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Functionally intact in vitro preparation generating respiratory activity in neonatal and mature mammals

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Abstract. The present report describes a novel rhythmically active brainstem slice preparation that generates respiratory activity spontaneously in both mice and rats of varying maturational states. The brainstems of neonatal (0–4 days) and mature (3–8 weeks) mice and rats were isolated and a 600- to 750-μm thick slice cut to include the dorsomedial and the ventrolateral regions of the complete rostro-caudal extent of the medulla. This plane of section we have termed "*tilted-sagittal*". Rhythmically discharging neurones were recorded extracellularly from both the dorsal and ventral regions of the slice. The recording sites of these neurones were found in the hypoglossal motonucleus (XII) and in areas of the ventrolateral medulla that includes the ventral respiratory group (VRG) region. Histological examination revealed the preservation of neuronal structures important for cardiorespiratory regulation and reflex control including the nucleus of the solitary tract as well as the nucleus ambiguus. In addition, pontine structures including the A5 region were also preserved. Rhythmic activity was found only in slices where the ambiguual column was preserved in its entirety. The mean frequency of discharge of XII neurones was 20 and 10 bursts per minute in neonates and mature rodents respectively. In preparations of mature animals we demonstrate that this frequency increased significantly ($P < 0.05$) by either raising temperature from 29°C to 38°C (54%), elevating extracellular potassium concentration from 4 to 7.5 mM (52%), blocking potassium channels (20%) or decreasing pH from 7.4 to 7.0 (18%). The burst duration to frequency ratio of XII and VRG rhythmic neurones was similar and therefore indicative of a common brainstem oscillator. Consistent with this finding was that rhythmic activity in the VRG persisted despite removal of the dorsomedial region of the slice. In contrast, rhythmic XII neurones became tonic following mechanical disconnection of the VRG.

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Introduction

Our aim is to directly analyse the cellular and synaptic mechanisms underlying respiratory rhythm generation in the developing mammal. One approach might be to study maturation of the respiratory generator as a way of revealing underlying mechanisms of developmental adjustment. Thus, the purpose of the present approach was to develop a brainstem slice preparation containing a functionally intact respiratory network that generates respiratory activity spontaneously in both neonatal and mature rodents.

Our current opinion for central respiratory rhythm generation is based on data from a variety of preparations in both adult and neonatal animals. In adult mammals these include in vivo (for example [2, 4, 6, 9, 20, 22–24]), the in situ [10], isolated perfused brainstem [1] and transverse brainstem slice [3, 5, 13] and in neonates there are in vitro brainstem preparations of rats such as the brainstem-spinal cord [15, 27, 30], en bloc brainstem [29] and rhythmic transverse slice [8, 28]. However, in both these adult and neonatal preparations there are technical limitations. For example, the resolution of synaptic and cellular data from in vivo and perfused brainstem approaches is restricted by an inability to gain direct access to the extracellular environment. This makes pharmacological approaches, to identify specific synaptic and intrinsic cellular mechanisms, more difficult. The transverse brainstem slice of the adult mammal is not rhythmically active, making cellular characterization uncertain and systems analysis impossible. Although all the above-mentioned neonatal brainstem preparations do generate respiratory activity spontaneously, the mechanisms involved appear to be distinct from those in the mature mammal. Indeed, evidence suggests differences

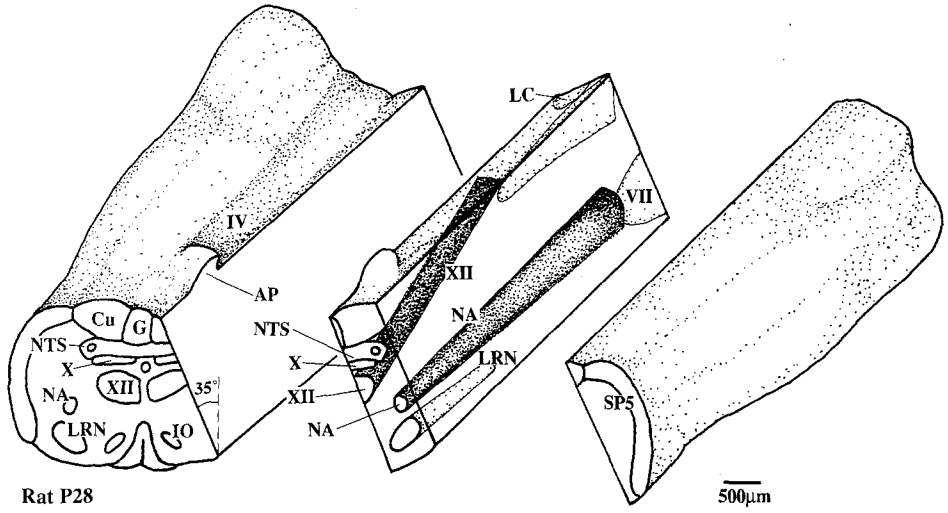

Fig. 1. Schematic representation of the brainstem of a mature rat (4 weeks) to demonstrate the angle and anatomical structures contained within a 700-μm tilted-sagittal slice. The angle is between 35° and 40° relative to the midline vertical axis of the brainstem. Abbreviations of anatomical structures: *AP*, area postrema; *Cu*, lateral cuneate; *G*, gracile; *IO*, inferior olive; *LC*, locus coeruleus; *LRN*, lateral reticular nucleus; *NA*, nucleus ambiguus; *NTS*, solitary tract nucleus; *SP5*, spinal trigeminal; *IV*, fourth ventricle; *VII*, facial; *X*, dorsal vagal motor; *XII*, hypoglossal motor

in both metabolic [1], synaptic [21, 25] and intrinsic membrane mechanisms [32]. Thus, the role of the neonatal rat in enhancing our understanding of the mechanisms for respiratory rhythmogenesis in the maturing and adult mammal is uncertain.

The present report describes a novel preparation that contains a functionally intact respiratory network of both neonatal and mature mice and rats. In the mature rodent (3−8 weeks old), we show that the rhythmic motor output pattern is adult-like, sensitive to changes in the extracellular environment (for example, potassium concentration, temperature and pH) and dependent upon the integrity of the ventrolateral medulla.

Aspects of this work have been published as abstracts [17−19].

Materials and methods

In vitro experiments

Conception of the "tilted-sagittal" slice

The original idea of the tilted-sagittal slice was based on the assumption that respiratory rhythm generation in the mature mammal is the product of synaptic interactions between a network of neurones located in the ventrolateral medulla known as the ventral respiratory group (VRG; see Fig. 1, [7, 14]). Thus, we postulated that in order to generate rhythmic activity spontaneously, the VRG region must be preserved within a slice of an adult mammal. In addition, it was also desirable to include the dorsomedial medulla to include the nucleus of the solitary tract (NTS), the central termination site of cardiorespiratory afferents, and the XII nucleus so that a motor output from the respiratory network could be directly accessed. Since the VRG and XII nucleus are both columnar in structure and extend rostro-caudally within the medulla, it was necessary to tilt the brainstem so that both the dorsomedial and ventrolateral surfaces of the brainstem could be included in a single slice (Fig. 1). This plane of section has been termed "tilted-sagittal" and was measured to be between 35−40° relative to the midline vertical axis of the brainstem (Figs. 1, 2). As a result, the tilted-sagittal slice contains the essential circuitry for central regulation and reflex control of respiratory function.

Slice preparation

Male and female mice (MRI-1 and Bahabor; 0−56 days) and rats (Sprague Dawley; 0−42 days) were deeply anaesthetized with ether. Once the withdrawal reflex of a limb was totally suppressed, animals were decapitated at the C3/C4 spinal level. The entire brain and upper cervical spinal cord segments were carefully extracted from the cranium in ice-cold artificial cerebrospinal fluid (a-csf) which was bubbled continuously with carbogen (95% O_2 and 5% CO_2). The brainstem was isolated by transecting the neuraxis at the level of the inferior colliculus and the cerebellum removed. A pre-prepared agar mould (see below) glued to a glass block was used to tilt and support the brainstem. The brainstem was positioned horizontally and secured in the agar mould using cyano-acrylate. The glass block was secured in a vibratome for slice manufacture. The first 200 ± 50 μm (neonates) or 500 ± 100 μm (mature) of the lateral edge of the brainstem were discarded (Fig. 1). The rhythmic slice was contained within the next 600−750 μm of tissue. It was only possible to obtain a single rhythmically active slice per brainstem. The slice was transferred onto a fine nylon mesh which was semi-submerged in a-csf bubbled with carbogen and covered to provide a humidified atmosphere. Following a 60- to 90-min stabilization period the slice was placed into a recording chamber. All recordings were made from submerged slices in a stream of a-csf bubbled with carbogen at a flow rate of 9−11 mls/min at 29°C. Since relatively thick slices were used we have measured the tissue partial pressure of O_2 (PO_2) at depths within the tilted-sagittal slice. In some experiments bath temperature was lowered to 27°C and elevated to 38°C (in 1-°C increments) in order to assess the effect on rhythmic discharge.

Agar mould manufacture

Agar moulds were used to tilt and support the brainstem during slicing, which allowed consistent reproducibility of rhythmically active slices. Due to the age-dependent change in the size of the brainstem, moulds of different sizes were manufactured. The first step was to make an acrylic template which was used repeatedly to generate agar moulds. The following outlines the procedure for making acrylic templates.

The first step is to make an acrylic brainstem model. A fresh brainstem was completely immersed in silicone dental impression compound (ESPE Permadyne, Germany). Once the impression compound had set, the fresh brainstem was removed and the impression created filled with a fast setting acrylic compound (ESPE Protemp II, Germany). The result was an acrylic model of the

brainstem which was removed from the impression compound. The brainstem model was suspended horizontally dorsal side up in a suitable container and rotated approximately 35° about the *y*-axis. Silicone compound was poured into the container to a level that half covered the model brainstem. Once the silicone material had set, the brainstem model was removed. The resultant silicone impression is analogous to a "*positive*" and forms the basis on which the acrylic template (the "*negative*") is made and used for manufacturing the agar moulds. The silicone impression positive was next submerged in acrylic compound to form the template. Once set, the resulting acrylic template (or negative) was filled with a 4.5% solution of agar made in a sugar-free a-csf solution (see below). The agar mould was turned out of the template by careful prying.

Recording methods

Electrophysiological. Recordings were made using extracellular electrodes manufactured from filamented glass (Clarke GC150-F; tip diameter 1−3 μm). These electrodes were filled with either 3 M NaCl (input resistance 3−8 MΩ) or a sodium acetate/pontamine sky blue solution (input resistance 5−11 MΩ). The latter permitted marking of recording sites by ionophoretic deposition of the dye (−500 μA; 800-ms pulse; 10 min). In many experiments, a-csf-filled glass macroelectrodes (tip diameter 20−40 μm) were used to record multiple unit activity and in addition, suction electrodes (tip diameter 200−300 μm) were employed in some neonatal experiments to record XII rootlet activity. Electrodes were positioned under visual guidance of a binoccular microscope and aimed into regions of the slice known to contain either the core of the respiratory network (for example, the VRG) or the XII. The XII is a motor output nucleus since it contains many neurones which receive inspiratory-related synaptic drive from VRG premotor neurones [33]. Superficial XII neurones were visible on the surface of the slice through the microscope. Electrodes were advanced through the slice in steps of 1.6 μm using a nano-stepper. Electrical signals were amplified using an AC pre-amp and filtered (3 Hz to 8 kHz; NPI Electronics: module DPA 2F) before being displayed on an oscilloscope (Gould DSO 420). Raw signals were recorded on VCR tape after passing through an interface (Instrutech VR100; sampling rate 26 kHz).

Tissue oxygen measurements. Depth profiles of the PO_2 within the tilted-sagittal slice of mature rats and mice was measured using a PO_2-sensitive electrode (tip diameter 40 μm) and a chemical microsensor meter (Diamond General). Following a 1-h polarization period (−750 nA) the PO_2 electrode was calibrated (in mm Hg) using two a-csf solutions satured with either carbogen or a gas mixture containing 95% N_2 and 5% CO_2. The PO_2 electrode was driven into areas of the slice including the XII and VRG region using the nano-stepper. Oxygen tensions were measured at 50-μm intervals. In order to demonstrate that the brain tissue was metabolizing aerobically, sodium cyanide (5 mM) was added to a HEPES-buffered perfusate (see below).

Lesion and cooling experiments

To assess the importance of different regions of the slice for the rhythmic motor discharge recorded in the XII nucleus, localized areas of the slice were either lesioned or reversibly inactivated using a cooling probe as described previously [16]. Lesions were made with a fragment of a razor blade (0.5−1.0 mm wide) glued to a steel rod and positioned with a hand-driven micromanipulator. The cooling probe consisted of a drilled-out stainless steel cone (tip diameter 800 μm) which was cooled by pumping ethanol from a reservoir chilled to −70°C using dry ice. The temperature of the tip of the cooling probe was controlled by the perfusion rate of the pump and was maintained at 4°C. During cooling experiments the slice was positioned so that the VRG region was "down stream" of the XII nucleus thereby avoiding direct cooling effect on the cell under study. The probe was lowered onto the surface of the slice using a manually operated micromanipulator.

Histological procedures

Following the end of an experiment slices were fixed in a solution containing 4% paraformaldehyde and 4% glutaraldehyde for at least 24 h before transferring to a solution containing 30% sucrose. Using a freezing microtome, serial sections were cut (50 μm thick) which were stained using crysol violet. Camera lucida drawings were made of all sections from individual slices. The nuclei contained within rhythmic slices and the location of recording sites were documented. The neuronal structures preserved were compared between rhythmically active preparations and slices where rhythmic activity was not found.

Solutions

The normal a-csf constituents in mM were: 1.25 KH_2PO_4; 1.25 $MgSO_4$; 25 $NaHCO_3$; 5 KCl; 125 NaCl; 2.5 $CaCl_2$; 20 dextrose. Fresh a-csf was made for every experiment and bubbled continuously with carbogen to maintain the pH at 7.4. The osmolarity of this solution was 290 ± 5 mosmol/kg H_2O. To examine the effect of potassium on rhythmic discharge, modified a-csf solutions were made containing a range of potassium concentrations (3−8 mM). In addition, 5−10 mM tetraethylammonium chloride (TEA) was added to the perfusate in some experiments to block potassium channels. The effect of pH on rhythmic firing was assessed by lowering it to 7.0, by decreasing the HCO_3^- concentration of the a-csf to 12.5 mM; the osmolarity was corrected by an appropriate addition of 5 M NaCl. Following the PO_2 measuring experiments, the HCO_3^--containing a-csf was switched to a HEPES (25 mM)-buffered Ringer solution so that sodium cyanide (5 mM) could be perfused to block aerobic metabolism. A HEPES Ringer was used to prevent cyanide gas formation.

In vivo experiments

The effect of temperature on the inspiratory period and the rate of breathing was assessed in five mature conscious mice. Breathing was measured by monitoring the thoracic movement using a pressure transducer connected to a detached finger of a surgical glove. Mice were placed ventral side down on the glove which was semi-inflated. The changes in pressure with each breath indicated the inspiratory time and frequency of ventilation and were displayed on a chart recorder (Gould TA 420). A thermocouple was placed adjacent to the animal to measure the ambient air temperature. Experiments were performed on a stainless steel heating plate connected to a water bath and pump. The temperature of the animal was controlled by adjusting the temperature of the circulating water in the heating plate and use of an infra-red lamp. Measurements were made over a range of recorded temperatures (25−40°C).

Analysis

In the in vitro experiments, a quantitative analysis was carried out to measure the burst duration, burst frequency and peak intra-burst frequency using the storage and cursor facilities of a digital oscilloscope (Gould DSO 420). The mean values quoted for each cell were calculated over ten cycles. Instantaneous intra-burst frequency versus time plots were compiled using a computer and custom-written software or calculated manually. The present data

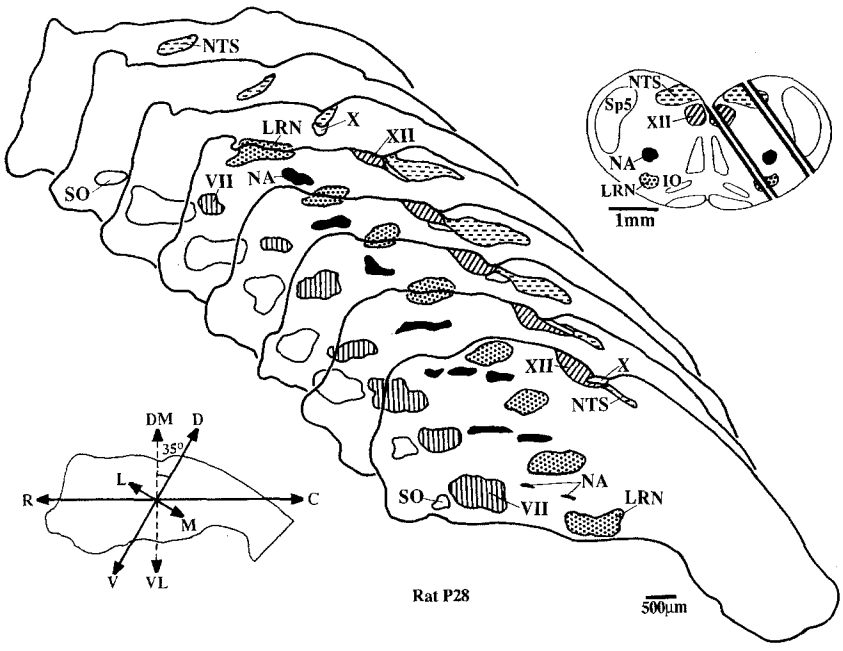

Fig. 2. Camera lucida drawings of alternate sections (50 μm thick) from a rhythmic tilted-sagittal slice of a rat (P28). Rhythmic activity was dependent upon the integrity of the NA which was preserved in its entirety within the slice. *Top right* shows a schematic transverse section of medulla to illustrate the cutting angle and regions contained within the tilted slice. *Bottom left* shows a scheme showing the orientation of the tilted-sagittal slice. For abbreviations refer to Fig. 1, plus: *C*, caudal; *D*, dorsal; *DM*, dorsomedial; *L*, lateral; *M*, medial; *R*, rostral; *SO*, superior olive; *V*, ventral; *VL* ventrolateral; *VRG*, ventral respiratory group. Note, for clarity the LC mesencephalic trigeminal and additional pontine nuclei have not been included but are shown in Figs. 1 and 3

were obtained from 150 slice preparations (700 μm thick) that showed spontaneous rhythmic activity in XII and VRG regions at 29°C and 7.5 mM potassium. This rhythmicity was found in approximately 80% of slices of both mice and rats. In the in vivo experiments the breath rate and inspiratory period were measured over 20 cycles during quiet breathing episodes at different temperatures. All values quoted are the mean ± SD. The statistical significance of the data were calculated using a Student's *t*-test (Statview 512 +) on an Apple Mac computer. Hard copies of the neural activity were made using a thermal array recorder (Gould TA 240).

Results

The primary purpose of this study was to develop a preparation that contained a functionally intact respiratory network of maturing mammals. Thus, the bulk of the experiments performed were on slices of mature mammals. However, data are presented to show that the tilted-sagittal slice of neonatal rodents is also rhythmically active. The characteristics of the rhythm generated in the neonate have been analysed previously using a different preparation [27].

In vitro experiments

Anatomical considerations

Neural structures preserved in rhythmic tilted-sagittal slices. The tilted-sagittal slice included neural structures in the dorsomedial and ventrolateral aspects of the medulla. Figure 2 shows camera lucida drawings of a representative tilted-sagittal rhythmic slice to illustrate the neuronal structures present. In the dorsomedial medulla the gracile, NTS, dorsal vagal motonucleus (X) and XII were found caudally whereas rostrally, the medial vestibular, locus coeruleus and mesencephalic trigeminal nuclei were identified and shown in Fig. 3. In the ventrolateral medulla, both the loose and compact (dorsal and ventral) divisions of the nucleus ambiguus were preserved together with the lateral reticular and facial nuclei. Anatomical reconstruction of the slice showed that these latter structures were centralized within the slice and preserved intact. In addition, the superior olive, lateral lemniscus and the ventrolateral pontine nucleus were also present more rostrally (Fig. 2).

In neonates of both species it was possible to include the XII rootlets within the slice without changing the tilt angle of the brainstem. Suction electrode recordings from XII rootlets revealed the presence of rhythmic activity. In contrast, following the manufacture of a rhythmic slice from a mature rodent neither the XII nor X rootlets were preserved. Indeed, examination of the remaining pieces of brainstem revealed that the medial tilted-sagittal surface was approximately 500 μm lateral to the XII rootlets, whereas the X rootlets were 200–300 μm lateral to the lateral most surface of rhythmically active slices.

In an attempt to preserve either the XII or X rootlets in slices of mature rats and mice the tilt angle of the brainstem was altered. However, in all slices containing these rootlets no rhythmic activity was recorded from the rootlet itself, or from the XII motonucleus, or VRG region. Indeed, histological analysis revealed that the ambiguual column was severed and incomplete either in the medio-lateral or rostro-caudal dimensions. Thus, the angle at which the brainstem is tilted appears critical for preserving the neuronal structures essential for generating rhythmic activity. This angle was measured and found to be between 35° to 40° rotation about the midline vertical axis of the brainstem (see Figs. 1, 2).

Location of recording sites. Figure 3 shows a schematic drawing of a tilted-sagittal slice and the location of recording sites of rhythmically firing neurones as deter-

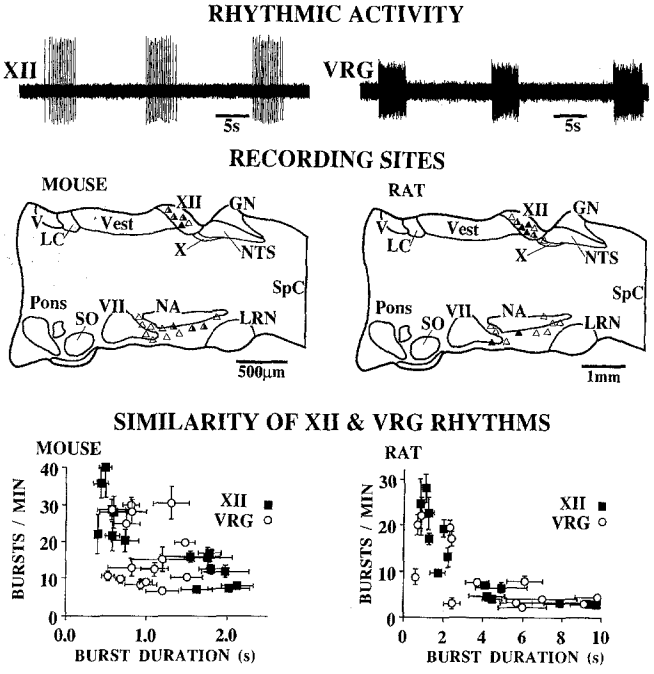

Fig. 3. The *top panel* is a representative example of rhythmic XII and VRG neurones recorded extracellularly from slice of mature rats (P28 and 35). Note the similarity in the frequency of bursts. Recordings made at 29°C and 7.5 mM potassium. The *middle panel* shows the location of recording sites, as determined by iontophoretic deposition of pontamine sky blue, of rhythmically firing neurones for both mice and rats and symbolized by *triangles*. The schematic representation of the tilted-sagittal slice was constructed from a series of camera lucida drawings so that all anatomical structures present within the slice are shown. *Key to triangles: open*, 1 cell; *half-filled*, 2 cells; *filled*, 3 cells. The *bottom panel* illustrates the similarity in rhythmic activity recorded from XII (*solid squares*) and VRG neurones (*open circles*) graphically as plots of burst duration versus burst frequency. There was no significant difference in the burst duration and frequency of burst relationship between XII and VRG neurones in either mouse and rat ($P > 0.42$)

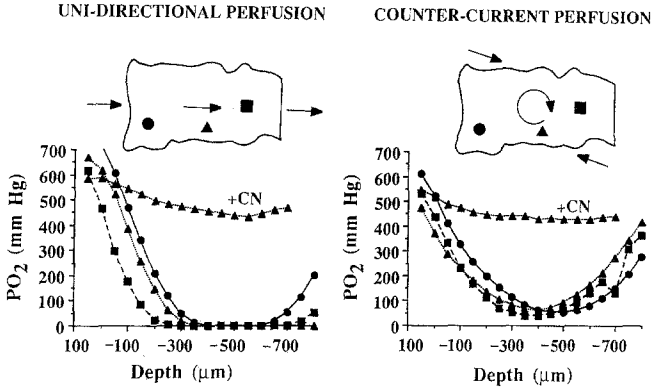

Fig. 4. Comparison of the O_2 tension (PO_2) levels within 700-μm thick tilted-sagittal slices from mature mice during uni-directional and counter-current perfusion. During uni-directional perfusion the level of PO_2 was a function of distance from the inflow site, and as a result much of the middle depths of the slice were hypoxic. However, PO_2 levels were maintained above 20 mmHg in 700-μm thick slices when a counter-current perfusion system was adopted, which had the effect of increasing the surface PO_2 level. See text for further details. Following addition of 5 mM cyanide (*CN*) to the perfusate PO_2 levels were maintained above 430 mmHg throughout all depths of the slice

mined from the ionophoretic deposition of potamine sky blue dye (see Materials and methods). In the dorsomedial medulla, recording sites of rhythmic neurones were found in the XII motonucleus. These rhythmic neurones did not appear to be localized to any particular region of this nucleus. Rhythmically active cells were also recorded throughout the rostro-caudal extent of the ventrolateral medulla extending from the level of the facial nucleus to the rostral pole of the lateral reticular nucleus (Fig. 3). These recording sites were either within the ambiguual column or ventral to it. In addition, rhythmically discharging neurones were found between the ventral border of the caudal region of the facial nucleus and the ventral surface. Despite searching we failed to locate rhythmic activity in other regions of the slice, including the NTS and regions of the reticular formation between the NTS and VRG.

PO_2 depth profile of mature tilted-sagittal slices

The direction of flow of a-csf was critical for maintaining adequate PO_2 delivery to the slice. Using a recording chamber with an uni-directional flow system (Fig. 4) the PO_2 fell dramatically as the depth beneath the slice surface increased. Indeed, the depth profiles indicate that at 250 μm below the surface the oxygen tension was zero. Furthermore, with the uni-directional chamber it was apparent that the surface PO_2 of the slice also decreased with increased distance from the inflow (Fig. 4). This is analogous to the Krogh effect and indicative of oxygen usage. In order to counteract the Krogh effect and to increase PO_2 levels at the surface of the slice, a counter-current perfusion system was adopted (Fig. 4). Under these conditions, surface PO_2 levels were higher and uniform across the slice surface. As a result the level of oxygen was maintained at greater than 20 mmHg at all depths of the slice (Fig. 4). These observations underlie the importance of measuring PO_2 in in vitro preparations and demonstrate that the tissue oxygen levels depend on the type of perfusion system employed. Furthermore, it should be realized that in different regions of a slice there are likely to be hyperoxic, normoxic and hypoxic regions. Following administration of 5 mM cyanide to the bath perfusate (see Materials and methods), to block aerobic metabolism, oxygen levels were maintained above 430 mmHg at all depths of the slice. The difference between the latter data and those obtained under control conditions represents the oxygen usage.

Characteristics of rhythmic activity

Rhythmically firing XII and VRG neurones were often found intermingled with tonically firing cells (1–25 Hz). In some slices "pools" of rhythmically active neurones were found in both the XII and VRG regions making it possible to record from more than one cell with a single electrode. Figure 3 shows an example of

Table 1. The burst duration and frequency of rhythmic discharges recorded from XII neurones are compared for neonatal and mature mice and rats

Parameter	Mouse		Rat	
	Neonate ($n = 7$)	Mature ($n = 23$)	Neonate ($n = 3$)	Mature ($n = 25$)
Burst duration (s)	0.56 ± 0.29 (0.24–1.01)	2.61 ± 1.6 (0.4–6.0)	0.26 ± 0.09 (0.11–0.36)	3.25 ± 3.2 (0.8–9.8)
Burst frequency (per min)	20.8 ± 2.9 (12.0–24.8)	9.9 ± 4.8 (4.3–26.0)	19.5 ± 4.8 (12.2–28.8)	10.3 ± 6.7 (3.3–24.0)

The data indicate the mean ± SD and the range

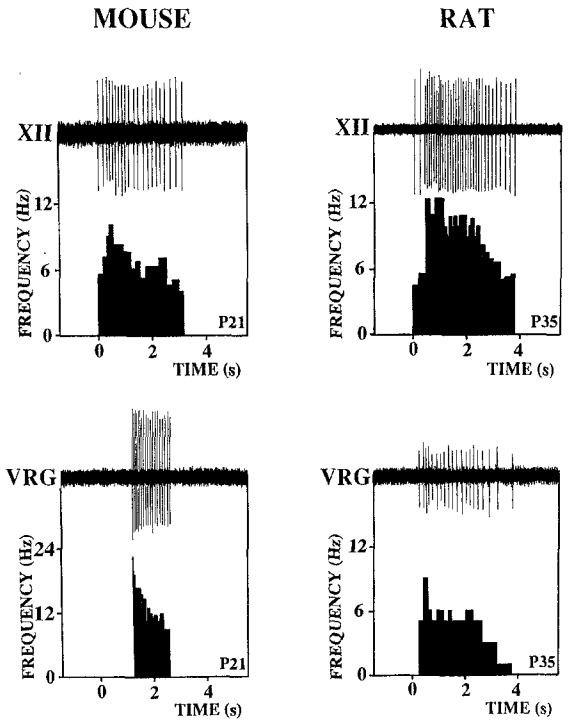

Fig. 5. A comparison of the XII and VRG discharge pattern recorded extracellularly from tilted-sagittal slices of mature mice and rats. Note the three components in the burst of XII neurones: augmenting, plateau and decrementing, which was distinct from the rapid onset-decrementing pattern recorded in the neonate (not shown). In the VRG there was a heterogeneity in the firing patterns and burst duration of rhythmically active cells in both species. Recordings made at 29°C and 7.5 mM potassium

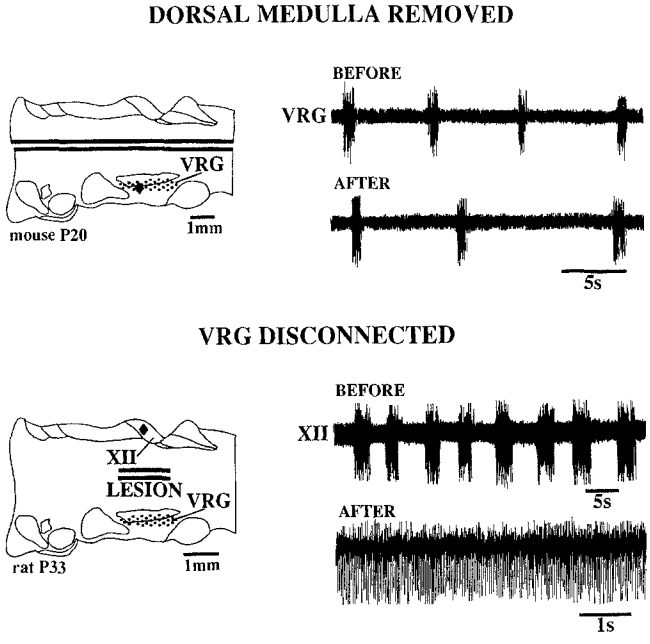

Fig. 6. The origin of the rhythmic activity was found to be within the ventrolateral medulla. This was based on two experimental observations: removal of the dorsomedial portion of a tilted-sagittal slice did not abolish rhythmic activity recorded in the VRG (*top*) whereas the rhythmic discharge of XII neurones was abolished following a restricted lesion placed between the XII and VRG (*bottom*). Similar results were obtained from slices of neonates. *Shaded region* indicates the location of the VRG. *Solid symbol* indicates recording site

rhythmic activity in a XII and VRG neurone which was stable for up to 16 h.

Comparision of rhythmic activity in neonatal and mature rodents. The mean frequency of rhythmic XII bursts was 9.9 ± 4.8 and 10.3 ± 6.7 bursts per minute in the adult mouse and rat respectively (Table 1). In neonatal rodents rate of occurrence of bursts was higher (mouse 20.8 ± 2.9 bursts/min; rat 19.5 ± 4.8 bursts/min; see Table 1). Table 1 compares the mean (± SD) and range for the burst duration and frequency of the rhythm recorded in slices from neonatal and mature mice and rats.

Instantaneous intra-burst frequency versus time plots revealed that in mature rats and mice XII bursts were comprised of multiple components including those that were augmenting (i. e. time delay to peak intra-burst frequency), and slowly decrementing (Fig. 5 upper panel); in some neurones there was also a plateau in the firing frequency as seen in Fig. 5 (upper right panel). In contrast, there was a heterogeneity in the firing patterns and burst duration of rhythmic VRG neurones in both mature species (Fig. 5 lower panel). In both neonatal mice and rats the firing pattern of rhythmically active XII neurones was distinct from the mature rodents, comprising a rapid onset and quickly decrementing burst of short duration (see Table 1) and was similar to that described

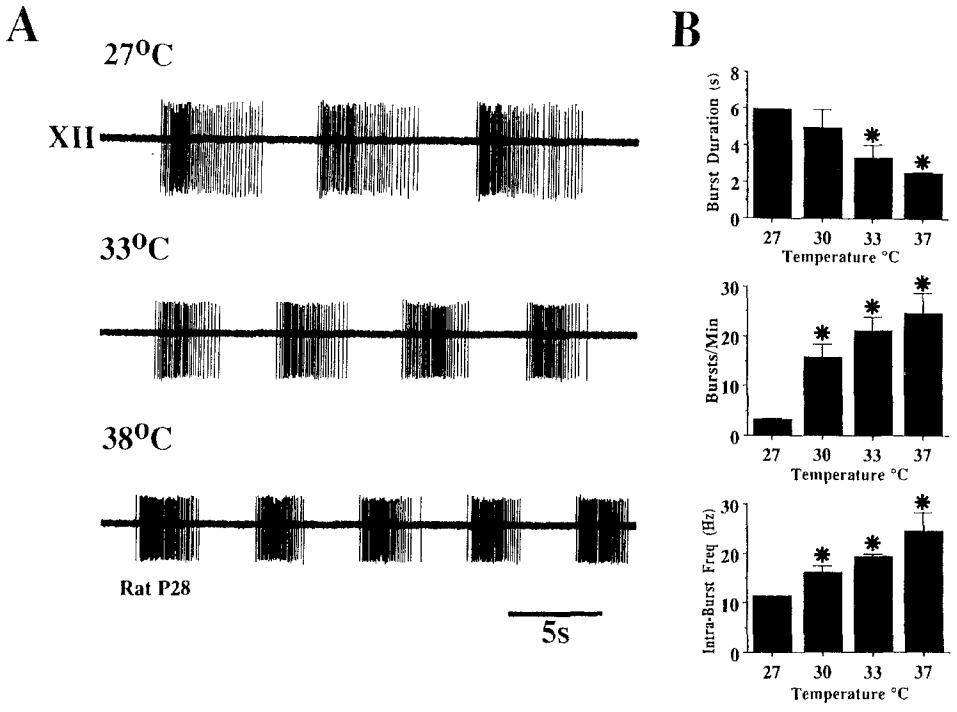

Fig. 7 A, B. The effect of increasing temperature on a rhythmic XII neurone resulted in a decrease in burst duration, an increase in burst frequency and peak intra-burst frequency. **A** A representative recording of a XII neurone, and **B** the group data ($n = 5$). *Asterisks* indicate a significant change from the value at 27°C ($P < 0.05$). Recordings made using 7.5 mM potassium

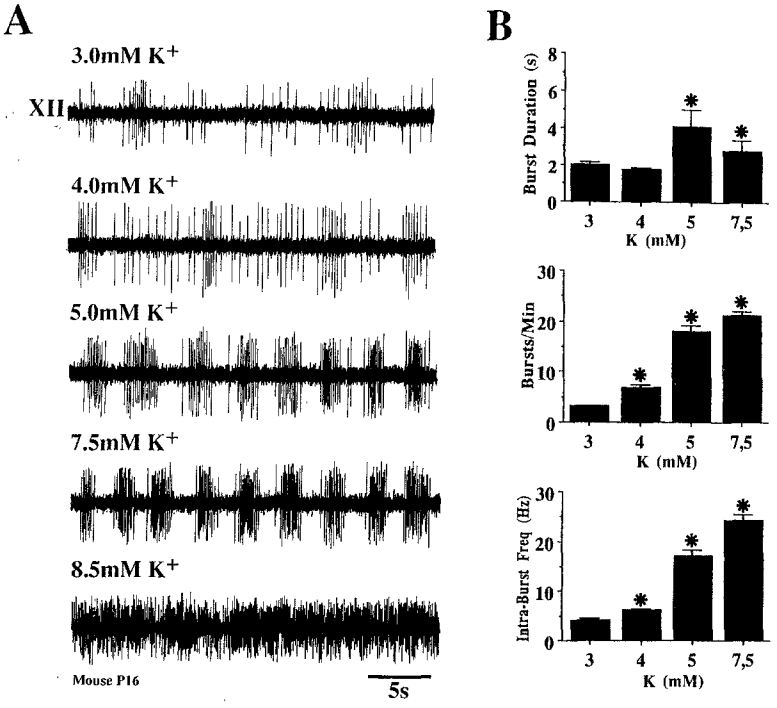

Fig. 8 A, B. The effect of elevating extracellular potassium from 3 mM to 7.5 mM produced an increase in the rate of rhythmic activity and peak intra-burst firing frequency of a rhythmic XII neurone. An original recording (**A**) and the group data of five cells is shown (**B**). Tonic discharge was produced when potassium concentrations exceeded 8 mM. Significant differences from values at 3 mM potassium are indicated by *asterisks* ($P < 0.05$). Recordings made at 29°C

previously [15, 27, 30]. In addition, peak intra-burst frequency was reached immediately in neonates. A comparison of the rhythmic behaviour of XII and VRG neurones was made by plotting the burst duration versus frequency in bursts per minute (Fig. 3). We found variations in the burst duration/frequency relationship of XII and VRG neurones which might reflect a heterogeneity in the excitability state between preparations. However, taken together, the total population of rhythmic neurones sampled from both the XII and VRG regions was found not to be significant in both species ($P > 0.42$). These data are indicative of a common rhythm generator.

Localization of the rhythm generating network. In three slices of both neonatal and mature mice the entire dorsomedial half of the preparation was removed using a razor blade (Fig. 6). Despite this, rhythmically active cells were found in the VRG. The frequency of rhythmic firing was reduced by 15–23% compared to intact preparations (see above) which might reflect removal of a

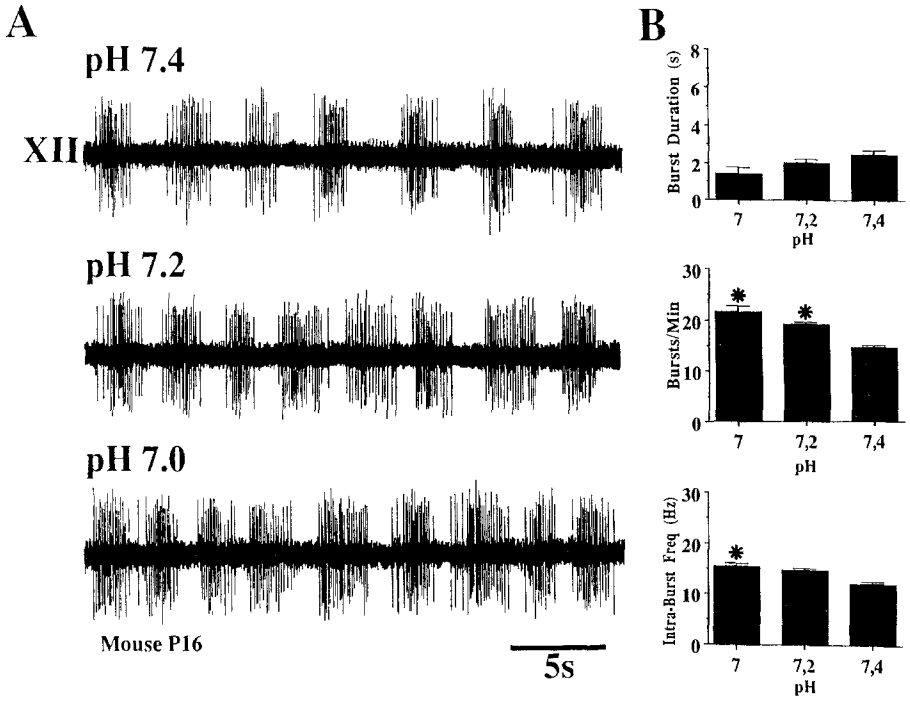

Fig. 9. A representative example of a rhythmic XII cell and its response to changes in pH is shown in **A**. Decreasing pH lowered burst duration but elevated the frequency of occurrence of bursting and peak intra-burst frequency. **B** The group data are shown for three neurones. *Asterisks* show significant differences from the values at pH 7.4 ($P < 0.05$). Recordings made at 7.5 mM potassium and 29°C

tonically active excitatory input. This result indicates that the rhythm generating network was localized within the ventrolateral medulla in both neonates and mature mammals.

Rhythmic drive from the VRG projected onto XII neurones as indicated by the following experiments. First, the VRG was mechanically disconnected from the dorso-medial portion of the slice by making a localized lesion (approximately 500 μm long) between the XII motonucleus and the VRG (see Fig. 6) in three slices from mature rats. Following this lesion, previously rhythmic XII neurones fired tonically (Fig. 6). The frequency of the tonic discharge (40−45 Hz) was higher than the intra-burst frequency (peak frequency range: 15−30 Hz) indicating the removal of both a phasic and tonically active inhibitory drive impinging on XII neurones. Similarly, rhythmic XII neurones became tonic or their rhythmic discharge severely slowed and disrupted during cooling of the VRG ($n = 5$). This effect was relatively specific since cooling the caudal most extent of the VRG, or the lateral reticular nucleus had no obvious effect on the frequency or burst duration of rhythmic XII neurones. Furthermore, control experiments were performed which cooled regions of the slice closer to the recording site and included the gracile nucleus and reticular formation. In all cases rhythmic XII activity was unaffected. Taken together the evidence supports the claim that the rhythmic discharge of XII neurones was a product of the respiratory network located within the VRG.

Sensitivity of rhythmic activity to TEA and changes in temperature, extracellular potassium and pH. Alterations in the extracellular environment were tested on XII neurones recorded from slice of mature rodents (see Figs. 7−9). The effect of raising temperature from 27 to 38°C resulted in significant changes in burst duration, frequency and peak intra-burst frequency of XII neurones which are shown in Fig. 7 ($P < 0.05$). The greatest effect of increasing temperature was on the rate of rhythmic firing which increased by 60% from 29°C to 37°C ($P < 0.05$). Increases in temperature above 38°C resulted in quiescence or production of tonic discharge in XII neurones. Rhythmic activity recorded from slices of mature rodents was also sensitive to elevations in extracellular potassium up to 7.5 mM (Fig. 8); concentrations of greater than 8 mM potassium resulted in tonic discharge as seen in Fig. 8. The lowest level of potassium at which rhythmic activity was detectable was at 3 mM. However, more stable and robust rhythmic discharge was reliably observed at 4 mM potassium (Fig. 8). Graded elevations of potassium of 3−7.5 mM resulted in an increase in the frequency of rhythmic firing (53.1%) and peak intra-burst firing rate (43.4%). The effect on burst duration was only significant at the higher concentrations of potassium (Fig. 8; $P < 0.05$). The enhancing effects of increased potassium probably reflect a direct depolarizing action on the respiratory network. Addition of TEA to the bath perfusate (5−10 mM), to block potassium channels, increased burst frequency by 20% in three slices. There were also small increases in both burst duration and peak intra-burst frequency (15%). These latter effects may be due to elevations in neuronal input resistance and increased sensitivity to synaptic input within the respiratory network.

The rhythmic discharge of XII was also effected by lowering pH. A reduction in pH from 7.4 to 7.0 increased burst frequency (44.6%) and peak intra-burst frequency (30.6%) significantly ($P < 0.05$), whereas burst duration showed no obvious change (Fig. 9). These data indicate either a direct excitatory action of protons

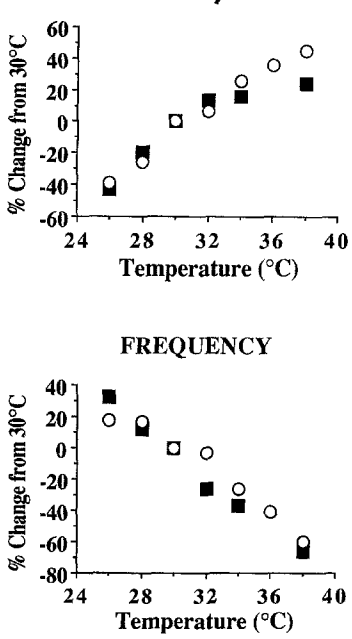

Fig. 10. The effect of temperature on respiration in the conscious mouse (*open circles*) and on the motor pattern and rate of a rhythmically discharging XII neurone recorded in vitro (*solid squares*) are compared. The graphs were compiled from representative data which was normalized and indicates a similarity in the percentage changes from 30°C in both inspiratory time and burst duration (*top*) and rate (*bottom*). In vitro recordings were made in an artificial cerebrospinal fluid perfusate containing 7.5 mM potassium

on respiratory neurones, or activation of central chemoreceptors resulting in an excitatory synaptic input into the respiratory network.

In vivo experiments

The in vivo experiments were undertaken to compare the effect of raising temperature on breathing rate and inspiratory duration with data collected from rhythmic XII neurones recorded in vitro (see above). At an ambient temperature of 25°C the respiratory rate during quiet breathing in conscious mature mice was 1.3 ± 0.2 Hz and inspiratory time 84.5 ± 19 ms ($n = 5$; $P > 20$ days). Elevating the temperature from 24 to 38°C produced a linear increase in rate of breathing to 3.7 ± 0.5 Hz and a reduction in the inspiratory time (down to 40.7 ± 5.7 ms). In order to directly compare these data with our in vitro findings, results were normalized by taking the percentage change relative to its value at 30°C. Figure 10 contains the normalized data to compare the effect of temperature on the rate and pattern of breathing recorded in vivo (inspiratory period and breathing rate) with a rhythmic XII neurone (burst duration and frequency of bursting) recorded in vitro. These graphs further support our claim that the rhythmic activity recorded in the tilted-sagittal slice preparation is a product of the central respiratory rhythm generator.

Discussion

The present report describes a novel rhythmically active brainstem slice preparation for studying the central mechanisms controlling respiratory rhythm generation and function in the developing mouse and rat. The plane of section of the slice has been described by us as "tilted-sagittal" since it contains both the dorsomedial and ventrolateral aspects of the entire rostro-caudal extent of the brainstem. Rhythmic activity was recorded from the XII and VRG and was stable for up to 16 h. The data presented indicate that the spontaneous rhythmic activity recorded in tilted-sagittal slices of neonatal and mature rodents is the product of the respiratory network located in the ventrolateral medulla.

Evidence for a functionally intact respiratory network in the tilted-sagittal slice

Our claim that the rhythmic activity recorded in the XII and VRG in the neonatal and mature rat and mouse is a product of the respiratory rhythm generator is based on a number of experimental findings. First, the anatomical structures present in rhythmic tilted-sagittal slice preparations included the entire rostro-caudal extent of the ambiguual column. This is consistent with our current opinion [26] that the nucleus ambiguus is essential for rhythm generation. Second, the recording sites of rhythmically active neurones were restricted to the XII (a motor nucleus of the respiratory network) and throughout regions of the ventrolateral medulla in areas coinciding with the VRG. Furthermore, exploration of other regions of the slice including the NTS, vestibular, and lateral reticular nucleus did not yield rhythmically active units. Third, the existence of multi-unit phase-locked activity in the XII and VRG revealed the phasic organization of the rhythm, implying the presence of a network. This was supported by the finding that rhythmicity persisted in the VRG after disconnection of the dorsomedial medulla and that mechanical disconnection or cold block of the VRG abolished the rhythm in XII neurones. Fourth, an analysis of the burst duration/frequency of bursting ratio of XII and VRG neurones indicated considerable overlap, suggesting a common medullary oscillator in the VRG. Finally, the effect of temperature on rhythmic XII neurones recorded in vitro were comparable with the changes seen in the rate and pattern of breathing measured in vivo. While temperature may affect neuronal function in a general way, it is argued that the effect of temperature on a complex network generating both rhythm and pattern may be more specific.

In the present experiments in mice and rats we failed to find rhythmically active neurones in the ventral NTS, an area analogous to the described dorsal respiratory group (DRG) region. The presence and importance of the DRG for respiratory rhythm generation in the rat is at this time controversial [11, 12]. Indeed, the lack of rhythmicity in the ventral NTS of tilted-sagittal slices, together with the observation of rhythmically active cells in the VRG of slices without a dorsomedial medulla, is

suggestive of a relatively minor role for the DRG in central respiratory rhythm generation in the tilted-sagittal slice preparation.

The characteristics of the XII rhythm recorded from a tilted-sagittal slice of a mature rodent are distinct from the rapidly decrementing short duration bursts observed by us in the present study, and others in rhythmically active neonatal preparations [15, 27, 28, 30]. Indeed, the frequency, duration and pattern of XII discharge in the present study (including augmenting, plateau and decrementing components) together with the cycle length versus burst duration relationship are comparable to motor outflows recorded from adult mammals in vivo [26, 33], in situ [10] and in vitro [1]. Thus, we suggest that the XII activity recorded in the tilted-sagittal slices of mature mice and rats reflects that of a mature respiratory network.

Advantages and potential of the tilted-sagittal slice

To our knowledge the tilted-sagittal slice is the first preparation that permits an analysis of respiratory rhythm generation of adult mammals in vitro. To date, the neonatal rat has been used as a model to gain further insight into respiratory rhythmogenesis but the results might not be comparable to the mechanisms operating in the developing and mature mammal. Three types of preparation that generate respiratory activity spontaneously have been used and these include the rhythmically active slice [28], the en bloc brainstem [29] and the brainstem-spinal cord preparation [15, 29, 30]. However, accumulating evidence indicates that the mechanisms for respiratory rhythm generation in the neonatal are distinct from the maturing and adult rat. Smith et al. [28] indicated that pacemaker neurones were important for rhythm generation in the rhythmically active slice from a neonatal rat. However, in the adult mammal the integrity of glycinergic and GABAergic (γ-aminobutyric acid) mechanisms are essential for respiratory rhythm generation and for the production of inhibitory post-synaptic potentials (IPSPs) observed from medullary respiratory neurones [2, 9, 10, 17, 21, 23, 24]. In addition, the metabolic processes of the neonatal respiratory network are different. Recent evidence shows that the respiratory network of the neonate is surprisingly resistant to hypoxia and continues to function in the absence of oxygen [1, 31]. In contrast, in the adult hypoxia produces apnoea which is currently thought to be due, in part, to a blockade of synaptic transmission preferentially effecting IPSP production [21, 25]. Taken together, the evidence indicates that the mechanisms of respiratory rhythm generation in the neonatal rat are not comparable with those in the mature mammal. Indeed, preliminary findings suggest that these developmental changes occur within 15 days (J. F. R. Paton, J.-M. Ramirez, D. W. Richter, unpublished data).

A further advantage of the tilted-sagittal slice is that it is rhythmic in both neonatal and mature mammals. The relevance of this is that for the first time the ontogenetic changes underlying the maturation of respiratory rhythm generation can be studied in in vitro conditions. This will permit a direct comparison of cellular and synaptic mechanisms for respiratory rhythmogenesis between neonates and adults in identical preparations. In this regard, preliminary evidence from our laboratory has indicated pronounced changes in the sensitivity to both bicuculline and strychnine between newborn and 2-week-old mice (J. F. R. Paton, J.-M. Ramirez, D. W. Richter, unpublished data). In addition to isolating the central pattern generator for respiratory rhythmogenesis, the tilted-sagittal slice preparation also appears to contain the central circuitry mediating its reflex control (J. F. R. Paton, J.-M. Ramirez, D. W. Richter, unpublished findings). For instance we have observed modulatory influences on rhythmic XII and VRG cells evoked by stimulating the NTS, the central terminal site for respiratory afferents. Since the NTS is the site for a wide variety of visceral afferents it is likely that other reflexes, such as those controlling cardiovascular function and cardiorespiratory coupling, might also be preserved in the slice and this is under investigation.

The introduction of the mouse as a species for studying respiratory rhythm generation may have distinct advantages over the rat since it appears more mature at birth based on its metabolic rate (D. Singer, personal communication). Further, the size of the brainstem of an adult mouse is significantly smaller than that of 3- to 6-week-old rat (see Fig. 3) which has obvious advantages for slice preparations. In addition, the mouse is used extensively in producing mutant strains and further development along this line might yield a useful model for revealing underlying neuronal mechanisms for respiratory rhythmogenesis, for example.

The tilted-sagittal slice offers a new preparation to directly compare the mechanisms for respiratory rhythm generation (and its reflex control) in neonatal, developing and mature mammals in vitro. The important finding that preparations of neonatal rodents are rhythmic will also permit ontogenetic studies into respiratory rhythmogenesis. This type of approach may be invaluable clinically for understanding respiratory-related disorders in newborn infants as well as adults. Thus, the tilted-sagittal slice has the potential of permitting simultaneous systems, cellular and molecular analysis of respiratory rhythm generation.

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