Pflügers Arch – Eur J Physiol (1995) 430:115–124


ORIGINAL ARTICLE

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# Maturational changes in the respiratory rhythm generator of the mouse

Received: 16 May 1994/Received after revision: 1 August 1994/Accepted: 13 October 1994

**Abstract** The changes in motor activity of the respiratory rhythm generator were quantitatively analysed in mice (from birth to at least 56 days old) in both awake and anaesthetized preparations, as well as in vitro to define the age at which the respiratory network is mature. In awake and anaesthetized spontaneously breathing mice respiratory-related thoracic movements were recorded and revealed an age-dependent increase in both inspiratory time (45 %) and cycle length (22 %) over the first 15 days of life. Similarly, the pattern of phrenic nerve activity recorded from anesthetized animals also changed from a short, rapid onset and offset burst, without a post-inspiratory phase (0–10 days old), to a discharge of longer duration which included both ramp and post-inspiratory components ( > 15 days). This pattern was comparable to that seen in adult mice ( > 56 days old). A recently developed tilted-sagittal brainstem slice preparation containing an isolated, but functionally intact, medullary respiratory network was employed in our *in vitro* studies. Since this preparation generates respiratory rhythmic activity spontaneously in both neonatal and mature mice ( > 56 days old) it has permitted a direct comparison of the respiratory motor output pattern, recorded from the hypoglossal (XII) motor nucleus, during post-natal development in similar preparations. Consistent with our *in vivo* findings there was an age-dependent change in the motor pattern. The rhythmic burst of XII neurones recorded from slices of neonates (0–10 days old) was short in duration and decremented whereas a longer discharge (increase of 625 % compared to neonate) containing a plateau component was seen in animals more than 15 days old. In addition, the cycle length of rhythmic XII neurones increased (143 %) and, together with the changes in burst duration, reached a steady-state value over a similar time course to the maturational changes in phrenic nerve activity recorded *in vivo*. Our *in vivo* and *in vitro* data indicate that the central respiratory network of the mouse appears to be mature at post-natal day 15.

**Key words** Ventral respiratory group · Phrenic nerve · Rhythmic hypoglossal motor neurones · Motor pattern · In vivo · In vitro

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## Introduction

The central neuronal mechanisms underlying the generation of the respiratory rhythm are unknown in both neonatal and mature mammals. One approach to enhance our understanding might be to investigate the maturational changes that occur in the respiratory network over the first weeks of life. The aim of this study was to compare the time course over which the respiratory network of the mouse develops in awake and anaesthetized animals with a recently developed brainstem slice preparation shown to generate respiratory rhythmic activity spontaneously in both neonatal and mature rodents [9]. Only when the time of maturation of the respiratory network is known can further analysis of the developmental changes in synaptic and cellular properties be made.

Previous studies have shown differences in the motor output pattern of the respiratory generating network of adult and neonatal mammals. In the mature respiratory network of the rat [15] and cat (for example, [12, 13]) there are three distinct phases, (inspiration, post-inspiration and expiration). However, in the neonatal rat there was a distinct absence of post-inspiratory activity in the phrenic nerve recorded either in in vivo or in an in vitro brainstem spinal cord preparation [16]. In comparison, although immature, the three phase rhythm was

present in the newborn cat [5] indicating that the respiratory rhythm generating network is more mature in the kitten compared to the rat. In the present study we have adopted the mouse as an animal model which may have numerous advantages over other species. For instance, in addition to the small size of the brainstem, which is advantageous for in vitro preparations, this species is used to produce different mutant strains. Thus, an appropriate strain of mouse could potentially provide a powerful means to a greater understanding of the central neuronal mechanisms for respiratory rhythm generation.

We report that the respiratory motor pattern of the mouse undergoes ontogenetic changes and appears to be mature at postnatal day 15 (P 15). A preliminary report of this study has been published [10].

## Materials and methods

Mice of either sex (MRI-1 and Bahabor) aged between 0 and > 56 days were used in both in vivo and in vitro experiments.

### In vivo experiments

Changes in the breathing/motor pattern were analysed in awake and anaesthetized mice of different ages.

#### *Anaesthetized animals*

Mice were anaesthetized with sodium pentobarbitone (Nembutal; 60 mg/kg; i.p.) and placed on a thermostatically controlled water-heated stainless steel table. Animals were supported in a cradle of plasticine, ventral side up and were maintained at 38 °C with an infrared lamp. Body temperature was measured using a thermistor probe placed either under the animal (neonates) or into the rectum (mature mice). The trachea was cannulated below the larynx and a stream of pure oxygen directed across the open end of the cannula. Animals breathed spontaneously throughout the duration of the experiment.

Central respiratory activity was monitored by recording phrenic or hypoglossal nerve discharge in some neonates and in all mature animals. The left phrenic or hypoglossal nerve was isolated via a ventrolateral approach and rhythmic activity recorded via a suction electrode (see below) connected to a differential amplifier (Tektronix AM 502). The nerve discharge was integrated using a custom-built leaky integrator (decay time constant of 100 μs). In all experiments the ventilatory movement of the thorax was measured non-invasively by a pressure transducer (Bell and Howell, type 4-327-I) connected to a semi-inflated balloon secured across the chest of the animal. The change in pressure with every breath gave a direct measure of the inspiratory-related movement of the thorax. From this, both the inspiratory (i.e. time to peak of pressure change) and expiratory (time between the peak of pressure change and the start of the next breath), times as well as the rate and a relative indication of the change in tidal volume were measured. These measurements paralleled precisely the changes in rate, duration and amplitude of integrated phrenic and hypoglossal nerve activity. All recorded signals were stored on magnetic tape (Racal V-Store) for subsequent off-line analysis and displayed on a thermal array recorded (Gould TA 2000).

#### *Awake animals*

Breathing was measured in neonatal and mature mice by monitoring thoracic movements using a non-invasive method described above. The only difference was that animals were placed ventral side down on the balloon. Following an acclimatization period of 5–10 min, measurements were made during quiet episodes of breathing when there was no obvious motor activity.

### In vitro experiments

The recently developed tilted-sagittal slice was employed since it generates respiratory rhythmic activity spontaneously in both neonatal and adult mice. Full details of this preparation have been published and only a brief description is given here (see Fig. 1; [9]).

Mice were anaesthetized deeply with ether and decapitated. The entire brain was removed in ice-chilled artificial cerebrospinal fluid (a-csf) which was bubbled continuously with carbogen (95 % oxygen plus 5 % carbon dioxide). The pons, brainstem and first segment of the spinal cord were isolated and glued (cyanoacrylate) horizontally, with the dorsal side up, in an agar mould which was mounted on a glass block. The mould both tilted and supported the brainstem at 35° about the midline vertical axis during slicing (Fig. 1). A tilted-sagittal slice (600–700 μm thick) was prepared after the lateral edge of the brainstem had been removed (approximately 200 μm in neonates and 500 μm in mature mice) using a vibratome. The slice was transferred to a recording chamber and placed onto a stainless steel mesh grid and superperfused with a continuous stream of carbogenated a-csf (11–12 ml/min) at 29 °C using a counter-current perfusion system (see [9]). The slice was held in place by fine nylon strands secured to a horseshoe-shaped platinum wire form. The strands were positioned away from the ventrolateral and dorsomedial regions of the medulla typically over pontine and spinal cord regions of the slice. A 30- to 60-min stabilization period elapsed before recording began.

### Recording procedures

In neonates and mature mice rhythmic respiratory activity was recorded from both the hypoglossal (XII) motor nucleus and neurones in the ventral respiratory group (VRG). In addition, in some neonates, rhythmic discharge was recorded from the XII rootlet. The XII motor nucleus was identified anatomically with reference to the shape of the dorsomedial medulla. Extracellular recordings of a single units were made using either glass microelectrodes (1–7 MΩ; tip diameter 2–4 μm) filled with 3 M NaCl or a-csf. In some experiments insulated tungsten steel electrodes (1–3 MΩ; tip diameter 2–3 μm) were used. A XII rootlet was recorded via a suction electrode (tip diameter 200–350 μm) filled with a-csf. XII rootlets were present in slices of neonatal, but not mature, mice. Electrodes were placed into the XII motor nucleus or VRG under visual guidance of a binocular microscope and driven into the tissue using a nano stepper (1.5-μm steps). Extracellular signals were amplified and filtered (3 Hz to 8 kHz; NPI Electronics pre-amp type 2F). Intracellular recordings were made with either fine-tipped glass microelectrodes (60–80 MΩ; tip diameter < 1 μm) or patch pipettes. Pipettes were manufactured using a two-stage pulling programme (Sutter Instruments, type P-87) and filamented capillary tubes (glass type: Science Products GC 150 F10). Pipette internal tip diameters were between 1 and 2 μm and when filled (see below), gave a resistance of 1.5–5 MΩ. A positive pressure (60–95 mmHg or 8–12.7 kPa) was applied to the pipette to prevent blocking while advancing through the tissue. Offset potentials were nulled using the DC offset control. While searching for and sealing onto neurones, a short current pulse was generated (AMPI Master 8; 60 ms; 0.5–1.0 nA) so that the quality of the seal could be measured.

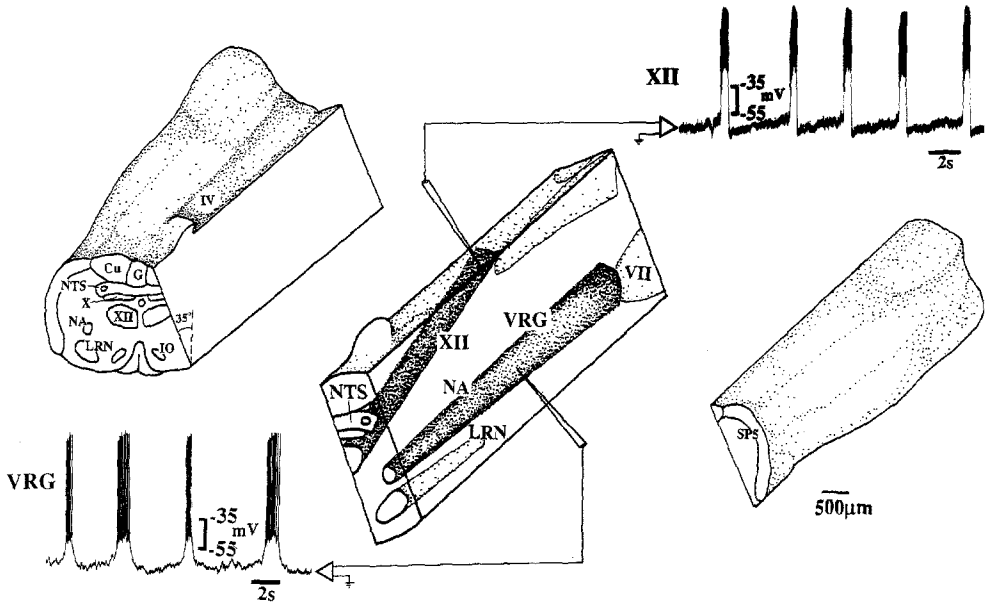


**Fig. 1** The tilted-sagittal slice contains the dorsomedial and ventrolateral medulla and is approximately 35° to the vertical midline axis. It includes the XII motor nucleus, nucleus of the solitary tract (*NTS*) and the nucleus ambiguual column (*NA*) in their entirety (see [9]). The primary respiratory oscillator resides in the ventrolateral medulla co-existing with the NA and is termed the ventral respiratory group (*VRG*). Respiratory rhythmic activity was present in both VRG neurones and XII motor neurones; activity in the latter nucleus representing the motor output of the respiratory network. Whole-cell recordings are depicted for a XII motor neurone and a VRG neurone from mature mice (P24, P31). Note that the large synaptic drive potentials in this XII motor neurone resulted in an inactivation of the fast spikes. Membrane resistance ($R_M$) values for these neurones were 190-MΩ (XII motor neurone) and 215 MΩ (VRG neurone). (Other structures include; *Cu* cuneate, *G* gracile, *IO* inferior olive, *LRN* lateral reticular, *Sp5* spinal trigeminal tract, *IV* fourth ventricle, *VII* facial nucleus, *X* dorsal vagal motor nucleus)

High-resistance seals (6–9 GΩ) were obtained on removing the positive pressure and applying suction. Following "break through", capacitive transients were fully compensated. Signals were amplified using an NPI SEC-10L amplifier and head stage before being displayed on an oscilloscope (Group DSO 420) and stored on magnetic tape via an ADC interface (Instrutech VR 100; sampling rate 26 kHz).

## Stimulation procedures

In three neonates and three mature mice, either the right or left vagus nerve was isolated below the larynx and placed on a bipolar silver wire stimulating electrode. Short train (1–2 s) stimuli were generated (Hi-Med, UK; 20–25 Hz; 0.1 ms) and delivered via an isolator unit (AMPI Iso Flex: 0.5–2 V) to assess the presence and potency of afferent vagal feedback on respiration. The possibility that connectivity between the nucleus of the solitary tract (NTS), the central site of termination of vagal afferent fibres [6] and the primary respiratory oscillator, located in the ventrolateral medulla, was preserved in the tilted-sagittal slice was examined. The evoked response of rhythmic XII motor neurones following NTS stimulation in slices was compared with the effect of electrical stimulation of the vagus nerve in vivo. In slices of both neonates and mature animals, a bipolar concentric stimulating electrode (tip diameter 200 μm) held in a micromanipulator was used to stimulate the regions of the NTS known to receive cardio-respiratory vagal afferents [6]. Trains of stimuli were generated (AMPI Master Eight; 20–100 Hz; 0.1–0.2 ms) and delivered via an isolated stimulator unit (AMPI Iso Flex; 10–50 μA) while recording rhythmic XII motor neurone activity. In addition, effective sites of the NTS were activated chemically by microinjecting 2-methyl serotonin maleate (2 mM; 50–100 nl), a $5HT_3$ (where 5HT denotes 5-hydroxytryptamine) receptor agonist, from a glass pipette (tip diameter 20–30 μm) held in a second micromanipulator. This compound is known to depolarize vagal afferent terminals specifically by activating pre-synaptic autoreceptors [11]. Microinjections were made at depths within the NTS using positive pressure generated from a syringe connected to the pipette with a length of polyethylene tubing. The volume injected was assessed by measuring the distance moved by the fluid meniscus using a binocular microscope equipped with a reticule.

## Solutions

A bicarbonate-buffered Ringer was prepared daily and continuously bubbled with carbogen to maintain the pH at 7.4. The normal a-csf contained in mM: 1.25 $KH_2PO_4$, 1.25 $MgSO_4$, 25 $NaHCO_3$, 3.5 KCl, 125 NaCl, 2.5 $CaCl_2$, 20 dextrose. Prior to the onset of a recording session extracellular potassium concentration was raised to 7.5 mM to enhance respiratory rhythmic activity. The constituents of the patch pipette solution were in mM; 140 K gluconate, 10 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 0.2 ethylenebis(oxonitrilo)tetraacetate (EGTA), 7.7 NaCl, 2 $K_2ATP$, pH adjusted to 7.3 using 1 M KOH.

## Data analysis

In all experiments 10–20 cycles of rhythmic activity were quantitatively analysed. In in vivo experiments in awake mice where recordings of inspiratory-related thoracic movements were

**Table 1** The mean ± SD of the inspiratory time, cycle length and, where appropriate, the post-inspiratory phase are indicated for neonatal (P0–10) and mature mice (P > 15 days) under different experimental conditions. Data presented are from both in vivo experiments on awake and anaesthetized mice, where thoracic movements and phrenic nerve activity were evaluated respectively, and in vitro preparations. In tilted-sagittal slices, rhythmic discharges recorded from XII motor neurones were taken as an index of the inspiratory period. The ratio of the inspiratory time or burst duration to cycle length are compared for the different experimental preparations. In in vitro experiments bath temperature was 29 °C and extracellular potassium was 7.5 mM.

| Variable | In vivo (38 °C) | | | | | |
|---|---|---|---|---|---|---|
| | Awake | | Anaesthetized | | | |
| | Thoracic movement | | Thoracic movement | | Phrenic nerve discharge | |
| | Neonate ($n = 10$) | Mature ($n = 7$) | Neonate ($n = 7$) | Mature ($n = 7$) | Neonate ($n = 8$) | Mature ($n = 14$) |
| Inspiratory time (ms) | 62.8 ± 9 | 96.0 ± 8 | 76.0 ± 8 | 140 ± 16 | 93.4 ± 13 | 174 ± 42 |
| Post-inspiratory time (ms) | | | | | — | 107 ± 34 |
| Cycle length (ms) | 409 ± 34 | 499 ± 14 | 645 ± 36 | 939 ± 38 | 724 ± 71 | 926 ± 131 |
| Ratio of IT:CL | 1:6.5 | 1:5.2 | 1:8.5 | 1:6.7 | 1:7.8 | 1:5.3 |

| | In vitro (29 °C) | |
|---|---|---|
| | Rhythmic XII neurone discharge | |
| | Neonate ($n = 11$) | Mature ($n = 18$) |
| Burst duration (ms) | 325 ± 68 | 2032 ± 560 |
| Cycle length (ms) | 2449 ± 627 | 5964 ± 815 |
| Ratio of BD:CL | 1:7.5 | 1:3.0 |

recorded, the inspiratory time and frequency were measured, whereas in anaesthetized mice inspiratory and post-inspiratory durations and frequency of breathing were obtained from phrenic nerve discharge. In addition, the rate of rise of inspiratory activity was measured from integrated phrenic nerve and XII nerve discharge and qualitatively compared between neonates and mature mice. The frequency of bursting, burst duration and intra-burst firing frequency were measured from rhythmic XII motor neurones in in vivo experiments. Measurements were either made manually or using the trigger and cursor facilities of a digital oscilloscope (Gould DSO 420). All values quoted are the mean ± standard deviation.

## Results

### Age-dependent changes in respiratory motor pattern

Age-dependent changes in respiratory activity were compared between experiments on awake and anaesthetized mice and with data from rhythmic XII motor neurones recorded from tilted-sagittal slices. In this study neonate indicates an animal between P0 and P10 whereas a mature mouse was more than 15 days old.

#### *In vivo experiments*

In in vivo experiments of both awake ($n = 10$ neonates; $n = 7$ mature) and anaesthetized neonatal ($n = 15$) and mature mice ($n = 21$), the inspiratory time and cycle length were measured from recordings of either inspiratory-related thoracic movement or phrenic nerve activity (see Table 1, Figs. 2, 3). There was good agreement between the post-natal changes in inspiration, expiration and cycle length measured from thoracic movements with those obtained from phrenic nerve activity in comparable preparations (Table 1, Figs. 2–4). It is evident from the traces of respiratory-related thoracic movement shown in Fig. 2 that there was a 150- to 200-ms delay, as measured from the start of the ramp inspiratory activity recorded from the phrenic nerve. The reason for this was a technical one reflecting the low pressure within the balloon and the "light" contact between the balloon and the animals' chest. The latter was important in preventing a restriction of thoracic movement.

In the neonate, the phrenic nerve discharge had a rapid onset with a short plateau component followed by a rapid decrementing pattern. The burst was found to occupy 12.9 % of the total respiratory cycle, whereas the quiescent phase lasted 87.1 % (Fig. 2). Over a maturational period of 15 days the inspiratory time increased by 45 %, whereas the expiratory phase decreased by 24.8 % (Table 1, Figs. 2 and 4). This increase in the inspiratory phase was accompanied by a change in the motor pattern which included a ramp component (Fig. 2). In addition, post-inspiratory activity was first detected around P10 and by P15 its

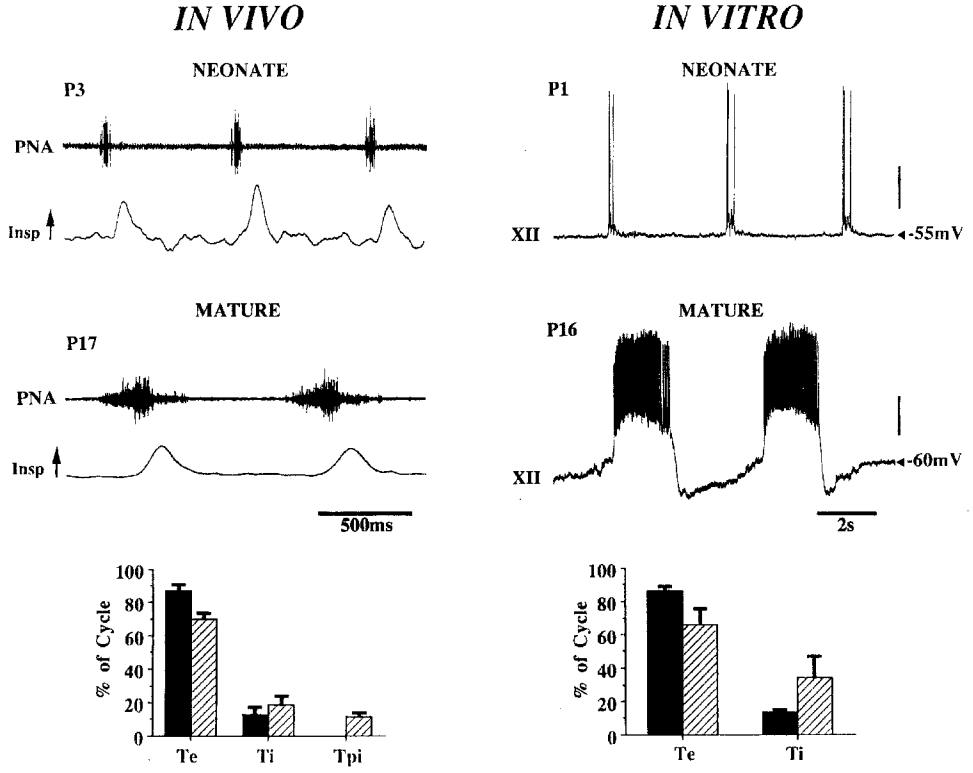


**Fig. 2** Comparison of the respiratory motor output pattern recorded from the phrenic nerve (*PNA*) and XII motor neurones of neonates and mature mice. In *in vivo* experiments, animals were anaesthetized and breathed spontaneously. In addition to phrenic nerve discharge, the inspiratory-related movement of the chest was monitored (*arrow* indicates direction of inspiration; *Insp*). Note the delay in the detection of inspiratory-related thoracic movement. The reason for this was a technical one reflecting the low pressure within the balloon and the "light" contact between the balloon and the animals' chest. Whole-cell recordings show the difference in the firing pattern of rhythmic XII motor neurones between P1 and P16 mice as recorded from titled-sagittal slices, $R_M$ values were 245 MΩ (P1) and 205 MΩ (P16). *Solid bar* indicates 20 mV. Histograms below compare the relative mean ratios of the inspiratory and expiratory phases as a percentage ( ± SD) of the total respiratory cycle in mature and neonatal mice (10 respiratory cycles; $N = 5$ each). The post-inspiratory component observed in mature mice is also included. Neonates, *solid bars*; mature mice, *hatched bars*

amplitude and duration were analogous to that seen in adult mice (P > 56; Table 1, Fig. 2). Also the frequency of breathing decreased significantly ($P < 0.05$) in both awake (18.1 %) and anaesthetized mice (26.6 %) during the first 15 days of life (see Table 1, Figs. 2 and 4). Figure 4 compares the time course of changes in inspirtory time and cycle length for both awake and anaesthetized animals. Since the phrenic nerve discharge in P15 mice was not significantly different ($P > 0.2$) from that seen in more mature and adult animals (Fig. 4), the respiratory rhythm generator seems to be mature at 15 days after birth. This result was supported by comparable age-dependent changes in XII nerve recordings (neonates, $n = 3$; mature mice, $n = 2$) and by our in vitro data described below.

*In vitro experiments*

In slice experiments rhythmic XII motor neurone activity (and rhythmic XII rootlet discharge in three neonates) was taken as an index of central inspiratory activity, since it is known that many XII motor neuorones receive innervation from pre-motor inspiratory neurones located within the VRG ([7] for additional refs. see below). Since the inspiratory-related discharge appears to be relatively homogeneous between respiratory-modulated XII motor neurones in both neonatal (rat: [2, 3, 16, 17]) and adult mammals (mice and rat: [8–10]; cat: [4, 20]) it permitted a qualitative analysis of the changes that occurred post-natally.

As seen in vivo there were also age-dependent changes in rhythmic XII motor neurone discharge (burst duration and cycle length) recorded in vitro which stabilized after approximately the 15th post-natal day (Table 1, Figs. 2–4). The burst duration of XII motor neurones, as measured at 29 °C, became prolonged from a mean of 0.325 ± 0.07 s in neonates to 2.03 ± 0.56 s in mature mice, which represented a 625 % increase (Table 1, Figs. 2–4). Further more, the rapid onset decrementing pattern changed to a burst containing a plateau component (Figs. 2, 3). In mice more than 15 days old, the plateau component of the XII motor neurone burst was preceded by an incrementing component of 200–400 ms duration

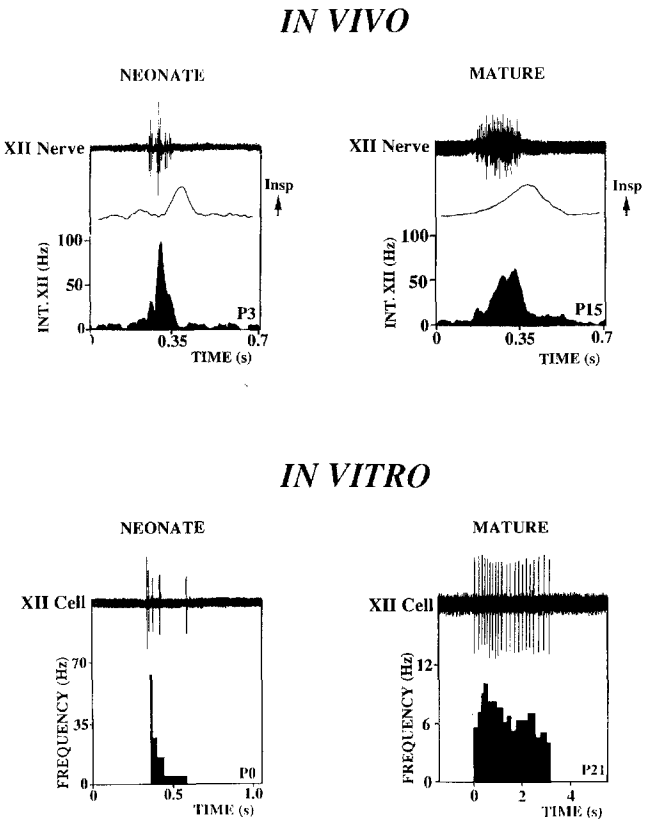


**Fig. 3** Developmental changes in the respiratory motor pattern recorded from the XII nerve in vivo (upper part) and two XII motor neurones recorded extracellularly in slices (lower part). In in vivo upper part experiments, the XII nerve activity was integrated and is shown together with the inspiratory-related thoracic movements (*arrow* indicates inspiration, *Insp*). Note that the rate of rise and duration of the integrated XII nerve discharge is reflected in the chest movement

(Fig. 3). In addition, the frequency of inspiratory-related XII motor neurone bursts showed an age-dependent decrease, such that the cycle length was prolonged by 143 % (see Table 1; Fig. 4) in mature (P > 15) compared to neonatal mice. From Table 1 it is evident that there was a major difference between the in vivo and in vitro data. This concerns the changes in the ratios of burst duration or inspiratory time to cycle length, which were significantly more depressed in in vitro than in vivo during maturation.

Unlike the uniformity of discharge of rhythmic XII motor neurones in mice of comparable ages, we found a heterogeneity in the burst duration and firing pattern of VRG neurones recorded in slices of neonatal and mature animals. VRG neurones displayed either ramping, decrementing or augmenting-plateau patterns of rhythmic discharge of variable duration, or slowly ramping activity interrupted by short (300–700 ms) silent periods. For this reason a quantitative comparison of the age-dependent changes was not performed. However, consistent with XII motor neurones described above, there was an age-dependent decrease in the frequency of rhythmically discharging VRG neurones.

### Presence and potency of reflex vagal afferents on respiration in vivo and in vitro

In three anaesthetized neonatal (P3) and three mature mice (P > 15 days) electrical stimulation of either the left or the right vagus nerve (0.5–1.0 V; 20–50 Hz; 0.1 ms) induced apnoea which was maintained throughout the stimulation period (Fig. 5) and, in some mice (neonatal, $n = 1$; mature, $n = 2$), for a 300- to 500-ms period after the end of the stimulus period. Stimulation of the vagus nerve coincident with the onset of an inspiratory burst could terminate it prematurely or prolong expiration if timed to arrive during the interburst interval. In all three neonatal animals bilateral vagotomy resulted in either a prompt and sustained arrest of breathing ($n = 2$), or a significant slowing and increase in amplitude. In this latter animal breathing was only maintained for 3–4 min before central apnoea. In contrast, in mature mice bilateral vagotomy decreased respiratory rate and elevated inspiratory depth, but did not cause apnoea.

In tilted-sagittal slices of both neonatal ($n = 3$) and mature mice ($n = 5$), activation of the NTS either electrically or chemically inhibited rhythmic activity recorded in the XII motor nucleus. The responsive zone of the NTS was assessed by electrically stimulating different sites of the NTS. The inhibitory effect was evoked from the ventral NTS localized to approximately the level of the obex (Fig. 5); stimulation at sties more than 250 μm rostral or caudal to the obex were ineffective. Stimulation produced early termination of inspiration or prolongation of the interburst interval depending on the time of the stimulus relative to the rhythmic cycle (Fig. 5). In order to selectively depolarize vagal afferent endings we microinjected 2-methyl serotonin maleate (50–100 nl; 2 mM), a specific $5HT_3$ agonist, into regions of the NTS where electrical stimulation resulted in cessation of rhythmic activity of XII motor neurones. (Unlike microinjecting glutamate this compound avoided the complication of activating NTS neurones that did not receive a vagal afferent input; see [11]. Following a microinjection of 2-methyl serotonin maleate a response qualitatively similar to that seen with electrical stimulation was elicited and included a slowing of XII motor neurone rhythmic activity (Fig. 5) and a reduction in intraburst frequency. In addition, rhythmic VRG neurones recorded from slices of mature mice were either inhibited or excited during activation of the NTS. The excitatory response of VRG neurones was seen as an evoked burst of activity followed by tonic discharge which declined over several seconds.

## Discussion

In order to understand the meaning of physiological data it is necessary to compare the results of in vitro

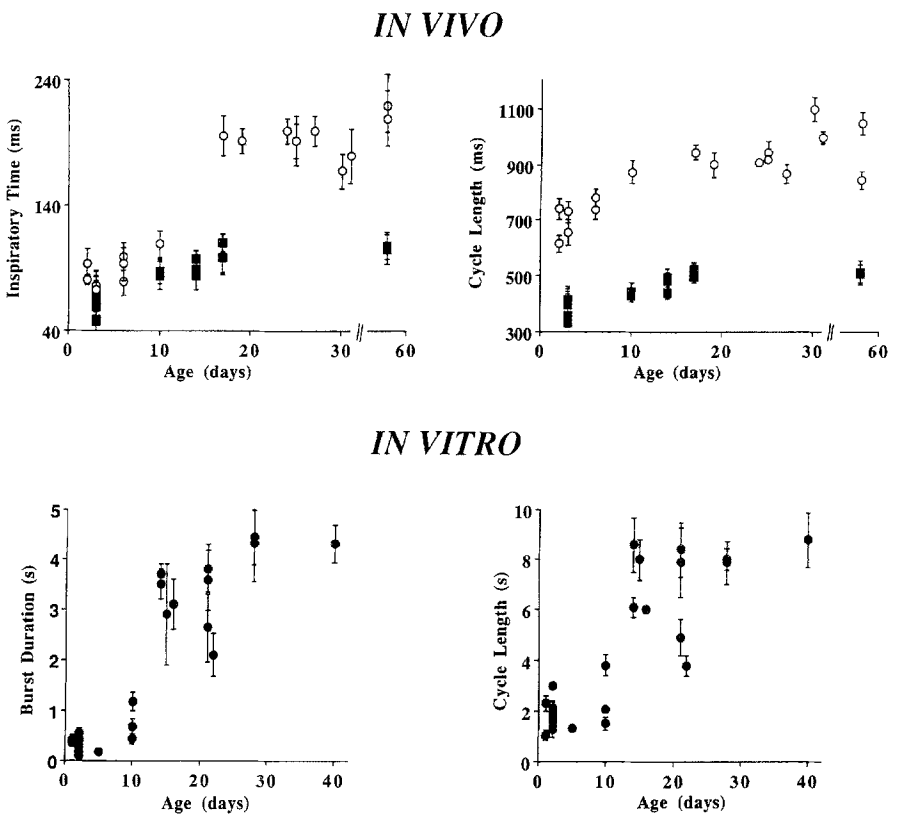


**Fig. 4** The maturational changes in respiratory activity are compared for mature and neonatal mice using data from *in vivo* (upper part) and *in vitro* (lower part) experiments. In *in vivo* experiments the age-dependent changes in inspiratory time and cycle length are plotted for both awake ($N = 17$; respiratory-related chest movement; *solid squares*) and anaesthetized animals ($N = 14$; phrenic nerve activity; *open circles*). In slices, (lower part) data are taken from 29 rhythmically active hypoglossal cells. Both data sets indicate that the respiratory rhythm generator is mature around P 15. Note that measurements made in *in vivo* experiments were at 38 °C but at 29 °C in slices. Values represent the mean ± SD of 12 cycles of respiratory activity from a single mouse

studies with those from in vivo experiments. In this report we present the first detailed analysis of the developmental changes occurring within the respiratory rhythm generating network. Although Smith et al. [16] noted developmental changes of the respiratory motor pattern in the rat, they did not fully describe or quantify the variety of ontogenetic changes.

## Maturational changes in respiratory motor output pattern

The similarity of our in vivo and in vitro findings gives us good reason to believe that the respiratory network of the mouse develops over a 15-day post-natal period. In in vivo experiments, phrenic nerve discharge, taken as an index of the output from the VRG, consisted of a short decrementing discharge in newborn animals and developed into a burst of longer duration which was qualitatively consistent with the developmental changes of XII motor neurones seen in vitro (see below). Between the ages of 10 and 15 days the development of a ramp and post-inspiratory components occurred which are characteristic of the phrenic nerve activity recorded in other adult species (for example: cat, [12, 13]; rabbit [14]; rat, [15]). In addition, the time course of the increase in cycle length of phrenic nerve activity paralleled the reduction in rate of rhythmically firing VRG neurones recorded in vitro.

In vitro recordings of rhythmic XII motor neurones showed that the motor pattern and burst duration to cycle length ratio matured over the first 15 days of life. Furthermore, the pattern of discharge of rhythmic XII motor neurones is comparable with previous in vitro and in vivo studies in other species; the short decrementing burst observed in XII motor neurones of neonatal mice is similar to that reported for rats of the same age [2, 16, 17]. In contrast, in slice preparations of mature mice, the burst of rhythmic XII motor neurones was longer, showed a tendency to augment and included a plateau phase and in this respect was analogous to the discharge pattern of inspiratory-related XII motor neurone discharges reported in the anaesthetized and paralysed adult cat in vivo [4, 20]. The developmental changes in XII motor neurone discharge pattern recorded from tilted-sagittal slices were qualitatively similar to changes recorded from the XII nerve in vivo. It should also be recognized that since tilted-sagittal slices of neonatal and mature mice were approximately the same thickness there will be differences in the neuroanatomical structural content which may contribute to the observed changes in XII motor neurone pattern during development.

It is apparent that the variance of rhythmic activity (duration and frequency) in some XII motor neurones in slices of mature mice is greater than those in neonatal slices (see Fig. 4). The possibility that this reflects a difference in the anatomical structures present between different slices cannot be ruled out. However, we have previously described that the thickness and angle of a tilted-sagittal slice, as well as its relative position within the medulla, is critical for

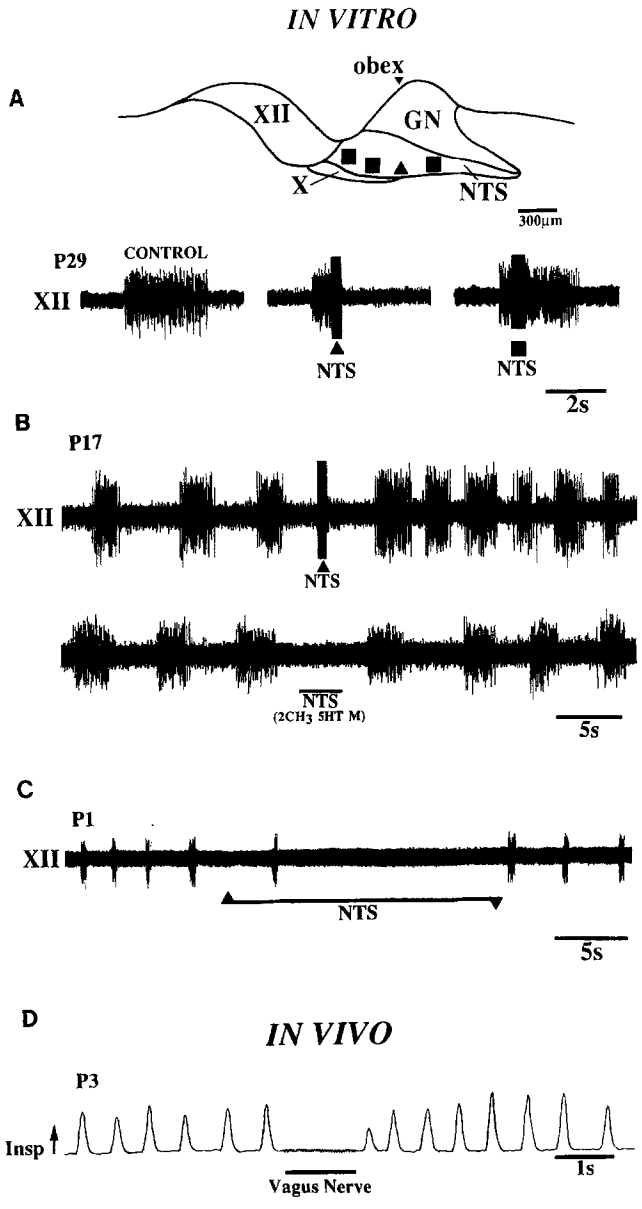


**Fig. 5A–D** Comparison of the effect of stimulating the NTS *in vitro* and the vagus nerve *in vivo* on respiratory activity. **A** A schematic representation of a 50-μm section of part of the dorsomedial region of a tilted-sagittal slice illustrating the structures present and the sites of stimulation (see [9] for full 3-D serial section reconstruction of a tilted-sagittal slice). In tilted-sagittal slices the effective region of the NTS was estimated to be around the level of the obex ± 150 μm rostro-caudal; *solid triangle*) and within regions known to contain vagal afferent terminals. In this example of a slice from a mature mouse, short train stimulation of the NTS (300–500 ms; 100 Hz; 0.1-ms pulse; 25 μA) abruptlly terminated a rhythmic burst recorded from a XII motor neurone. Stimulation at sites > 250 μm rostral or caudal to this sensitive region (*solid square symbols*) had no effect. **B** A comparison of the response evoked either by electrical stimulation of the NTS (100 Hz; 0.1-ms pulse; 30 μA) or during microinjection of 2-methyl serotonin maleate (2 mM; 50 nl) on the same rhythmic XII motor neurone from a slice of a mature mouse (P17). 2-Methyl serotonin maleate is a $5HT_3$ agonist that selectively depolarizes vagal afferent endings by a pre-synaptic mechanism (see [11]). Note the excitatory rebound effect following the inhibition of rhythmic discharge may reflect the multimodal pathways excited. **C, D** Compare *in vitro* and *in vivo* data from neonatal mice. The effect of activating the NTS electrically (28 s train; 1 Hz; 0.1 ms pulse; 40 μA) in a tilted-sagittal slice and vagal nerve stimulation (1.1-s train; 50 Hz; 0.1 ms pulse; 1 V) in an anaesthetized animal is shown. Note that the inhibitory reflex vagal afferents are present and powerful at birth. **D** The *arrow* indicates the inspiratory direction (*Insp*). *GN* Gracile nucleus, *NTS* nucleus of the solitary tract, *X* dorsal vagal motor nucleus, *XII* hypoglossal motor nucleus, *$2CH_35HT$ M*, 2-methyl serotonin maleate, *P* post-natal age in days).

obtaining a rhythmic preparation [9]. Indeed, in rhythmic preparations, we found that the anatomical structures present were consistent between slices of mature mice and any alteration led to a failure to find rhythmic activity within both the XII motor nucleus and the VRG; this was normally due to a severing of the ambiguual column [9]. Furthermore, removal of pontine regions from tilted-sagittal slices did not result in an increase in variance of rhythmic XII motor neurone discharge, but a reduction in burst duration and an increase in frequency [9]. This latter observation tends to dismiss the possibility of the presence of pontine regions in some, but not all, preparations. It is suggested that since the greatest variance of XII motor neurones occurs in mice around P15 it may reflect a time of instability that occurs during the transitional period from the immature to the mature respiratory network.

## Comparison of the respiratory cycle in vivo versus in vitro

The present results show that the inspiratory time (or burst duration) to cycle length ratio in both in vivo preparations (i.e. awake and anaesthetized mice) and in rhythmic XII motor neurones recorded from slices increased over a similar time course of maturation. However, the relative duration of the burst of XII motor neurones recorded in slices of mature mice was significantly longer than the inspiratory time recorded in vivo. This resulted in a significant reduction in the ratio of burst duration to cycle length of the respiratory activity measured from mature mice in vitro compared to in vivo data of mice more than 15 days old. This might be explained by differences in experimental conditions. First, slice experiments were performed at temperatures 8 °C lower than in vivo. Interestingly, although increasing the bath temperature from 27 °C to 38 °C reduced burst duration and increased frequency of rhythmic XII motor neurones [9], the burst duration to cycle length ratio increased. Second, under in vitro conditions, reducing potassium levels from 7.5 mM (the level used in this study) to 3–4 mM significantly reduced burst duration and increased cycle length [9]. This latter finding would again contribute to increasing the duration of the rhythmic burst to cycle length ratio. Third, since in anaesthetized cats it was demonstrated that almost half of the respiratory-related XII motor

neurones discharge during post-inspiration [20] the prolonged burst duration of some XII motor neurones in tilted-sagittal slices may, in part, reflect an additional post-inspiratory-related component. If the relative mean percentages of the inspiratory and post-inspiratory phases of phrenic activity recorded in vivo in the present study are summed (i.e. 18.7 % + 11.5 % = 30.2 %; see Fig. 2) this begins to approach the value of the inspiratory period/burst duration recorded in vitro (i.e.: 34.1 %). Finally, the lack of peripheral vagal afferent feed-back from pulmonary stretch receptors may also increase the burst duration to cycle length ratio of XII motor neurones in mature slices as is the case in anaesthetised vagotomized animals (for example, [19]).

### Vagal afferent control of respiration

The in vivo data presented indicate that vagal afferent reflex pathways that suppress ventilation are fully developed and mature at birth and are comparable with other species (for example, rabbit, [18]). Although the origin of vagal afferents was not determined in these in vivo experiments, the inhibitory effect evoked is likely to originate from either the heart, lungs or lower airway since the stimulating electrode was positioned well below the larynx. It is clear that in anaesthetized neonates, but not in mature mice, vagal afferent activity was essential for maintaining respiration since sectioning of both vagal nerves resulted in a central apnoea indicating a failure of the respiratory rhythm generator. This finding suggests a maturational change in the role of reflex vagal afferent control of ventilation in the mouse. This is in contrast to the findings of Smith et al. [16] where anaesthetized neonatal rats continued to breathe spontaneously following bilateral vagotomy. The fact that vagal afferents appear essential for breathing in the anaesthetized neonatal mouse prompts discussion as to the central mechanisms for rhythm generation in this species. Since there is good evidence for the existence of pacemaker cell activity in the respiratory network of the neonatal rat, as recorded under in vitro conditions [17], the role of pacemakers in the neonatal mouse must be considered. One might expect that if respiration in the neonatal mouse were dependent upon pacemaker neurones, bilateral vagotomy should not induce apnoea. Although we have no data to exclude the presence of pacemakers we suggest that if they are present in the respiratory network of the neonatal mouse they appeared functionally ineffective under our experimental conditions of anaesthesia and bilateral vagotomy. This might result from the voltage dependency of pacemaker neurones requiring excitatory synaptic drive to maintain membrane potential at a voltage where oscillatory behaviour is produced. Assuming that the vagi provide a net excitatory synaptic input into the medullary respiratory network, vagotomy may hyperpolarize pacemaker neurones to a voltage where they cannot oscillate. The reason for the difference between neonatal rats and mice is unclear and may reflect species-related differences or differences in development.

Since electrical and chemical activation of the NTS in vitro produced a similar pattern of response to vagal stimulation in vivo it is suggested that the afferent pathways involved in the control of respiratory rhythm are intact in the tilted-sagittal slice. The region of the NTS that mediated this response was localized to ventral sub-divisions at a rostro-caudal level coinciding with the obex. Taking into consideration the size of the stimulating electrode and current spread, we estimate that the area of the NTS actually activated is likely to span the obex by at least 150 μm rostro-caudally. This area coincides well with reports detailing the termination of afferents from pulmonary (i.e. slowly and rapidly adapting) and respiratory tract receptors (see [6] for a review). Indeed, the pattern of evoked response recorded in tilted-sagittal slices resembles that described by Bonham and McCrimmon [1] during microinjection of excitatory amino acids into regions of the NTS that were critical for mediating the Hering-Breuer reflex.

In conclusion, to the best of our knowledge, this is the first ontogenetic study to directly compare in vitro data with functional changes in vivo. Moreover, the good agreement between the time course of muturational changes in phrenic nerve activity, observed in anaesthetized animals, with the motor output pattern of rhythmic XII motor neurones recorded in vitro indicates that the tilted-sagittal slice is a viable preparation for studying the central mechanisms underlying development of the respiratory rhythm generator in the mouse.

**Acknowledgements** J.F.R.P. was in receipt of an Alexander von Humboldt Fellowship. The financial support of the Deutsche Forschungsgemeinschaft is acknowledged.

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