J. exp. Biol. ***197****, 201–213 (1994)*
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RESPIRATORY RHYTHM IN THE ISOLATED CENTRAL NERVOUS SYSTEM OF NEWBORN OPOSSUM

DONG-JING ZOU*

Department of Pharmacology, Biozentrum der Universität Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

Accepted 20 July 1994

Summary

1. Respiratory activity was recorded from spinal ventral roots in the isolated intact central nervous system (CNS) of newborn opossum, *Monodelphis domestica*. These signals occurred in synchrony with movements of the ribs and the electromyogram (EMG) recorded from the intercostal muscles during inspiration. Rhythmical activity could be recorded for more than 6 h in acute preparations.

2. The rhythm-generating region was shown to be located in the lower brain stem by perfusing different CNS regions with medium containing 20 mmol l^{-1} Mg^{2+}, which blocks synaptic transmission reversibly in the opossum CNS. The conclusion that respiration was generated by neurones in the lower brain stem was further confirmed by selective ablation of part of the CNS.

3. Recordings were made from 128 neurones in the respiratory region of the lower brain stem with activity related to the respiratory rhythm. They consisted of two inspiratory groups and two expiratory groups. In the groups of inspiratory units, recordings were made from 69 early inspiratory and 38 inspiratory units. In the groups of expiratory units, recordings were made from 17 post-inspiratory and 4 expiratory units. The sites of 22 respiratory neurones were marked in 4-day-old animals by injecting Pontamine Sky Blue. These neurones were distributed from 175 μm anterior to 525 μm posterior to the obex, from 225 to 450 μm lateral to the midline and from 175 to 425 μm deep to the ventral surface of the brain stem.

4. The respiratory rhythm recorded in the isolated CNS was influenced by pH and neurotransmitters. The respiratory rate decreased by about 26 % at high pH (7.7) and increased by about 33 % at low pH (7.1). Bath application of noradrenaline (30–100 μmol l^{-1}) decreased the respiratory rate and increased the amplitude of the rhythmic bursts significantly. All these effects were reversible.

5. The results presented here indicate that the isolated intact CNS of newborn opossum offers advantages for exploring mechanisms responsible for generating the respiratory rhythm.

*Present address: Cold Spring Harbor Laboratory, PO Box 100, 1 Bungtown Road, Cold Spring Harbor, NY 11724, USA.

Key words: opossum, respiration, central nervous system, *in vitro* tissue culture, *Monodelphis domestica.*

Introduction

Extensive studies have been made in adult cats on the mechanisms by which respiratory rhythms are generated (for reviews, see Cohen, 1979; Richter, 1982; von Euler, 1983) and on the connectivity of central respiratory neurones to respiratory motoneurones (Kirkwood and Sears, 1978; Davies *et al.* 1985). Similar work has recently been performed in adult rats (Saether *et al.* 1987; Ezure *et al.* 1988; Ellenberger and Feldman, 1988, 1990; Schwarzacher *et al.* 1991). In whole-animal experiments, pulsations of the blood vessels and the side-effects of anaesthesia can make it difficult to analyze cellular mechanisms. The introduction of the neonatal rat brain stem/spinal cord preparation (Suzue, 1984) has made it possible to study in greater detail the anatomical organization of the respiratory centre (Smith *et al.* 1990), the properties of respiratory neurones (Onimaru and Homma, 1987; Onimaru *et al.* 1988) and the modulation of the respiratory rhythm by pH and by neurotransmitters (Harada *et al.* 1985; Hilaire *et al.* 1989; Errchidi *et al.* 1990, 1991; Morin *et al.* 1990; Greer *et al.* 1991). In addition, use has been made of even simpler preparations, such as the foetal rat CNS (Di Pasquale *et al.* 1992; Greer *et al.* 1992) or medullary slices that are capable of generating respiratory-like oscillating signals (Smith *et al.* 1991).

The opossum, a marsupial, offers certain advantages because the pup is born at a very immature stage. At birth, the central nervous system is underdeveloped; the cerebral cortex has only two layers of cells and is therefore equivalent to that of a 14-day-old embryonic rat; there is little sign of a cerebellum and the spinal cord is not fully formed (Saunders *et al.* 1989). The isolated CNS of the newborn opossum remains electrically excitable for over a week when maintained in culture. Even the fine structure of the spinal cord appears normal after 5 days in culture (Stewart *et al.* 1991). These long-term cultures have made it possible to study the plasticity of synaptic connections and receptors. For example, $GABA_B$ receptors have been shown to be regulated by the chemical environment and neural activity (Zou *et al.* 1991). Restoration of electrical conduction and growth of axons occur through the injured sites in the spinal cord in culture (Treherne *et al.* 1992). The newborn animal breathes and sucks, so the circuit for respiration must be fully functional. Indeed, rhythmical respiratory activity can be recorded from the spinal cord in the isolated CNS (Nicholls *et al.* 1990).

In the present experiments, the region generating respiration was shown to be located in the lower brain stem, and units related to respiration were identified in the respiratory centre.

Materials and methods

Newborn opossums, *Monodelphis domestica* (3–10 days old), were removed from their mothers in the breeding colony (Nicholls *et al.* 1990). The pups were anaesthetized by cooling on ice and then immersed in BME medium (Basal Medium with Eagle's salts, no. 041-01010, Gibco, Life Technologies Ltd, Scotland, UK) containing (in $mmol\,l^{-1}$): NaCl, 116.3; KCl, 5.4; $NaHCO_3$, 26.2; $NaH_2PO_4{\cdot}H_2O$, 1.0; $CaCl_2$, 1.8; $MgSO_4{\cdot}7H_2O$, 0.8; D-glucose, 5.5; as well as amino acids and vitamins. The medium was continuously gassed with O_2/CO_2 (95 %:5 %, pH 7.4). The entire CNS was removed, sometimes together with the rib cage, and kept at room temperature (23–25 °C).

Electrical recordings

Just before electrical recording, the rib cage was removed and dorsal root ganglia (DRG) in the cervical and upper thoracic spinal cord were dissected so as to obtain long ventral roots. The preparation was transferred to the recording chamber and perfused continuously with gassed BME medium. Spontaneous activity from a spinal ventral root was recorded by a glass suction electrode with a tip diameter of 30–40 μm. Unit activity from the brain stem was recorded extracellularly by a glass microelectrode filled with 1 mol l^{-1} NaCl, with a resistance of 5–10 MΩ. Electrical signals were amplified by low-noise differential amplifiers (Almost Perfect Electronics, Basel, Switzerland), bandpass filtered at 3–3000 Hz, displayed on an oscilloscope and a pen recorder (Clevite Corporation, Brush Instruments Division, Cleveland, Ohio, USA) and digitized using a VR-10 digital recorder (Instrutech Corp., Mineola, NY, USA) for storage on videotape.

Perfusion of different CNS regions with high-Mg^{2+} medium

In order to determine at which CNS level respiration is generated, different CNS regions were perfused with BME medium containing 20 mmol l^{-1} Mg^{2+} (Mg^{2+} was added to normal BME medium, the pH remained at 7.4) to inhibit synaptic transmission. The perfusing chamber was a Petri dish separated into two parts by a thin film partition; Vaseline was used to seal the partition around the preparation. One part of the chamber was first perfused with normal BME medium and then switched to high-Mg^{2+} BME medium, while the other part was continuously perfused with normal BME medium. Addition of Fast Green as an indicator to the perfusing fluid showed that there was no diffusion from one part of the chamber to the other. The position of the preparation was adjusted relative to the partition so that different regions of the CNS could be perfused.

Marking the position of the extracellular electrode

All the experiments for revealing the extracellular recording sites were performed on the isolated CNS of 4-day-old animals. Glass extracellular electrodes filled with 2% Pontamine Sky Blue 6BX (BDH Chemicals Ltd, Poole, England) in 0.5 mol l^{-1} sodium acetate (Boakes *et al.* 1974) had resistances of 3–7 MΩ. When the activity of a unit in the brain stem was synchronized with the rhythmic respiratory discharges recorded from a spinal ventral root, the neurone was identified as ‘respiratory’. Having recorded such activity, the position of the recording site was labelled by pressure injection of the dye. The CNS was fixed for 4–6 h in a solution of 4% paraformaldehyde in 0.1 mol l^{-1} phosphate buffer (pH 7.4) and rinsed three times in the same buffer. The brain stem region was transferred to 0.1 mol l^{-1} phosphate buffer containing 25% sucrose. Serial 25 μm cross sections were cut in a cryostat (Cryocut 1800, Reichert-Jung, Cambridge Instruments GmbH, Nussloch, Germany). The recording site, which appeared as a blue spot with a diameter of 20–30 μm in 1–3 adjacent sections, was drawn with a *camera lucida* and photographed. Sections were counterstained with 0.1% Cresyl Violet (Sigma, St Louis, MO, USA) in 0.2 mol l^{-1} acetate buffer (pH 5.0) to reveal the position of different nuclei.

Results

Respiratory rhythm recorded from the isolated CNS preparation

Spontaneous rhythmical movements of the ribs were observed in the isolated CNS preparation with attached rib cage. These rhythmical movements were monitored by recording the electromyogram (EMG) from intercostal muscles. Spontaneous neural activity was simultaneously recorded by a suction electrode on the contralateral spinal ventral root. Discharges recorded from the ventral root occurred in synchrony with the EMG signals (Fig. 1A,B) and were observed only when the rib cage was moving in a caudal to rostral direction. These discharges therefore corresponded to inspiration.

In the preparation without ribs, rhythmical respiratory activity was recorded from the cervical and upper thoracic spinal ventral root and from the vagus nerve. Fig. 1C shows that these rhythmical respiratory discharges continued regularly and reliably for more than 6 h in BME medium. In the isolated CNS of 4-day-old animals, the average rate of the respiratory rhythm was 34±1.1 discharges min^{-1} (N=35, mean ± S.E.M.). The rate in 8-day-old animals was 47±4.3 discharges min^{-1} (N=5), indicating that, during development, the frequency increased significantly (t-test, P<0.025).

Origin of respiratory rhythmicity

In the following experiments, medium containing 20 mmol l^{-1} Mg^{2+} was perfused over different regions of the CNS to observe at which level the respiratory rhythm was abolished (Fig. 2). When the partition was at the junction of the brain stem and spinal cord, high-Mg^{2+} medium perfused in the upper part of the CNS stopped the respiratory rhythm within 2 min. After changing the bathing fluid back to normal medium, the respiratory rhythm resumed within 10 min (Fig. 2A). When the partition was placed at the midbrain level, perfusion of the cortex and midbrain with high-Mg^{2+} medium for more

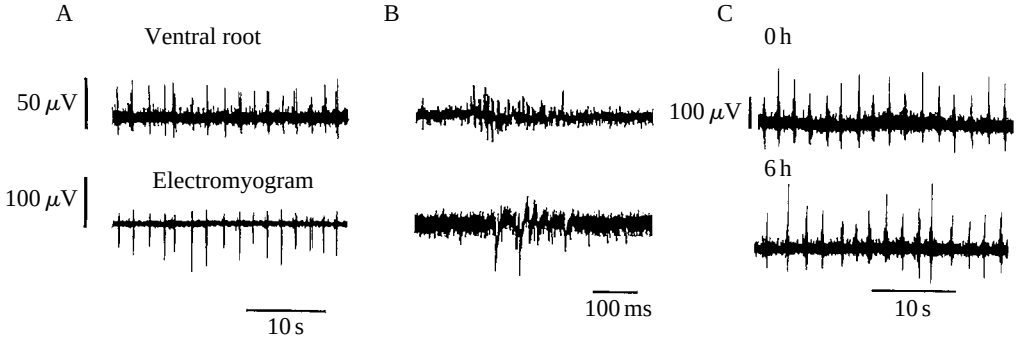

Fig. 1. Rhythmical respiratory activity recorded from spinal ventral roots. (A) Simultaneous recordings of electrical activity in the third thoracic (T3) ventral root (upper trace) and in the intercostal muscles (lower trace) from a 4-day-old opossum. (B) Similar recordings shown at a faster time scale. Signals recorded from the T3 ventral root (upper trace) began about 50 ms before the EMG signals (lower trace). (C) The respiratory rhythm was stable for more than 6 h. Upper trace, spontaneous rhythmic activity recorded from the first thoracic (T1) ventral root of a 3-day-old opossum 1 h after dissection. Lower trace, the rhythm after 6 h of continuous recording.

than 5 min had no effect on the respiratory rhythm (Fig. 2B). The respiratory rhythm was stopped reversibly when structures rostral to the medulla were perfused with high-Mg^{2+} medium (Fig. 2C).

Selective transection experiments were also made in order to confirm the location of neurones generating the respiratory rhythm. When the forebrain, midbrain and rudimentary cerebellum were removed by transection, the pattern of respiratory discharges recorded from a spinal ventral root was virtually unchanged. When transection was made at the border of the pons and the medulla, the respiratory rhythm was disturbed. In some cases, the abnormally high activity caused by ablation masked any rhythmic respiratory activity recorded from spinal ventral roots. In other experiments, the rate of the respiratory rhythm was decreased. When the cut was made at the level of the obex, the respiratory rhythm disappeared.

Classification of respiratory related neurones

In the lower brain stem, 128 units with activity related to the respiratory rhythm were recorded in 58 isolated CNS preparations (ranging from 3 to 10 days old). These units were all situated on the ventral medulla and displayed a variety of discharge patterns. They were classified into two inspiratory groups and two expiratory groups. The classification was based on the phase (inspiration or expiration) in which the discharge was dominant, and then on the time that firing started and stopped in relation to the respiratory discharges recorded from a spinal ventral root. 107 inspiratory neurones were early inspiratory or inspiratory; 21 expiratory neurones were post-inspiratory or expiratory. These groups, shown in Fig. 3, can be described as follows. Early inspiratory neurones (N=69) started discharging before the onset of the inspiratory phase and stopped bursting before the end of inspiration (Fig. 3A). They were the most prevalent neurones. Inspiratory neurones (N=38) started bursting after the onset of the inspiratory phase and ended at the end of inspiration (Fig. 3B). Post-inspiratory neurones (N=17) fired after inspiration, but did not continue to fire throughout expiration (Fig. 3C). Expiratory neurones (N=4) discharged throughout expiration and did not show activity during inspiration. They were the type of respiratory neurone least frequently encountered (Fig. 3D).

Particular types of respiratory neurones did not appear at particular times of development (for animals aged between 3 and 10 days). More respiratory neurones were encountered in older (8- to 10-day-old) than in younger (3- to 6-day-old) preparations.

Distribution of respiratory neurones in the brain stem

In 13 isolated CNS preparations from 4-day-old animals, the sites of 22 respiratory units were marked by pressure injection of Pontamine Sky Blue from the recording electrode. These cells were distributed over a well-defined region of the ventral medulla, extending from 175 μm anterior to 525 μm posterior to the obex, from 225 to 450 μm lateral to the midline and from 175 to 425 μm deep to the ventral surface of the brain stem (Fig. 4). Most of the respiratory neurones were detected caudal to the obex in a region in and around the nucleus ambiguus. No respiratory neurones were recorded in the dorsal aspect of the medulla or in the pons.

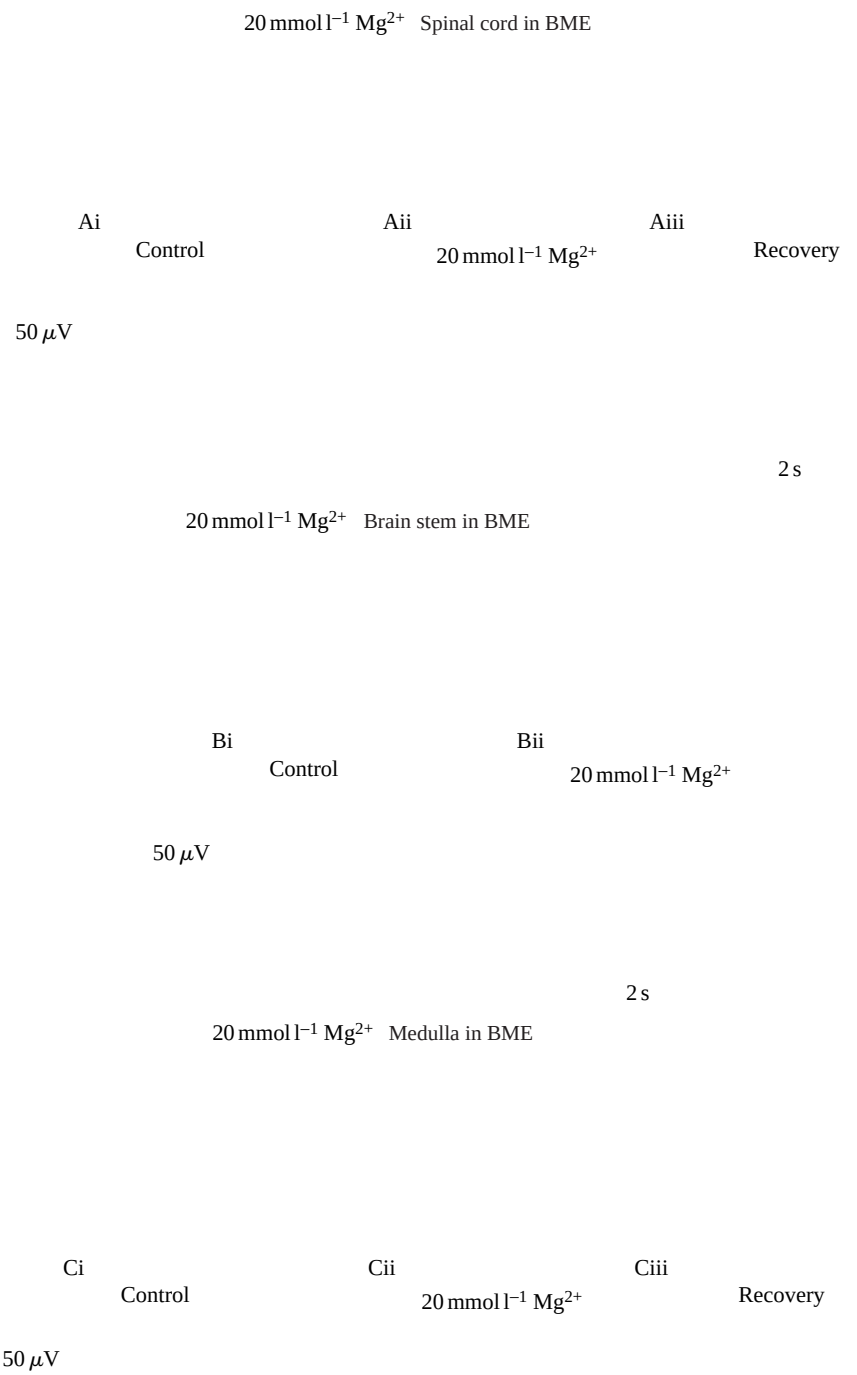
20 mmol l−1 Mg2+ Spinal cord in BME
Ai
Aii
Aiii
Control
20 mmol l−1 Mg2+
Recovery
50 μV
2 s
20 mmol l−1 Mg2+ Brain stem in BME
Bi
Bii
Control
20 mmol l−1 Mg2+
50 μV
2 s
20 mmol l−1 Mg2+ Medulla in BME
Ci
Cii
Ciii
Control
20 mmol l−1 Mg2+
Recovery
50 μV
2 s

Fig. 2

Modulation of the respiratory rhythm

Effects of pH

To determine whether chemical stimuli influence respiration in the isolated CNS, the pH of the perfusing medium was adjusted by changing the concentration of bicarbonate. The pH for normal BME, which contains 26 mmol l^{-1} HCO_3^-, was 7.4. In medium with 6 mmol l^{-1} or 40 mmol l^{-1} HCO_3^-, the pH values were 7.1 or 7.7, respectively. When the CNS of 4-day-old opossums was perfused with high-pH medium, the rate of respiration was significantly decreased by 26±2.7 % (N=5) (t-test, P<0.01). This effect was reversed after the high-pH medium had been changed back to normal BME (Fig. 5A). The rate of respiration was significantly increased by 32±5.0 % (N=4) (t-test, P<0.01) after perfusion with low-pH medium. This effect was also reversible (Fig. 5B). Similar results were observed when the O_2:CO_2 ratio in the gas with which the normal BME medium was continuously bubbled was changed.

Effects of noradrenaline

Noradrenaline applied to the bathing fluid significantly decreased the rate of the respiratory rhythm (t-test, P<0.01). 30 μmol l^{-1} noradrenaline reduced the rate of respiration in 4-day-old opossum CNS by 48±3.2 % (N=5). 100 μmol l^{-1} noradrenaline had stronger effects, reducing respiration rate by 71±0.4 % (N=4). Meanwhile, the amplitudes of the spontaneous bursts (i.e. the respiratory driving force) increased dramatically (Fig. 6). The effects of noradrenaline on respiration were reversible after 20 min in normal BME.

Discussion

In the present investigation, spontaneous rhythmic respiratory activity was recorded stably for several hours from the isolated CNS of neonatal opossum. Different patterns

Fig. 2. Definition of the site of generation of the respiratory rhythm, determined by perfusion of separate regions with BME medium containing 20 mmol l^{-1} Mg^{2+}. (A) A partition was placed at the junction of the brain stem and spinal cord. The CNS rostral to the spinal cord (shaded area) was perfused with high-Mg^{2+} medium. (i) The respiratory rhythm recorded from a spinal ventral root in normal BME medium. (ii) Rhythmic respiratory activity stopped within 2 min of changing the normal BME to high-Mg^{2+} medium, whereas spontaneous activity remained. (iii) Replacing high-Mg^{2+} medium with normal BME restored the respiratory rhythm in 10 min. (B) The partition was at the midbrain level. The CNS rostral to the midbrain (shaded area) was perfused with high-Mg^{2+} medium. (i) Respiratory rhythm recorded from a spinal ventral root in normal BME medium. (ii) Rhythmic respiratory activity was not disturbed after changing the normal BME to high-Mg^{2+} medium for more than 5 min. (C) Respiration originated in the lower brain stem. Here, the partition was at the junction of the pons and medulla. The CNS rostral to the medulla (shaded area) was perfused with high-Mg^{2+} medium. (i) The respiratory rhythm recorded from a spinal ventral root in normal BME medium. (ii) Respiratory activity stopped within 3 min of changing the normal BME to high-Mg^{2+} medium. (iii) Replacing the high-Mg^{2+} medium with normal BME restored the respiratory rhythm in 10 min. All of these experiments were made in a single isolated CNS preparation from a 4-day-old opossum. Similar results were obtained in two other experiments.

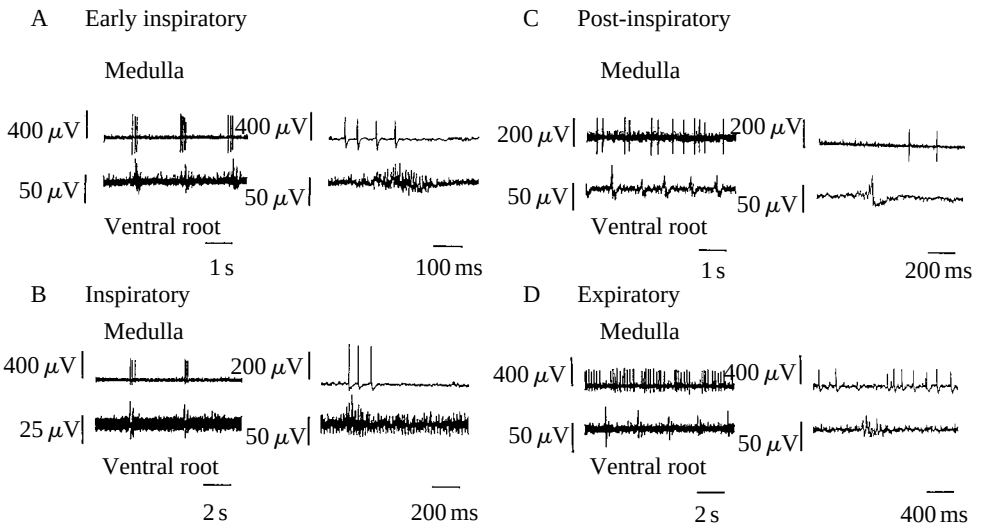

Fig. 3. Examples of discharge patterns of two types of medullary inspiratory and two types of medullary expiratory neurones (upper traces) shown in relation to the respiratory rhythm recorded from a spinal ventral root (lower traces). Recordings are shown at slow (left-hand traces) and fast (right-hand traces) chart speeds. (A) Early inspiratory neurone. (B) Inspiratory neurone. (C) Post-inspiratory neurone. (D) Expiratory neurone.

of respiratory neurone activity were characterized. The region responsible for generating respiration was shown to reside in the lower brain stem. Several lines of evidence indicate that the rhythmic activity recorded from spinal ventral roots is a respiratory rhythm. (1) Activity was well synchronized with the contractions of intercostal muscles. (2) The rhythm originated in the lower brain stem, as was demonstrated by perfusing high-Mg^{2+} medium and by ablation experiments. (3) Respiratory unit activity could be detected in a defined lower brain stem region that in other mammals is known to be rich in respiratory neurones. (4) Factors such as pH and neurotransmitters, known to modulate respiration in other mammalian preparations, affected the rhythm in a characteristic manner.

In 4-day-old opossum CNS preparations *in vitro* at room temperature, the average frequency of the respiratory rhythm was 34 discharges min^{-1}. That of 8-day-old pups was 47 discharges min^{-1}. This increase of respiration rate in older pups might reflect increasing levels of metabolism during development. A similar increase has been obtained in neonatal rat isolated brain stem/spinal cord preparations in which the mean respiratory frequency increased from 3.8 discharges min^{-1} (0- to 1-day-old) to 5.5 discharges min^{-1} (2- to 4-day-old) (Errchidi *et al.* 1991). The respiratory rhythm was shown to be generated in the lower brain stem by locally perfusing the preparation with BME medium containing 20 mmol l^{-1} Mg^{2+}. This concentration of Mg^{2+} has been shown to block synaptic transmission effectively and reversibly in the isolated CNS of opossum (Nicholls *et al.* 1990). When the lower brain stem, but not other higher structures, was perfused with high-Mg^{2+} BME medium, the respiratory rhythm recorded from the spinal

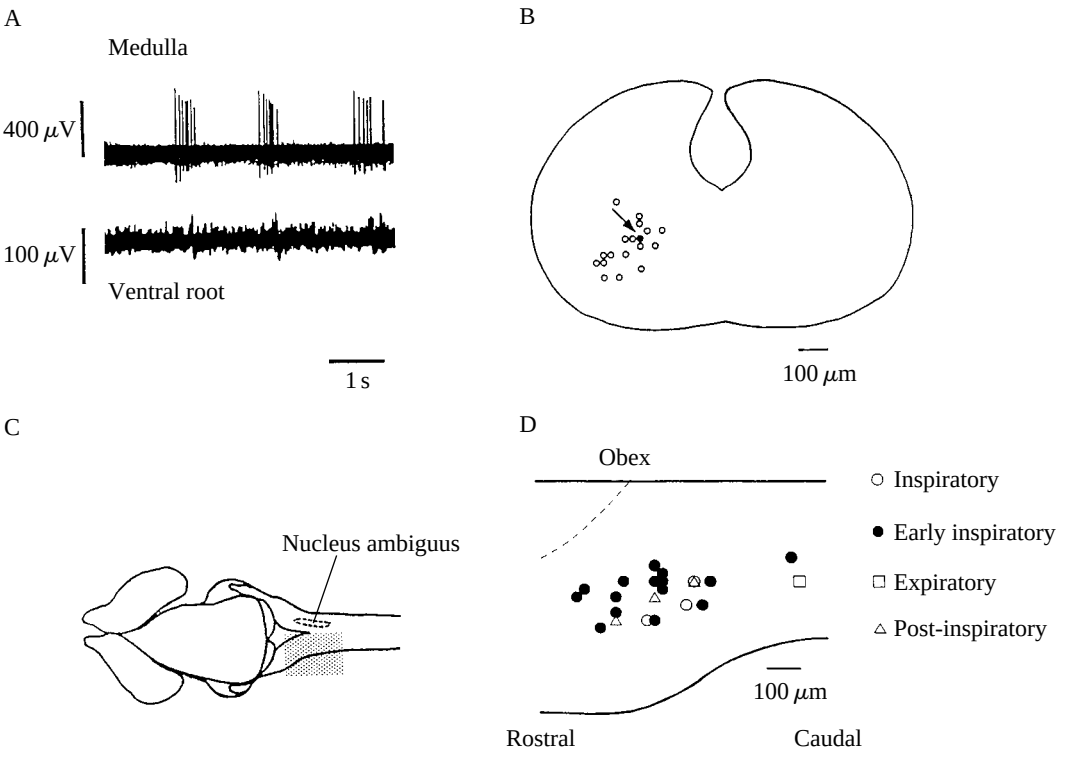

Fig. 4. Respiratory neurones in the lower brain stem. (A) Synchronization of unitary activity recorded from the medulla with the respiratory rhythm recorded from the spinal ventral root C4. These units were early inspiratory neurones. (B) Drawing of the recording site (filled circle indicated by the arrow) revealed by injection of Pontamine Sky Blue. The open circles are other recording sites projected to this section. (C) A schematic drawing of the dorsal view of a 4-day-old opossum CNS. The dashed line outlines the nucleus ambiguus. The respiratory neurones were recorded from the shaded area, which is enlarged in D. (D) Locations of 22 respiratory neurones in the brain stem viewed from the ventral aspect. The dashed line is the border of the fourth ventricle, as if viewed from the dorsal side. The distribution of the neurones was projected onto a horizontal plane. The dimensions of the drawing were averaged from thirteen 4-day-old animals in which the respiratory neurones had been recorded and successfully marked.

cord was abolished reversibly. Selective ablation experiments showed that when the cut was made at the level of the obex, the respiratory rhythm disappeared. These results indicate that the region for generating respiration is located in the lower brain stem and that this region might involve the rostral medulla.

The four types of respiratory bursting patterns recorded from neurones in the ventral aspects of the lower brain stem of the neonatal opossum are generally similar to those recorded from other mammalian preparations. These include adult cats (Cohen, 1979; Richter, 1982; von Euler, 1983), adult rats (Saether *et al.* 1987; Schwarzacher *et al.* 1991) and opossum *Didelphis marsupialis* (Faber, 1988) *in vivo*, as well as the neonatal rat brain

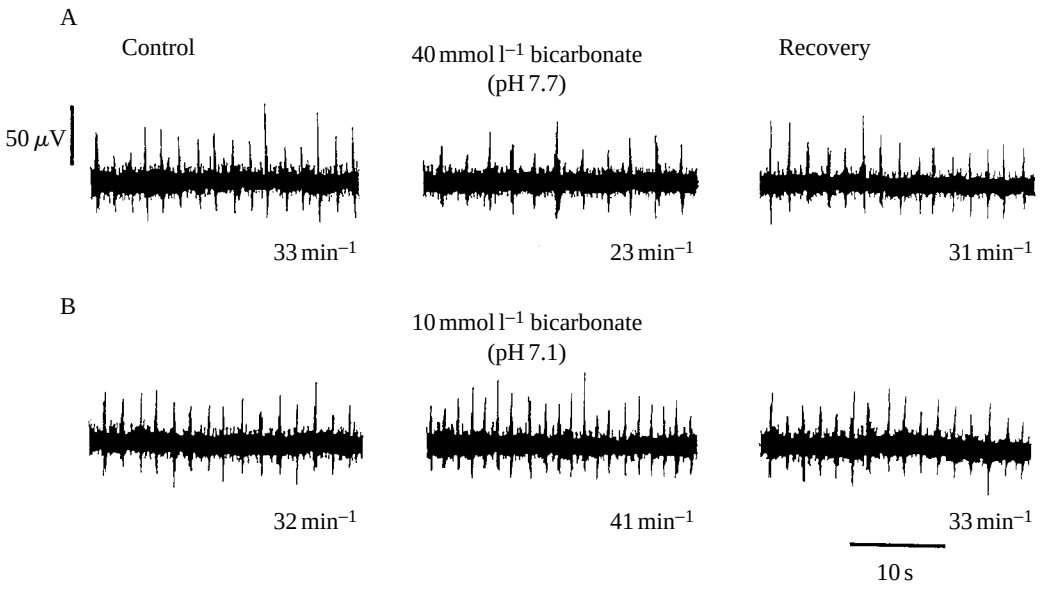

Fig. 5. Effects of pH on respiration rate. (A) The effects of high pH. The rate of respiration decreased from 33 discharges min^{-1} in the control recording (normal BME; 26 mmol l^{-1} HCO_3^-, pH 7.4; left-hand trace) to 23 discharges min^{-1} after changing to high-pH medium (40 mmol l^{-1} HCO_3^-, pH 7.7; middle trace) for 2 min. The rate of respiration returned to the original level 5 min after changing back to normal BME (right-hand trace). (B) The effects of low pH. The rate of respiration increased from 32 discharges min^{-1} in the control recording (left-hand trace) to 41 discharges min^{-1} after changing to low-pH medium (10 mmol l^{-1} HCO_3^-, pH 7.1; middle trace) for 3 min. The rate of respiration returned to the original level 10 min after changing back to normal BME (right-hand trace).

stem/spinal cord preparation *in vitro* (Smith *et al.* 1990). However, in isolated CNS preparations from neonatal opossum at this stage of development (3–10 days old), phase-spanning respiratory units were not detected. As only spontaneously active units were sampled, it is possible that respiratory neurones that were silent under the current experimental conditions of low temperature and artificial medium were missed. The opossum medullary respiratory neurones have a low firing frequency, typically 2–5 spikes per breath for inspiratory neurones (Fig. 3). Similar patterns were observed in the opossum *Didelphis marsupialis*, in which bulbospinal inspiratory and expiratory–inspiratory neurones recorded from animals younger than 60 days fired an average of 1.9 spikes per breath (Faber, 1988).

In my experiments, as in neonatal rat brain stem/spinal cord preparations (Smith *et al.* 1990), respiratory neurones were detected only in the ventral aspects of the medulla. This finding differs from those in anaesthetized adult cats, in which respiratory neurones are found on both ventral and dorsal aspects of the brain stem (Cohen, 1979). In neonatal opossum, the respiratory neurones revealed by marking the recording sites were concentrated in a narrow column in the ventral medulla. This region is in and around the

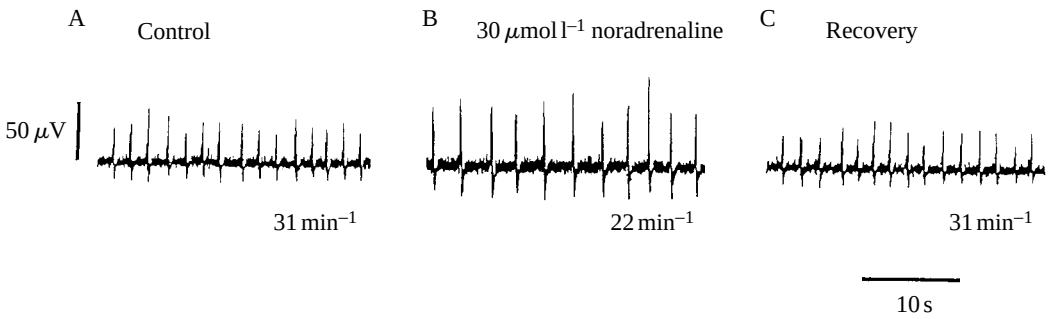

Fig. 6. Noradrenaline decreased the rate of respiration and increased the amplitude of spinal respiratory discharges. (A) A control recording of the respiratory rhythm in normal BME medium. (B) The rate of respiration decreased from 31 to 22 discharges min^{-1} after changing the bathing medium to BME containing 30 μmol l^{-1} noradrenaline for 5 min; and the amplitudes of spinal respiratory discharges increased. (C) The rate of respiration returned to the original level 20 min after the bathing medium had been changed back to normal BME.

nucleus ambiguus which, as in other mammals, is rich in respiratory neurones (Cohen, 1979; Saether *et al.* 1987; Ezure *et al.* 1988; Faber, 1988; Smith *et al.* 1990).

In isolated CNS preparations, alterations of the respiratory rhythm induced by changes in the pH or by neurotransmitters in the bathing fluid are mediated centrally. Besides reducing the rate of the respiratory rhythm, noradrenaline also increased the amplitude of respiratory bursts. Similar results have been obtained in neonatal rat preparations *in vitro*, where noradrenaline modulates respiration through the activation of α_2 noradrenaline receptors in the medulla (Errchidi *et al.* 1990, 1991). Central chemosensitive areas have been found in discrete regions of the ventral medulla in cats (Loeschcke, 1982).

Two types of mechanism have been proposed to explain respiratory rhythmogenesis. (1) Pacemaker mechanisms, in which respiratory neurones have pacemaker properties. (2) Network interaction mechanisms, in which the respiratory rhythm arises from synaptic interactions between different types of neurones that do not themselves possess intrinsic rhythmicity (for reviews, see Cohen, 1979; Richter, 1982; von Euler, 1983). Neonatal opossum may provide a good preparation for analyzing these mechanisms and the development of a respiratory network. Intracellular recording is difficult, because neurones in the respiratory region in neonatal opossum are small (10–15 μm diameter), but possible (J. Eugenin, unpublished results). Patch-clamp recording in a whole-cell configuration may have advantages over conventional intracellular recording techniques (Smith *et al.* 1992). One can hope that the application of such techniques will promote progress in understanding mechanisms of respiratory rhythmogenesis.

I thank Professor J. G. Nicholls for his support, encouragement and critical help with the manuscript. I thank Eric Hardman, Marlies Thalmer and Urs Berglas for maintaining the opossum colony, Dr W. B. Adams for electronics and Paul Bättig for photography. This work was supported by the Swiss Nationalfonds to J. G. Nicholls.

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