBötC) neurokinin-1 receptor (NK1R) expressing neurons are required for eupnea in adult awake rats

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Normal respiratory rhythm in mammals is hypothesized to be generated by NK1R neurons in the preBötC. Ablation of these neurons in adult rats was predicted to severely perturb normal breathing rhythm. We directly injected substance P conjugated saporin (SP-SAP) into the preBötC to selectively kill NK1R neurons [1]. Four to five days postinjection in rats with greater than 75% bilateral loss of preBötC NK1R neurons, spontaneous breathing rhythm transformed from normal, ie, eupnea, to ataxia, with greatly reduced ventilation and consequent disturbances in blood gases and pH. Hypoxic or hyperoxic challenges produced prolonged, often fatal, apnea. PreBötC NK1R neurons are therefore necessary for generation of the eupneic breathing rhythm.

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2.7

The neural mechanisms involved in the generation of three types of respiratory activities in the transverse slice preparation of mice

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The transverse medullary slice preparation of mice expresses three distinct types of fictive respiratory activity patterns, that are generated within the pre-Bötzinger complex [1]. Under normoxic conditions these patterns include both fictive eupneic and sigh activity. Sighs occur at a lower frequency than eupnea. Respiratory neurons in the pre-Bötzinger complex that are active during eupneic activity also burst during the sigh. The sigh burst was selectively blocked by DL-AP5 (50 μM) and low concentrations of cadmium (4 μM). In contrast, the generation of eupneic bursts was not abolished at these concentrations. Thus, although there is an overlap between the neural populations underlying the generation of both activities our data suggest that different burst generating mechanisms underlie eupneic and sigh bursts.

During prolonged hypoxic conditions sigh and eupneic activity cease and the respiratory network continues to generate a rhythmic activity, which we refer to as fictive gasping. The duration and rise time of integrated inspiratory activity decreases significantly during the transition from fictive eupnea to gasping. Further, phasic synaptic inhibition in expiratory neurons is suppressed and post-inspiratory neurons are transiently activated in phase with inspiratory activity. We postulate that the same neuronal network in the pre-Bötzinger complex generates both eupneic and gasping activity *via* a network reconfiguration that involves a selective suppression of synaptic inhibition. This suppression leads to changes in the shape of inspiratory activity, a suppression of phasic hyperpolarization in expiratory neurons, and an inspiratory activation of post-inspiratory neurons.

In the presentation, we will discuss the interaction of pacemaker and network properties in the generation of the frequency, shape, and amplitude of the different respiratory activities. We will present evidence indicating that non-NMDA, NMDA, glycinergic, GABAergic and electric synaptic mechanisms are all essential for the generation of fictive eupneic activity. In contrast, synaptic inhibitory mechanisms appear not to be essential for the generation of gasping activity.

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ORAL PRESENTATIONS – SESSION 3 Plasticity

3.1

Serotonin-dependent respiratory plasticity

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Serotonin initiates neuroplasticity in a number of invertebrate and vertebrate experimental models. The first report of serotonin-dependent plasticity in respiratory motor control was a long-lasting facilitation of phrenic activity following episodic stimulation of chemoafferent neurons [1], a phenomenon now known as long-term facilitation (LTF). Recent progress has contributed considerably towards an understanding of the mechanisms and manifestations of this potentially important model of respiratory plasticity. In this presentation, recent progress in understanding the mechanism of LTF will be reviewed. In all studies, we exposed awake or anesthetized Sprague Dawley rats to episodic hypoxia as an experimental model of LTF. Both awake and anesthetized rats express LTF following episodic hypoxia. Intermittent, but not continuous hypoxia elicits LTF, indicating remarkable pattern sensitivity in its underlying mechanism. Both episodic chemoafferent activation by stimulation of the carotid sinus nerve and episodic hypoxia in carotid denervated rats elicit LTF, suggesting that at least two discrete mechanisms contribute to LTF in anesthetized rats. Hypoxia-induced LTF requires serotonin receptor activation during, but not following episodic hypoxia, indicating that serotonin is necessary to initiate but not maintain LTF. Phrenic LTF following episodic hypoxia is blocked by intrathecal administration of a serotonin receptor antagonist (methysergide) or protein synthesis inhibitors (cyclohexamide, emetine) to the cervical spinal cord. On the other hand, intraspinal drug administration had no effect on hypoglossal LTF. Thus, the relevant serotonin receptors in phrenic LTF are within the spinal cord, suggesting a location within the respiratory motor nucleus itself. These observations form the basis of our working hypothesis that LTF is initiated by episodic activation of 5-HT_2 receptors on respiratory motoneurons, thereby initiating a cell-signaling cascade leading to new protein synthesis. Although the specific protein(s) necessary for LTF is (are) unknown, we recently found that episodic hypoxia and LTF are associated with elevations in ventral spinal concentrations of brain derived neurotrophic factor (BDNF). The elevation in BDNF following episodic hypoxia is blocked by local application of methysergide, suggesting that it may be a causal agent in LTF. Although the physiological (or pathophysiological) role of LTF is uncertain, it may reflect a general mechanism whereby intermittent activation of raphe serotonergic neurons elicits plasticity in respiratory motoneurons. Thus, the same fundamental mechanism may be operational in a number of physiological (eg. altitude or repetitive exercise) or pathophysiological (eg. lung disease or neural injury) states.

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3.2

Age and gender effects on serotonergic innervation and modulation of the hypoglossal motor nucleus

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Aging results in structural, functional and neurochemical alterations in the respiratory system. Serotonin (5HT) plays a major role in breathing and the control of upper airway function. We tested the hypothesis that with increasing age there is a selective decrease in serotonergic modulation of respiratory motoneurons, in particular hypoglossal motoneurons to the tongue in male rats. We used light microscopic immunocytochemistry to study the distribution of 5HT

axons and boutons throughout the hypoglossal nucleus in young and old rats male and female rats. Aged male rats (>12 months) had fewer serotonin immunoreactive axons and boutons in the hypoglossal nucleus than young male rats (<6 months). In contrast, 5HT immunoreactivity in the hypoglossal nucleus in female rats was higher than in age-matched males, and increased with age. In order to assess the functional consequences of this anatomical reorganization, we measured long term facilitation (LTF), a serotonin-dependent, long lasting increase in respiratory motor output following episodic hypoxia. LTF in the hypoglossal motor output was significantly reduced in aged male rats by comparison with young male rats [1]. In contrast, hypoglossal LTF increased in aged female rats in diestrus, but not in estrus. Taken together, these data suggest that in males, but not females, normal aging may result in decreased serotonergic facilitation of hypoglossal motoneurons that could result in reduced airway patency.

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3.3

Integral-differential calculus computations by short-term potentiation and depression in NTS-pontine pathways

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Neural integrators are a class of neural networks that afford extended temporal summation of neural activity or, equivalently, low-pass frequency filtering of neurotransmission [1]. In the respiratory system, a biphasic neural integrator in the carotid chemoreflex response is demonstrated by the classic short-term potentiation (STP) and afterdischarge of phrenic activity [2]. Recently, a new class of neural networks called *neural differentiators*, which afford high-pass frequency filtering of afferent inputs through short-term depression (STD) of neurotransmission, has been identified in the vagal-carotid control of the expiratory off-switch [3]. The differentiator response is characterized by: 1) a habituation-like adaptation to the vagal Hering-Breuer (HB) reflex lengthening or carotid chemoreflex shortening of expiratory duration; 2) secondary adaptive effects in the form of pontine-mediated and NMDA receptor-dependent post-stimulus rebound and recovery of these vagal or carotid [4] (or hypoxia [5]) mediated responses.

To elucidate the mechanism of the habituation of the HB reflex, we obtained extracellular field potentials (FPs) in the medial and commissural regions of the nucleus tractus solitarius (NTS) evoked by electrical vagal stimulation (1 min) in urethane-anesthetized, paralyzed and mechanically ventilated rats ($n=17$) *in vivo*. FPs from vagal A-fibers (stimulus current, 20–70 μA, response latency, 4–5 ms) revealed a stimulus frequency-dependent (20–80 Hz) STD similar to the synaptic accommodation observed *in vitro* [6] and the habituation of HB reflex *in vivo* [4]. FPs from C-fibers (150–350 μA, 20–45 ms) also demonstrated a frequency-dependent (5–20 Hz) STD during afferent activation but their amplitude remained depressed for >50 s post-stimulation. Microinjection of the NMDA receptor antagonist D-APV abolished the short-term memory following C-fiber activation but not the frequency-dependent STD with A- or C-fibers. The characteristics of the vagal A- and C-fiber mediated responses are reminiscent of the type I and type II NTS neurons found *in vitro* [7].

To elucidate the mechanism of the pontine-mediated adaptation of the HB reflex, we obtained extracellular recordings of pontine neuronal activity simultaneously with phrenic nerve recording during electrical vagal stimulation (1.5 × T, 80 Hz, 1 min). Neurons with tonic and respiratory modulated activity were found within or near the parabrachialis (PB) and Kölliker-Fuse (KF) complex. Most tonic neurons in KF ($n=11$) were depressed during and/or shortly after vagal stimulation that elicited the characteristic biphasic adaptation of the HB reflex in the phrenic activity, whereas most tonic neurons in medial PB ($n=22$) were not affected. Activity of respiratory modulated neurons ($n=4$) recorded in KF remained in phase with phrenic activity during vagal stimulation. Results showed that vagal input induced STD of tonic neurotransmission in KF.

These findings support a dual-process nonassociative learning model of integral-differential calculus computations in the brain [8] through activity-dependent STP and STD of neurotransmission in primary and secondary afferent pathways.

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3.4

Activity-dependent plasticity in desecending synaptic inputs to spinal motoneurons in an *in vitro* turtle brainstem-spinal cord preparation

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Long-term (>1 h) and short-term (sec-to-min) activity-dependent synaptic plasticity have been proposed to contribute to the patterning of rhythmic network activity. There is, however, very little experimental evidence to support this hypothesis. Previously, we demonstrated that electrically-evoked descending synaptic inputs to respiratory-related spinal motoneurons express long-term depression (LTD) or long-term potentiation (LTP) following spinal stimulation at different frequencies in an *in vitro* turtle brainstem-spinal cord preparation [1]. For example, evoked potentials in pectoralis (expira-

tory) nerves express LTD following 1 and 10 Hz spinal stimulation (900 pulses), while potentials in serratus (inspiratory) nerves express LTP following 100 Hz spinal stimulation (900 pulses).
In this study, we hypothesized that activity-dependent synaptic plasticity is expressed in identified synaptic connections within the respiratory control system of adult turtles (*Pseudemys*), and that LTD is expressed in respiratory descending synaptic inputs to pectoralis motoneurons following 10 Hz spinal stimulation. Using *in vitro* turtle brainstem-spinal cord preparations ($n=6$), the lateral funiculus at spinal segment C5 (rostral to pectoralis and serratus motoneurons) was electrically stimulated (10 Hz, 900 pulses, 400 μA) while the preparation spontaneously produced rhythmic respiratory motor output. One application of spinal stimulation immediately decreased respiratory burst amplitude by ~75% on both pectoralis and serratus ($P<0.05$), but amplitudes returned to pre-stim levels within 30 min. Thus, 10 Hz spinal stimulation produces short-term depression of expiratory and inspiratory motor output. In separate experiments ($n=5$), three episodes of 10 Hz conditioning stimulation (separated by 5 min) nearly abolished pectoralis and serratus burst amplitudes during stimulation ($P<0.05$). Pectoralis bursts returned to pre-stimulus levels within 30 min, but serratus bursts returned to pre-stimulus levels within 20 min and tended to exceed pre-stim levels by 30–40% at 50 min after conditioning ($P>0.05$). Closer examination showed that serratus burst amplitudes at 50 min post-stim were depressed by ~20% in 3/5 preparations, and potentiated by 100–150% in 2/5 preparations. Spinal stimulation did not change hypoglossal burst amplitude.
In conclusion, 10 Hz spinal stimulation did not exclusively elicit LTD in spontaneous expiratory activity in pectoralis nerves, suggesting that LTD is expressed in nonrespiratory-related descending synaptic inputs to pectoralis motoneurons, or that spontaneous respiratory activity in pectoralis motoneurons overrides the expression of LTD. We hypothesize that the activity-dependent plasticity observed with spinal stimulation is due to spinal mechanisms because hypoglossal respiratory activity was unaltered.

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3.5

Carotid body dopaminergic mechanisms during acclimatization to hypoxia

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Increased carotid body sensitivity to hypoxia has been found to be an important component of the mechanism of ventilatory acclimatization to chronic hypoxia. Considerable attention has been focused on the potential role of dopamine in the mechanism of increased carotid body sensitivity to hypoxia. This is related to the likely important role dopamine plays in carotid body function. Dopamine has a well-established role as having an inhibitory modulatory effect on the carotid body. For example, dopamine infusion inhibits carotid body responses to hypoxia and dopamine D_2 receptor blockade causes an increased response of the carotid body to hypoxia. Thus, it has been hypothesized that a down-regulation of dopaminergic inhibition could be occurring within the carotid body making it more responsive to hypoxia during ventilatory acclimatization to prolonged hypoxia. A study in cats [1] supported this hypothesis. The investigators used domperidone, a peripheral dopamine D_2 receptor antagonist, and found that it was no longer effective in increasing the ventilatory and carotid body responses to hypoxia after acclimatization, suggesting that dopamine inhibition had been abolished. However, similar studies failed to support this finding in goats [2] or human subjects [3].
In goats and dogs dopamine has a biphasic effect on carotid body activity, eg a bolus intra-carotid infusion of dopamine causes a burst of excitation followed by prolonged inhibition of afferent discharge frequency. A low affinity excitatory carotid body dopamine receptor has been postulated [4]. We made the hypothesis that there could be a facilitated dopaminergic excitation within the carotid body during acclimatization to hypoxia. This hypothesis would be compatible with the greatly increased metabolism of dopamine that occurs in the carotid body during chronic hypoxia [5]. If the dopamine-mediated excitation could be blocked, then one could test this hypothesis. After an extensive search of dopaminergic antagonists, we found that the dopamine excitatory activity was mediated by the serotonin type 3 receptor ($5HT_3$) and that this excitatory activity could be blocked by specific $5HT_3$ antagonists such as tropisetron. Tropisetron blocked not only the excitatory activity induced by serotonin, but also that produced by dopamine and by the specific $5HT_3$ agonist, chlorophenylbiguanide, in the goat carotid body.
Carotid sinus nerve recording studies showed that the response of the goat carotid body to acute hypoxia was significantly attenuated by tropisetron. Further studies in awake goats were carried out in order to test the hypothesis that $5HT_3$ antagonists could block ventilatory acclimatization to hypoxia. Blockade with tropisetron failed to modify the time-dependent increase in ventilation that occurs in goats during ventilatory acclimatization.
Our data provide no evidence to support the hypothesis that carotid body dopamine acting *via* either dopaminergic or $5HT_3$ receptors mediates ventilatory acclimatization to hypoxia in the goat.

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3.6

Circadian patterns of breathing

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Life has evolved on a planet with rotation around itself and the Sun. A fundamental mechanism of adaptation is the capacity of time-keeping, such that daily and seasonal events can be antici-

pated and prepared for. Many physiological variables have a circadian pattern, which in mammals is controlled by a biological clock with its own period of 24 h, placed in the suprachiasmatic nucleus of the hypothalamus. Among the most studied are the patterns of activity (Act), body temperature (Tb) and metabolic rate (oxygen consumption, VO_2, and carbon dioxide production, VCO_2). In the rat, a mostly nocturnal animal, all these variables increase during the dark hours of the night. Since all of them are known to influence pulmonary ventilation (VE), it is expected that also the breathing pattern, and possibly its controlling mechanisms, will present circadian oscillations. In rats chronically instrumented for measurements of Tb and Act by telemetry, VO_2 and VCO_2 were measured continuously for several days by an open-circuit method, while VE was monitored by a modification of the barometric technique [1]. All variables of the breathing pattern (tidal volume VT, frequency f, and VE) increased in the dark (D) compared to the light (L) hours, with minor L-D differences in VE/VO_2. The L-D differences in VE**,** and in all other parameters, persisted when comparisons between the L-D phases were made for the same level of either very low or very high Act, indicating that the oscillations in breathing pattern do not depend on Act. Indeed, ongoing experiments on rats in which circadian patterns are disturbed by sudden phase shifts between D and L, indicate a very poor correlation between levels of Act and breathing, which is much better correlated with Tb.

Sustained hypoxia (10% O_2) blunted the amplitude of the circadian oscillations of all variables, Act being the least affected, and Tb the most [2,3]. In constant L ('free running' conditions), in which the natural period of the clock is unmasked, the effects of hypoxia on the Tb oscillations were not accompanied by a change in the clock period [2], and were not abolished by sino-aortic denervation [4], suggesting that hypoxia does not affect the clock itself but acts elsewhere centrally to affect the circadian patterns, possibly at the level of the hypothalamic thermoregulatory centers. Alterations in Tb patterns were also observed in men living intermittently at high altitude [5]. Preliminary observations in adult rats seem to indicate that the hypoxic effects on the oscillations of Tb are less marked in females than males.

Sustained hypercapnia had minimal effects on Tb, activity, VO_2 and VCO_2, and, as observed during sustained hypoxia [3], the degree of hyperventilation (percent increase in VE/VO_2) was essentially independent of the time of the day.

In conclusion, the existence of a biological clock implies the oscillations of numerous variables known to affect the breathing pattern; indeed, VT, f, and VE present daily oscillations. The hyperventilatory responses to hypoxia and hypercapnia, however, remain constant, despite the fact that hypoxia and hypercapnia can have major and differential effects on numerous physiological variables and their circadian patterns.

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3.7

Site(s) and mechanism of changes in arterial chemo-sensitivity after carotid (CBD) and/or aortic (AOD) denervation

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Through injections of NaCN at various locations in awake piglets and rats, we gained insight into the plasticity of arterial chemosensitivity. Less than 8 day old piglets exhibited both carotid and aortic chemosensitivity. The aortic chemosensitivity persisted after 8 days only if CBD had been performed, but CBD after 8 days of age resulted in restoration of aortic chemosensitivity. At the site of aortic chemosensitivity, there was greater serotonin (5-HT) immunoreactivity in CBD than in carotid intact piglets and this increase was dependent upon intact aortic innervation. Intravenous injections of the 5-HT5a receptor antagonist, methiotepin, prior to the NaCN injection eliminated aortic chemosensitivity. Western blots indicated the expression of the 5-HT5a receptors at this aortic site and the protein existed equally in CBD and carotid intact piglets. AOD + CBD resulted in chemosensitivity in the left ventricle which was attenuated by prior injection of the 5-HT5a receptor antagonist. Neonatal and adult rats also developed aortic chemosensitivity after CBD. These data indicate there are multiple sites for plasticity in arterial chemosensitivity, which appears to involve upregulation of serotonin acting at the 5-HT5a receptors.

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ORAL PRESENTATIONS – SESSION 4
Intracellular signalling and synaptic modulation

4.1

Activating convergent signal pathways in respiratory neurons of the ventral medullary group

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Opiates are known to disrupt the respiratory rhythm by binding to μ- and δ-type opioid receptors of respiratory neurons within the ventral medullary group and by activating signal pathways that induce depression of neuronal excitability and synaptic interaction. In previous experiments we demonstrated that opioid depression of respiration can be treated by a variety of drugs that increase intracellular cAMP levels.

In the present study, we investigated how activation of μ-type opioid receptors can be counteracted and respiratory depression be treated by activation of convergent signal pathways targeting the same second messenger systems of the neurons. Our starting point was the observation that a compensatory effect can be achieved with Buspirone, a drug purported to activate $5\text{-}HT_{1A}$ receptors and, consequently, to reduce intracellular cAMP levels [1]. If these effects were confirmed, this observation would indicate a profound non-specificity of the 5-HT-1A directed drug. The task then would be to identify the serotonin receptor isoforms responsible.

In various preparations, including the anaesthetized *in vivo* cat, the perfused mouse or rat brain stem and the brain stem slice of the mouse or rat, we performed current and voltage clamp measurements with fine tipped or patch electrodes to measure electrophys-

iological parameters. RT-PCR methods were applied to identify the mRNA encoding for serotonin receptor isoforms, while immunocytochemical techniques were used to verify receptor expression.
We verified the stabilizing effect of cAMP [2,3] and confirmed the protective effect of 5-HT_{1A}-receptor agonists 8-OH DPAT and Buspirone against opioid depression of neural respiratory activity [4].
A detailed inspection of the results obtained with the commonly used 5-HT_{1A}-agonists, 8-OH-DPAT and Buspirone, indicated ambiguous effects of these drugs. Our conclusion was that these drugs probably act on other serotonin receptor isoforms which are expressed in addition to the known 5-HT_{1A} and 5-HT_{2A} subtypes.
Therefore, we developed two novel antibodies and demonstrated the expression of 5-HT_4 and 5-HT_7 receptors in neurons of the VRG and the hypoglossal nuclei.
These immunocytochemical findings were verified by quantitative RT-PCR analysis of the VRG region and confirmed in single cell RT-PCR analysis on identified respiratory neurons.
Finally, we demonstrated that these findings provide a basis for novel strategies for the treatment of respiratory depression induced by opioids.
The same strategy seems to be efficient in the treatment of respiratory disturbances induced by barbiturates.

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4.2

Oscillations in inspiratory synaptic inputs: role in controlling the repetitive firing behaviour of inspiratory motoneurons

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The transformation of neuronal input into patterns of action potential output, a key element of signal processing in the brain, is determined by the interaction between synaptic and intrinsic membrane properties. Traditional use of rectangular, ramp-like or sinusoidal waveforms to stimulate neurons has focussed attention on the role of intrinsic membrane properties and their modulation in determining repetitive firing behaviour. However, such studies do not address the role that dynamic features of endogenous synaptic inputs play in controlling output. Oscillations are prominent features of synaptic currents/potentials in many networks including those that provide rhythmic excitatory drive to motoneurons involved in behaviours such as locomotion and respiration [1–3]. Phrenic motoneurons (PMNs), for example, receive inspiratory currents of brainstem origin that oscillate with peak power in the 20–120 Hz bandwidth. These oscillations are ubiquitous throughout the respiratory network, *in vivo* [2,4] and *in vitro* [3,5], and although well-characterized, their function, if any, is not known.

Using rhythmically active brainstem spinal cord preparations from neonatal rat and recently developed stimulation techniques [6], we explored the physiological significance to motor control of such oscillations by activating PMNs with native inspiratory synaptic currents that oscillate in the 20–50 Hz bandwidth. Action potentials arose predominantly from peaks of the current oscillations and the timing of spikes within trains was reproducible within 2%. Activation of neurons with low-pass filtered currents produced spike trains with considerably more variability; filtering also reduced the number of action potentials by 35%. Finally, the excitatory neuromodulator, phenylephrine, which significantly increased instantaneous firing frequency responses to filtered inspiratory or square-wave stimuli, had no effect on frequency evoked by endogenous (unfiltered) synaptic waveforms.
Results indicate that oscillations in synaptic inputs generated by central respiratory circuits maximise neuronal output, and play a dominant role in controlling the timing of action potentials during behaviourally relevant repetitive firing, even in the presence of neuromodulators.

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4.3

Physiological and pharmacological properties of GABA-ergic gain modulation of canine ventral respiratory group (VRG) neurons

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In vivo local application of the selective $GABA_A$ receptor antagonist bicuculline methochloride (BIC) to respiratory neurons in the caudal VRG of dogs produces a profound increase in their discharge frequency (F_n) pattern. The resulting F_n pattern is an amplified replica on the underlying control F_n pattern even when the pattern is reflexly altered, for example by lung inflation, or enhanced by changes in chemodrive [1]. These results suggested the presence of a tonic GABAergic gain modulation (GM) that normally

attenuates the F_n pattern to typically less than 50% of the unblocked pattern. This multiplicative process can be modeled as: $F_{out}=(1-\alpha)*F_{in}$, where F_{out} and F_{in} are the instantaneous F_ns in the presence and absence of GABAergic inhibition, respectively, and α is the relative magnitude of tonic inhibition. The pharmacology of this mechanism is unusual in that picrotoxin, a noncompetitive $GABA_A$ receptor antagonist, does not produce GM, but is able to block the silent phase inhibition [2]. Also, recent *in vitro* studies have shown that the methyl derivatives of bicuculline block spike afterhyperpolarizations (AHPs) mediated by small conductance Ca^{2+} activated K^+ channels (SK) [3]. The main objective of this study was to compare the *in vivo* effects of BIC with those of the SK channel blocker apamin on endogenously- (spontaneous) and exogenously-induced neuronal activities to discern the mechanism for BIC effects.

Multibarrel micropipettes were used to record single unit activity from cVRG neurons in decerebrate dogs before and during picoejection of agonists and antagonists. Cycle-triggered histograms were used to quantify the F_n patterns and to determine the drug-induced changes in the gain and offset of the spontaneous F_n patterns. For the exogenous aspect of the study, the net increase in F_n due to repeated short duration picoejections of the glutamate receptor agonist, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), was quantified before and during locally induced antagonism of 1) $GABA_A$ receptors by BIC or 2) SK channels by apamin.

BICm and apamin produced similar marked increases in gain, but different offsets. During maximum SK channel block with apamin, BICm produced an additional 112 ± 22% increase in peak F_n. Conversely, apamin produced an additional 176 ± 74% increase in peak F_n during the maximum BICm-induced response. The net AMPA-induced increases in F_n were not significantly altered by BIC, but were amplified in accordance with the gain increase produced by apamin block of AHPs.

These results suggest that the BIC-sensitive GM of canine cVRG neurons is not due to a nonspecific block of AHPs, but is due to a GABAergic mechanism that modulates endogenously-, but not exogenously-induced activity. GABAergic GM, acting possibly *via* a shunting inhibition, may be functionally isolated from the soma/spike initiation zone to allow multiple nonrespiratory behaviors to be expressed by the same neurons, while providing adaptive respiratory gain control.

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4.4

Contribution of cholinergic systems to state-dependent modulation of respiratory networks

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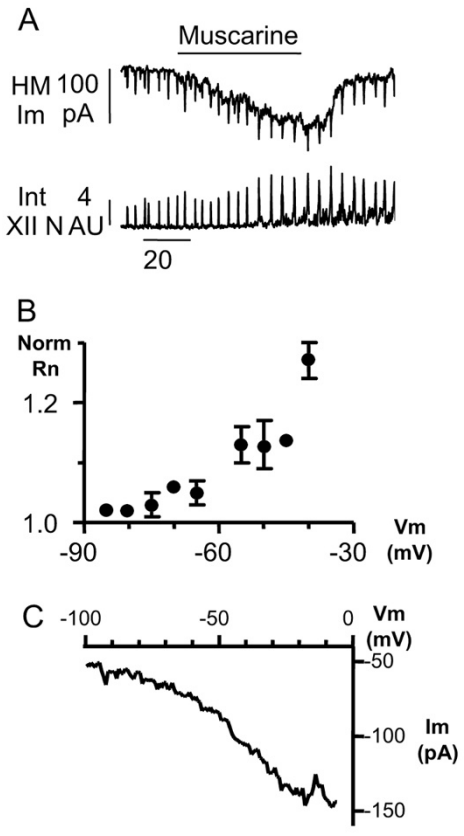
The sleep/wake cycle is controlled by reciprocal inhibition between cholinergic and aminergic cell groups in the pons and brainstem [1]. In rapid eye movement (REM) sleep, cholinergic neurone discharge is at its highest level while aminergic discharge is at the lowest level across all behavioural states [1]. These changes are accompanied by alteration of respiratory pattern and in the output of some respiratory motoneurone pools [1]. While effects of aminergic receptors on respiratory pattern and motor output have been extensively investigated, less is known of the effects of cholinergic receptors. Disease syndromes such as obstructive sleep apnoea in adults or infants, or sudden infant death syndrome may involve abnormal cholinergic receptor-mediated responses.

Here we report network and cellular effects of muscarinic acetylcholine receptors (mAChRs) on respiratory pattern and motor output in hypoglossal motoneurons (HMs) using *in vitro* slice preparations from mouse. Rhythmically active brainstem slices were made from mice (P0–4 days) and the mAChR agonist, muscarine was bath applied (10 μM, $n=6$). As illustrated in Fig. A, integrated hypoglossal nerve (Int XII N) burst amplitude increased by

Figure



80 ± 24% (mean ± SE) while burst frequency decreased (–34 ± 7%). Local injection of muscarine (100 μM, $n = 14$) over the hypoglossal nucleus (nXII) produced an increase in Int XII N burst amplitude (96 ± 23%) but no change in burst frequency (5 ± 4%). Injections of muscarine ($n = 3$) in the pre-Bötzinger complex (PBC) decreased Int XII N burst frequency (–20 ± 5%) and increased burst amplitude (60 ± 12%). These effects were blocked by bath application of atropine ($n = 3$, 1 μM). In whole cell recordings from HMs ($n = 14$) local nXII or bath application of muscarine evoked an inward current (–29 ± 5 pA) in voltage clamp at –60 mV (see Fig. A for example) and depolarization in current clamp. This current was associated with increased neuronal input resistance (Rn) which was greater at voltages positive to –60 mV (Fig. B), suggesting that muscarine inhibited a voltage-dependent outward current. Muscarinic inhibition of K^+ currents elicited by slow (2 s) depolarizing voltage ramps in the presence of TTX and Cd^{2+} was much greater at voltages from –70 to –20 mV (Fig. C), a voltage range typically occupied by the voltage- and ligand-dependent K^+ current I_M but not by other voltage-dependent K^+ currents.

These results suggest that mAChRs have potent effects both on respiratory rhythm generation and HM motor output by distinct actions on both PBC neurones and HMs. We hypothesize that excitatory effects of mAChRs on HMs is partly due to muscarinic inhibition of the potassium current I_M, causing depolarization and increased Rn and thus enhancing HM firing.

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4.5

Convergent effects of multiple modulators on two-pore-domain 'leak' potassium channels in respiratory-related neurons

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The neuronal membrane conductance at rest is dominated by channels that preferentially conduct potassium ions. These resting or 'leak' K^+ channels drive the membrane potential toward the potassium equilibrium potential, away from spike threshold and provide a shunt conductance to diminish voltage responses to synaptic currents. Interestingly, a number of modulators act to dynamically regulate neuronal excitability by increasing or decreasing activity of these channels.

Although resting K^+ channels represent a major target for neuromodulators, the molecular basis for this class of channels has remained elusive. Recently, however, a novel gene family of putative leak potassium (KCNK) channels was identified by molecular cloning [1,2].

Among those, a subgroup of so-called TASK channels generate currents with a unique constellation of properties. Thus, in heterologous systems, TASK-1 and TASK-3 currents are persistent and time-independent, and they display a weak rectification in asymmetric physiological K^+ conditions that obeysconstant field predictions for an open, K^+-selective pore (ie, they are instantaneous open-rectifiers). Moreover, cloned TASK channel currents are inhibited by extracellular protons in a physiological pH range and activated by inhalation anesthetics at clinically relevant concentrations [1,2].

We have used histochemical and whole cell electrophysiological approaches *in vitro*, taking advantage of the unique properties of TASK channels, to establish functional expression of native TASK currents in brainstem neurons, including those associated with respiration. As described below, we find that TASK channels underlie a pH-, anesthetic- and transmitter-sensitive K^+ current in respiratory-related motoneurons; they also contribute to pH-sensitive responses in presumptive respiratory chemoreceptor neurons of the locus coeruleus (LC) and medullary raphe.

Using *in situ* hybridization, we found high levels of TASK-1 and TASK-3 mRNA in cranial and spinal motoneurons and accordingly, hypoglossal motoneurons expressed a pH-sensitive K^+ current under voltage clamp with kinetic and voltage-dependent properties of TASK channels (ie, instantaneous open-rectification). In addition, this motoneuronal pH-sensitive, open-rectifier K^+ current was inhibited by a number of neurotransmitters (serotonin, norepinephrine, SP, TRH) and activated by halothane and sevoflurane with an EC_{50} identical to that of their anesthetic effects.

By combining *in situ* hybridization with immunohistochemistry, we found that TASK channel transcripts are expressed in catecholaminergic LC neurons and serotonergic raphe neurons, although at moderate levels. In those aminergic cells, pH-sensitive currents appear to involve multiple ionic mechanisms. Nevertheless, a contribution from native TASK channels was readily revealed by taking advantage of their combined pH- and halothane-sensitivity; thus, the pH-sensitive halothane-induced K^+ current in LC and raphe neurons had the properties of an instantaneous, open rectifier.

In summary, TASK channels represent a molecular substrate for convergent effects of multiple modulatory mechanisms. By virtue of their pH-sensitivity and cell-type expression in respiratory-related neurons, TASK channels may contribute to integrated central respiratory responses to alterations in brain acid-base status; this could involve effects on chemoreceptor neurons in LC and raphe – and/or directly on respiratory motoneurons. Inhibition of TASK channels by transmitters associated with behavioral arousal may provide an excitatory bias to motoneurons, and support well known state-dependent differences in motor activity; TASK channel activation in motoneurons and aminergic brainstem neurons likely contributes to immobilizing and hypnotic anesthetic effects.

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ORAL PRESENTATIONS - SESSION 5
Chemoreception: peripheral mechanisms and genetic determinants

5.1

Time domains of the sympatho-respiratory response to hypoxia: plasticity in phrenic and sympathetic nerve activities

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Powell *et al.* defined short- and long-term time domains in the respiratory response to both single and repetitive hypoxic exposures [1]. Aspects of these time domains reflect a neural 'plasticity' in the network generating the respiratory pattern and are evident in both the timing and amplitude of the cycle. Studies from our laboratory have focused on the plasticity evoked by hypoxia, in particular, the role of the lateral pons in modulating the cycle's timing [2,3]. Because of respiratory modulation of sympathetic nerve activity (SNA), we hypothesized that comparable time domains are also evident in SNA's response to hypoxia and that the lateral pons also modulates plasticity in SNA.

We recorded phrenic nerve activity (PNA) and splanchnic SNA in anesthetized (Equithesin), paralyzed, vagotomized, thoracotomized, adult male rats (Sprague-Dawley, Zivic Miller). We generated cycle-triggered averages of PNA and sSNA before, during and after hypoxic exposures (8% O_2/92% N_2, 45 s duration). In a series of experiments, we have analyzed the hypoxic-evoked plasticity before and after ventrolateral (vl) pontine interventions as well as before and after multiple exposures to hypoxia.

Before hypoxic exposures, SNA was modulated weakly by respiration with a tendency for activity to increase just after the phase transition from inspiration (I) to expiration (E). During the 1st brief hypoxic exposure, SNA increased during stage-II E. Immediately after hypoxia, sSNA became quiescent during the prolonged E associated with short-term depression of respiration. Following vl pontine interventions, baseline respiratory modulation of SNA was attenuated, the shift or increase in stage-II "expiratory" sSNA did not occur during hypoxia but the post-hypoxic decrease of sSNA remained. After the 10th hypoxic exposure, both SNA and PNA increased their activity; in particular, respiratory-modulated SNA coincident with expiration remained increased after hypoxia. This increase in activity was sustained for more than 60 min. The increase in SNA was reflected by a greater response to injected cyanide compared to the baseline response prior hypoxia. We conclude that long-term facilitation (LTF) is elicited in SNA after repetitive hypoxic exposures and, thus, the interaction between the sympatho-respiratory control systems may be a mechanism for the increased SNA associated with sleep apnea. Further, we interpret the results of these studies as supporting an 'activity-dependent' plasticity not only in respiratory but also sympathetic networks [4].

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5.2

Hypoxic chemosensitivity of the cardiorespiratory regions of the rostral ventrolateral medulla (RVLM)

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Recently, unique cardiorespiratory regions in the RVLM have been found to be oxygen sensitive [1,2]. However, the mechanism of sensing O_2 in these RVLM regions is unknown. Since heme oxygenase (HO) has been shown to be involved in the hypoxic responses of the carotid body and pulmonary artery, the aim of our work has been to determine if HO is present in the RVLM, whether expression of HO is altered by chronic hypoxia, and whether HO is necessary for the excitatory response of RVLM neurons. RT-PCR of the RVLM of rats exposed to hypoxia (10% O_2) or normoxia for 10 days, revealed that HO-2 is expressed in the RVLM constitutively during both normoxia and hypoxia and that HO-1 is induced during chronic hypoxia [3]. Immunocytochemistry localized these HO isoforms to the C1 region and pre-Bötzinger complex (pre-Böt) of the RVLM. Thus, HO isoforms are present in the RVLM cardiorespiratory regions under control and hypoxic conditions, consistent with a potential role for HO in the O_2 sensing function of these cardiorespiratory regions. To determine whether HO is important for the oxygen sensitivity of the RVLM, we examined the response of cultured RVLM neurons to chemical hypoxia (NaCN), before and after blocking HO with SnPP-IX, and correlated the hypoxia-excited response with the expression of HO-2 using immunocytochemistry. Primary cultures were prepared from neonatal rats and studied using the whole-cell perforated patch clamp technique. The hypoxia-induced depolarization of these RVLM neurons in response to NaCN was abolished after blocking HO. Examination of these hypoxia-excited neurons showed that they were immunoreactive for HO-2. Further phenotyping found that RVLM neurons that express HO are located in the tyrosine hydroxylase rich C1 sympathoexcitatory region as well as within the neurokinin-1 receptor (NK-1R) rich pre-Böt. In fact, 70% of the neurons expressing HO-2 in the pre-Böt co-express NK-1R suggesting that oxygen sensitivity may reside in some respiratory rhythm generating neurons in the pre-Bötzinger complex. Thus, HO is present in the cardiorespiratory regions of the RVLM, HO-1 is induced by chronic hypoxia, and HO is necessary for the excitatory response to chemical hypoxia in RVLM neurons consistent with the presence of HO-2 in these excited neurons. These findings support an important role for HO in the oxygen sensitivity of cardiorespiratory neurons in the RVLM.

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5.3

CNS changes in chronic hypoxia

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Chronic hypoxia increases the hypoxic ventilatory response (HVR) in awake humans and animals [1] and we have shown that this involves changes in the "CNS gain of the HVR". The ventilatory or phrenic nerve response to pharmacological stimulation of carotid bodies or electrical stimulation of the carotid sinus nerve is significantly increased in awake and anesthetized rats, respectively, after 2–7 days at $P_{I_{O2}}$=74 Torr [2]. We hypothesize that changes in neurotransmitters and their receptors along the HVR reflex pathway, and specifically in the nucleus tractus solitarius (NTS), increase the CNS gain of the HVR during chronic hypoxia. Previously we found that dramatic time-dependent changes in dopaminergic neurotransmission occur in the NTS during chronic hypoxia but they cannot explain the changes in the HVR [2]. Now we are testing the hypothesis that NMDA receptors in the NTS are necessary for the increased CNS gain of the HVR during chronic hypoxia.

We previously reported no significant changes in mRNA for NMDA-R_1 receptors during 7 days of hypoxia [3] but further experiments are planned to quantify changes in actual receptor protein. In awake unrestrained rats held in normoxic conditions (n=6), chronic administration of a non-competitive NMDA receptor antagonist to the caudal NTS (25 µL/hr of MK-801 for 7 days *via* an Alzet miniosmotic pump) did not significantly change the HVR measured with barometric pressure plethysmography after 2 or 7 days of treatment. This is in contrast to the decrease in the HVR observed by other laboratories performing acute microinjections of MK-801 to the NTS [4]. However, acute systemic injections of MK-801 (3 mg/kg ip.) decreased the HVR in our laboratory (n=8) in agreement with published results [5]. These results suggest time-dependent changes in glutamate neurotransmission with chronic blockade of NMDA receptors and we are testing this by repeating experiments using acute microinjections of MK-801. Experiments with acute or chronic blockade of NMDA receptors after chronic hypoxia will be compared with the former results to determine the independent effects of hypoxia versus NMDA receptor activation on the CNS gain of the HVR.

Future experiments will investigate the role of GABA in determining the CNS gain of the HVR. Chronic hypoxia changes mRNA levels for GABA-R1α [3] and GABA is reported to modulate the gain of preinspiratory bulbospinal neuron responsiveness to other neural inputs [6]. This change in gain instead of set point is similar to that observed for the HVR during chronic hypoxia.

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5.4

Cytochromes, reactive oxygen species and responses of the oxygen sensing signal pathway

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The heterogeneous oxygen partial pressure (PO_2) distribution in mammalian organs requires for triggering gene expression and ion channel conductivity an O_2-sensing signal cascade with three responses: optimizing of cellular functions during normoxia, adaptation of cell function under hypoxia and survival of cell function to withstand anoxia. It is not known whether these different responses are induced by various O_2 sensing signal cascades. One signal casade, however, is supposed to consist of mitochondrial and/or non-mitochondrial heme proteins sensing oxygen with subsequent second messenger formation such as reactive oxygen species (ROS), in particular hydroxyl radicals (OH) which influence transcription factor stability as well as ion channel pore formation. NADPH oxidase isoforms with different gp91phox subunits as well as an unusual cytochrome c oxidase (CYT) with a hemea/hemea3 relationship of 9:91 as detected by light absorption photometry in the carotid body as well as human liver tumor cells (HepG2) will be discussed as putative oxygen sensor proteins. Whereas CYT might be of special importance for the carotid body, NADPH isoforms generating ROS in dependence on PO_2 are considered as more general cellular O_2 sensors. The formation of $OH^{\bullet}$ by a perinuclear Fenton reaction from hydrogen peroxide (H_2O_2) is imaged three dimensionally by 1 and 2 photon confocal laser microscopy in HepG2 cells using dihydrorhodamine 123 or 2′7′dichlorodihydrofluorescin as indicators. Hot spots of $OH^{\bullet}$ generation are seen predominantly in the endoplasmatic reticulum (ER) but also in mitochondria (MIT). It is concluded, that heme and non-heme iron binding proteins in the ER and MIT compartment represent the perinuclear hot spots which degrade H_2O_2 originating from NADPH oxidase isoforms.

5.5

Regulation of tyrosine-hydroxylase (TH) gene expression by hypoxia and von Hippel-Lindau tumor suppressor complex

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Loss of von Hippel-Lindau (VHL) gene function leads to a familial cancer syndrome, VHL disease, characterized by CNS and retinal hemangioblastomas, renal clear cell carcinomas, and pheochromocytomas. Pheochromocytomas, tumors of the chromaffin cells of the adrenal medulla, secrete large amounts of catecholamines. This results from enhanced expression of TH, the rate-limiting enzyme in catecholamine biosynthesis. Overexpression of exogenous pVHL in PC12 cells lead to a substantial repression of TH

protein and mRNA [1]. Here we examined the role of endogenous pVHL in the regulation of TH gene expression. We developed PC12 clonal cell lines that stably express VHL antisense RNA and have reduced levels of endogenous pVHL by 5–10 fold. In these cells, TH protein and mRNA were increased 2–3 fold as compared to control cells. Nuclear run-on analysis revealed an increase in the overall TH gene transcription. In addition, we measured augmented efficiency of the TH transcript elongation in the distal part of the gene. Transient co-transfections of −773 kb TH-CAT promoter-reporter constructs with the VHL antisense RNA resulted in an approximately 2–3 fold increase in reporter gene activity. TH is a hypoxia-inducible gene. The overall accumulation of TH mRNA and the rate of TH gene transcription were substantially enhanced in cells with decreased levels of pVhl exposed to 1% O_2 for 16 h. These results indicate that endogenous pVhl constitutively represses TH gene expression in PC12 cells.

TH promoter contains a functional hypoxia-responsive element (HRE) - like sequence. Transient co-transfections of the HIF1α and HIF2α expression vectors with the wild type, but not mutated TH promoter revealed significant 3–4 fold induction of the reporter gene activity. Gel-shift analysis showed presence of the hypoxia-inducible complexes associated with the wild type but not mutated TH HRE. Electroelution of protein complexes from the hypoxia-inducible complex showed presence of the HIF2α, and to lesser extent of the HIF1 subunit. pVHL is part of the E3 ubiquitin ligase complex that targets the HIF alphas for ubiquitination and degradation. Changes in the levels of the pVHL in PC12 cells by either overexpression of pVHL or expression of VHL antisense RNA resulted in respectively decreased and increased HIF-and hypoxia-mediated activation of the TH promoter. Stable changes in the pVHL levels in PC12 cells resulted in accumulation of HIF alphas during normoxia in cells with decreased amounts of pVHL, and in decreased expression of HIF alphas during hypoxia in cells overexpressing pVHL. The changes in HIFα protein accumulation induced by pVHL resulted from ubiquitination of HIF alphas by pVHL.

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ORAL PRESENTATIONS - SESSION 6
Chemoreception: peripheral mechanisms and genetic determinants

6.1

Conditional repression of a calcium-activated potassium channel reveals its role in the hypercapnic ventilatory response

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An increase in tidal volume is an important feature of the ventilatory response to hypercapnia. The neural substrate for tidal volume augmentation is the frequency of action potentials per inspiratory burst. Small-conductance, calcium-activated potassium channels, which are gated by the increases in intracellular calcium during activation, contribute to the interval between action potentials by generating an afterhyperpolarization current. The generation of mice in which one member of this class of potassium channels (SK3) can be regulated by dietary doxycycline (dox) [1] has enabled its role in the ventilatory response to carbon dioxide to be examined. Experiments were carried out in awake mice at 2 weeks of age. A control period in 100% oxygen was followed by 5 min in 5% CO_2, 95% O_2. Animals targeted for repression of SK3 (SK3 T/T) on dox showed a greater increase in tidal volume than either SK3 T/T not exposed to dox or wild type. There was no difference in the increase in respiratory rate between the three groups of animals. These results show that SK3 is an important regulator of action potential frequency during CO_2 stimulation. They also suggest that medullary neurons which contribute to respiratory pattern may be characterized by SK3 channels while those responsible for rhythm may not.

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6.2

On synchronizing pH-sensitive subthreshold oscillations of membrane potential in locus coeruleus (LC) neurones

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The LC is composed for the most part (ca. 90%) of pH-chemosensitive neurones which under *in vitro* conditions show a strong tendency to synchronize their discharge. The object of this study was to explore some of the mechanisms involved in such synchronization. In neonatal LC neurones the most prominent feature contributing to spike synchronization is a low frequency subthreshold rhythmic oscillation (SRO) of membrane potential which has been attributed to gap junction-mediated electrical coupling. In the present experiments we took advantage of the fact that in the *en bloc* isolated neonatal brainstem-spinal cord LC neurones receive a respiratory-phased innervation [1]. It was thus possible to examine the influence on the electrically coupled network of a naturally occurring rhythmic synaptic input. In simultaneous whole cell and extracellular recordings from LC neurones in this preparation there was a strong tendency towards the synchronous occurrence of spikes and, when examined in paired whole cell recordings, it was apparent that this was due to strong synchronization of their SRO. Under conditions of intact chemical synaptic transmission, current injection into one neurone failed to influence membrane potential, SRO frequency or the timing of spike discharge in the other neurones.

The timing of the respiratory cycle, simultaneously recorded as the efferent discharge on a phrenic root, influenced the timing of the SRO: the occurrence of a phrenic burst triggered a wave of depolarization and the insertion of one or a small number of extra spikes; this was followed by a delay to the onset of the next spontaneously occurring oscillation. Addition of QX314 (4 mM) to the pipette solution to block voltage-gated Na^+ conductances suppressed the discharge of large, rapid spikes and revealed that the underlying

SRO was composed of a slow depolarizing wave with superimposed small depolarizing transients. These transients, which we have previously shown to be blocked by Cd^{2+} and which are therefore presumably Ca^{2+} spikes, were synchronized with the full sized extracellularly recorded spikes of a second LC neurone. The effect of the respiratory-phased synaptically-mediated input was, first, to trigger a short sequence of synchronized Ca^{2+} spikes throughout the network, and then for a short period to inhibit the network, ie, to delay the next SRO cycle, *via* an α_2-adrenoceptor-mediated mechanism. This last suggests that the inhibition depends on noradrenaline released either by LC neurones themselves or by respiratory-phased afferent input. Raising the CO_2 concentration (2–10%) eliminates the inhibition and increases SRO frequency on average by a factor of 2 (equivalent to a spike frequency increase of about 50%) [1].

Following partial or complete replacement of extracellular Ca^{2+} with Ba^{2+}, and in the presence of TTX, the synchronized SRO was transformed into a large amplitude oscillation. The frequency of this rhythm was partly set by a ramp-like growth of depolarization which initiated each cycle of oscillation, and which was sensitive to pH, the frequency of oscillation increasing by about 30% when the CO_2 concentration was raised from 2 to 10%.

These observations suggest that synchronizing mechanisms within this network normally include both chemical synaptic input and electrical coupling of a pH-sensitive Ca^{2+}-dependent rhythm (SRO), which does not itself depend on such input.

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6.3

Cellular mechanisms of chemosensitivity in serotonergic raphe neurons

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Serotonergic neurons of the medullary raphe nuclei are strongly stimulated by acidosis [1,2], and are putative central respiratory chemoreceptors. We have examined the relationship between serotonergic neurons and blood vessels in the adult rat brain by using immunohistochemistry for tryptophan hydroxylase (TpOH) and injections of arteries with fluorescent albumin in gelatin. Serotonergic neurons within the raphe were closely associated with the basilar artery and its large penetrating branches. There were also homologous serotonergic neurons near large arteries of the rostral and caudal ventrolateral medullary surface (VLMS). We propose that serotonergic neurons on the VLMS contributed to the ventilatory response to application of acidic solution in classical *in vivo* experiments that localized the chemoreceptors to that region. However, these neurons may not be specifically sensing CSF pH, but rather the PCO_2 of blood in proximal arteries that happen to be located adjacent to the CSF space. In addition, a large number of homologous chemosensitive neurons are also located within the midline raphe nuclei.

Perforated patch clamp recordings were made from raphe neurons in tissue culture, and the responses to respiratory acidosis, metabolic acidosis and isohydric hypercapnia were quantified. From a baselin firing rate of 0.2 Hz to 1.5 Hz, an increase in PCO_2 from 5% to 9% and a decrease in pH_o from 7.4 to 7.17 (at a constant [$NaHCO_3$] of 26 mM) induced a mean increase in firing rate of 1.38 Hz (to 285% of control; $n=11$). Metabolic acidosis (5% PCO_2, 15 mM [$NaHCO_3$], pH_o 7.16) induced a mean increase in firing rate of 1.15 Hz (to 309% of control). Isohydric hypercapnia (9% PCO_2, 40 mM [$NaHCO_3$], at constant pH_o) induced a mean increase in firing rate of 1.01 Hz (to 384% of control). These neurons also increased their firing rate in response to a decrease in pH_o in the absence of CO_2 and bicarbonate (in HEPES buffer). The only acid/base change that is shared by these four manipulations is a decrease in pH_i, suggesting that this is the primary stimulus for these neurons – as is true for other chemosensitive neurons, including carotid body glomus cells.

Using voltage clamp recordings, we have identified a novel ionic current in serotonergic neurons that is highly pH sensitive. This current was activated by calcium influx during depolarization. The major charge carrier was K^+ under physiological conditions, but it also had a high permeability to Na^+, with a permeability ratio for K^+:Na^+ of 4 : 1. It was not blocked by apamin (400 nM), charybdotoxin (240 nM), or TEA (15 mM). The current was extremely sensitive to changes in pH centered near 7.3. The current at pH 7.61 was 95% of the maximum current, while the current at pH 6.99 was 2% of the maximum. To our knowledge, a current with these properties has not been reported previously. This current would be predicted to induce changes in membrane potential that have been observed during whole-cell current clamp recordings.

Serotonergic medullary raphe neurons are strongly stimulated by a decrease in pH_i due to the presence of a novel highly pH-sensitive calcium-activated cation channel. The degree of chemosensitivity of these neurons is large enough to make a major contribution to the changes in ventilation observed in whole animals in response to respiratory acidosis. The location of serotonergic neurons next to blood vessels and their widespread projections suggest that these neurons are chemoreceptors that modulate a variety of brain functions, including but not limited to respiratory control, and thus contribute to pH homeostasis.

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6.4

CO_2 chemotransduction in central neurons: role of intracellular pH (pH_i) and extracellular pH (pH_o)

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We have been studying the role of changes of pH_i as part of the signaling pathway of hypercapnia in neonatal rat brainstem neurons from both chemosensitive (NTS-nucleus tractus solitarius, VLM-ventrolateral medulla, LC-locus coeruleus) and non-chemosensitive (IO-inferior olive, Hyp-hypoglossal) regions. We are testing the model of chemotransduction that proposes that hypercapnia acidifies neurons, which leads to inhibition of K^+ channels, resulting in neuronal depolarization and increased firing rate. We measure pH_i in individual neurons within brain slices using fluorescence imaging microscopy [1,2]. We developed a new technique to measure pH_i and V_m simultaneously by loading pH-sensitive fluorescent dyes using perforated patch (amphotericin B) pipettes in LC neurons [3]. All studies were done at 37°C.

Neurons from all brainstem regions have high intrinsic buffering power, around 45 mM/pH unit for NTS, VLM, IO and Hyp neurons [1] and about 90 mM/pH unit for LC neurons [3]. The only pH-regulating transporter available for recovery from an acid load in these neurons is Na^+/H^+ exchange (NHE) [1,2]. The only HCO_3-dependent transporter present is the acidifying Cl^-/HCO_3^- exchanger, which has been found in VLM, IO, Hyp and some NTS neurons [1]. Interestingly, the NHE in neurons from chemosensitive regions (eg, NTS and VLM) is more sensitive to inhibition by decreased pH_o than neurons from non-chemosensitive regions (eg, IO and Hyp) [1].

We observed different responses to hypercapnia in neurons from chemosensitive vs. non-chemosensitive regions. Hypercapnic acidosis (HA–10% CO_2, pH_o 7.15) resulted in a maintained fall of pH_i in neurons from chemosensitive areas whereas pH_i recovery from acidosis was evident in neurons from non-chemosensitive areas [2]. Under conditions of isohydric hypercapnia (IH–10% CO_2, 52 mM HCO_3^-, pH_o 7.45), pH_i recovery was seen in neurons from all regions [2], indicating that pH-regulating mechanisms are present in all these neurons but that they are inhibited more fully by decreased pH_o in neurons from chemosensitive regions. The same pH_i responses to HA (15% CO_2, pH_o 6.8) and IH (15% CO_2, 77 mM HCO_3^-, pH_o 7.45) were seen in LC neurons where pH_i and V_m were measured simultaneously. The changes of pH_i occurred before the changes in neuronal firing rate [3]. In LC neurons, HA resulted in a larger fall of pH_i (0.27 vs. 0.15 pH unit) and a larger increase in firing rate (1.31 vs. 1.06 Hz) than seen with IH. Notably, increased firing rate was accompanied by membrane depolarization (2.5 mV) in response to HA but by membrane *hyperpolarization* (2.3 mV) in response to IH. Other acid challenges involving decreased pH_o (acidified HEPES; isocapnic acidosis–5% CO_2, 7 mM HCO_3^-, pH_o 6.9) resulted in increased firing rate with depolarization while acid challenges involving constant pH_o (50 mM propionate, pH_o 7.45) resulted in slightly increased firing rate with membrane hyperpolarization.

In summary, the response of V_m to a fall of pH_i is dependent on whether pH_o changes or not and is not well correlated with increased firing rate in LC neurons. The parameter that best correlates with increased firing rate in response to an acid challenge is the change of pH_i, indicating that pH_i is likely involved as part of the chemosensitive signaling pathway in LC neurons.

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6.5

Central chemosensitivity, sleep, and wakefulness

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Central chemoreceptors are widespread within the brainstem. This conclusion is based on *in vitro* evidence of neurons from many locations that are responsive to pH changes and on *in vivo* evidence of respiratory responses to focal acidification at these same locations. Why are there so many chemoreceptor sites? As one possibility we hypothesize that function at different sites varies with arousal state. In unanesthetized rats, we produce focal acidification at single sites with microdialysis (probe tip = 1 mm × 240 μm diameter) using artificial cerebrospinal fluid equilibrated with 25% CO_2. Tissue acidosis, measured at the region of the retrotrapezoid nucleus (RTN) in unanesthetized rats, is approximately equivalent to that observed with end-tidal PCO_2 = 7–8 mmHg above the eupneic value. Focal acidification of the retrotrapezoid nucleus (RTN) increased ventilation by 24% only in wakefulness *via* an increase in tidal volume [1]. In the medullary raphé, the excitatory effect of such focal acidification was observed only in sleep (defined by EEG and EMG criteria); ventilation and frequency increased by 15–20% in NREM sleep and frequency by 15% in REM sleep. There was no effect in wakefulness [2]. In our most recent study, focal acidification of nucleus tractus solitarius (NTS) increased ventilation by up to 20% in both wakefulness and NREM sleep due to an effect on both tidal volume and frequency. The increase in ventilation is greater with focal acidification in the caudal part of NTS at the level of the area postrema. Acidification of more rostral parts of the NTS produced variable results.

The medullary raphé contributes to chemoreception in sleep, the RTN in wakefulness, and the cNTS in both sleep and wakefulness. Central chemoreceptors at these three different locations do appear to vary in effectiveness with arousal state. The response at each site is only a fraction of the response attributable to all sites being stimulated at the same intensity. Hypocapnia resulting from single site stimulation can lower the contribution of other sites minimizing the apparent effectiveness of single site stimulation.

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6.6

Normobaric CO_2 and hyperbaric O_2 stimulate the same neurons in the solitary complex

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We recently reported that neurons in the dorsocaudal chemosensitive area, the solitary complex (SC), are stimulated by reactive oxygen species (ROS) during acute hyperbaric oxygen (HBO_2), suggesting that oxidative stress modulates cardiorespiratory networks [1,2]. Hyperoxia is known to have various effects on cardiorespiratory function [3,4], and it has been proposed that central hypoventilation syndromes, for example, SIDS, are due in part to increased oxidative stress of neurons after birth [5]. Thus, we have tested the hypothesis that excitability of CO_2-chemosensitive neurons in the caudal SC is altered during oxidative stress at normobaric and hyperbaric pressures.

Intracellular recordings were made from SC neurons in 300 μm thick medullary slices prepared from weaned and adult rats. Control medium was equilibrated with 95% O_2 –5% CO_2 at barometric pressure (P_B) ~1 atmosphere absolute (ATA) (medium PO_2 ~720 Torr, PCO_2 ~38 Torr). A slice was maintained in a hyperbaric

chamber and exposed to one or more of the following conditions (~37°C): 1) normobaric hypercapnia (8–20 mins 15% CO_2 in O_2) to test for CO_2 chemosensitivity; 2) normocapnic HBO_2 (10–20 mins 98.3% O_2 –1.65% CO_2 at P_B 3.3 ATA) and 3) pro-oxidants at P_B ~1 ATA (8–10 mins 500 µM Chloramine-T or 1 mM N-chlorosuccinimide) to test for the effects of ROS; and 4) hypercapnic HBO_2 (10–20 mins 95% O_2 –5% CO_2 at P_B 3.2 ATA) to test for the effects of hypercapnic acidosis on sensitivity to HBO_2 [6,7,9]. During HBO_2, tissue PO_2 (mean ± S.E.) at the core of the slice (150 µm depth) increased from 291 ± 20 to 1517 ± 15 Torr [6].

Focusing on the caudal SC, 62% (18/29) of the neurons tested were depolarized by normobaric hypercapnia. Of these, 78% (14/18) were stimulated by normocapnic HBO_2 and pro-oxidants. When hypercapnia and HBO_2 were combined (hypercapnic HBO_2), the firing rate response was greater to both stimuli than to the sum of their individual responses. Most neurons (9/11) that were CO_2 insensitive were also unresponsive to HBO_2 and/or pro-oxidants.

We conclude that acute exposure to oxidative stress, either by increased tissue PO_2 at hyperbaric pressure or pro-oxidants at normobaric pressure, stimulates CO_2-chemosensitive neurons in the SC, suggesting that central chemosensitivity may likewise be affected by oxidative stress. It is unclear, however, if the strong neuronal excitation observed when hypercapnic acidosis and HBO_2 are combined is due to increased lipid peroxidation during intracellular acidosis [7,8,9] or to the effect of increased ROS on CO_2 chemosensitivity.

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POSTERS

P1

Substance P reconfigures the neural respiratory network in ventrolateral medulla in the neonatal rat brainstem *in vitro*

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The effects of SP on the respiratory activity in the rat (0–4 days old) neonatal brainstem-spinal cord preparation were investigated. Respiratory activity was recorded from C4 ventral roots and intracellularly, from three types of respiration-related neurons: pre-inspiratory (Pre-I or biphasic E, $n=19$), three subtypes of inspiratory (InspI-III, $n=18$), expiratory (Exp, $n=11$), and tonic neurons ($n=11$) in the ventrolateral medulla (VLM).

There were two types of respiratory rhythm in respiration-related neurons and in integrated C4 nerve activity under normal experimental conditions, with marked similarities to normal eupneic respiration and sighs. These two types of bursts were similar to patterns described recently in rhythmic brainstem slices in neonatal mice [1]. In control, the frequency of eupneic-like bursts varied in a range of 7–11 bursts per minute, whereas the frequency of the sigh-like activity was 10–30 times slower at 0.2–0.6 bursts per minute.

Bath application of SP (10 nM–1 µM) caused a biphasic effect on eupneic-like respiratory frequency. There was a pronounced dose-dependent decline of burst rate (by 48% from control level of 9.35 ± 1.8 (mean ± SEM) bursts per minute, $n=28$) after the onset the SP application (phase P1), followed by a weaker dose-dependent increase (by 17.5%) in burst rate (phase P2) ($P<0.001$, two-way ANOVA). The biphasic effect of SP on inspiratory burst rate was associated with sustained membrane depolarization (in a range of 0.5–13 mV) of respiration-related and tonic neurons. C4 bursting before and after SP application was synchronized with neuronal activity of respiration-related neurons.

Unlike the biphasic effect shown in the eupneic-like activity, the sigh-like burst frequency was considerably and gradually increased. This elevated sigh-like activity could reset normal respiration rhythm, so that in some cases the respiration activity was represented exclusively by the sigh-like pattern. As with the first pattern, this frequency increase depended on SP concentration ($P<0.001$, one-way ANOVA), though the range of the changes was an order bigger as compared to normal respiratory pattern, and reached 650%. There was significant correlation ($R=0.537$, $P<0.0001$) between the eupneic-like frequency decrease during P1 and the sigh-like rhythm increase, and P1 phase corresponded in time to maximum slope of increase in sigh-like frequency. In turn, the P2 phase in the eupneic-like bursting coincided in time with maximum sigh-like rhythm developed during SP application. Thus, the biphasic effect of SP on eupneic-like respiratory activity was superimposed on a monotonic increase of the sigh-like pattern frequency.

Our results suggest that a) a single respiratory network in the VLM might reproduce or generate two different respiratory patterns; b) within this respiratory network SP exerts a general excitatory effect on respiration-related and tonic neurons; c) the transient changes in neuronal activity in the VLM produced by SP may underlie the biphasic effect in the eupneic-like bursting, as well as the changes in the sigh-like pattern frequency, and d) SP can transform the particular type of the interaction of eupneic-like and sigh-like patterns, which compose the normal respiratory activity in the neonatal brainstem, and thus, reconfigure the neural network controlling respiratory rhythm generation.

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P2

Evidence for multiple rhythmogenic networks in the rat *en bloc* preparation

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The pre-Botzinger Complex is coextensive with a population of SP and DAMGO-sensitive inspiratory neurons, retained in the transverse slice preparation, which has been extensively used to study mechanisms for respiratory rhythm generation. Here, qualitatively different effects of peptidergic modulation were observed in two preparations *reduced*, and *complete*. The reduced preparation was either the standard transverse slice, or an *en bloc* preparation whose rostral margin corresponded with the rostral margin of the transverse slice. The complete preparation was an *en bloc* preparation whose rostral margin extended just past the vagus rootlets, approximately 600 µm past the rostral margin of the reduced preparation. In the reduced preparation, periods following peptidergic modulation were distributed continuously, while in the complete preparation, periods were distributed multimodally. These observations suggest that additional rhythmogenic circuitry is retained in the complete preparation.

P3

Substance P and NK_1 receptor activation within the ventral medullary respiratory network prolongs expiratory time

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Substance P exerts different effects when applied to the medullary respiratory network. *In vivo* microinjection of substance P into the ventrolateral medulla prolongs expiratory time and augments tidal volume [1,2] whereas respiratory frequency is enhanced in rhythmic *in vitro* preparations of immature rat [3,4]. Here, we microinjected substance P and an NK_1 receptor agonist (GR 73632) into the ventral respiratory group of an arterially perfused, *in situ*, working heart-brainstem preparation of mature rat [5]. We wished to determine (i) the effects of NK_1 receptor activation in the ventral medullary respiratory network of this perfused preparation and (ii), the neuronal mechanism that could account for any changes in respiratory motor outflow.

Unilateral microinjection of substance P (1000 pmol) into the ventral respiratory group prolonged phrenic nerve activity cycle length from 2.0 ± 0.2–4.0 ± 0.7 s ($P < 0.01$; $n = 7$ sites). Qualitatively a similar response was observed with GR 73632 (100 pmol). At these responsive sites, CP-99,994, an NK_1 receptor antagonist, failed to affect respiratory motor output. Recordings from the central end of the vagus nerve indicated both inspiratory and post-inspiratory discharge (ie, stage I expiratory activity) in control. Following a microinjection of substance P, post-inspiratory discharge was both augmented and prolonged. Extracellular recordings were made from 23 respiratory neurones using compound microelectrodes for pressure ejection of drugs. Substance P (10 mM) excited 66% of post-inspiratory neurones raising both the peak firing frequency and total number of action potentials per cycle by 15% and 56% respectively; both effects were prevented by antagonising NK_1 receptors.

Our findings indicate that substance P acting on NK_1 receptors in the ventral respiratory network promotes a prolongation of expiratory time *via* an excitatory effect on some post-inspiratory neurones.

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P4

The pre-Bötzinger complex and phase-spanning neurons in the adult rat

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Recent *in vitro* studies indicate that neurons in the pre-Bötzinger (pre-Bot) complex of neonatal rats play an essential role in respiratory rhythm generation. In the adult rat, however, the location and physiology of pre-Bot neurons is less clearly understood. The present study aims to investigate the firing patterns of neurons that are located between Bötzinger and rVRG area, and the precise location of this transition zone in relation to other medullary nuclei. Sprague-Dawley rats (weighted between 400–550 g) were anaesthetised with 72 mg/kg sodium pentobarbitone and 0.4 mg/kg atropine (i.p.), and paralysed with 1 mg/kg pancuronium dibromide (i.v.), followed by additional doses as required. Extracellular recordings were made from 302 respiratory units located between 0 and 1.6 mm caudal to the facial nucleus and ventral to the nucleus ambiguus. As expected, expiratory units were mostly recorded from the rostral medulla (80%, 125/157) and inspiratory units were concentrated in the more caudal area (80%, 36/45). However, we report here that, between the Bötzinger expiratory and rVRG inspiratory units, there exists a transition zone containing a mixture of phase-spanning units (41%, 41/100) as well as inspiratory (37%) and expiratory units (22%). The phase-spanning units are active across the expiratory-inspiratory phase, or vice versa. The rostrocaudal extension of this transition zone is about 400 µm, with the caudal end at the level of the caudal pole of the ambigual compact formation. Our preliminary data suggest that this transition zone could be the locus of pre-Bot neurons in the adult rat.

P5

Network interactions in inspiratory (I) and expiratory (E) neuron populations as indicated by high-frequency oscillations

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Fast rhythms are present in neural discharges of many motor systems. In the decerebrate cat, high-frequency oscillations (HFO, range 50–100 Hz) are ubiquitous in the discharges of I motoneurons (phrenic, recurrent laryngeal, external intercostal) and of medullary I neurons. The amplitude and frequency of HFO are greater when respiratory drive is increased. Coherence spectral analysis shows that the various discharges (population and unit) are significantly correlated at the HFO frequency. This indicates that the common rhythm arises in the brainstem and is transmitted to cranial motoneurons *via* propriobulbar projections and to spinal motoneurons *via* bulbospinal projections.

HFO phase relations of medullary I neuron discharges. Cross correlation analysis was used to measure the phase lag between unit and phrenic discharges. The distributions of unit-phrenic lag times were distinctly different between I unit types classified by overall discharge pattern. The I-augmenting (ramp) units showed a higher proportion of positive unit-phrenic lags (0.0 to 8.8 ms), whereas the I-decrementing and I-plateau units showed a higher proportion of negative lags (–7.6 to 0.0 ms), ie, peak unit HFO lagged peak phrenic HFO. Laryngeal I motoneurons fell into the latter group. The existence of lag distributions that distinguished populations of I neurons suggests that the HFO arises from feedback loops between different populations.

Intracellular recordings from medullary E neurons. For both augmenting E neurons and decrementing laryngeal E neurons, the hyperpolarization during the I phase had superimposed HFOs, which presumably are based on rhythmic IPSP inputs from I neurons.

HFOs in rat. In adult rat *in vivo* preparations, HFOs were present in I discharges, but these were in a higher frequency range (100–150 Hz) than those in the cat (50–100 Hz).

Expiratory HFOs (EHFOs). In some cat preparations during spontaneous respiration, strong oscillations (range 24–74 Hz) were observed in recurrent laryngeal (RL) E nerve and unit discharges. These EHFOs were highly coherent between unit and nerve discharges and between bilateral nerve discharges. Similar oscillations were consistently obtained during fictive vocalization (FV) elicited by midbrain periaqueductal gray electrical stimulation, which produces a large increase of RL E discharge. These were intrinsic rhythms (range 50–70 Hz), since the spectral frequency was different from the stimulation frequency. Thus, when the RL E network is subjected to strong excitation, the neuronal interactions result in generation of strong coherent rhythms in the population.

Abdominal E discharges. During FV, there was a large increase of lumbar (L1, L2) nerve discharges. However, spectral analysis revealed that no intrinsic rhythm was generated; the only rhythm observed was locked to the stimulus frequency. Thus the medullary premotor system cannot readily generate correlated rhythmic activity.

Synchronization mechanisms. The observations suggest that HFO (of both I and E types) is an emergent property of the respective networks. Interspike interval distributions indicate that modal interval durations may occur in a 1 : 1, 2 : 1, or 1 : 2 relation to the HFO period. The heterogeneity of phase lags for different populations suggests that the oscillation may arise from feedback loops in the brainstem.

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P6

Differential modulation of inspiratory motoneurons and respiratory rhythm generating circuits by ATP

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Adenosine triphosphate (ATP) gates a diverse family of P2 ($P2X_{1-7}$ and $P2Y_{1-11}$) receptors that are expressed throughout the CNS, including respiratory regions of the brainstem [1]. Activation of ATP receptors potentiates activity of inspiratory motoneurons (MN) [2], modulates respiratory behaviour *in vivo* when applied to medullary respiratory nuclei and, *via* activation of pH-sensitive $P2X_2$ receptors, may contribute to central respiratory chemosensitivity [3]. Aims of this study were to: i) determine the effects of ATP receptor activation within the pre-Bötzinger complex (preBötC, proposed site of rhythm generation) on respiratory behaviour; ii) test the hypothesis that respiratory networks are endogenously modulated by ATP; and iii) compare the sensitivity of inspiratory MN pools and rhythm generating networks to purinergic modulation.

To determine the effects of ATP on respiratory rhythm and inspiratory motor output, ATP was pressure-injected into the pre-BötC and XII nuclei of rhythmically-active medullary slice preparations from neonatal rats, while monitoring XII nerve and MN output. Effects on phrenic MNs were determined by locally applying drugs over the phrenic MN pool of brainstem spinal cord preparations. ATP (10 s, 0.01–1 mM) caused up to a 4-fold, suramin-sensitive (0.05–1.0 mM), increase in frequency, that was followed by a brief (22 ± 5%) reduction. To test whether this post-ATP inhibition was due to hydrolysis of ATP to adenosine and activation of adenosine receptors, we applied ATPγs, a non-hydrolyzable ATP analogue. Peak potentiation of frequency by 0.1 mM ATPγs (3.20 ± 0.3 fold increase, $n = 7$) was similar to that evoked by ATP (3.30 ± 0.3, $n = 7$), but the effects of ATPγs were longer lasting (102.9 s ± 10.64 vs 29.3 s ± 2.06 for ATP). The secondary reduction in frequency was also absent following ATPγs. Since P2 receptor antagonists also antagonize glutamate receptors which are essential for rhythm generation, the role of endogenous ATP in modulating respiratory rhythm was investigated *via* bath application of ectoATPase inhibitors (DEPC, pCMPS) and an allosteric modulator of $P2X_2$ receptors (Cu^{2+}). DEPC (100 μM, $n = 4$) and pCMPS (30 μM, $n = 3$) increased respiratory frequency 1.30–1.40 fold, while Cu^{2+} (10–50 μM, $n = 6$) increased frequency ~1.5-fold.

Local application of ATP (1–10 mM) over XII and phrenic nuclei produced a biphasic response comprising an initial potentiation of burst amplitude (1.40 ± 0.20 of control and 1.22 ± 0.7 respectively) followed by a decrease in burst amplitude (to 0.82 ± 0.05 and 0.90 ± 0.05 of control respectively) that was theophylline-sensitive and absent following application of ATP-γ-s. The doses of ATP required to potentiate burst amplitude by 1.20–1.40 of control [2] were ~100 times higher than required to increase rhythm 2.5- to 4-fold.

Results show that exogenous ATP potently increases respiratory frequency and that rhythm generating networks are significantly more sensitive to ATP than respiratory motoneurons. In addition, effects of ectoATPase inhibitors and allosteric modulators of ATP receptors suggest that respiratory networks are endogenously modulated by ATP. The differential sensitivity of rhythm generating elements and motoneurons provides an opportunity to explore the physiological significance of P2 receptor diversity to respiratory control.

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P7

Phase-coupling of the respiratory network by somatosensory receptors during locomotion

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Phase-coupling of the respiratory rhythm to locomotor activity has been reported in quadrupeds and humans [1]. Ventral respiratory group (VRG) neurons are involved in respiratory rhythm generation due, in part, to both intrinsic and synaptic interactions between pre-Botzinger and Botzinger complex neurons [2], as well as the influence of extrinsic synaptic drive to the VRG network. The aim of this study was to determine the role of neurogenic input from somatosensory receptors on the bursting patterns of respiratory neurons in the VRG network. We hypothesized that neural input from contraction-sensitive somatosensory receptors entrained respiration by modulating the phasic activity of VRG neurons. Experiments were performed using the working heart-brainstem preparation [3]. Rats (70–100 g) were anesthetized, decerebrated pre-collicularly, paralyzed and perfused with a Ringers solution plus an oncotic agent. Somatosensory afferents were stimulated by intermittent contraction of the forelimb (3 ms pulses, 90–150 V, 10–15 g of developed tension). Phrenic (PNA) and central vagus (CVA) nerve activities were recorded using suction electrodes and the extracellular single-unit activity of VRG neurons was recorded using glass microelectrodes (tip resistance: 5–15 mΩ). Neurons were classified based on their bursting patterns relative to the phase of the respiratory cycle as inspiratory (I; $n=18$), post-inspiratory (PI; $n=21$) or augmenting expiratory (E-AUG; $n=12$). Intermittent activation of somatosensory afferents entrained respiratory motor outflow to the frequency of forelimb contraction and generated a 1 : 1 phase-coupled rhythm. In order to determine the effect of somatosensory receptor activation on the bursting patterns of respiratory neurons, forelimb contraction was evoked during specific phases of the respiratory cycle. Once a respiratory neuron was identified and its ongoing activity was characterized, somatosensory afferents were activated by a single twitch contraction of the forelimb. When somatic afferents were activated during early PI phase 67% of PI neurons were inhibited ($n=14$); whereas, 58% of E-AUG neurons were excited ($n = 7$). In contrast, only 33% of I neurons were inhibited when the forelimb was contracted during early inspiration ($n=6$). From these findings, we conclude that the phase-locking of respiratory rhythm to repetitive muscle contraction is mediated by somatosensory-evoked excitation of E-AUG neurons and inhibition of PI neurons. Our findings suggest that peripheral feedback from somatosensory afferents modulates respiratory network activity in discrete phases of the respiratory cycle during locomotion.

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P8

Role of nucleus parabrachialis complex in inspiratory off-switch studied in isolated brainstem-spinal cord preparation from neonatal rat

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The nucleus parabrachialis complex (NPB) of the pons is known as a respiratory modulating center. The NPB and the medullary respiratory center are mutually interconnected and vagus nerve afferents from pulmonary stretch receptors project to the NPB [1]. There is much evidence in *in vivo* preparations from adult mammals [2] that the NPB plays a crucial in the inspiratory off-switch. In the present study, we examined how the NPB participates in the inspiratory off-switch using brainstem-spinal cord preparations obtained from 0–4 days old rats. We hemi-sectioned the pons keeping one side intact.

First, we examined the effects of NPB electrical stimulation on C4 ventral nerve inspiratory activity. The electrical stimulation (0.1 ms duration, 1–5 trains, 20 ms interval) applied at various times (100–300 ms after the onset of C4 inspiratory activity) induced a transient depression or termination in C4 inspiratory activity. This depression was stronger when the stimulation was applied near the end of inspiratory activity but less conspicuous when it was applied at around the peak of inspiratory activity. Thus, activation of the NPB neuronal elements could produce termination of inspiratory activity in this *in vitro* preparation similar to the *in vivo* preparation.

Previous *in vivo* experiments in adult mammals suggested that an NMDA receptor- mediated off-switch mechanism is present in the inspiratory termination [3]. Then, we examined whether a similar mechanism is involved in the above-observed inspiratory off-switch in the *in vitro* preparation. It was shown that the inhibition of C4 inspiratory activity induced by the NPB stimulation was greatly reduced by perfusion of NMDA antagonists (MK-801, APV) and that the inhibition was blocked by perfusion of a $GABA_A$-antagonist (Bicuculline). To determine the location of NMDA receptors responsible for this inhibition by the NPB stimulation, an NMDA-antagonist MK-801 (1 mM) was microinjected into the NPB using a glass capillary placed in the vicinity of the stimulation electrode. The injection of MK-801 reduced the inhibition of C4 inspiratory activity by the NPB stimulation, indicating that NMDA receptor-mediated processes take place within the NPB.

Since neurons and/or neuron networks responsible for inspiratory off-switch are expected to exist in the NPB, we tried to find respiratory neurons and analyzed their distribution and firing patterns. Neurons were recorded from the cut-surface of the intact half of the pons using whole cell patch clamp method. We found several types of respiratory neurons: inspiratory, tonic inspiratory, expira-

tory and inspiratory-expiratory (I-E) neurons. The population of I-E neurons was the largest, being more than 50% of the recorded neurons. I-E neurons fired from middle of C4 inspiratory activity through the early expiratory phase and were inhibited at the early part of C4 inspiratory activity. Perfusion of MK-801 (10 M) decreased significantly the driving potential and burst duration of I-E neurons. In addition, this perfusion decreased also the amplitudes of EPSPs induced by vagal nerve stimulation in the I-E neurons.
In conclusion, 1) the NPB is involved in the inspiratory off-switch in *in vitro* preparations, 2) NMDA receptors within the NPB mediate, at least partly, the NPB-related inspiratory off-switch, and 3) a relatively large population of I-E neurons of the NPB could be responsible for the NPB-related inspiratory termination.

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P9

Distribution and classification of respiratory neurones in the nucleus tractus solitarius of the rat medulla and their responses to central and peripheral stimuli

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In the cat the dorsal respiratory group comprising the nucleus tractus solitarius (NTS) is regarded as an important respiratory centre. However the relevance of this nucleus in the control of respiration in the rat is still unclear. The present work aimed to identify and localize the various neurons involved in respiration in the nucleus tractus solitarius region of the rat medulla. Further, we intended to study the responses of these identified respiratory neurons to central and peripheral stimulation, in particular: 1) effect of stimulation of midbrain periaqueductal gray (PAG) matter and, 2) effect of stimulation of the vagus (vagal feedback).
Nembutal-anaesthetised (80 mg/Kg), spontaneously-breathing rats (n=60, 300–400 g) were used for the study. Neurons firing in relation to diaphragm electromyogram (dEMG) were located between 0.6–1.5 mm lateral to the midline, extending 0.5 mm caudal to 1.8 mm rostral to the posterior end of the area postrema (AP). Four different populations of respiratory-related neurons were recorded. They have been classified (with reference to dEMG) as: expiratory-inspiratory phase-spanning cells, early inspiratory neurons (eI-cells), cells that fire only in phase with diaphragm (I-all cells), and expiratory cells. The phase-spanning and eI-cell populations were located both in the ventrolateral and ventromedial NTS rostral to the area postrema, while the other cells were found predominantly in the more lateral regions. eI-cells were observed to start firing up to 20 ms prior to dEMG and ceased firing about one third of the way through diaphragm activity. They also showed a decrementing discharge pattern.
Excitatory amino acid (DLH, 15–60 nl of 0.2 M) stimulation of PAG along its dorso-ventral axis produced dose-dependent changes in the cardiorespiratory parameters: increased respiratory frequency, decreased inspiratory and expiratory durations, increased blood pressure (BP) and heart rate. PAG stimulation elicited increases in activity in both phase-spanning and eI-neuronal populations in the NTS. The PAG sites evoking an increase in the phase-spanning cell activity were located more laterally than the PAG sites that led to increased eI-cell activity. The increase in the responses of the phase-spanning neurons was not accompanied by any changes in the respiratory frequency, whereas the PAG stimulation giving rise to an increase in eI-neuronal activity also elicited simultaneous increases in dEMG and the respiratory frequency.
The central end of the cut vagus was electrically stimulated to activate pulmonary stretch-receptor afferents. Vagal stimulation caused depression of inspiratory cell activity along with bradycardia and a fall in BP. Both eI-neurons and I-all cells were inhibited. These cells exhibited a 'vagal escape' phenomenon ie, resuming activity whilst vagal stimulation continued. Phase-spanning cells and expiratory cells are yet to be tested for responses to vagal stimulation.
The behaviours of these respiratory-related cells of the NTS, their temporal relationships with the dEMG, firing patterns, their responses to central and peripheral stimuli are discussed and a possible role for each in the brainstem control of respiration is proposed.
Similar studies have also been carried out to elucidate the roles of ventrolateral column (VLM)/ventral respiratory group (VRG) neurons in the control of respiration in the rat. The findings of these studies are presented in an accompanying abstract.

Acknowledgement: This work was approved by the University of Sydney Animal Care and Ethics Committee.

P10

Respiratory-related neuronal activity in the nucleus ambiguus-retroambigualis complex and their responses to peripheral and central stimulation in the rat

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This study aimed to locate respiratory-related neurons in NA/NRA complex within the ventrolateral medulla (VLM), characterise them, and study their responses to the stimulation of midbrain periaqueductal gray matter (PAG) and vagal afferents.
Stereotactic mapping of the medullary NA/NRA complex using stainless steel microelectrodes was undertaken in Nembutal-anaesthetised (80 mg/kg), spontaneously-breathing rats (n=60, 300–400 g). Individual cells and cell populations showing respiratory-related firing patterns were recorded along the VLM, 2.5 mm rostral –2.1 mm caudal to the obex, 1.4–2.2 mm lateral to midline and 1.5–3.00 mm below dorsal medullary surface. The cells were classified according to their temporal relationship with the diaphragm electromyogram (dEMG). Early inspiratory cells (eI cells) were found to start firing up to 20 ms before dEMG activity and to cease activity through mid-inspiration, exhibiting a decrementing discharge pattern. Late inspiratory (lateI) cells commenced their activity midway through inspiration and stopped firing prior to the cessation of dEMG. These cells exhibited an incrementing discharge pattern. Cells firing in phase with the dEMG have been classified as I-all cells and exhibit a steady impulse pattern throughout inspiration. Neurons active during the expiratory phase have been categorised as early expiratory cells (early E), late expiratory cells (late E) and E-all neurons according to their discharge pattern in relation to dEMG.

Cells in the dorso-lateral PAG were activated with microinjections of excitatory amino acid D,L-homocysteic acid (DLH 0.2 M, 10–60 nl) and VLM respiratory neuronal and muscle responses were studied. PAG stimulation induced dose-dependent changes to respiratory output. Inspiratory and expiratory durations were shortened, respiratory frequency and dEMG activity increased along with increases in heart rate and blood pressure. Concurrently PAG stimulation led to increased activation of eI cells, I-all cells and E-all cells found in the NA/NRA complex. Abdominal muscle activity was not evident during quiet breathing though expiratory neuronal activity was seen. However, activation of PAG elicited synchronous aEMG bursts along with an increase in E-all cell activity.

Unilateral vagal nerve stimulation, in order to simulate activation of the pulmonary stretch receptors, was undertaken. I-all cell and dEMG activity were inhibited upon stimulation of the vagus. However, I-all neuronal firing as well as the diaphragm activity resurfaced whilst vagal stimulation was prolonged. Expiratory cells are yet to be tested for responses to vagal stimulation.

Our previous studies [1] demonstrated a physiological link between the PAG and NTS in the rat. The current investigation shows that chemical activation of PAG influences the discharge patterns of the VLM respiratory neurons within the NA/NRA complex suggesting a possible physiological link between the two centres. These results indicate that both NTS and NA/NRA respiratory neurons participate in the mediation of PAG induced respiratory changes. Roles for NA/NRA neurons in the brainstem control of respiration in the rat and the interactions between the neural centres in the midbrain are discussed.

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P11

Hypothermia and recovery from respiratory arrest in a neonatal rat *in-vitro* brainstem preparation

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In mammals, progressive hypothermia leads to loss of reflex responses, respiratory arrest, and finally, cardiac arrest and death. Under acute conditions in neonatal mammals, if progressive rewarming occurs soon enough, both the heart beat and breathing will resume spontaneously and they will recover fully but in adult rats, once respiratory arrest occurs, the animals must be artificially resuscitated. The mechanistic basis of the initial respiratory arrest in hypothermia as well as the ontogenetic changes in tolerance and in the ability of the system to autoresuscitate on rewarming are poorly understood. Onimura and Homma [1], demonstrated that when temperature was lowered from 26 to 24°C in an *en bloc* brainstem-spinal cord preparation from newborn rats, respiratory rate was depressed and bursts of activity in Pre-I neurons were not always followed by bursts of inspiratory activity in respiratory motor neurons. This suggested that either the number of active Pre-I neurons decreased and were no longer sufficient to activate the motor neuron pools, or that the threshold for the generation of inspiratory-modulated efferent activity in the motor neuron pools was raised, or both. This in turn suggested that respiratory arrest might occur at the level of pre-motor or motor neurons rather than at the level of the rhythm generator itself.

This study was designed to examine whether respiratory arrest during hypothermia occurs at the level of pre motor or motor neurons rather than at the level of the central rhythm generator itself. Specifically we sought to determine the consequences of hypothermic cooling until respiratory arrest, and subsequent re warming, on neurons in the preBötzinger Complex (*via* field potential recordings), as an indication of the output of the entire rhythmogenic network; and from cervical spinal (phrenic) ventral roots (*via* suction electrode recording), as an indication of motor neuron output, in an *in vitro* neonatal rat brainstem-spinal cord preparation. With this preparation, progressive cooling slowed the frequency of fictive breathing with the cycle period increasing exponentially until respiratory related rhythmic activity ceased. There was no decrease in the peak, integrated sum or duration of the field potential during this process; ie, while slowing was progressive, arrest was not. The same was not completely true of the phrenic motor output. During progressive cooling there was a 14% fall in peak amplitude and a 32% fall in the integrated sum of the activity associated with each fictive burst. There were also rare instances at low temperature during both cooling and recovery where bursts of activity in the field potential were not accompanied by any increase in activity in the phrenic motor output. These data suggest that there must have been some increase in the threshold for generation of activity in the phrenic motor neuron pool relative to the neuron pool from which the field potential was recorded. Ultimate arrest, however, appears to occur at the level of the central rhythm generating network giving rise to complete and abrupt cessation of activation of the motor neuron pool(s).

On rewarming, the motor output often was fractionated suggesting that changes occurred in network synchronization either during cooling or during reactivation following hypothermic arrest.

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P12

Effects of hypoxia and hypothermia on the end-inspiratory pause and Hering-Breüer reflex in the neonatal tammar wallaby

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When cooled in normoxia newborn rats decreased body temperature (Tb), increased metabolic rate and weakened the intensity of the vagally mediated Hering-Breüer inspiratory inhibition reflex (HB reflex) [1]. On the other hand cooling in hypoxia decreased both Tb and metabolic rate and had no effect on HB reflex intensity [2]. This suggested that in newborn rats the effect of cooling on the strength of the HB reflex was not attributable to Tb *per se* but to the corresponding changes in metabolic rate [2]. Nevertheless, in normoxia the drop in Tb with cooling, *via* the Q_{10} effect, could oppose the changes in metabolic rate induced by thermogenesis, and the hypoxia *per se* could be expected to provide a 'drive' through its effect on the peripheral chemoreceptors. To clarify the effects of hypothermia and hypoxia on the HB reflex in the absence of thermogenesis we examined the ectothermic neonatal tammar wallaby (*Macropus eugenii*). As newborn marsupials breathe with a pronounced end-inspiratory pause [3,4], the result of vagally con-

trolled laryngeal closure [3], the effect of cooling and hypoxia on the end-inspiratory pause was also examined.

Neonates (aged 2–3 weeks, mass ~2.5 g) were studied at Tb of 36.5 (normothermia), 32, 28 and 20°C in normoxia or hypoxia (10% O_2). The rate of oxygen consumption ($\dot{V}O_2$), breathing pattern and ventilation ($\dot{V}_E$) were measured as previously described [4]. Applying a vacuum across the body until the first inspiratory effort induced the HB reflex, quantified as the inhibitory ratio (IR) of the expiratory time during lung inflation compared to the expiratory time during spontaneous breathing (TE).

At 36.5°C in normoxia the tammar neonate exhibited a marked IR to maintained lung inflations at –5 and –10 cm H_2O. Acute hypoxia invoked a hyperpnea (+42%) and hypometabolism (–30%) and reduced the IR by ~50% at –10 cm H_2O and abolished it at –5 cm H_2O. This is in agreement with the effects of hypoxia on hyperventilation and the HB reflex in 8-day-old rat pups [5] which possess a greater chemosensitivity than newborn rats. In normoxia decreased Tb was associated with a decrease in $\dot{V}O_2$ yet $\dot{V}_E/\dot{V}O_2$ remained unchanged (~32) to that at 36.5°C; the decrease in $\dot{V}_E$ was contributed to by an increase in TE, primarily the end-inspiratory pause. Associated with the increase in expiratory time was a failure to elicit a HB reflex at either –5 or –10 cm H_2O. At lower temperatures exposure to hypoxia maintained the hyperventilation ($\dot{V}_E/\dot{V}O_2$ ~62) seen at 36.5°C, though at these temperatures this was achieved solely through a hypometabolic response. Whether the lack of hyperpneic response was affected by the affect of hypothermia on chemo-afferents [6] is unknown but maintenance of $\dot{V}_E/\dot{V}O_2$ during hypothermia in the 2–3 week old tammar wallaby suggests that, relative to metabolic rate, respiratory gain, which is centrally controlled, is not depressed. Additionally, hypothermia prolongs the end-inspiratory pause presumably through temperature effects on neural function [7] and that these appear to predominate the vagally mediated HB reflex.

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P13

Developmental changes of the response of *in vivo* ventilation to acute and chronic hypercapnia in rats

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As part of our studies of the role of changes of intracellular pH (pH_i) in central chemosensitive neurons, we began studying the *in vivo* ventilatory response to increased inspired CO_2. Our hope was to identify distinct sub-populations of rats with widely different CO_2 sensitivity of *in vivo* ventilation so that we could study the cellular responses to hypercapnia (pH_i changes and increased chemosensitive neuron firing rate) and look for a correlation with the *in vivo* ventilatory patterns.

Ventilation in neonatal Sprague-Dawley rats (P1–P21) was studied using a dual chamber Respiromax Plethysmograph. Weight specific minute ventilation (V_E) was measured as a function of inspired CO_2 over the range of 0–5%. V_E increased linearly with increasing CO_2 due entirely to increased tidal volume (no change in respiratory frequency) [1]. The V_E response to increased CO_2 varied among individuals and from day to day in a given individual. The CO_2 sensitivity of ventilation (determined as the slope of the V_E vs. inspired % CO_2 curve) showed a biphasic relationship with age. CO_2 sensitivity was high in newborn rats (P1) and decreased to a minimum value at P7–P8. It increased again to reach a level at P14 that was very similar to the CO_2 sensitivity in adults [1]. The basis for this developmental pattern is unknown but it is not due to changes in the CO_2 responsiveness of chemosensitive neurons from the locus coeruleus, since these neurons show a constant increased firing rate of about 44% in response to hypercapnia (10% CO_2).

In an attempt to vary CO_2 sensitivity, we reared neonatal rats in a chronic hypercapnic (CH) environment [2] and studied the effect of this treatment on V_E and on CO_2 sensitivity of V_E. We reared time pregnant mothers in an environment of constant CH (7% CO_2) for 1 week prior to the birth of the pups and maintained the mother and pups in the CH environment until the neonates were tested (at least 6 additional days). These neonates exhibited retarded growth (smaller by 1–2 g) for the first 2 weeks of life but attained the same weight as control rats (reared in room air) by P16. These CH rats exhibited higher CO_2 sensitivity than control rats at days P6–P9 and then showed lower values that were indistinguishable from control rats from P10–>P19. Other litters were exposed to severe CH (10%) using the same protocol as for the 7% CH rats. Litters were culled in these severe CH rats (1/3 of the litter sacrificed at birth), but even so these rats showed marked growth retardation that got worse with increasing age. These rats exhibited an even higher CO_2 sensitivity than control or 7% CH rats at P6–P9 and then showed values that were similar to control rats at P10–>P19. V_E increased with increasing exposure to CH from (mean ± SE; n = 14) 892 ± 100 (control) to 960 ± 109 (7% CH) to 1127 ± 108 (10% CH) ml-min^{-1}-kg^{-1}. We suggest that CH results in a slowing of development so that CO_2 sensitivity of V_E remains elevated longer after birth.

The biphasic developmental pattern suggests that after birth rats display a *neonatal pattern* of chemosensitivity that decreases during the first week of life and is replaced after the second week by a more adult form of chemosensitivity. A critical period of low CO_2 sensitivity of V_E in seen between these two periods. The mechanistic basis for this biphasic pattern is unknown as is the effect of CH on the properties of central chemosensitive neurons and both of these questions should be fruitful areas of future work.

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P14

Selective inhibition of the sodium proton exchanger subtype 3 (NHE3) as a tool to study central chemosensitivity

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In vitro studies on organotypic medullary cultures of new born rats (OMC) have shown that CO_2/H^+-sensitive neurons of the ventrolateral medulla oblongata (VLNcs) increase their bioelectric activity upon a decrease of intracellular pH (pHi), while changes of extracellular pH are of minor importance [1]. Therefore, acid extrusion of VLNcs appears to be a key element for the control of steady state pHi and hence neuronal activity. To extrude H^+, ventrolateral medullary neurons use sodium proton exchange but not Na^+-dependent Cl^-/HCO_3^- exchange [2]. Our previous studies have shown that NHE3 is expressed in ventrolateral parts of OMC (obex level). Furthermore, the NHE3 inhibitor S1611 (1 µmol/l) decreased pHi and mimicked the bioelectric response of VLNcs to hypercapnia [3]. Due to these findings we hypothesized that NHE3 as a membrane transporter plays an important role for the control of chemosensitivity and breathing. Aim of this study was to test a new brain permeant NHE3 inhibitor (S8218) for effects on pHi, bioelectric activity and phrenic nerve activity *in vivo*.

For *in vitro* studies OMC were prepared and kept in R16 medium for 2–3 weeks [3]. Fluorimetric measurements of pHi were carried out after loading neurons with BCECF AM as described [3]. Membrane potentials were recorded with sharp microelectrodes. S8218 at concentrations of 1–2 µmol/l lowered the steady state pHi of a subset of ventrolateral neurons (the IC50 of S8218 for NHE3 = 0.81 µmol/l). In these cells pHi regulation after an ammonium prepulse (20 mmol NH_4Cl/l, 3 min) was also impaired by S8218. In contrast, cells whose steady state pHi was not altered by S8218 showed normal pHi recovery after ammonium prepulse. This finding strongly suggests that NHE3 expressing cells in culture use the NHE3 for maintainance of their steady state pHi. Accordingly, S8218 also changed the bioelectric activity of VLNcs: firing rates increased by up to 200% and periodic discharges appeared. Changes of the bioelectric activity resembled the neuronal responses to hypercapnia. In general, the effect was smilar to the effects of S1611 and S3226 [cf. 3].

For *in vivo* studies anaesthetized, ventilated and vagotomized rabbits were used. By means of RT-PCR, NHE3 mRNA was detected in homogenates of the rabbits' brainstems. Indirect immunocytochemistry showed that NHE3 immunoreactive protein was expressed in ventrolateral areas of the brainstem. Cumulative doses of S8218 (9.2 ± 1.1 mg/kg) resulting in plasma concentrations of 0.9 ± 0.2 µg/ml increased integrated phrenic nerve activity (IPNA, measured as IPNA*f_R) by 51 ± 6.4% ($n = 7$, $P < 0.0001$). Phrenic nerve responses to increased $PaCO_2$ were also changed: at a plasma concentration of 0.3 µg S8218/ml the $PaCO_2$ at the apneic threshold was lowered by 0.43 ± 0.1 kPa ($n = 7$, $P < 0.01$). In 4 out of 7 animals even strong hyperventilation failed to suppress phrenic nerve activity completely suggesting an ongoing activity at least of parts of the respiratory network. In the range of $PaCO_2$ being 1–6 kPa above the apneic threshold we observed a 38 ± 8.5% increase of IPNA*f_R (35 measurements in 7 animals).

The data underline the role of NHE3 for central chemosensitivity both *in vitro* and *in vivo*. We suggest that also *in vivo* a decrease in intracellular pH of NHE3 expressing neurons precedes the augmentation of the respiratory drive. Since NHE3-inhibitors act on central chemoreception of rats, rabbits and also piglets [cf. 4], these drugs may become useful tools to study mechanisms of central chemosensitivity.

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P15

Respiratory responses to pH of neonatal hamster and mouse *in vitro* compared with that of opossum

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In the isolated CNS from neonatal opossum Monodelphis domestica, low pH superfusion increases both respiratory rate and amplitude. One unusual control mechanism in the isolated Monodelphis CNS is that the frequency of respiration depends primarily on changes in the duration of the expiratory rather than the inspiratory phase [1]. To reveal whether this is a characteristic common to other species, we analyzed in isolated hamster and mouse CNS the respiratory responses to pH in terms of variables for amplitude and for timing.

Newborn Golden hamsters and CF1 mice (0–5 days old) were anesthetized with ether and cooled on ice. Their CNS were removed and decerebrated by pontobulbar transections. A vaseline seal at the level of the spinal cord segment C1 allowed selective superfusion of the brainstem. Basal conditions were obtained during brainstem superfusion with a continuos flow of 0.8 ml·min^{-1} artificial cerebrospinal fluid (aCSF) gassed with 95% O2 and 5% CO2, at 20–24°C (pH 7.35–7.40). Fictive respiration was recorded from C3–C5 ventral roots with suction electrodes. Acidification (pH 7.3 or lower) of the brainstem superfusion was attained by reducing the aCSF's bicarbonate final concentration.

Acidification of the brainstem superfusion (from pH 7.4–7.3) increased the frequency and decreased the amplitude of respiration in both hamster and mouse ($P < 0.01$, Wilcoxon test). A common feature of these results is that changes in rate are brought about solely by changes in the duration of the expiratory phase. The inspiratory phase remains constant in duration. Thus, cycle duration can be represented by a linear function exclusively depending on the expiratory duration. The slope of the straight line is very close to 1.

These results indicate that the timing of the respiratory cycle in the neonatal hamster and mouse, as well as in the opossum, are controlled by an expiratory instead of an inspiratory "off-switch". Increase in respiratory amplitude in response to chemical stimulation is a distinctive feature of the isolated CNS preparation obtained from newborn opossum.

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P16

Postnatal development of neural chemoafferent pathway and respiration is altered following prenatal nicotine exposure in rats

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Maternal smoking during pregnancy increases the risk of Sudden Infant Death. A defect in cardiorespiratory control has been suggested. Chronic exposure to nicotine during fetal development of rat induced postnatal developmental disorders on central neural pathways [1], autonomic function [2], carotid body chemoreceptors [3], ventilatory response to hyperoxia [4]. The interrelation between all these sparse data has to be investigated. We hypothesized that exposure to nicotine might impair or delay the development of respiratory control pathways, ie, of the carotid body chemoafferent pathway, causing an abnormal response to ventilatory challenges. On the 5th day of gestation, pregnant Sprague-Dawley rats received a transdermal patch delivering, either 50 mg of nicotine free base over 21 days, or excipient. At birth, male pups were selected and analysed at postnatal day 3, 7, 11, 14, 21 and 68. The *in vivo* tyrosine hydroxylase activity was determined in offspring carotid bodies and brainstem areas involved in cardiorespirarory events (A2C2 in the nucleus tractus solitarius, A1C1 in the ventrolateralmedulla, A5 and A6 in the pons) by measuring the endogenous DOPA accumulation after blockade of DOPA decarboxylase with NSD (m-hydroxybenzylhydrazine) 100 mg/kg, 20 min. Ventilation was measured in awake pups using a barometric plethysmograph, at rest and in response to 10% hypoxic challenges. The response to hypoxia was recorded at 1, 4, 7 and 10 min of hypoxic exposure. Concerning ventilation, prenatal nicotine induced a significant increase in resting ventilation of the 7- and 11-day-old offspring and an altered response to hypoxic challenge characterised by an instability of the response within the first 2 weeks and an increased amplitude of response. Concerning neurochemistry, the tyrosine hydroxylase activity is reduced in brainstem areas (A5, A1C1, A6) until 11 days and the decrease in the TH activity observed in the control carotid body until 7 days of age is delayed until 11 days in the prenatal nicotine offspring. In conclusion, prenatal nicotine altered development of the chemoafferent pathway. The long-lasting hypoxic ventilatory response of neonate type might be in relation with the delay in the carotid body resetting. This could lead to increased vulnerability during the early postnatal life, by reducing the ability to face up to environmental stresses. These data might be relevant for SIDS physiopathology.

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P17

Prenatal nicotine exposure increases apnoea and attenuates nicotinic potentiation of hypoglossal output in mice

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Maternal smoking is associated with an increased risk of Sudden Infant Death Syndrome (SIDS) and nicotine is implicated as a causative agent due to adverse effects on central nervous system development [1]. The consequences of these changes for respiration are uncertain, but there is growing consensus that altered respiratory control contributes to the reduced ability of nicotine-exposed animals to tolerate hypoxia [2,3]. Most studies have focussed on overall changes in ventilation, but paid minimal attention to breathing pattern. In addition, responses have rarely been examined in the earliest neonatal periods when immature control mechanisms are more likely to contribute to unstable breathing, frequent apnoeas and hypoxic episodes. We therefore examined the effects of prenatal nicotine exposure on the development of breathing pattern and the ventilatory response to hypoxia (7.4% O_2) *in vivo* using whole-body plethysmography at postnatal days 0, P3, P9, P19 and adult. To determine whether differences observed *in vivo* were due to altered activity of medullary respiratory networks, motoneurons (MNs) controlling the airway, or MN responsive-ness to nicotinic modulation, we recorded *in vitro* hypoglossal (XII) nerve and MN activity in rhythmic medullary slice preparations from control and nicotine-exposed P3 mice. Foetal mice were exposed to nicotine using osmotic micropumps implanted in the dam at gestational day 10.

Developmental changes in respiratory behaviour *in vivo* were delayed in the youngest nicotine-exposed animals. The high level of apnoea present during normoxia in P0 control animals (frequency of apnoea, f_A, $6.7 \pm 0.7\ min^{-1}$, percent of time apnoeic, T_A, $29 \pm 6\%$) and nicotine-exposed groups (f_A $8.1 \pm 1.7\ min^{-1}$, T_A $25 \pm 5\%$) persisted until P3 in the nicotine group but fell significantly in control animals (f_A $2.2 \pm 0.7\ min^{-1}$, T_A $5 \pm 2\%$). At the onset of hypoxia, f_A and T_A fell rapidly and remained low throughout hypoxia except in P0 nicotine-exposed animals where they declined initially (f_A 1.8 ± 0.5, T_A $4 \pm 2\%$) but then rose progressively during the 12 min hypoxic period to final values of $7.1 \pm 2.9\ min^{-1}$ and $17 \pm 6\%$ respectively. During recovery, apnoea increased in both groups at P0 (f_A 10.8 ± 0.8 and $11.3 \pm 1.3\ min^{-1}$; T_A 50 ± 6 and $49 \pm 5\%$). By P3, the absolute magnitude of this posthypoxic increase was reduced in control (f_A $5.3 \pm 0.1\ min^{-1}$; T_A $30 \pm 7\%$) but not nicotine-exposed animals (f_A $8.5 \pm 1.0\ min^{-1}$; T_A $44 \pm 8\%$).

The frequency and variability of the inspiratory-related output in medullary slice preparations from control and nicotine-exposed animals were indistinguishable. The pattern of the inspiratory-related XII nerve burst and inspiratory synaptic currents recorded from XII MNs were also similar.

Local application of nicotine (10–100 μM) over the XII nucleus produced a small, TTX-resistant inward current in MNs that reversed near 0 mV, and potentiated XII nerve inspiratory burst amplitude ($25 \pm 5\%$). Both actions were hexamethonium-sensitive, suggesting nicotinic receptor involvement in upper airway control. This potentiation was significantly lower in nicotine-exposed animals ($14 \pm 3\%$).

Results indicate that prenatal nicotine exposure in mice delays development of breathing pattern, increasing incidence of apnoea in P0–P3 animals before, during and after hypoxia. This increased apnoea does not reflect alterations in the baseline behaviour of

rhythm generating or pattern forming circuits, but may result from reduced nicotinic modulation of XII MN activity.

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P18

Ionic currents and action potential induced by electrical stimulation, ACh and ATP in petrosal ganglion neurons

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The glossopharyngeal nerve contains the sensory fibers of the petrosal ganglion (PG) neurons that innervate the carotid body and sinus, through the carotid sinus nerve, and the tongue and the pharynx, through the glossopharyngeal branch. Based on the shape of the action potential, two populations of PG neurons can be recognized. However, little is known about the ionic currents underlying these action potentials. The application of ACh and ATP, transmitters putatively involved in the communication between the glomus cells and PG neuron terminals, to the isolated PG increase the frequency of discharge of the carotid sinus nerve, while only ATP increases discharges in the glossopharyngeal branch. The above suggests the presence of at least two neuronal populations.

Petrosal ganglia were excised from cats anesthetized with sodium pentobarbitone (40 mg/kg, ip), dissociated, and the neurons cultured for 3–16 days. Using whole-cell patch-clamp technique, we recorded from isolated PG neurons both action potentials and ionic currents evoked by electrical stimulation, and by application of ACh and ATP.

Neurons presented either fast action potentials (F-type) or slower spikes with an inflexion in the repolarizing phase (H-type). The F-type neurons had fast inward and a sustained outward current. The spike and inward currents were reversibly blocked when choline replaced Na^+ or in the presence of 3 μM tetrodotoxin (TTX). The outward current was partly blocked by 5 mM tetraethylammonium (TEA). The H-type neurons presented an inward current with at least two components, and an outward current with a fast -partly inactivating- and a slower sustained component. When external Na^+ was replaced by choline, or when 3 μM TTX was added, the amplitude of the spike and the inward current were reversibly decreased. Similarly, when intracellular K^+ was replaced by Cs^+, or when 5 mM TEA was added, the amplitude and duration of the spike increased and the outward current was reduced.

Under voltage-clamp, ATP induced a dose-dependent inward current that was partly desensitized during 20 s application pulses. The ATP-induced current had a threshold of 100 nM, and saturated between 20–50 μM. ACh also induced a fast, inactivating inward current, with a threshold between 10–50 μM, and saturated about 1–5 mM. From 34 neurons tested for ACh and ATP, 9% responded only to ACh, 24% to ATP only, 50% responded to both agents, and the remaining 17% were unresponsive. In current-clamp, ATP and ACh depolarized the neurons and may induce action potentials.

Our results show that H- and F-type PG neurons express different voltage-gated ionic and receptor-gated currents, which are activated either by ACh or ATP, with a half population responding to both putative transmitters.

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P19

Chemosensory activity from single or few-fibres of the carotid sinus nerve in the guinea-pig

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Humans who are born and raised at high altitude possess a blunted breathing response to hypoxia. The guinea-pig also has a blunted breathing response to hypoxia [1], which may be linked to its high altitude origin in South America. Our laboratory has shown that the carotid bodies of the guinea-pig are not required for the breathing response to hypoxia [1]. Whether the carotid bodies can actually detect hypoxia is uncertain because the central depressive effects associated with hypoxia may attenuate the breathing response to an otherwise potent carotid body stimulant. In a recent study, we looked for evidence of chemoreceptor activity in the whole carotid sinus nerve (CSN), but found that the whole CSN activity was dominated by baroreceptor afferent activity [2].

In this study activity was recorded from single or few-fibre CSN filaments during application of the chemoreceptor stimulants NaCN (200 μg kg^{-1} i.v.), 8% CO_2 (60 s) and 8% O_2 (60 s), from guinea-pigs (n=10; ~420 g; ~50 days old) that were anaesthetised with ketamine/xylazine (20/1 mg kg^{-1}).

Of the 10 guinea-pigs, only 8 fibre preparations containing chemoreceptor activity were successfully obtained from 4 guinea-pigs. In general, most of the isolated nerve filaments were multi-fibre preparations. Basal chemoreceptor activity could not be detected. However, NaCN and hypercapnia increased activity in 5 of the fibre preparations that were otherwise silent, or that contained 1 or 2 baroreceptor fibres. The magnitude of response varied considerably between filaments because of the different number of individual fibres in each preparation and, thus, the difference in the actual count of action potentials. Only one of the fibre preparations responded to hypoxia with an increase in activity; no fibres responded with a decrease in activity.

The physiological relevance of the latter results could be similar to that of the aortic depressor nerve (ADN) in the rat. Brophy *et al.* revealed that, in contrast to the general consensus, the rat ADN does contain chemoreceptor afferent fibres, and that such fibres are stimulated by chemoreceptor stimulants [3]. Kobayashi *et al.* agreed that chemoreceptor fibres were present, but concluded that the rat ADN does not contain a functionally significant number of chemoreceptor afferent fibres that could appreciably contribute to the generation of chemoreflexes [4].

The present study has shown that the carotid bodies of the guinea-pig can detect NaCN and hypercapnia, as expected, and also hypoxia, although the latter was a comparatively weak stimulant. In conclusion, the physiological relevance of the carotid body of the guinea-pig for the ventilatory responses to hypoxia remains uncertain.

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P20

Prenatal hypoxia impairs the early postnatal development of the carotid chemoafferent pathway

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Rat carotid bodies (CB) are considered as the main component that initiates the Hypoxic Ventilatory Response (HVR). Most of these chemoafferent fibres project into discrete areas of the medulla oblongata *via* the petrosal ganglion (PG), mainly in the caudal part of the nucleus Tractus Solitarius (NTS), and, to a lesser extent, in the ventrolateral medulla (VLM) [1]. Both medullary areas contain two major respiratory cell groups: the dorsal respiratory group, in the ventrolateral subset of the solitary tract, and the ventral respiratory group, in the VLM. These two medullary respiratory groups are closely associated with catecholaminergic neurones that belong to, the A2C2 cell group and the A1C1 cell group respectively. There is growing evidence that medullary catecholaminergic neurones participate in the chemoreflex response to systemic hypoxia. During the two first weeks of life, the sensitivity of the carotid bodies adapts to the comparatively hypoxic environment of the foetus and then reset to the higher PaO_2 of the newborn. This environmental change in oxygen leads to an increase in the carotid body sensitivity, due in part to a decrease in the release of dopamine [2]. The postnatal maturation of the carotid body sensitivity depends on the level of environmental oxygen during the early postnatal period.

The aim of the present study is to investigate the effects of prenatal hypoxia (last 15 days of gestation in 10% 0_2) on the neurochemical and functional development (postnatal day 0, 3, 7, 14, 21 and 68) of the chemoafferent pathway. We thus assessed the development of *in vivo* tyrosine hydroxylase (TH) activity, the rate-limiting enzyme in the catecholamine synthesis [3], in the CB, PG and the A1C1, A2C2 cell groups. In the same way, TH mRNA was evaluated in the CB, PG structures. Moreover, we evaluated the functional maturity of the chemoreflex pathway by measuring the HVR. We attempted to address four main questions. First, can prenatal hypoxia induce a neurochemical impairment of the CB resetting? Second, could the neurochemical impairment be related to the mRNA level? Third, are central and peripheral structures affected in the same manner? Fourth, are the neuronal impairments reflected at the functional level?

Our results show that 1) prenatal hypoxia amplifies the neurochemical resetting of the peripheral chemoreceptors; 2) a part of the neurochemical impairment is explained at the mRNA level; 3) central and peripheral structures exhibit opposite impairment; 4) prenatal hypoxia modifies the HVR pattern.

In conclusion, prenatal hypoxic exposure during the two last weeks of gestation impairs the carotid body chemoreflex pathway at the functional, the neurochemical and the molecular level, affecting the CB resetting process.

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P21

Concomitant effect of dopamine and acetylcholine on carotid body chemosensory response to hypoxia in the cat

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Dopamine (DA) and acetylcholine (Ach) are released from the carotid body chemoreceptor cells under basal conditions and stimulation by hypoxia. The role of DA and Ach in the control of the carotid sinus nerve chemosensory discharge (CSND) are still under debate. Here we tested the hypothesis that DA is inhibitory to the CSND and the effect is reversed by Ach. The CSND was recorded in 2 groups of anesthetized and artificially ventilated adult cats. The treatment group received alpha-methyl-paratyrosine (250 mg/kg) and reserpine (5 mg/kg) administered intraperitoneally, respectively 2.5 h and 12 h prior to the experiment in order to minimize the contribution of endogenous DA. The control group was injected with normal saline. CSND and arterial blood pressure (ABP) were recorded 1) at baseline 2) with a continuous iv infusion of DA, 5 µg/kg/min and 3) with a continuous infusion of Ach (25 µg/kg/min), while DA infusion was ongoing. DA & Ach infusions were initiated in room air. At baseline and 10 min into each infusion, the F_IO_2 was switched from room air to 8% O_2 in N_2 for 2–3 min and then to 100% O_2 for 2–3 min.

One min into DA infusion, CSND was decreased from 3.1 ± 0.4–0.8 ± 0.4 imp/s ($P < 0.01$) in controls and from 3.2 ± 0.8–0.3 ± 0.2 (imp/s, $P < 0.01$) in the treatment group. Up to 10 min into DA infusion, the CSND was restored in controls but still significantly inhibited in treated cats in room air and hyperoxia. The mean ± SEM CSND (impulses/sec) and ABP (mmHg) at steady state are listed in Table 1 overleaf. Compared with DA alone, DA+ACh appears to increase the CSND in control cats breathing room air as well as in treated cats breathing room air or 100% O_2. Compared with DA, ABP was particularly depressed during DA+Ach in the treatment group breathing room air. The CSND in hypoxia was unchanged throughout the experiment.

It is concluded that endogenous DA regulates the basal CSND and Ach may be capable of restoring the activity previously inhibited by DA. The effect of Ach appears to be independent from ABP changes in room air for control cats and in hyperoxia for treated cats. In the experimental conditions, neither DA nor DA+Ach appear to modify the CSND response to hypoxia.

Table 1

		Non treated cats (n=6)			Treated cats (n=6)		
FiO_2 (%)		21	8	100	21	8	100
Baseline	CSND	3.4±0.5	35.5±5.9	0.5±0.1	4.2±1.0	30.2±4.1	0.5±0.1
	ABP	123.6±6.8	99.7±13.9	115.5±7.5	99.9±6.9*	72.0±13.0	117.8±7.4
DA	CSND	2.5±0.6	38.9±7.6	0.4±0.1	0.7±0.4 +*	28.8±6.3	0.1±0.01 +*
	ABP	106.2±7.5	85.3±8.2	113.2±7.1	93.6±5.9	62.2±12.3	130.6±9.6
DA+ACh	CSND	4.1±0.6$	39.1±7.1	0.5±0.2	2.4±1.1$	35.9±6.3	0.4±0.05$
	ABP	92.3±6.0+	84.3±18.0	98.1±5.3	57.3±9.5+*$	47.9±9.6	83.2±13.7

+$P<0.01$ vs baseline; *$P<0.04$ vs non-treated; $$P<0.04$ vs DA.

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P22

Neonatal maternal separation alters development of the hypercapnic ventilatory response in awake adult rats

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Mother-pup interactions during early life serve to program the circuitry orchestrating the diverse responses to stress in the offspring [1,2,3]. Long periods of daily separation from the mother (ie, 3–6 h) alter behavioural, neural, and endocrine responses to stress which persist throughout life, owing to altered development and regulation of neurones orchestrating the stress response [3,4]. These neurones include the paraventricular nucleus of the hypothalamus (PVH) and noradrenergic neurones of the locus coeruleus (LC) [3,5]. Because neurones affected by maternal separation also play an important role in ventilatory control, we tested the hypothesis that maternal separation disrupts development of the respiratory control system. This study 1) compared ventilatory responses to hypercapnia between control animals and rats subjected to maternal separation during the neonatal period, and 2) determined whether hypercapnic activation of LC and PVH neurones (as indicated by Fos mRNA expression) is affected by neonatal separation. Newborn rats were separated from their mother 3 h/day for 10 consecutive days (P3–P12). Pups were then reared in standard conditions until adulthood. Ventilatory activity was measured in awake rats using whole body plethysmography under baseline (normoxic normocapnia) and hypercapnic conditions (5% CO_2 in air). At the end of the experiment, animals were sacrificed and brains were collected for subsequent quantification of Fos mRNA in the PVH and LC by *in situ* hybridization.

Plethysmographic measurements show that the hypercapnic ventilatory response is 30% lower in separated rats ($n=6$) than in controls ($n=13$). This attenuation of the response is due exclusively to a reduced increase in tidal volume, as the frequency component of the response was not affected by the neonatal treatment. In separated rats, the blunted responsiveness to hypercapnia was associated with a lack of increase in Fos mRNA levels in the PVH. However, hypercapnic exposure nearly doubled Fos mRNA expression in control rats, thereby suggesting that PVH neurons play a role in the hypercapnic ventilatory response. Conversely, hypercapnia decreased Fos mRNA in the LC equally in both groups.

These data suggest that a neonatal stress, such as disruption of mother-infant interactions, which is not directly relevant to respiratory homeostasis may have a significant impact on development of the hypercapnic ventilatory response. These results may point to a new factor in the etiology of delays in developmental maturation in respiratory control and subsequent of respiratory disorders.

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P23

Serotonergic modulation of respiratory neural activity during tadpole development

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The respiratory physiology of bullfrogs undergoes important modifications during development [1,2,3]. The dramatic changes in the partitioning of gas exchange function between lungs and gills during development require important alterations in the neural mechanisms controlling ventilation [4,5]. Yet, the mechanisms at the basis of these changes in respiratory motor behaviour remain poorly understood.

Hilaire and Duron proposed that maturation of the mammalian respiratory network may be defined, at least partly, by serotonergic modulatory processes [6]. To test the hypothesis that serotonergic modulation of respiratory neural activity changes during tadpole development, the effects of serotonin (5-HT) on neural correlates of respiratory activity were assessed using an *in vitro* brainstem-spinal cord preparation.

Preparations from tadpoles of developmental stages varying between TK stages VI and XXV were superfused with mock CSF containing 5-HT concentrations ranging from 0–25 μM. Neural correlates of gill and lung ventilation were recorded extracellularly from cranial nerve rootlets V and X.

In younger tadpoles (facultative air breathers; TK stages VI-XV) 5-HT bath application attenuated the frequency and amplitude of respiratory-related motor output (both fictive gill and lung ventilation), an effect most notable at high concentrations. In more mature animals (obligate air breathers; TK stages XVI-XXV), the effects of 5-HT bath application on fictive lung ventilation was bi-phasic. Fictive lung ventilation frequency was enhanced at low 5-HT concentrations (0.5 μ) and was depressed by higher 5-HT concentration. Moreover, 5-HT-induced attenuation of fictive gill ventilation was stronger in obligate air breathers than in preparations from younger tadpoles.

These results indicate that serotonergic modulation of respiratory activity changes substantially during tadpole development. Such changes in modulatory influences may contribute to the maturation of the respiratory control system in this species.

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P24

Chronic intermittent asphyxia impairs rat upper airway muscle responses to acute hypoxia and asphyxia

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Sleep disordered breathing (SDB) is characterised by chronic intermittent asphyxia (CIA) due to repetitive apnoeas. We tested the hypothesis that CIA affects upper airway muscle activity and responses to acute hypoxia and asphyxia.

Wistar rats were placed in restrainers with their heads in hoods ventilated with air (controls, $n=6$) or an air/N_2/CO_2 mixture intermittently (treated group, $n=6$) for 8 h per day, 5 days per week for 5 weeks using timed solenoid valves as previously described [1]. In the treated group, every 30 s, N_2 and CO_2 were added to the airflow for 15 s reducing hood O_2 to a minimum of 6–8% and increasing CO_2 to a maximum of 12–14% followed by removal of the N_2 and CO_2 and recovery to room air values. After 5 weeks, animals were anaesthetised (chloralose and urethane, 100 and 1000 mg/kg respectively IP). Sternohyoid (SH) electromyogram (EMG) activity was measured breathing air, 7.5% O_2 in N_2 (hypoxia) and 7.5% O_2 and 3% CO_2 (asphyxia). EMG data were expressed as % of peak activity breathing 9% CO_2 (% of reference).

Baseline SH EMG activity was significantly elevated in the treated group (28.4 ± 0.3% of reference in the controls vs. 48.5 ± 0.5% of reference in the treated group; means ± SEM, $P<0.05$ ANOVA). CIA significantly reduced SH EMG responses to hypoxia and asphyxia. Thus, the increase in EMG activity from baseline values (ΔEMG) during the first minute of hypoxia was +46.2 ± 5.8% in control rats vs. +30.4 ± 6.1% in treated rats. Similarly, ΔEMG during the first minute of asphyxia was +66.3 ± 5.9% in control rats vs. +41.1 ± 11.1% in treated rats.

We suggest that the elevated upper airway muscle activity associated with SDB is due to CIA. We propose that a reduction in the response of upper airway dilator muscles to acute asphyxia following upper airway obstruction is likely to cause further asphyxic insult leading to a vicious feed-forward cycle. We further propose that CIA contributes to the pathophysiology of SDB and other conditions of intermittent asphyxia.

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P25

Associative conditioning of the exercise ventilatory response in humans

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In goats, repeated exposure to moderate steady-state exercise with an added deadspace has been shown to alter the ventilatory response to subsequent exercise tests [1], a phenomenon termed long-term modulation (LTM). There are few relevant studies in humans, but it has been proposed that LTM can be achieved by a form of associative conditioning involving exercise and hypercapnia [2]. However, such data as there is would appear to be conflicting [3,4]. We have further investigated this phenomenon in humans in an attempt to resolve the disparity.

After providing informed consent as approved by the Local Ethics Committee, and familiarisation, 16 subjects (9 female, age 22.4 ± 2.7 years) were randomly divided into two groups; a control group (8 subjects, 5 female) and an intervention group (8 subjects, 4 female).

The Intervention protocol consisted an initial (pre) steady-state exercise bout (100W, 5 min) on a cycle ergometer, followed by a 'conditioning session' of 10 repeated bouts of steady-state exercise (100W) while breathing through an additional deadspace (+VD = 1.35 l). Two post-conditioning steady-state exercise tests (100W, 5 min) were performed, one 7 min after (post1) and one 1 h after (post2) the end of the last conditioning bout. Each bout was interposed by a 7-min period of unencumbered rest.

Table 1

Changes in exercise response following conditioning.

	Intervention		Control	
	Pre vs Post1	Pre vs Post2	Pre vs Post1	Pre vs Post2
ΔV_E (%)	−2.92	−4.95	−0.62	1.33
Δf^R (%)	12.85†	9.06‡	11.13‡	8.47†
ΔV_T (%)	−11.31†	−12.30†	−10.63§	−7.16§
$\Delta PetCO_2$ (mmHg)	−1.67†	−1.30‡	−1.72‡	−1.92‡

†$P<0.05$; ‡$P<0.01$; §$P=0.07$.

The control group protocol was identical to the Intervention protocol, except that at no time was exercise associated with an added deadspace.

Breath-by-breath recordings for ventilatory parameters and respired gases (including PetCO2) were measured during exercise (Morgan Benchmark, Kent). The results are given in Table 1.

Post-conditioning ventilation was not augmented in either group. Respiratory frequency was increased significantly in both groups post-conditioning, whilst both tidal volume and $PetCO_2$ were reduced in both groups. The magnitude of the changes in the pattern of breathing and the $PetCO_2$ were similar between groups at both post-conditioning assessments. The mechanisms underlying these changes are not immediately obvious, but the results do not support the concept of a LTM of exercise ventilation dependant on associative conditioning.

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P26

The effect of volitional breathing on the breathlessness-ventilation relationship during progressive exercise in healthy subjects

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The current hypothesis about the generation of breathlessness sensation is that a conscious awareness of reflex activation in the brainstem, rather than sensory feedback from peripheral receptors, is the major factor that gives rise to this sensation.

This hypothesis was based on work that showed subjects and patients were less breathless when volitionally copying a hypercapnic breathing pattern [1]. However, later studies have shown no effects of volitional breathing on breathlessness [2].

To investigate the contribution of the cortical drive on modulation of the reflex drive, experiments on volitional breathing *per se* were conducted during progressive exercise.

Sixteen subjects undertook a progressive exercise test on a bicycle ergometer, to a symptom-limited maximum during the course of which breathlessness was estimated each minute using the Visual Analogue Scale (VAS). All subjects had completed control exercise experiments to reach reproducible breathlessness estimations. A control study was followed, 7–10 days later, by a study of volitional breathing.

The subjects (all males) had a mean age of 29.6 years (SD ± 9.3). The breathing pattern of a control exercise test (control) was recorded. Following this, an 'appropriate volitional tracking' experiment was conducted in which the subjects attempted to copy the previous control breathing pattern during another control exercise test. The subjects were told to follow the breathing pattern by adjusting a cursor, which was driven by flow through a pneumotachograph (PK Morgan, UK).

In control and volitional tracking experiments, ventilation (V_E), tidal volume (V_T) and respiratory frequency (f_R) were plotted against time. Breathlessness estimations were plotted against ventilation, from which slope and intercept values were derived. Mean changes in the saturation of oxygen in arterial blood (S_aO_2) found at the end of each exercise test were also recorded (Biox IIA, USA). Comparisons were made using a paired t-test. All t-tests were two-tailed and the level of probability taken as significant was 5% ($P<0.05$) (Table 1).

Table 1

Breathlessness index	Control (mean ± SEM)	Volitional (mean ± SEM)	*P*
VAS/V_E slope (mm min l^{-1})	0.86 ± 0.10	0.97 ± 0.11	$P=0.307$
VAS/V_E Intercept (l min^{-1})	45.50 ± 4.29	54.34 ± 4.91	$P=0.009$

The mean slope and intercept of V_E/time, V_T/time and f_R/time relationships for both experiments were not significantly different. Mean S_aO_2 levels also showed no significant changes. Mean slopes of the VAS/V_E relationship were also not significantly different. However, the mean intercept of the volitional experiment was significantly increased relative to control.

The major finding in this study was that the onset of breathlessness was significantly delayed, though there were no significant changes in any objective physiological data set between control and volitional experiments. We postulate that this delay is due to cortical drive bypassing the medullary rhythm generator, with possible inhibition of the reflex efferent drive projecting to the sensory cortex. This is consistent with the hypothesis that the medullary reflex activity is responsible for the genesis of breathlessness sensation.

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P27

Reconstruction of motoneuronal morphology using two-photon molecular excitation microscopy

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Neuronal somato-dendritic morphology is an important determinant of the conduction properties of neurons, and thus the way neurons process information. Current semi-automated methods of neuronal tracing by wide-field light microscopy of diaminobenzidine (DAB) labelled neurons suffer a number of limitations. Firstly, the required fixation introduces a variable spatial distortion. Secondly, under optimal conditions the spatial resolution is at best diffraction limited and these conditions are rarely achieved. Current estimates of dendritic diameter indicate a lower limit of ~0.5 μm [1]. This is close to the limit of resolution, and the diameter of smaller dendrites may therefore have been overestimated. Thirdly, the requirement for extensive, time-consuming user-input limits the practicality of the procedure.

In this study, two-photon molecular excitation microscopy (2PM) was used to reconstruct the morphology of living, cytoplasmically-labelled motoneurons (MNs) from the hypoglossal (XII) nucleus of neonatal mice. Using the improved imaging capabilities of 2PM in light-scattering tissues and combining it with advanced image analysis we overcome the limitations of conventional light microscopy described above. MNs in isolated transverse brainstem slices containing the XII nucleus [2] were visualised using infra-red differential contrast microscopy. Selected MNs were whole-cell voltage-clamped and fluorescent dyes iontophoresed *via* the patch-pipette. Slices were then transferred to an inverted confocal microscope modified for 2PM, and 3D stacks of images were acquired at diffraction-limited resolution. Custom-written analysis routines were used to assess dendrite diameters at selected points. Fluorescence intensity was measured along a line orthogonal to the dendrite at these points and diameter defined as full width at half maximum intensity. While the resolution of this method is also limited by the wavelength of light, we overcame this limitation by using the fact that in 2PM the signal from structures smaller than the resolution limit (0.4 μm in x-y, 0.8 μm in z) is proportional to the illuminated volume. It is therefore possible to relate the recorded fluorescence intensity to the local diameter of sub resolution dendrites if they are modelled as cylinders [3]. By combining this approach with fully automatic tracing we can quantify the morphology and local diameters of intact functioning MNs and unequivocally clarify the existence of small diameter (<0.5 μm) dendrites.

To date, reconstructions have been completed for the proximal regions of the somato-dendritic tree (<250 μm from the soma). These are comparable in branching pattern, complexity and local diameters (0.8–4 μm) to reconstructions of XII MNs made using wide-field microscopy [1]. Significant progress has also being made towards completion of a fast, robust, fully automatic system for tracing and quantifying the morphology of whole neurons from 2PM volume data.

The functional significance of somato-dendritic complexity is a rapidly expanding research field. Accurate reconstructions of specific somato-dendritic morphologies, and subsequent identification of the generalised principles describing dendritic morphology, is essential to developing models of neuronal behaviour and to elucidating the roles neurons play in information processing.

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P28

Synaptic interactions of retrogradely labeled genioglossus motoneurons with substance P-like immunoreactive terminals in the cat: a double labeling immunoelectron microscopic study

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The genioglossus muscle (GM) is the principal protruder of the tongue and comprises a major portion of the caudal body of the tongue. The position of the GM is a major determinant of upper airway resistance. Our laboratory is interested in identifying the neurochemical microcircuitry of functionally identified hypoglossal motoneurons. We have found that substance P (SP) makes synaptic contacts with hypoglossal motoneurons that innervate intrinsic tongue muscles [1] in the cat. The purpose of the present study was to investigate whether there are synaptic interactions between motoneurons that innervate the GM and SP-immunoreactive nerve terminals utilizing double labeling immunoelectron microscopy. Cholera toxin B conjugated to horseradish peroxidase (CTB-HRP) was injected into the right GM of three cats. Two days later, the animals were deeply anesthetized and perfused with an acrolein/paraformaldehyde mixture. Cells containing CTB-HRP were labeled with a histochemical reaction utilizing tetramethylbenzidine (TMB) as the chromogen. TMB forms a crystalline reaction product that is very distinct at the electron microscopic level. The tissues were then processed for immunocytochemistry using an antiserum against SP. The chromogen used in this case, diaminobenzidine, yields an amorphous reaction product. At the light microscopic level, retrogradely labeled cells were observed primarily ipsilaterally to the injection site. These neurons were located predominantly in the ventral and ventrolateral portions of the nucleus primarily at the level of the area postrema. At the electron microscopic level, numerous SP-labeled terminals were observed some of which made synaptic contacts with retrogradely labeled GM's. SP terminals formed synapses with retrogradely labeled dendrites and perikarya. SP-labeled terminals also synapsed on unlabeled dendrites. These are the first ultrastructural studies demonstrating synaptic interactions between hypoglossal motoneurons that innervate an extrinsic tongue muscle and SP nerve terminals. These studies demonstrate that motoneurons that innervate the GM are modulated by SP and that SP appears to play a role in the control of tongue movement and GM activity.

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P29

Abstract not submitted for publication

P30

Postnatal changes in the noradrenergic system modulating hypoglossal motoneurons

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Appropriate activation of hypoglossal (XII) motoneurons (MNs) during inspiration is essential for maintaining upper airway patency. Morphological and electrophysiological properties of XII MNs, as well as numerous modulatory systems affecting these MNs, undergo significant change postnatally. These changes must be properly coordinated to ensure that breathing continues uninterupted throughout development. We have previously described an α1 noradrenergic receptor-mediated potentiation of XII nerve activity that increases approximately 1.8-fold over the first three postnatal days and 2.5-fold over the first two postnatal weeks [1]. To examine the development of the noradrenergic system underlying this potentiation, we combined electrophysiological and immunohistochemical approaches. Mechanisms underlying the potentiating effects of α1 receptor activation were assessed using whole-cell recordings from XII MNs in rhythmically-active medullary slice preparations from P0 (postnatal day zero) and P3 mice. The development of the catecholaminergic cell phenotype and the time course over which norepinephrine (NE) immunoreactive terminals appear in the XII nucleus were examined immunohistochemically using a monoclonal antibody against tyrosine hydroxylase (TH; Boehringer Mannhiem) and a polyclonal antibody against NE (Chemicon) in mice ranging from P0 to adult. Tissue from all age groups was processed simultaneously and labelled using a standard DAB/H_2O_2 protocol. Images were captured using a CCD camera and optical density of immunolabelling was measured using NIH Image software.

Phenylephrine (PE) induced an inward current or a depolarization in all inspiratory XII MNs tested (n=21). At a holding potential of −60 mV, currents induced by PE (100 μM) increased from 2.9 ± 0.7 pA/pF in P0 (n=9) to 4.7 ± 1.1 pA/pF in P3 (n=12) MNs. Application of PE did not affect input resistance (R_N) in P0 MNs (n=7) but increased R_N by 11 ± 4% in P3 MNs (n=11). PE potentiated inspiratory synaptic currents, and the magnitude of this potentiation was significantly greater in P3 (peak current 25 ± 7%; charge transfer per burst 43 ± 7%, n=8) relative to P0 (peak 17 ± 7; charge transfer per burst 28 ± 11%, n=6) XII MNs. Involvement of post-synaptic receptors was indicated by persistence of the responses in TTX (1 μM, n=7). PE did not affect repetitive firing elicited by injected current in P0 MNs tested (n=2). However, the frequency/current relationship was significantly shifted to the left in 3/5 P3 MNs.

Immunohistochemical data showed that labelling for TH in the A5, A7 and LsC (Locus subCoeruleus) cell groups was already strong in P0 and increased approximately 30% by P14, before decreasing to adult levels which were similar to P0 values. Immunolabelling for NE in these cell groups was also apparent at P0. A developmental increase in NE immunolabelling was modest, except in LsC where it increased up to 140% by P7, before decreasing to adult levels (that were half those observed in P0 animals). The number of NE-positive fibers and varicosities within XII nucelus was low in P0 mice, but increased dramatically by P14 where dense innervation was present along the entire rostro-caudal extent of the nucleus. In adults, NE-positive terminals/varicosities were dense over only a limited rostro-caudal region.

These data suggest that the factors contributing to the developmental increase in the noradrenergic potentiation of XII MN activity include a proliferation of NE-containing terminals within the XII nucleus and maturation of postsynaptic α1 receptor mechanisms.

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P31

Abdominal muscle spindle discharge does not affect abdominal motoneuronal discharge in decerebrate cats

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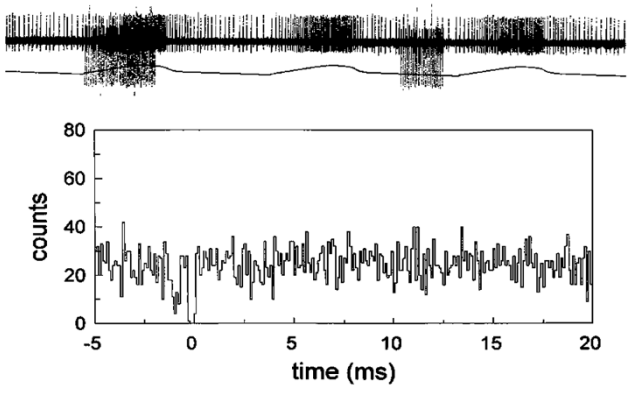
Despite their importance to respiration under a variety of circumstances (see [1] for review), the segmental control of abdominal motoneuron discharge has not been studied intensively. I [2] recently described diverse and often asymmetric responses of abdominal efferents of both the same and other segments to electrical shocks to the caudal branch of the left caudal iliohypogastric (L2) nerve and suggested that these responses subtend postural rather than respiratory reflexes. In this study, I tested the hypothesis that spontaneous abdominal muscle spindle activity affects the excitability and therefore discharge of abdominal motoneurons during eupneic activity.

In decerebrate, paralysed and ventilated cats, I recorded the discharge of six abdominal muscle spindle afferents from a filament of the caudal branch of left L2 while simultaneously recording the motor discharges of another branch of the same nerve, of the contralateral L2, and of left L1. Afferents were identified by an increased discharge during lung inflation, motoneurons by aug-

Figure 1



menting activity during the inter-phrenic burst intervals. Cross-correlation of afferent activity to all motor activities revealed no changes in probability of motoneuron discharge. This was true even in one case in which the filament contained a single afferent and a single efferent (Fig. 1).

These results, based on cross-correlation analysis, indicate that the discharge of abdominal respiratory motoneurons is not affected by activity of spindle afferents pro- jecting to the same or different segmental levels. The reasons for this are unclear but may be due to multiple intervening interneurons and/or preferential projection of spindle afferents to motoneurons serving postural rather than respiratory reflexes and which are inactive under these experimental conditions. Future studies of the segmental control of abdominal muscles should be directed at evaluating connections using the more sensitive method of spike-triggered averaging and determining if postural and respiratory abdominal motoneurons constitute distinct pools in terms of reflex control.

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P32

Differential expression of voltage-activated calcium channels in functionally distinct brainstem motoneurons controlling airway and extraocular muscles

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Voltage-activated calcium channels are important in controlling motoneuron (MN) excitability and repetitive firing behaviour through their effects on calcium-dependent potassium channels, and the after-hyperpolarization (AHP), in particular the medium AHP (mAHP). Differential expression of voltage-activated calcium channels may therefore be important in establishing the task-specific firing properties of functionally distinct motoneuron pools. Differential expression of these channels may also be involved in the variable susceptibility of MNs to disruptions in calcium homeostasis, which are implicated in the pathology of neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS).

To further explore the expression of calcium channels in brainstem MNs and the possibility that differential expression of calcium channels predisposes some MNs to degeneration, we compared voltage-activated calcium channels in hypoglossal (XII) and oculomotor (III) MNs, that differ not only in their firing behaviour, but in their vulnerability to degeneration in ALS. XII MNs, which innervate the genioglossus muscle of the tongue and degenerate in ALS, produce rhythmic bursts of action potentials during inspiration that last ~200–400 ms. Discharge frequency peaks between 25–50 Hz [1]. Oculomotor motoneurons, which innervate extraocular muscles and are resistant to degeneration in ALS, can produce sustained firing rates of 300 Hz during fixation and peak discharge rates of 600 Hz for up to 25 ms during saccades [2].

Voltage-activated calcium channel expression was compared between XII and III MNs electrophysiologically in neonatal rat brain slices using whole-cell patch-clamp recording techniques, and immunohistochemically using antibodies to different channel subtypes.

Current densities of low voltage-activated (LVA) calcium currents were similar in XII (-5.9 ± 1.1 pA/pF, $n=9$) and III (-4.6 ± 0.8 pA/pF, $n=13$) MNs. Immunolabelling for the α1G subunit of the LVA calcium channel was also similar between MN pools. In contrast, high voltage-activated (HVA) calcium current density was two-fold greater in XII (-38.2 ± 3.0 pA/pF, $n=33$) compared with III (-19.5 ± 1.4 pA/pF, $n=40$) MNs. Use of HVA calcium channel antagonists (nimodipine and nifedipine for L-type; ω-agatoxin-TK for P/Q-type; ω-conotoxin-GVIA for N-type) revealed that the majority of this difference reflected greater P/Q-type currents in XII MNs (XII: -15.4 ± 0.9 pA/pF, $n=6$; III: -4.4 ± 1.2 pA/pF, $n=12$). Immunohistochemical analysis of P/Q channel expression supported greater expression in XII MNs. N-type currents were not significantly different in XII (-4.3 ± 1.2 pA/pF, $n=9$) and III MNs (-8.0 ± 1.7 pA/pF; $n=11$). L-type currents, defined by nimodipine or nifedipine sensitivity, were not detected.

These data suggest that differential expression of voltage-activated calcium channels may not contribute to the distinct firing properties of XII and III MNs since activation of the mAHP is primarily associated with calcium flux through N-type channels [3] and these channels do not differ between XII and III MNs. However, our data do support the possibility that greater expression of HVA calcium channels may predispose XII MNs to degeneration during periods of metabolic stress as seen in ALS. The greater density of P/Q-type HVA calcium channels in XII MNs would allow more calcium to enter MNs upon depolarisation and increase the likelihood of interaction with calcium channel antibodies found in some ALS patients.

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P33

Simple circuits to maintain isocapnia regardless of ventilation

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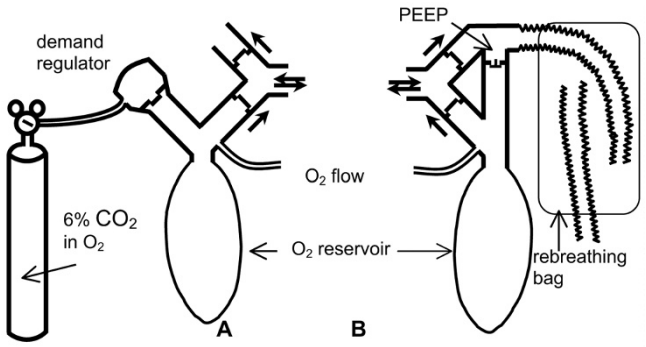
Many studies of respiratory control require a constant CO_2 to eliminate it as a confounding variable. Methods for controlling CO_2 generally rely on feedback circuits which are subject to phase lags and instabilities.

In 1998, we introduced a simple circuit ([1]; Fig. A) which stabilizes CO_2 levels regardless of increases in ventilation. It passively adds CO_2 to the circuit from an external source at a rate proportional to any increase in ventilation above control. Because the CO_2 in this reserve gas has a PCO_2 equal to that in mixed venous blood, it cannot contribute to pulmonary CO_2 exchange.

Figure



Alternatively, CO_2 can be supplied from previously exhaled gas. Banzett and colleagues [2] suggested one version. Our version (Fig. B) operates on the same principle but is more compact, relying on a simple bypass valve between the inspiratory and expiratory lines and which opens only when the fresh gas reservoir is exhausted. When ventilation exceeds the fresh gas flow, the 'extra' gas inhaled will be expired gas from the preceding expiration. Proportional rebreathing of expired gas with a PCO_2 approximately equal to that of mixed venous blood prevents hypocapnia. Moreover, if the fresh gas flow consists of a hyperoxic gas mixture, such as 100% O_2, partial rebreathing ensures that FIO_2 remains close to that of the administered gas.

Is an external gas source mandatory for maintaining isocapnia regardless of ventilatory level? The circuit in the black box contains no compressed gas or electronics. We challenge you to breathe through it and a) change your expired CO_2 level using any breathing pattern you want, and b) explain how our circuit works.

Possible applications for this "mystery circuit" include preventing hyperventilation-induced hypocapnia in acute mountain sickness and resuscitation of asphyxiated neonates [3].

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P34

Respiratory synchronisation of left and right medullary inspiratory neurons in decerebrate rats

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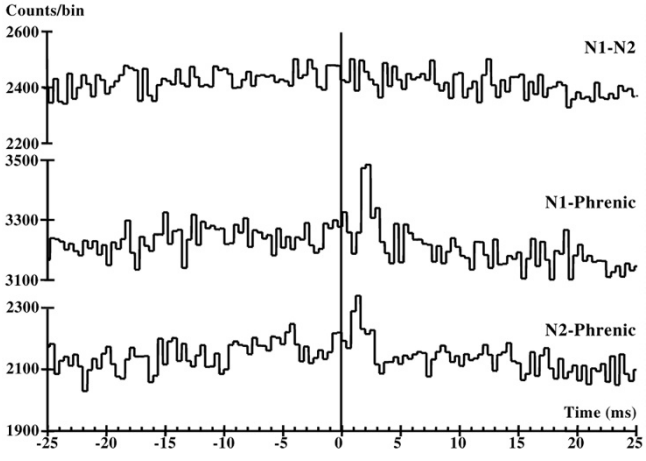
Introduction To be effective, inspiratory muscles on the left and right sides must contract together. In rats, the left and right halves of the diaphragm are synchronised because a bilateral population of medullary premotor neurones [1] simultaneously excites left and right phrenic motoneurones. However, transection studies demonstrate that each side of the brainstem is capable of generating respiratory rhythm independently [2], so that left and right medullary inspiratory neurones must themselves be synchronised. The interconnections and common excitation that accomplish such synchronisation are unknown in rats.

Methods To detect common activation and interconnections we used the cross-correlation technique [3]. We recorded inspiratory neurons on the left and right sides of the medulla with glass-insulated tungsten microelectrodes and the phrenic nerve with bipolar silver wire electrodes in decerebrated rats.

Results Inspiratory neurons were classified according to their firing pattern, and phrenic premotor neurones were identified by the presence of a narrow peak at short latency in their cross-correlogram with phrenic nerve discharge. The figure shows cross-correlograms computed for a left and right pair of phrenic premotor neurons (0.4 ms bins). Of 240 cross-correlograms, 73% were featureless, 6% displayed central peaks, and 6% displayed peaks at short latencies.

Figure



Discussion/Conclusions Central peaks denoting common activation were rarely detected for pairs of neurons of any type, as were peaks denoting excitatory cross-connections. These findings imply a mostly unilateral distribution for excitatory inputs to inspiratory neurones, such as those from central chemoreceptors. Also, that synchronisation of left and right side inspiratory neurones via excitatory cross-connections is weak.

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P35

Cross-correlation of respiratory neurons in transverse medullary slice preparations from neonatal rats

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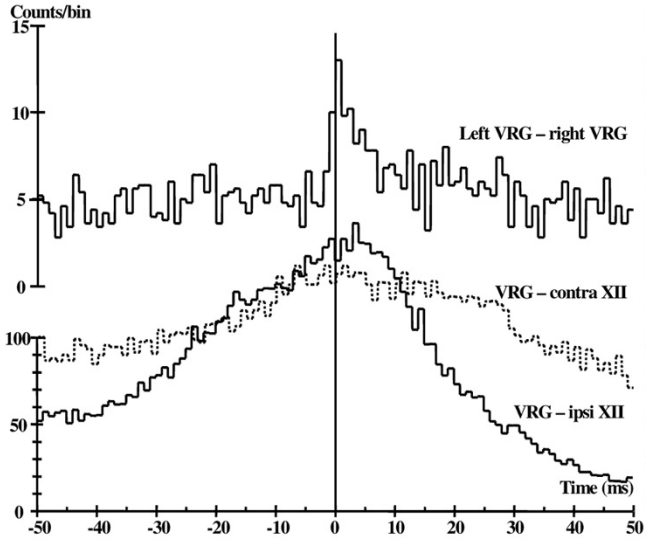
Introduction The respiratory rhythm of hypoglossal (XII) nerve discharge in transverse medullary slice preparations from neonatal rats is thought to originate in the region of the ventral respiratory group (VRG); generated there by a combination of "pacemaker" neurones [1] and their interactions with other respiratory neurones. Our goal was to discover interconnections between left and right VRG neurones as well as their connections to XII motoneurones.

Methods We used the cross-correlation technique [2] to detect interconnections. We recorded from VRG neurones and XII motoneurones with glass-insulated tungsten microelectrodes and from XII nerve rootlets with glass suction electrodes in transverse medullary brain slices from neonatal rats.

Results The figure shows example cross-correlograms computed with 0.4 ms bins. Those computed between left and right VRG neurones displayed broad central peaks. Those between VRG neurones and XII nerves displayed broader central peaks that were less defined for the contralateral side.

Discussion/Conclusions Direct interconnections were not detected. The central peaks in the left to right VRG cross-correlograms indicate common activations, likely shared excitation. The broad peaks of the cross-correlograms between VRG neurones and XII nerves VRG neurons indicate that VRG neurones drive XII motoneurones via polysynaptic pathways, with the ipsilateral pathway stronger than the contralateral.

Figure



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P36

The effect of CO on the ventilatory response to CO_2 in humans

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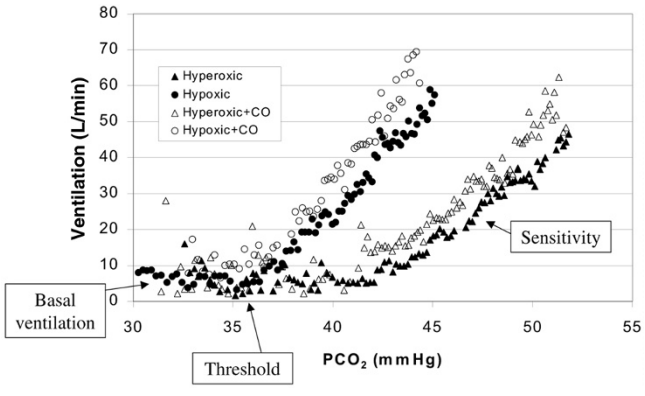
Introduction "Oxygen sensing by the carotid body is crucial for life in mammals and, in fact, may play a life-or-death role in situations involving acute hypoxia. However, the transduction and transmission mechanisms at the carotid body chemoreceptors are not well understood"[1]. Two main transduction hypotheses for oxygen sensing have been suggested [2]: Mechanism 1 involves oxygen-sensitive K^+ channels while mechanism 2 involves Heme proteins and/or reactive oxygen species [3]. In support of the latter mechanism, it has been found that carbon monoxide (CO) inhibits the discharge, recorded in-vitro, from the cat carotid body during hypoxia [2]. We hypothesized that exposure to exogenous CO in humans would also inhibit the carotid body and produce a decreased ventilatory response to hypoxia.

Methods We used hypoxic (50 mmHg) and hyperoxic (150 mmHg) modified rebreathing tests[2] given in a randomized order, with the subjects blinded as to the type of rebreathing test, before and after breathing ~1000 ppm CO until carboxyhemoglobin = 10–12%.

Results A representative subject's ventilatory responses to carbon dioxide during hyperoxia and hypoxia, with and without CO present is illustrated in the figure.

Figure



Two-way ANOVA analyses of basal ventilations, thresholds and sensitivities were done for 9 subjects. The two factors were oxygen level and CO level; CO was not a significant factor.

Discussion/Conclusion.Physiological COHb levels due to endogenous CO production are ~1%. Our subjects achieved levels 10 times greater. Yet during hypoxia, the ventilatory response to carbon dioxide was unchanged. This argues against our hypothesis and so against mechanism 2. We conclude that CO is not a key factor in the oxygen sensing apparatus.

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P37

Pharmacology of nicotinic receptors that mediate modulation of respiratory pattern by nicotine in preBötzinger complex

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Nicotine from cigarette smoke affects respiratory pattern and is a risk factor for some common disorders such as sudden infant death syndrome and sleep-disordered breathing. We have delineated mechanisms underlying regulation of respiratory pattern by nicotinic actions within preBötzinger Complex (preBötC), the hypothesized site for respiratory rhythm generation. Low concentrations of nicotine increases respiratory frequency by modulating excitatory synaptic transmission in preBötC inspiratory neurons [1]. Here, we investigated the receptor subtypes mediating these effects.

Using a medullary slice preparation from neonatal rat which contains the preBötC and generates respiratory-related rhythm *in vitro*, we patch-clamped preBötC inspiratory neurons and recorded respiratory-related rhythmic activity from hypoglosssal nerve simultaneously.

Bath application of low concentrations of nicotine (0.5 μM, equivalent to the arterial blood nicotine concentration after smoking a cigarette) increased respiratory frequency, decreased the amplitude of respiratory-related rhythmic motor activity, induced a tonic inward current, increased the frequency of spontaneous EPSCs during expiratory periods and decreased the amplitude of inspiratory drive inward current in preBötC inspiratory neurons. These effects could not be blocked by nicotinic antagonists α-bungarotoxin (200 nM), methyllycaconitine (MLA, 1 μM), but were completely blocked by dihydro-β-erythroidine (DH-β-E, 200 nM), hexamethonium (10 μM) and partially blocked by tubocurarine (10 μM). Bath application of RJR-2403 (0.5 m[ED]M), a nicotinic agonist selective for the α4β2 nicotinic receptor, or cytisine (0.1 μM), an agonist selective for the β4 subunit containing nicotinic receptors, induced effects similar to those induced by nicotine. The effects of RJR-2403 or cytisine were not blocked by MLA, but blocked by DH-β-E.

These results suggest that neither α7 nor α9 subunit homomers are involved in the modulatory actions of nicotine on preBötC inspiratory neurons and on respiratory pattern. The effects are likely mediated by the α4β2 nicotinic receptor. The β4 subunits may be also involved.

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