Role of fast inhibitory synaptic mechanisms in respiratory rhythm generation in the maturing mouse

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1. The importance of glycinergic and $GABA_A$ergic synaptic mechanisms for respiratory rhythm generation in the maturing mouse were investigated *in vivo* and in an *in vitro* slice preparation generating respiratory rhythmic activity spontaneously at all postnatal ages.

2. The effect on respiration of topical application of strychnine or bicuculline to the surface of the ventrolateral medulla was assessed in spontaneously breathing anaesthetized mice of different ages (postnatal (P) days 0 to >56). Glycine receptor antagonization with concentrations of strychnine up to 25 μM was ineffective in altering the breathing pattern in neonates (P1–P8). However, in mature mice (P > 15), low doses of strychnine (0·2–2 μM) abolished regular rhythmic discharge in the phrenic nerve. Bicuculline (0·5–50 μM) produced dose-dependent increases in inspiratory time, amplitude and cycle length of phrenic nerve discharge in anaesthetized neonatal mice whereas both cycle length and duration of inspiratory activity were reduced in mature animals. In addition, in both neonates and mature mice low concentrations of bicuculline (0·5–5 μM) abolished phrenic nerve discharge intermittently.

3. The response of respiratory-modulated hypoglossal (XII) neurones recorded in tilted sagittal slices from newborn and mature mice during blockade of glycine and $GABA_A$ receptors was similar to the phrenic nerve changes observed *in vivo*: in slices from neonates, the rhythmic activity of XII neurones was resistant to concentrations of strychnine up to 50 μM whereas low doses of strychnine (0·2–2 μM) abolished rhythmic activity in preparations from mature mice. Bicuculline (1–50 μM) produced a dose-dependent prolongation of burst duration and a slowing of rhythmic discharge in slices from neonatal mice whereas in mature mice rhythmic XII bursts were shortened and their frequency increased. At all maturational stages, bicuculline (1–50 μM) induced severe disruption of the regular rhythm of XII neurone activity causing maintained depolarizations and oscillations in membrane potential.

4. On-going inhibitory postsynaptic potentials of neurones located in the ventral respiratory group region of tilted sagittal slices from both immature and mature mice were sensitive to low concentrations of either bicuculline or strychnine (1–5 μM) indicating an absence of a maturational change in the sensitivity of $GABA_A$ and glycine receptors to their respective antagonists.

5. We conclude that over the first 15 days of life in the mouse there is a dramatic increase in the relevance of glycine receptors for respiratory rhythm generation, and change in the functional role of $GABA_A$ receptors within the respiratory network, which may provide a stabilizing influence on neurones within the respiratory network from birth onwards.

The underlying neural mechanisms for respiratory rhythm generation may be quite different in newborn and adult mammals. Indeed, there is a wealth of information describing maturational changes in both neuronal and synaptic mechanisms within the mammalian nervous system during the first weeks of life. An increase in presynaptic sprouting and number of synaptic contacts per neurone (e.g. Purves & Lichtman, 1978; Kraszewski &

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Grantyn, 1992) as well as clustering of postsynaptic receptors (St John & Stephens, 1993) have all been described. This prompts the question as to what the changes might be within the central respiratory network which is vital for the oxygen supply of the organism during postnatal life.

During the first postnatal days of life the integrity of glutaminergic receptors for respiratory rhythm generation in rats is essential (Smith, Ellenberger, Ballanyi, Richter & Feldman, 1991*a*; Greer, Smith & Feldman, 1991; Funk, Smith & Feldman, 1993). For instance, in the rhythmic transverse brainstem slice preparation of the neonatal rat, blockade of non-NMDA receptors abolished rhythmic activity in neurones of the ventral respiratory group (VRG) and in the hypoglossal (XII) rootlet (Smith *et al.* 1991*a*; Funk *et al.* 1993). In contrast, administration of either strychnine (Onimaru, Arata & Homma, 1990), bicuculline (Feldman & Smith, 1989; Onimaru *et al.* 1990), to antagonize glycine and $GABA_A$ receptors, respectively, or a chloride-free perfusate (Feldman & Smith, 1989; Onimaru *et al.* 1990) did not arrest rhythmic discharge in respiratory neurones or the phrenic nerve in the isolated brainstem–spinal cord preparation of newborn rats. Indeed, the notion of a pacemaker-driven oscillator restricted to a localized part of the ventrolateral medulla, termed the Pre-Bötzinger area, was postulated by Smith *et al.* (1991*a*) and substantiated by evidence of intrinsic bursting properties of some respiratory neurones within this region (Smith *et al.* 1991*a*; Smith, Funk, Johnson & Feldman, 1993). In addition, respiratory neurones located rostral to the Pre-Bötzinger region in neonatal rats have also been shown to produce bursts of spikes intrinsically (Onimaru, Arata & Homma, 1993). However, in adult mammals, the evidence to date does not support a role for a 'pacemaker'-based respiratory oscillator as the primary mechanism for respiratory rhythmogenesis but, rather, inhibitory synaptic interactions amongst a network of neurones (Hayashi & Lipski, 1992; Richter, Ballanyi & Schwarzacher, 1992).

Evidence for the involvement of fast chloride-mediated inhibition is based on studies using specific antagonists or agonists. Intracerebral ventricular administration of strychnine or bicuculline, for example, severely disrupted the phrenic nerve discharge often resulting in apneusis (Schmid, Böhmer & Gebauer, 1991*a*, *b*). Consistent with these findings was that respiratory rhythm generation was found to be dependent upon both bicuculline- and strychnine-sensitive mechanisms in the arterially perfused brainstem of the adult rat (Hayashi & Lipski, 1992). Moreover, activation of $GABA_A$ and glycine receptors for the production of spontaneous inhibitory postsynaptic potentials (IPSPs) in medullary respiratory neurones was demonstrated using bicuculline and strychnine applied ionophoretically onto characterized respiratory neurones in anaesthetized adult cats (Champagnat, Denavit-Saubié, Moyanova & Rondouin, 1982; Haji, Remmers, Connelly & Takeda, 1990; Haji, Takeda & Remmers, 1992). This is supported by the presence of both GABA and glycine immunoreactive boutons in close apposition to identified medullary respiratory neurones in the cat (Schwarzacher, Maschke & Richter, 1993). Indeed, models simulating central respiratory activity (see Richter, Ballantyne & Remmers, 1986; Ogilvie, Gottschalk, Anders, Richter & Pack, 1992; Richter *et al.* 1992) are based on spike-triggered averaging data, indicating reciprocal inhibitory synaptic connections between different populations of respiratory neurones (e.g. for review see Ezure, 1990), and the presence of profound IPSPs in different types of medullary respiratory neurones recorded from anaesthetized adult cats (e.g. Ballantyne & Richter, 1984). The latter allowed a comparison of the timing of changes in membrane voltages relative to the phrenic motor output pattern (Richter *et al.* 1986, 1992). Taken together, the evidence suggests that the neuronal mechanisms underlying rhythm generation for respiration in the adult mammal are distinctly different to those in newborns, as suggested by Hayashi & Lipski (1992).

Our approach was first, to investigate the role of glycine and $GABA_A$ receptors in ventilation in neonatal and mature mice under *in vivo* conditions and second, to assess any developmental changes in fast chloride-mediated synaptic inhibitory mechanisms for respiratory rhythmogenesis within the isolated, but functional, respiratory network *in vitro*. For this purpose, we have used a recently developed rhythmic tilted sagittal-slice preparation from both neonatal and mature mice (Paton, Ramirez & Richter, 1994*b*), which has for the first time permitted a direct comparison of maturational changes in analogous *in vivo* and *in vitro* preparations. We report that inhibitory synaptic mechanisms play an increasingly important role in respiration over the first 2 weeks of life.

Preliminary reports of part of this study have already been published (Paton, Ramirez & Richter, 1994*a*, *c* and *d*).

METHODS

Experiments were performed on MRI-1 and Bahabor mice of either sex and between the postnatal (P) ages of 0 to >56 days. Based on our previous data, the respiratory network was considered mature by P15 (Paton & Richter, 1995). Hence in the present study 'neonatal' animals were between P0–P6 days and 'mature' mice >15 days old.

In vivo experiments

Mice anaesthetized with sodium pentobarbitone (Nembutal; 60 mg kg^{-1}, I.P.) were placed in a cradle of plasticine on a thermostatically controlled, water-heated stainless-steel table

which was maintained at 38 °C. The trachea was intubated and the animals spontaneously breathed air enriched with oxygen .

Central respiratory activity was recorded from either the left phrenic or hypoglossal (XII) nerve. Nerves were isolated via a ventrolateral approach and activity recorded via a suction electrode connected to a differential amplifier (Tektronix AM 502). The nerve discharge was integrated using a custom-built leaky integrator (decay time constant of 100 ms). Ventilatory-related thoracic movements were measured non-invasively by a pressure transducer connected to a semi-inflated balloon secured across the chest. The change in pressure with every breath gave an indirect measure of inspiratory time (i.e. time from baseline to peak of pressure change), and expiratory time (peak of pressure change to start of next breath), as well as rate and a relative indication of tidal volume changes. These measurements precisely paralleled the recorded changes in phrenic or hypoglossal nerve activity. All variables were stored on magnetic tape (Racal V Store) for subsequent off-line analysis and displayed on a thermal array recorder (Gould TA 2000).

The ventral surface of the medulla was exposed by dividing the trachea and oesophagus. The larynx and pharynx were vascularly isolated and retracted rostrally to reveal the underlying muscles (longus capitis, longus colli and rectus capitis) which were removed from the anterior atlanto-occipital membrane and occipital bone. The anterior atlanto-occipital membrane was incised and the basilar part of the occipital bone removed to the level of the ponto-medullary junction. The dura and arachnoid were incised and retracted to expose the entire ventral medulla. Blocking drugs (see below), prepared in artificial cerebrospinal fluid (ACSF; see below), were applied topically to the ventral surface of the medulla.

In vitro experiments

Tilted sagittal slices were prepared from mice (P0–P > 56). This preparation was used since it generates respiratory rhythmic activity spontaneously at all postnatal ages in the mouse (Paton *et al.* 1994*b*).

Mice deeply anaesthetized with ether were decapitated and the entire brain removed in ice-cooled ACSF which was bubbled continuously with carbogen (95% oxygen–5% carbon dioxide). The brainstem was isolated and glued (cyanoacrylate) horizontally with the dorsal side up in an agar mould which was mounted on a glass block. The mould served to tilt and support the brainstem at 35 deg about the mid-line vertical axis during slicing (see Fig. 1 of Paton & Richter, 1995). A 600–700 μm thick tilted sagittal slice was prepared after the lateral edge of the brainstem was removed (approximately 200 or 500 μm from the lateral border in neonatal and mature mice, respectively) using a vibratome. Thinner slices were used from the neonatal mice in order to partially compensate for the difference in the size of the medulla between neonatal and mature mice. The slice was transferred to a recording chamber and placed on a fine stainless-steel mesh grid and superfused with a continuous stream of carbogen-gassed ACSF (11–12 ml min^{-1}) at 29 °C using a counter-current perfusion system (see Paton *et al.* 1994*b*). 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 respiratory regions and hypoglossal (XII) motor nucleus. Before recording began, a 30–60 min stabilization period was allowed. The slice preparations remained rhythmic for up to 14 h.

Recording procedures

Rhythmic activity was recorded from XII neurones or the XII rootlet as well as neurones in the VRG region. Activity from the XII rootlets which are present in slices from neonatal but not mature mice was recorded via a suction electrode (tip diameter, 200–350 μm) filled with ACSF. The XII motor nucleus was identified anatomically with reference to the characteristic shape of the dorsomedial medulla. Extracellular recordings from single units were made using either glass microelectrodes (1–7 MΩ; tip diameter, 2–4 μm) filled with 3 M NaCl or ACSF. In some experiments insulated tungsten steel electrodes (1–3 MΩ; tip diameter, 2–3 μm) were used. Electrodes were placed into the XII nucleus or VRG under visual guidance using a binoccular 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; pre-amp type 2F, NPI Electronics, Tamm, Germany). Intracellular recordings were made with either fine-tipped glass microelectrodes (60–80 MΩ; tip diameter, < 1 μm) or using patch pipettes. Patch pipettes were manufactured using filamented capillary tubes (glass type; GC 150 F10, Science Products, Frankfurt, Germany) and a two-stage pulling programme (type P-87; Sutter Instruments Co., Novato, CA, USA), which gave an internal tip diameter of between 1 and 2 μm with a resistance of 1·5–5 MΩ when filled (see below). A positive pressure (60–95 mmHg) was applied to the patch pipettes 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 (60 ms, 0·5–1·0 nA; Master 8, AMPI, Jerusalem, Israel) so that the quality of the seal could be measured. Seals of 2–8 GΩ were obtained on removing the positive pressure and applying suction (−40 to −140 mmHg). Following 'break-through' (or successful impalement when using microelectrodes) the bridge was balanced and capacitive transients fully compensated. Signals were amplified using an NPI SEC-10L amplifier and head stage before being displayed on an oscilloscope (Gould DSO 420) and stored on magnetic tape via an ADC interface (sampling rate 26 kHz; VR 100, Instrutech, Great Neck, NY, USA).

In addition to using 'blind' recording techniques in slices from neonatal and mature mice (sharp microelectrodes and patch pipettes), an infrared video system in conjunction with a fixed-stage upright microscope was also used to patch VRG neurones under visual control in slices from neonates. In these latter experiments, slices were placed in a small glass-bottomed recording chamber and viewed using a 40 × 0·75 numerical aperture water immersion lens (working distance of 1·9 mm; Carl Zeiss) and phase contrast (Phako I.V. Z7; Zeiss). The slice was illuminated from below with infrared light obtained by placing an infrared filter (wavelength, 780 nm; RG 9; Schott, Mainz, Germany) in the light path of the halogen lamp (6 V, 15 W) fitted in the microscope. Neurones to a depth of 120 μm were visualized using an infrared-sensitive camera and controller (C2400, Hamamatsu Photonics, Enfield, Middlesex, UK) and displayed on a standard black and white monitor (Panasonic WV 5410). Images were enhanced by adjustment of contrast and shadow controls of the Hamamatsu controller.

Solutions

ACSF was prepared daily and bubbled continuously with carbogen to maintain the pH at 7·4. The normal ACSF contained (mM): 1·25 KH_2PO_4, 1·25 $MgSO_4$, 25 $NaHCO_3$, 3·5 KCl, 125

Table 1. Baseline values (means ± S.E.M.) of inspiratory duration and cycle length for *in vivo* and *in vitro* experiments

	n	Inspiratory time (ms)	Cycle length (ms)	Burst duration (ms)
In vivo (38 °C)				
Thoracic movement				
Neonate	7	78 ± 4	648 ± 6	—
Mature	7	148 ± 7	944 ± 6	—
Phrenic nerve discharge				
Neonate	8	93·4 ± 2	720 ± 10	—
Mature	14	174 ± 3	929 ± 10	—
In vitro (29 °C)				
Rhythmic XII neurone discharge				
Neonate	11	—	2449 ± 63	325 ± 6·8
Mature	18	—	5964 ± 48	2032 ± 33

In anaesthetized mice note that the measurements of respiratory-related thoracic movements are comparable with those recorded from the phrenic nerve. In the tilted sagittal-slice experiments the cycle length and burst duration of respiratory rhythmic hypoglossal neurones are documented. The difference in the values between *in vivo* and *in vitro* preparations are discussed in Paton & Richter (1995).

NaCl, 2·5 $CaCl_2$, 20 dextrose. In many experiments the extracellular potassium concentration was raised to 5 or 7·5 mM to enhance respiratory rhythmic activity. The constituents of the patch pipette solution were (mM): 140 potassium gluconate, 10 Hepes, 0·2 EGTA, 7·7 NaCl, 2 K_2ATP (pH adjusted to 7·3 using 0·1 M KOH). Fast chloride-mediated inhibitory synaptic transmission was antagonized using bicuculline methiodide (0·1–50 μM; Sigma, Germany) and strychnine hydrochloride (0·1–50 μM; Sigma) whereas non-NMDA receptors were blocked using 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 0·5–10 μM; RBI Biochemicals, Mannheim, Germany). All drugs were prepared in ACSF (*in vivo* and *in vitro* experiments).

Data analysis

Rhythmic activity in both *in vivo* and *in vitro* experiments was quantitatively analysed over twenty cycles during control, peak response and recovery periods. The duration, frequency, amplitude and slope of phrenic nerve discharge were measured whereas the frequency of bursting, burst duration and intraburst firing frequency were measured from rhythmic XII neurones recorded *in vitro*. Changes in membrane potential and input resistance (R_m) of neurones recorded intracellularly were measured before and after drug application. The duration and amplitude of drive potentials and postsynaptic potentials were also analysed quantitatively. Measurements were either made manually or by using trigger and cursor facilities of a digital oscilloscope (Gould DSO 420). Quantitative data (means ± S.E.M.) are documented in Tables 1 and 2 whereas most values quoted in the text indicate the percentage change (means ± S.D.) unless otherwise stated. The statistical evaluation of the data was determined using Student's *t* test with Statview 512+ software (Abacus Concepts Inc., Berkeley, CA, USA) on a Macintosh computer.

RESULTS

Sensitivity of maturing respiratory network to glycine and $GABA_A$ receptor blockade

In order to study the role of fast chloride-mediated synaptic inhibition for respiratory rhythm generation, a range of concentrations of either strychnine or bicuculline (0·1–50 μM) were applied topically onto the ventral surface of the brainstem of mice *in vivo* or superfused *in vitro* to specifically antagonize glycine and $GABA_A$ receptors, respectively. Since the VRG region extends the rostro-caudal length of the brainstem and is localized relatively close to the surface of the ventrolateral medulla (see Ezure, 1990), it is argued that VRG neurones will be the primary mediator of respiratory responses during delivery of drugs to the ventral surface. In all these *in vivo* experiments, warmed ACSF was applied to the ventral surface of the brainstem as a control and was without effect.

Respiratory responses might also be due to direct effects of strychnine and bicuculline acting on central chemoreceptors described as residing in the superficial regions of the rostral ventrolateral medulla (for review see Loeschke, 1982). However, these effects, if any, are probably only likely in the mature animals since in neonatal rats central chemoreceptor activity was not found to be effective compared with adults (Ballanyi, Kuwana, Volker, Morawietz & Richter, 1992).

Table 2. Changes induced on central respiratory activity by blocking either glycine or $GABA_A$ receptors within the immature and developed medullary respiratory network

	n	Inspiratory time (ms)	Cycle length (ms)	Burst duration (ms)
In vivo				
Strychine data				
Neonate				
Control	5	90·7 ± 6	746 ± 17	—
Drug (10 μM)	5	91·9 ± 6	738 ± 8	—
Mature				
Control	7	170 ± 6	813 ± 20	—
Drug (0·5 μM)	7	110 ± 8	406 ± 38	—
Bicuculline data				
Neonate				
Control	5	91·4 ± 2	720 ± 28	—
Drug (5 μM)	5	97 ± 4	931 ± 34	—
Mature				
Control	5	184 ± 16	689 ± 30	—
Drug (5 μM)	5	118 ± 7	470 ± 17	—
In vitro				
Strychine data				
Neonate				
Control	10	—	3200 ± 300	520 ± 80
Drug (5 μM)	10	—	2900 ± 310	580 ± 60
Mature				
Control	7	—	6710 ± 1100	2400 ± 400
Drug (0·2 μM)	7	—	—	—
Bicuculline data				
Neonate				
Control	10	—	3600 ± 510	660 ± 60
Drug (5 μM)	10	—	7009 ± 1210	1400 ± 108
Mature				
Control	12	—	9110 ± 106	3460 ± 512
Drug (5 μM)	12	—	5560 ± 917	2484 ± 410

The means ± S.E.M. of inspiratory time and cycle length are given for *in vivo* experiments and the cycle length and burst duration for respiratory rhythmic hypoglossal neurones recorded in tilted sagittal slices. Since strychnine abolished regular rhythmic activity of hypoglossal neurones of mature mice *in vitro*, it was not possible to perform a meaningful quantitative analysis.

Since the respiratory rhythm generator is mature at P15 (Paton & Richter, 1995) we compared responses between neonatal mice (P0–P6) and mature animals (P > 15). Importantly, the response to blockade of glycine or $GABA_A$ receptors was qualitatively similar within these two age groups. In experiments on neonatal mice either chest movements and/or phrenic nerve activity were used to monitor alterations in respiratory activity. Table 1 shows the group data from the present experiments and includes control values of cycle length, inspiratory time (anaesthetized mice) and burst duration of XII neurone discharge from tilted sagittal slices. Measurements made from the respiratory-related thoracic movements correlated well with the response in phrenic nerve discharge (Table 1).

Role of glycine receptors for respiratory rhythm generation

The range of doses of strychnine used did not produce convulsive muscle activity in anaesthetized mice. However, based on visual inspection, we could produce convulsive motor activity only at concentrations in excess of an order of magnitude higher than those used in our experiments.

***In vivo* and *in vitro* experiments on neonatal mice.** Strychnine (0·1–20 μM) applied topically to either the ventral surface of the medulla ($n = 5$; *in vivo*) or to tilted sagittal slices from neonatal mice ($n = 10$) did not produce significant responses in either phrenic nerve activity/respiratory related thoracic movements or rhythmic XII

activity (rootlet, $n = 3$; unitary, $n = 7$), respectively (Table 2; Figs 1 and 2). In addition, strychnine did not significantly change resting membrane potential (control, -57 ± 3 mV; during application, -55 ± 4 mV) or input resistance (control, 193 ± 11 MΩ; during application, 195 ± 10 MΩ) in five rhythmic XII neurones recorded *in vitro*. When doses of strychnine exceeded 50 μM apneustic-like bursts (i.e. prolongation of inspiration) and increases in cycle length occurred intermittently in both *in vivo* and *in vitro* experiments (for *in vivo* results see Fig. 1, bottom graph), which probably reflects a non-specific action of strychnine at this relatively high dose.

In the absence of a response to strychnine *in vivo*, the sensitivity of the medulla to topical drug application was tested using comparable doses of CNQX (0·25–2 μM), a non-NMDA receptor blocker known to be essential for rhythm generation in neonatal rats (Smith *et al.* 1991*a*; Funk *et al.* 1993). Consistent with previous reports, CNQX produced a pronounced, but reversible, inhibition of respiration in three anaesthetized neonatal mice. Application of 2 μM CNQX induced an increase in cycle length from 788 ± 28 to 1013 ± 21 ms and a decrease in the rate of rise of inspiratory thoracic movement (from $0{\cdot}24 \pm 0{\cdot}01$ to $0{\cdot}06 \pm 0{\cdot}01$ mm ms^{-1}), which was accompanied by a $60 \pm 4\%$ reduction in amplitude (control, $24{\cdot}7 \pm 1{\cdot}5$ arbitary units; during CNQX application, $9{\cdot}9 \pm 1{\cdot}6$ arbitrary units). This indicated that the method of drug application was effective, at least with similar doses of CNQX.

***In vivo* experiments on mature mice.** In contrast to the absence of a response to strychnine in neonatal animals, respiratory rhythmic activity recorded *in vivo* and *in vitro* in the mature mouse was severely disrupted over a comparable dose range (0·1–2 μM).

In seven mature mice a quantitative analysis was only carried out during exposure to low doses of strychnine (0·1–0·5 μM; see Table 2) and before a severe deterioration of phrenic discharge developed (see below). Blockade of glycine receptors induced a decrease in the duration of inspiratory (8–42%) and postinspiratory activity (15–34%) and cycle length (7–39%) whereas phrenic amplitude was increased (18–58%). As seen in Fig. 3, strychnine induced a change in the inspiratory ramp of phrenic nerve discharge to a three-component pattern which included: (1) an initial step before, (2) a rapid onset burst, and (3) a plateau, which is consistent with

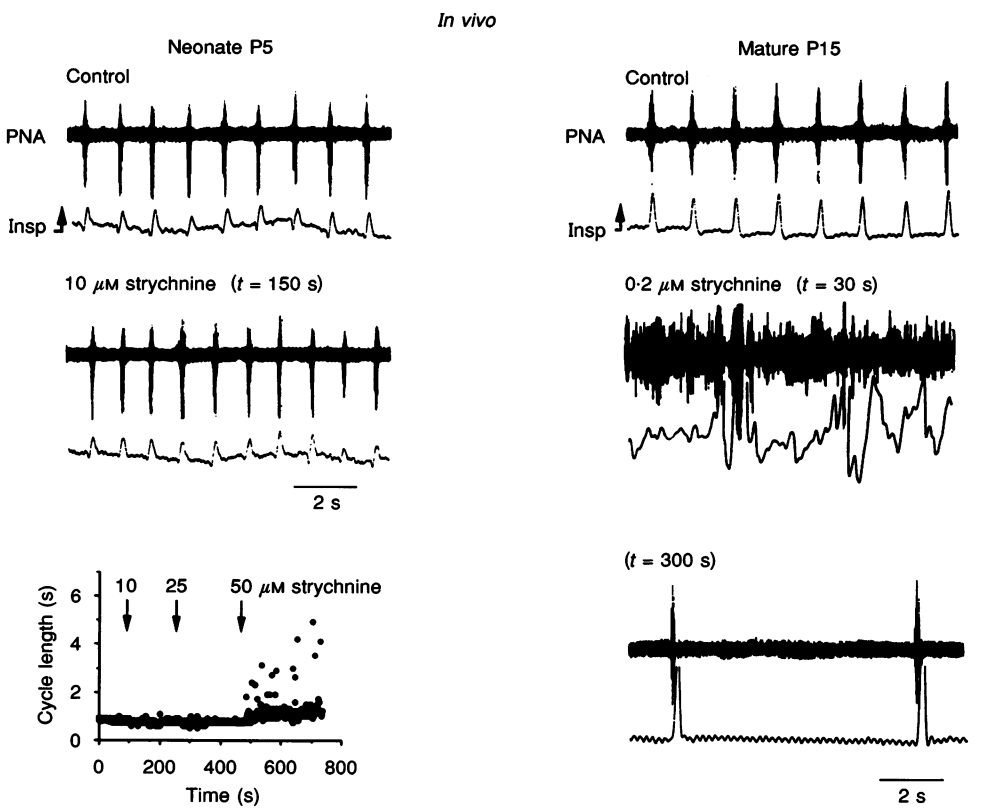

Figure 1. Contrasting effect of strychnine on respiratory rhythm generation for anaesthetized, spontaneously breathing neonatal and mature mice

In both animals, phrenic nerve activity (PNA) and respiratory-related thoracic movements are shown (arrow indicates inspiration, Insp). The effect on cycle length of subsequent doses of strychnine applied topically to the ventral surface of the medulla are also shown in the neonate (bottom graph). In both the neonate and mature animals *t* indicates time after drug application.

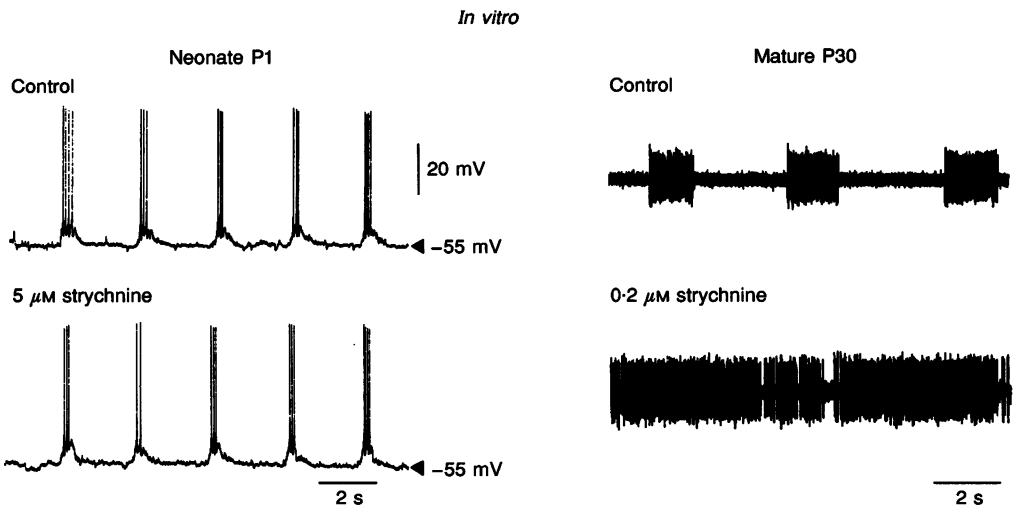

Figure 2. Comparison of the effect of strychnine on rhythmic hypoglossal (XII) neurones recorded from tilted sagittal slices from a neonatal and mature mouse

The XII motoneurone in the neonatal preparation was recorded with a patch pipette (R_m, 190 MΩ) whereas extracellular techniques were applied in the mature slice. Compare these data with the effects seen *in vivo* (Fig. 1.)

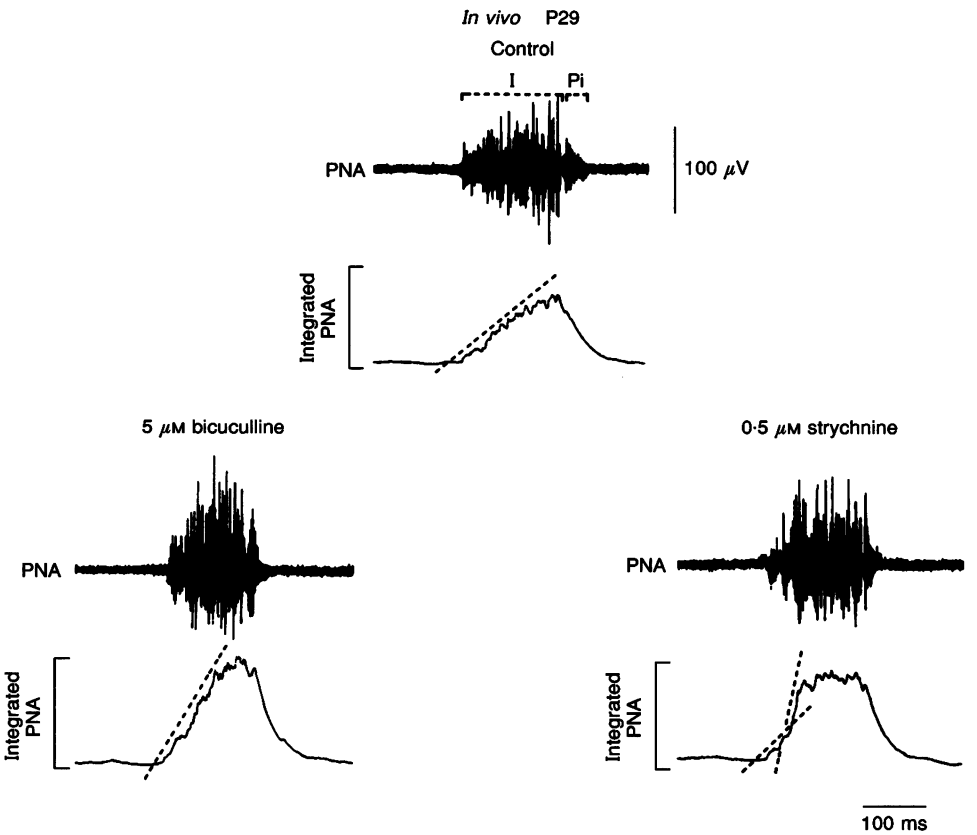

Figure 3. Comparison of the effects of $GABA_A$- and glycine-receptor blockade on the motor pattern of a single representative phrenic nerve burst recorded from an anaesthetized mature mouse

The ramp was greatly steepened and the inspiratory time reduced by bicuculline; this resulted in a plateau component in the inspiratory phase. In comparison, the ramp was almost abolished during strychnine administration. PNA, phrenic nerve activity; I, inspiratory phase; Pi, postinspiratory phase.

observations in the mature rabbit (Schmid *et al.* 1991*b*). In three animals these effects were reversible but in four a gradual deterioration in rhythmic activity resulted (Fig. 1).

Over a time course of 5–40 min, doses of 1–2 μM strychnine proved lethal. The effect included the occurrence of prolonged or apneustic-like discharge on which were superimposed high-amplitude bursts occurring at frequencies of 2–4 Hz (Fig. 1). During this time normal rhythmic discharge appeared absent. Subsequently there was a progressive increase in tonic discharge and a decrease in the amplitude of apneustic bursts and rate (Fig. 1). This led to either persistent tonic activity or apnoea followed by gasping as depicted in Fig. 1 (bottom right).

***In vitro* experiments on mature mice.** The effect of strychnine on seven rhythmic XII neurones was tested. Consistent with the effects seen in phrenic nerve activity *in vivo* (see above), strychnine induced severe disturbances in rhythmic activity at all doses used (0·1–1·0 μM; Fig. 2). Although there was a tendency towards increases in burst duration and cycle length, these effects were not consistent in all experiments. Prolonged exposure to strychnine induced tonic activity during the interburst interval, which increased and eventually resulted in the absence of rhythmic activity (Fig. 2). Similar effects were seen in rhythmic VRG neurones; these have been described elsewhere (Paton *et al.* 1994*c*).

Summary of strychnine effects

Antagonism of glycine receptors in the neonate did not appear to affect the respiratory rhythm generator at the cellular or systems level. In contrast, severe disruption and loss of respiratory rhythmic activity occurred in mature mice exposed to relatively low concentrations of strychnine.

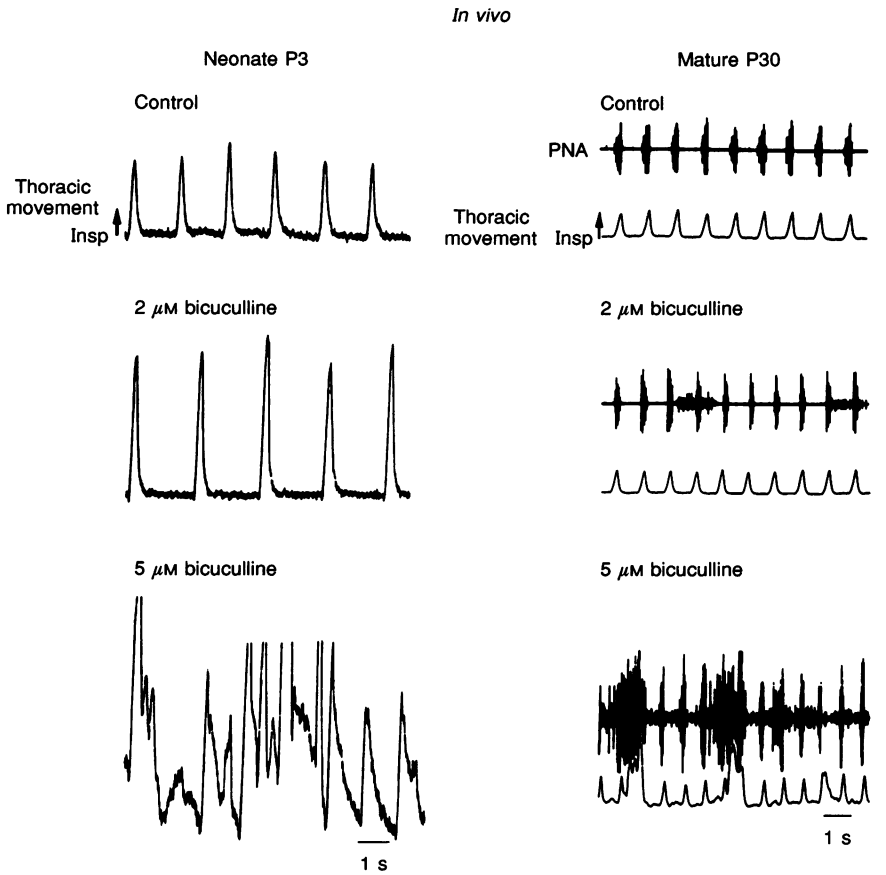

Figure 4. Effects of bicuculline administered topically to the ventral surface of the medulla on respiration are compared in anaesthetized, spontaneously breathing neonatal and mature mice

The respiratory-related thoracic movements were monitored in both animals (arrow indicates inspiration, Insp) and phrenic nerve activity (PNA) was also recorded in the mature mouse. Note the similarity in the disturbances of respiratory activity between the mice during exposure to 5 μM bicuculline.

Role of $GABA_A$ receptors for respiratory rhythm generation

In vivo experiments.

Neonatal mice. In five neonatal mice bicuculline (1–20 μM) applied to the ventral surface of the brainstem showed a tendency to increase inspiratory time but this was not found to be significant (Table 2; Figs 4 and 5). However, a dose-dependent increase in the amplitude of inspiration (4–175%) and cycle length (7–43%) were observed (Table 2; Figs 4 and 5). In all experiments, and at all doses of bicuculline (1–20 μM), intermittent disturbances and temporary loss of the normal respiratory activity were induced (Fig. 4). These became more frequent and pronounced at higher doses and included large-amplitude, short-duration breaths/bursts occurring at a frequency of 2–3·5 Hz and superimposed on a maintained inspiratory period. The data presented in Fig. 5 do not include these irregularities. All effects of bicuculline were fully reversible following irrigation of the medulla with ACSF. Doses of bicuculline below 20 μM are relatively specific in antagonizing the $GABA_A$ receptor without inducing adverse effects on membrane excitability (J. Champagnat, personal communication).

Mature mice. Bicuculline decreased the mean duration of the inspiratory phase in the phrenic nerve discharge (by 28%) whereas the amplitude and rate of rise of the inspiratory ramp increased by 47 and 21%, respectively, in a dose-dependent manner ($n = 5$; Table 2; Figs 3, 4 and 5). In addition, in all mature mice there was an enhancement of postinspiratory activity either in amplitude or duration (Fig. 3), and a dose-dependent decrease in cycle length (15–38%; Table 2; Figs 4 and 5). The resultant effect was a reduction in the length of the stage 2 expiratory phase. This pattern of response is similar to that reported in the mature rabbit (Schmid *et al.* 1991*a*). As with the neonate, bicuculline induced disturbances in phrenic nerve discharge which occurred at all doses. These disturbances were intermittent at low concentrations consisting of periods of tonic discharge spanning up to two and a half respiratory cycles (Fig. 4). However, at higher doses (>5 μM) the frequency of occurrence and amplitude of tonic discharge were

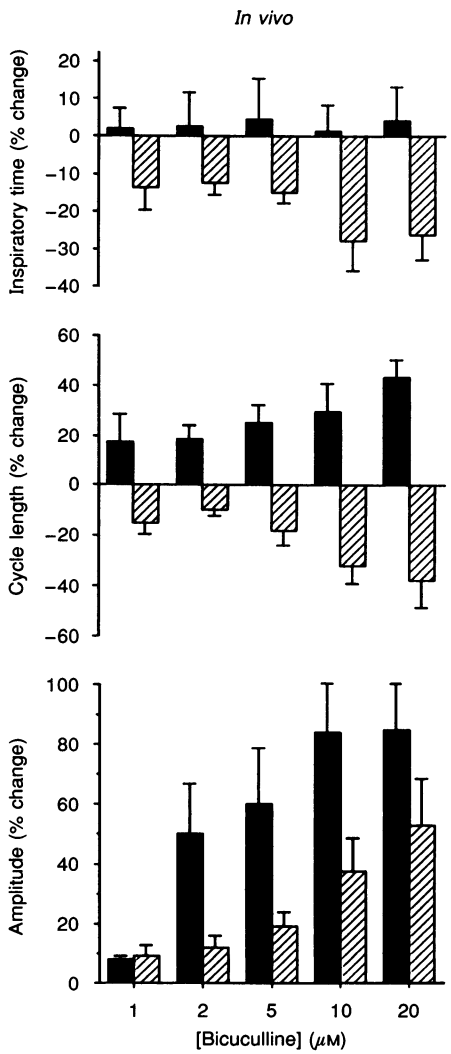

Figure 5. The dose dependence of the bicuculline-induced responses of respiratory variables from five neonatal and mature mice

The induced changes in respiration are expressed as percentage changes (± S.D.). ■, neonates (P2–6); ▨, mature mice (P16–32).

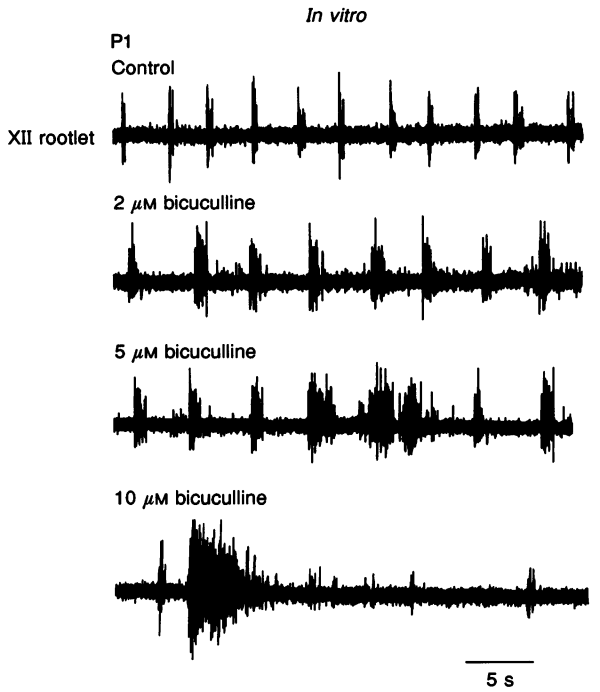

Figure 6. The *en masse* activity of a hypoglossal rootlet recorded from a tilted sagittal slice of a neonatal mouse shows the respiratory motor output response to bath administration of different doses of bicuculline

Note the increase in burst duration and slowing of the rhythm which were also seen *in vivo*. At relatively modest doses, severe disruption to the rhythm and pattern occurred and are consistent with effects seen *in vivo*. Compare with *in vivo* findings of Fig. 4 (left panel). XII, hypoglossal.

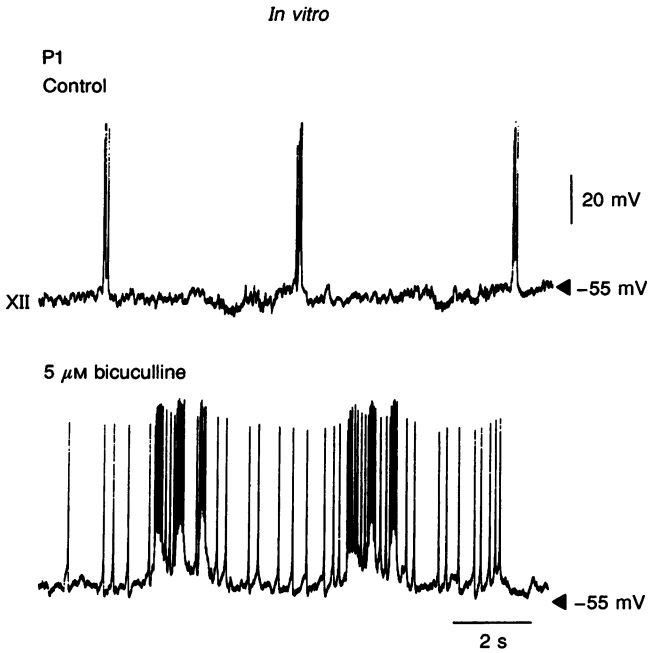

Figure 7. Whole-cell recording of a respiratory rhythmic hypoglossal cell (XII) from a neonatal mouse *in vitro*

The development of maintained depolarizing potentials and oscillating discharges was induced with relatively low concentrations of bicuculline. Compare with phrenic nerve responses of Fig. 4 (left panel). R_m, 210 MΩ.

increased (Fig. 4). In addition, prolonged inspiratory periods or apneustic-like discharge were also observed. As seen in Fig. 4, there were short, high-amplitude bursts superimposed on the apneustic discharge, occurring at a frequency of 3–5 Hz, which were also seen in thoracic movements.

***In vitro* experiments.** Measurements made with either sharp microelectrodes or patch pipettes revealed no consistent differences in the resting membrane potentials (range, −50 to −65 mV) or input resistance (range, 175–215 MΩ) of rhythmic XII neurones in slices from neonatal and mature mice. Furthermore, there was no correlation between the response of the neurone and the depth at which it was located within the slice.

Neonatal mice. The pattern of response of rhythmic XII neurones during exposure to bicuculline was similar under all recording approaches, i.e. extracellularly either as a single unit ($n = 3$) or *en masse* via a rootlet ($n = 3$), and intracellularly using a microelectrode or patch pipette ($n = 4$). The only measurable difference to the *in vivo* data described above was that, *in vitro*, the increase in burst duration (i.e. inspiratory period) was significant in the presence of bicuculline, reaching a maximal mean value of 66% with 10 μM (Table 2; Figs 6 and 9). Since *in vivo* there was no significant change in the duration of inspiratory activity this might reflect a major role of lung stretch afferents, not present *in vitro*, acting to terminate inspiration, which would be consistent with the powerful inhibitory vagal reflexes in newborn mice (Paton &

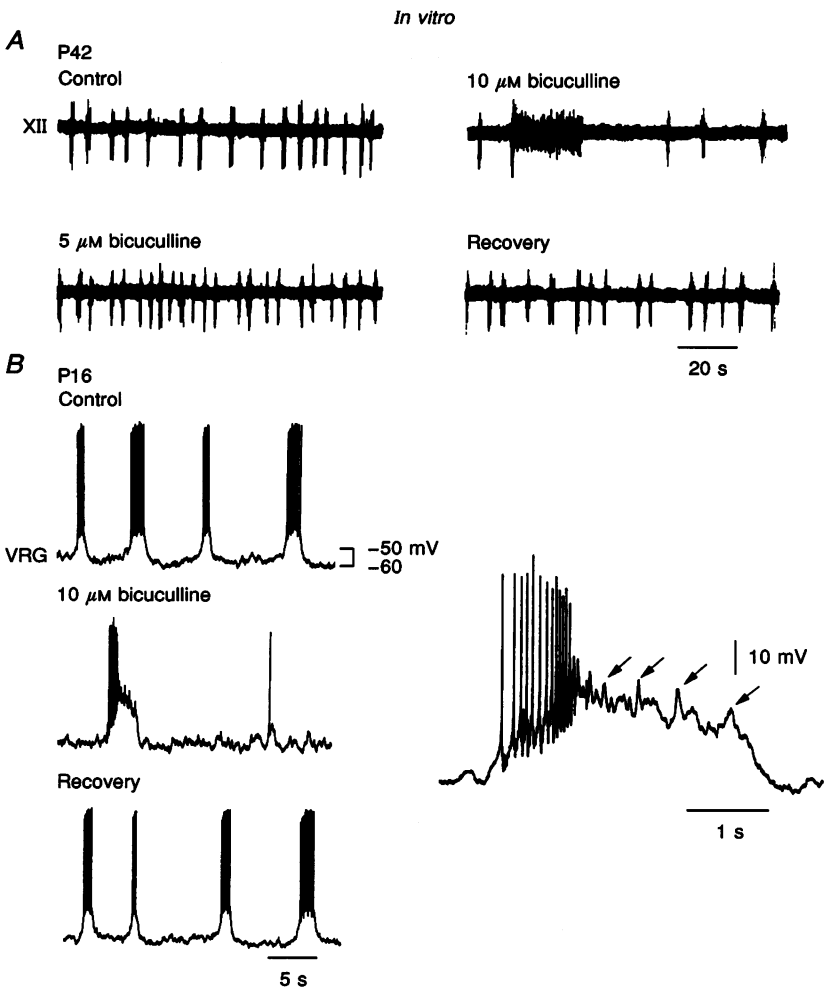

Figure 8. Effect of antagonizing $GABA_A$ receptors on rhythmic discharge of a hypoglossal (XII) and ventral respiratory group (VRG) neurone recorded in a tilted sagittal slice from mature mice

In *A*, the burst duration decreased and frequency increased at low concentrations (up to 5 μM), whereas there was a severe disruption of the rhythm at higher doses including 'apneustic' discharges followed by a prolonged interburst interval. *B* shows a similar response of a rhythmic VRG neurone recorded with a patch pipette (R_m, 180 MΩ). Note the maintained depolarization on which occurred small-amplitude, longer-duration spikes (arrowed). Note the similarity of the response to that seen *in vivo* (Fig. 4).

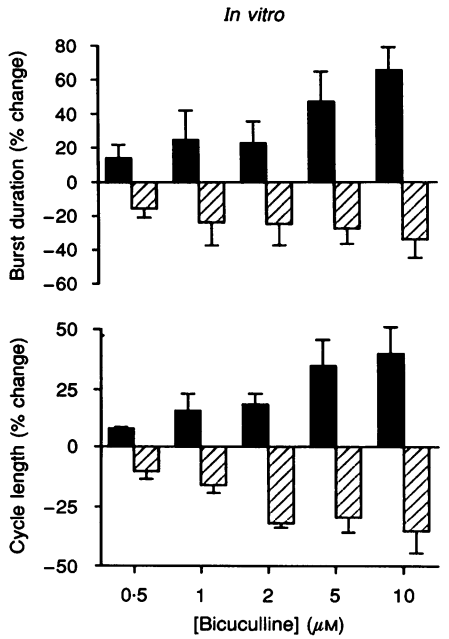

Figure 9. Burst duration and cycle length responses during bath administration of various doses of bicuculline to tilted sagittal slices from neonatal and mature mice

The data are normalized and show percentage changes (± S.D.) for neonates (■; $n = 10$) and mature mice (▨; $n = 12$). There were also comparable increases in intraburst firing frequency in neonatal (35%) and mature (33%) mice during administration of 5 μM bicuculline. Compare Fig. 5.

Richter, 1995). As found *in vivo*, cycle length increased by 16–42% in a dose-dependent manner in most units (Table 2; Figs 6 and 9) in tilted sagittal slices from neonates. In addition, intraburst firing frequency of single XII units increased from 34 ± 5 to 51 ± 8 Hz (35%) during exposure to 5 μM bicuculline. All doses of bicuculline induced both tonic and large-amplitude apneustic-like bursts in rootlet activity followed immediately by a loss of rhythmic activity over several cycles (Fig. 6). In units recorded extracellularly similar effects were seen and included an increase in intraburst firing frequency (20–35%) and a prolongation of burst duration. During apneustic-like discharges recorded from XII rootlets it was not possible to identify whether rhythmic activity was still present. Furthermore, it was apparent that the apneustic discharges appeared to reset rhythmic activity as shown by the constant delay before the onset of the following regular rhythmic burst (Fig. 6) and similar to that reported in the perfused isolated brainstem preparation of the adult rat (Morawietz, Kuwana & Richter, 1993). Intracellular analysis of rhythmic XII neurones exposed to bicuculline (0·5–5 μM) revealed an increase in the amplitude (2–4 mV) and duration of rhythmic drive potentials and an increase in amplitude and number of synaptic potentials, often leading to tonic activity in the interburst interval (Fig. 7). The membrane input resistance of rhythmic XII neurones decreased (by 15–22%), which might be explained, in part, by the increase in synaptic activity observed; similar, in this regard, to the effect of bicuculline on spinal motoneurones (Krejevic, Puil & Werman, 1976). Furthermore, the development of maintained depolarizations (5–22 mV), ranging from 4·8–25·6 s in duration (mean, 13·8 ± 5·9 s), on which were superimposed rhythmic oscillations (0·8–1·7 Hz, 10–20 mV amplitude), were also induced during bath administration of bicuculline (>2 μM; Fig. 7). These maintained depolarizations appeared to be triggered by respiratory rhythmic drive potentials since they occurred at regular intervals (Fig. 7), comparable to the plateau potentials evoked synaptically in lumbar motoneurones in anaesthetized cats (Conway, Hultborn, Kiehn & Mintz, 1988). Prolonged exposure to bicuculline resulted in greatly increased intervals between maintained inspiratory depolarizations/oscillations of XII neurones. These maintained periods of discharge were also seen in two VRG cells in which burst duration increased by 50–150% during exposure to similar doses of bicuculline.

Mature mice. The effects of bicuculline on the discharge of twelve rhythmic XII neurones recorded extracellularly were consistent and significant over a dose range of 0·1–10 μM (Table 2; Fig. 9). At concentrations >10 μM inconsistent responses in burst duration (increase, $n = 4$; decrease, $n = 3$; no change, $n = 2$) and cycle length (increase, $n = 4$; decrease, $n = 5$) were observed in nine rhythmic XII neurones tested. The response pattern at low doses was comparable to that seen *in vivo* and included a dose-dependent decrease in burst duration (up to 27%) and cycle length (up to 36%; see Table 2 and Figs 8 and 9). In addition, there was an increase in intraburst firing frequency from 9 ± 2 to 13·5 ± 3 Hz (60%) with 5 μM bicuculline. In addition, doses of bicuculline exceeding 2 μM induced intermittent but major disruption to rhythmic activity including bursts of long duration (18–25 s) and of a higher intraburst firing frequency compared with controls (Fig. 8*A*), followed by

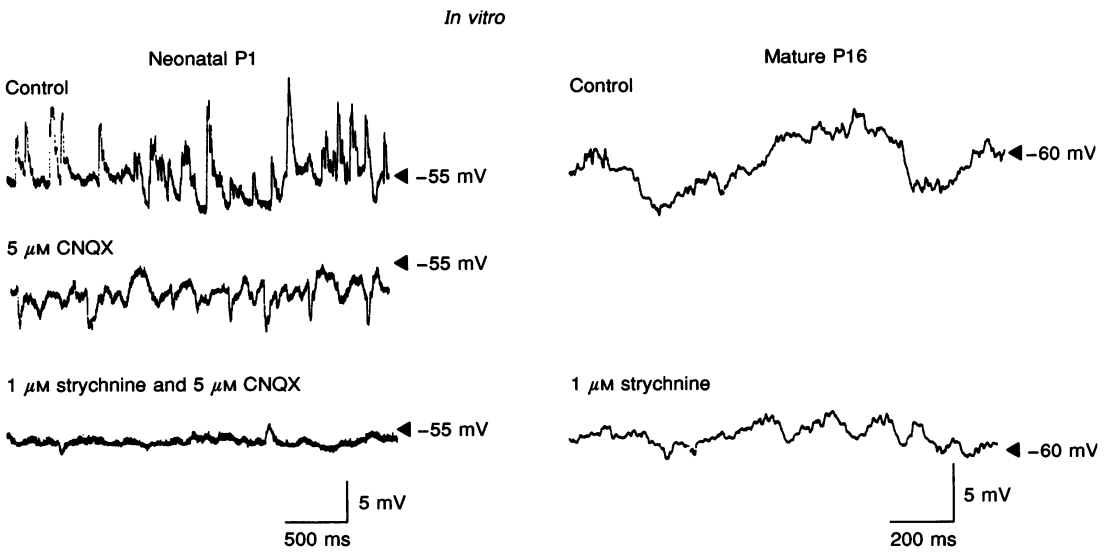

Figure 10. Whole-cell recordings from VRG neurones indicate that on-going IPSPs are sensitive to strychnine in slices prepared from both neonatal and mature mice

Note that in neonatal animals there is a predominance of EPSPs masking IPSPs which were only clearly visible following administration of 5 μM CNQX.

quiescence (i.e. no activity) lasting one and a half to four cycles. These intermittent disruptions were not included in the quantitative analysis of Fig. 9. Furthermore, these disruptions were not confined to XII neurones but were also seen in recordings from rhythmic VRG neurones (Fig. 8*B*). Intracellular analysis of VRG neurones showed that bicuculline induced maintained depolarizations causing an inactivation of fast spikes and the occurrence of low-amplitude (8–12 mV) long-duration spikes (55–95 ms; see Fig. 8*B*). These depolarizations were typically 3–7 s long, had a maximal amplitude of 25 mV and were followed by a period (typically the length of two to three cycles) without the occurrence of a rhythmic burst. All responses were fully reversible during washout of bicuculline.

Summary of bicuculline effects

The data described above indicate that respiration in both neonatal and mature mice is dramatically affected by relatively low concentrations of bicuculline. Antagonism of $GABA_A$ receptors under both *in vivo* and *in vitro* conditions produced alterations in respiration which were opposite in response patterns between neonatal (i.e. increase in burst duration and cycle length) and mature (decrease in burst duration and cycle length) mice. In addition, bicuculline induced maintained depolarizations in both immature and mature XII and VRG neurones, which might explain the apneustic-like discharges recorded from XII rootlets *in vitro* and phrenic nerve *in vivo*.

Sensitivity of on-going IPSPs in immature VRG neurones to strychnine and bicuculline

The sensitivity of on-going spontaneous IPSPs was studied in VRG neurones to assess whether there were any age-dependent changes in synaptic activity that might account for the contrasting effects of strychnine and bicuculline on respiratory rhythm generation in the maturing mouse.

In eleven immature VRG neurones recorded *in vitro* one of the most striking observations was the predominance of EPSP activity which in most neurones ($n = 9$) almost masked the occurrence of IPSPs (Fig. 10). (This observation was made from neurones recorded either using an infrared video system (see Methods) or blind-patch techniques.) In contrast, in slices from mature mice VRG neurones received equal numbers of IPSPs and EPSPs and the occurrence of summating IPSPs was more apparent ($n = 5$; Fig. 10).

In order to assess whether there was an age-dependent change in the sensitivity of IPSPs to bicuculline and/or strychnine, IPSPs were first unmasked and isolated pharmacologically by applying 1–5 μM CNQX to slices from neonatal mice. Following this intervention neurones hyperpolarized between 5–8 mV and most fast excitatory potentials were abolished (Fig. 10). Membrane input resistance increased from a mean of 190 ± 12 to 242 ± 18 MΩ (or 26·7%) indicative of a substantial α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor synaptic input. The reversal potential of IPSPs

was measured at -75 ± 2 mV. In nine cells tested the amplitude and number of IPSPs were reduced by either addition of strychnine (0·5–2 μM; see Fig. 10) or bicuculline (1–5 μM). A combination of both anatagonists abolished most IPSPs. These data indicate that glycine and $GABA_A$ receptors located on VRG neurones of neonatal mice are sensitivite to strychnine and bicuculline, respectively.

DISCUSSION

The present study has taken advantage of the recently developed tilted sagittal brainstem slice as a preparation that permits the study of maturational changes occurring within the isolated, but functionally intact, medullary respiratory network of mammals (Paton *et al.* 1994*b*). In an attempt to prove the functional significance of our *in vitro* findings, similar investigations were made *in vivo*. Our data demonstrate a profound increase in the dependence upon glycine-mediated inhibition for respiratory rhythm generation during maturation of the respiratory network. In addition, we found that $GABA_A$ receptors seem to play an important role from birth onwards in both rhythm and pattern generation as well as stabilizing the respiratory network.

Considerations of the tilted sagittal slice

Based on the relative size of the brainstem in neonatal and mature mice it should be recognized that there may be differences in the anatomical structures present within slices from mice of different ages. Although in the present study we partially compensated for this by employing thinner slices from neonatal mice (see Methods) we cannot rule out the possibility that the different responses observed following glycine and $GABA_A$ receptor blockade may reflect differences in anatomical content of the slices. However, this is strongly opposed by comparable findings *in vivo*. It should also be recognized that since we were employing thick slices there will be an oxygen gradient through the slice. This is pertinent since synaptic transmission is known to be sensitive to hypoxia and this might change with development (Richter & Ballanyi, 1994). However, our recent evidence indicates that the core of the tilted sagittal slice is not severely hypoxic when certain modifications are made to the slice chamber (see Paton *et al.* 1994*b*); this is thus unlikely to affect the responses observed during blockade of glycine and $GABA_A$ receptors.

Maturational changes in glycine-mediated inhibitory synaptic interactions within the respiratory rhythm generator

The possibility that our data reflect an age-dependent increase in strychnine or bicuculline sensitivity of glycine and $GABA_A$ receptors, respectively, is unlikely since IPSPs present in VRG neurones of neonatal mice *in vitro* were sensitive to low doses of their respective antagonists. Respiratory neurones of mice, therefore, seem to be different to cells within the spinal cord of rats where there was an increase in the sensitivity of the glycine receptors to strychnine during a 14 day postnatal period (Becker, Hoch & Betz, 1988). Our evidence, however, supports an increased involvement of glycine-mediated synaptic inhibition in respiratory rhythm generation in the maturing mouse. Interestingly, the time course for the increase in sensitivity of the respiratory rhythm generator to strychnine corresponds exactly with the developmental changes in the molecular subunit structure of the glycine receptor (Becker *et al.* 1988; Malosio, Pouey, Kuhse & Betz, 1991), known to accelerate the kinetics of the evoked chloride current (Akagi & Miledi, 1988; Takahashi, Momiyama, Hirai, Hishinuma & Akagi, 1992), and its clustering (St John & Stephens, 1993). These maturational changes would appear to augment the efficacy of glycine as a powerful inhibitory neurotransmitter in the mature mammal. In this context, glycine-mediated inhibition is known to play a major role in off-switch mechanisms resulting in the transition of different phases of the respiratory cycle in the mature mammal (Champagnat *et al.* 1982; Ballantyne & Richter, 1984; Haji *et al.* 1990, 1992; Hayashi & Lipski, 1992; Klages, Bellingham & Richter, 1993). Taken together the evidence supports the view of a greater dependence on glycinergic mechanisms for rhythmogenesis in the maturing respiratory network.

Role of $GABA_A$-mediated synaptic inhibition for respiratory rhythm generation in neonatal and mature mice

In neonatal mice the augmenting effect of bicuculline on burst duration in rhythmic XII neurones recorded *in vitro* and amplitude of inspiratory activity (*in vivo*) is consistent with previous studies in other species (opossum: Farber, 1993; rat: Feldman & Smith, 1989; Onimaru *et al.* 1990). Furthermore, in mature preparations the bicuculline-induced increase in frequency of rhythmic discharge observed *in vivo* and *in vitro*, and the enhanced amplitude and postinspiratory activity in the phrenic nerve recorded *in vivo*, are similar to reports in the adult rat (Hayashi & Lipski, 1992) and rabbit (Schmid *et al.* 1991*a*). The increase in the ramp inspiratory component of phrenic nerve discharge together with the reduction in inspiratory and expiratory phases seen in mature mice during bicuculline administration in the present *in vivo* and *in vitro* studies were also a feature of phrenic nerve responses in adult rabbit (see Schmid *et al.* 1991*a*). Based on these observations, $GABA_A$ receptors would appear to play an important part in pattern generation and in particular in termination of inspiratory activity.

A difference between the present study and previous investigations is our finding of a decrease in frequency of

respiratory activity in neonates produced with bicuculline (1–10 μM). Feldman & Smith (1989) applied high doses of bicuculline (500 μM) to the brainstem–spinal cord preparation of the neonatal rat and described no change in frequency of rhythmic C4 discharge whereas Onimaru *et al.* (1990) reported an increase in rate with 10 μM bicuculline. The reason for this discrepancy and the difference from the results of the present study remains unclear. Plausible explanations for a slowing of rhythmic activity during $GABA_A$ blockade might be: (1) disinhibition of a tonically active inhibitory input impinging on the rate-generating network, (2) inadequate membrane hyperpolarization for removal of inactivation of low-threshold calcium currents important for rebound excitation of rhythm-generating neurones (see Richter *et al.* 1992), (3) bicuculline-induced depolarization block of some respiratory neurones, as seen in the neonatal opossum (Farber, 1993), or (4) a preparation-related difference.

Since major disruption of rhythmic activity in XII and VRG neurones was seen at relatively modest concentrations of bicuculline (i.e. 1–5 μM), it is argued that these disturbances were due, at least in part, to the specific blockade of $GABA_A$ receptors. However, it is well known that high doses of bicuculline, such as those used by Feldman & Smith (1989; 500 μM), can produce epileptic discharge (e.g. Schwarzkroin & Prince, 1978). Thus, the non-specificity of this drug has made it difficult to assess accurately whether there is a maturational change in the bicuculline sensitivity of the $GABA_A$ receptor within the respiratory network. Nevertheless, the finding in both *in vivo* and *in vitro* experiments that low concentrations of bicuculline produced major disturbances to the respiratory activity (i.e. apneusis, tonic activity, rhythm resetting) in neonatal and mature mice implies that $GABA_A$ receptors play a role in the generation and stabilization of the respiratory network from birth onwards.

The bicuculline-induced apneustic and persistent tonic discharge seen in the phrenic nerve activity, and in the respiratory-related thoracic movements of both immature and mature anaesthetized mice *in vivo*, are consistent with the finding of maintained depolarization and oscillations induced in rhythmic XII and VRG neurones in slices from both neonatal and mature mice. Although we did not determine the underlying ionic basis for these changes, we suggest that they might be due to a blockade of tonic $GABA_A$ conductances. They might be triggered by inputs from VRG neurones since the persistent depolarizations occurred regularly and, in slices from neonatal mice, at a comparable frequency to the respiratory rhythm. These maintained depolarizations resemble synaptically evoked plateau potentials found in lumbar motoneurones of anaesthetized cats (Conway *et al.* 1988). In the latter study plateau potentials were due to intrinsic membrane properties (i.e. bistability) and large calcium influxes (see Kiehn, 1991). Indeed, the low-amplitude, long-duration spikes superimposed on plateau potentials of XII and VRG neurones observed in this study are suggestive of a dendritic calcium influx. Such a change in intracellular calcium could lead to activation of potassium and chloride conductances (e.g. Schwindt, Spain & Crill, 1992) and a shunting of rhythmic inputs, which might partly underlie the observed changes in neuronal behaviour.

Evidence for inhibitory synaptic organization of the respiratory rhythm-generating network in the postnatal period

Numerous findings in the present study suggest the development of a functional organization of inhibitory synaptic mechanisms within the respiratory network during the first 2 weeks of life in the mouse. First, the contrasting role of $GABA_A$ receptors between neonates and mature mice; second, the profound increase in strychnine sensitivity of the respiratory rhythm generator during maturation, which occurs over a similar time course to the development of postinspiratory activity and a ramp in the inspiratory phase of the phrenic nerve reported recently (see Paton & Richter, 1995); and third, the predominance of excitatory synaptic potentials seen in VRG neurones of neonates but not mature mice. Indeed, the latter observation suggests that, during maturation of the respiratory network, there is a reduction in excitatory synaptic mechanisms. Moreover, although summating IPSPs are present in some rhythmic VRG neurones of neonatal rats (e.g. preinspiratory neurones, Onimaru *et al.* 1992; tonic expiratory neurones, Smith, Ballanyi & Richter, 1992), these cells are not thought to be necessary for rhythm generation (Smith *et al.* 1991*a*; Smith, Greer, Liu & Feldman, 1991*b*).

The simultaneous development of functional glycinergic synaptic mechanisms, postinspiratory activity and a ramp in the inspiratory phase (see Paton & Richter, 1995), warrants discussion on their possible relationship. There is evidence suggesting an inhibitory role for postinspiratory neurones via a putative glycinergic synapse. A population of postinspiratory neurones (Richter, Ballantyne & Remmers, 1987) are postulated to be inhibitory interneurones and, together with late inspiratory neurones, function to terminate the ramp inspiratory neurones so delaying the onset of discharge of expiratory neurones (so called E2 neurones, see Richter *et al.* 1986, 1992). Furthermore, Champagnat *et al.* (1982) showed that synaptic influences coinciding with the postinspiratory phase on respiratory neurones recorded in the nucleus of the tractus solitarius were blocked with strychnine but not bicuculline. Although indirect, this evidence indicates that postinspiratory neurones are glycinergic. Finally, in the present study, strychnine changed the pattern of the

inspiratory activity from a ramp to a rapid-onset pattern in the mature mouse, characteristic of the *in vitro* neonate (Paton & Richter, 1995), and consistent with the inhibitory influence of postinspiratory activity on early inspiratory neurones in adult cats (Richter *et al.* 1986). Thus, an ontogenetic change in the neural mechanisms underlying respiratory rhythmogenesis might be the functional involvement of postinspiratory neurones; this being the case, a change in the neural organization of the respiratory network would result.

Relevance of increased importance of inhibition within the mature respiratory rhythm generator

Our results have prompted a number of questions concerning the greater involvement of inhibitory circuitry within the maturing respiratory rhythm-generating network. Specifically, the origin of postinspiratory activity remains unresolved and the possibility that postinspiratory neurones are responsible for the increased sensitivity to strychnine awaits experimental investigation. However, it appears that maturation of the respiratory rhythm generator involves an increase in the emphasis for fast inhibitory synaptic mechanisms during the first 2 weeks of life in the mouse.

The development of the respiratory network, i.e. becoming more dependent upon inhibitory mechanisms and including a postinspiratory phase, may provide both stabilization and plasticity simultaneously. This must be vital for the survival and adaptation of the maturing animal to its new and changing environment. Stabilization is essential since peripheral afferent traffic, exclusively excitatory, will increase during the maturational period. The introduction of a three-phase oscillator may permit more precise and versatile reflex adjustment of ventilation. In addition, the network might be more sensitive to neuromodulatory substances as well as permitting complex reflex changes (e.g. chewing/swallowing) and behavioural adjustment such as vocalization.

In conclusion, from a comparison of our *in vivo* and *in vitro* data, the tilted sagittal slice proves to be a valuable preparation for studying ontogenetic changes in both cellular and synaptic mechanisms in the developing mouse.

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