Postnatal changes in the mammalian respiratory network as revealed by the transverse brainstem slice of mice

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1. Spontaneous rhythmic activity in hypoglossal (XII) rootlets is generated at all postnatal stages from postnatal day (P) 0 to P22 in the transverse brainstem slice of mice containing the pre-Bötzinger complex (PBC). The PBC is known to be a region essential for respiratory rhythm generation. It contains neurones generating periodic bursts that occur in synchrony with rhythmic XII activity. This synchrony indicates that the rhythmic PBC activity generated by the transverse slice is the central respiratory rhythm.

2. The strength of coupling between XII bursts and PBC bursts decreased during early postnatal development. In younger mice (P0–4) each burst in XII rootlets corresponded to one burst in the PBC. In older mice (P5–18) one burst in XII rootlets occurred only every third to fourth burst in PBC neurones.

3. Cycle length and burst duration of rhythmic XII activity did not change significantly during the first three postnatal weeks. However, the pattern of XII bursts changed from decrementing (P0–7) to bell shaped (P8–18) while the rate of rise of XII bursts decreased significantly.

4. The rate of rise of rhythmic depolarizations in neurones of the PBC discharging in phase with XII bursts ('inspiratory neurones') decreased with postnatal development. During interburst intervals, membrane potentials of neurones of older mice (P6–18) were characterized by waves of synaptic input that were not observed in neonatal animals (P0–5).

5. Blockade of glycine receptors by strychnine increased the frequency of rhythmic XII activity in neonatal and older mice (P0–22). Although in expiratory PBC neurones glycinergic transmission was blocked at 10 μM strychnine, in inspiratory PBC neurones and XII rootlets even higher concentrations of up to 50 μM strychnine failed to abolish rhythmic activity.

A complex reorganization of neuronal structures characterizes the early postnatal development of the mammalian brain. Changes in the distribution of neurotransmitters, transmitter receptors and ion channels seem common, and for rats and mice it has also been demonstrated that the molecular composition of receptor and ion channels changes postnatally in many parts of the brain (Becker, Hoch & Betz, 1988; Betz, 1991; Kleckner & Dingledine, 1991; Laurie, Wisden & Seeburg, 1992; Takahashi, Momiyama, Hirai, Hishinuma & Akagi, 1992; Smart, Xie & Krishek, 1994). As a consequence of this molecular reorganization, physiological properties of various voltage- and ligand-gated receptors and channels change (Ben-Ari, Cherubini & Krnjevic, 1988; Ben-Ari, Cherubini, Corradetti & Gaiarsa, 1989; Mynlieff & Beam, 1992; Smart *et al.* 1994; Viana, Bayliss & Berger, 1994). This may have important implications for the postnatal development of brain functions. In fact it is well established that several brain functions do change postnatally. For example, the respiratory system undergoes drastic functional changes during postnatal development of various mammalian species. Alterations have been demonstrated in the motor pattern (Smith, Greer, Liu & Feldman, 1990), in the electrophysiological properties of phrenic and hypoglossal motoneurones (Cameron, Jodkowski, Fang & Guthrie, 1991; Viana *et al.* 1994), in chemoreflexes (Jansen & Chernick, 1983), in properties of the respiratory system during sleep and wakefulness (Guthrie, Standaert, Hodson

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& Woodrum, 1980; Haddad, Gandhi & Mellins, 1982; Gould, 1983), in ionic homeostasis (Trippenbach, Richter & Acker, 1990) and in the response of the respiratory system to hypoxia (Ballanyi, Kuwana, Völker, Morawietz & Richter, 1992). Although there is a great clinical and basic scientific interest in understanding neuronal mechanisms involved in the postnatal development of the respiratory system, currently only a few animal models exist which are useful for studying this issue under *in vitro* conditions. This is an important basis for performing detailed cellular, and eventually molecular, analyses of voltage- and ligand-gated receptors involved in the generation of respiratory rhythm generation. As yet most *in vitro* preparations have been used to study the regulation of central respiratory activity in neonatal mammals (e.g. Suzue, 1984; Hilaire, Monteau, Gauthier, Rega & Morin, 1990; Smith *et al.* 1990; Greer, Smith & Feldman, 1991; Smith, Ellenberger, Ballanyi, Richter & Feldman, 1991; Onimaru & Homma, 1992; Funk, Smith & Feldman, 1993). Two *in vitro* preparations introduced to study the older respiratory system (the tilted-sagittal slice and the perfused brainstem preparation) have certain experimental limitations. In the perfused brainstem preparation of adult rats (Ballanyi *et al.* 1992; Hayashi & Lipski, 1992) or guinea pigs (Morin-Surun, Boudinot, Sarraseca, Fortin & Denavit-Saubie, 1992) the situation is similar to *in vivo* conditions and it is impossible to control precisely the extracellular environment. Thus, developmental changes in sensitivity or specificity of receptors and channels cannot be assessed. Although easy access to the extracellular space is permitted in the tilted-sagittal slice of developing mice and rats (Paton, Ramirez & Richter, 1994; Paton & Richter, 1995*a*, *b*), this preparation does not include rootlets of cranial nerves to record rhythmic motoneurone population activity indicating medullary respiratory output activity (Suzue, 1984). Therefore, activities of single XII neurones have to be taken, which are not defined and difficult to relate to specific respiratory phases (Withington-Wray, Mifflin & Spyer, 1988). Identification of respiratory neurones is therefore questionable as respiratory phases remain unknown.

To overcome these limitations the transverse rhythmic slice, originally described by Smith *et al.* (1991) for neonatal rats, was adapted for mice. The transverse slice generates spontaneously rhythmic activity at all postnatal stages from P0–22 (Funk, Smith & Feldman, 1994; Ramirez, Quellmalz & Richter, 1995). This preparation captures the pre-Bötzinger complex (PBC), a region which is essential for the generation of the respiratory rhythm (Smith *et al.* 1991). Contained within this preparation is also the hypoglossal motor nucleus. Its output can be recorded as motoneurone population activity from rootlets of the XII nerve which are rhythmically active in a well-defined phase relationship to C4 spinal–phrenic nerve respiratory activity (see also Suzue, 1984; Smith *et al.* 1990). In this report, we demonstrate that rhythmic respiratory activity is maintained in a transverse brainstem slice from mice up to an age of 22 days. Over this time period changes occur in the respiratory motor pattern of the XII and in the pattern of activity of neurones within the respiratory network. We propose that these changes reflect ontogenetic changes of the central respiratory network which can be studied in the transverse slice under *in vitro* conditions.

METHODS

Preparation

Male and female mice (MRI-1 and Bahabor: postnatal day (P) 0–22) were deeply anaesthetized with ether and decapitated at the C3–C4 spinal level. The brain and upper cervical spinal cord were isolated in ice-cold artificial cerebrospinal fluid (ACSF) that was bubbled with Carbogen (95% O_2 and 5% CO_2). Following transverse transection of the neuroaxis at the level of the inferior colliculus, the cerebellum was removed to isolate the brainstem. The brainstem was glued with cyano-acrylate vertically with its rostral end up and its dorsal side attached to an agar block which was mounted on a glass block. The preparation was secured in a vibratome with the rostral end tilted at an angle of 20 deg to the plane of the blade. This angle was necessary to catch the projections from PBC to the XII nucleus and from the XII nucleus to the rootlets. The brainstem was sectioned serially from rostral to caudal. Upon reaching the caudal end of the aquaeduct, thin slices of 100–200 μm thickness were cut and discarded until the rostral boundary of the PBC was seen. This region was characterized by cytoarchitectonic landmarks clearly visible under the dissection microscope, such as inferior olive (IO), nucleus of the solitary tract (NTS), hypoglossal nucleus (XII) and nucleus ambiguus (NA), but there was no longer the facial nucleus. At all postnatal stages examined in this study, the rhythmic slice was contained within the next 650–700 μm of tissue (Fig. 1*A*). This slice was immediately transferred into the recording chamber, submerged under a stream of ACSF (temperature, 29 °C; flow rate, 10 ml min^{-1}). After stabilizing for 30 min in ACSF containing 3 mM K^+, first rhythmic discharges could be recorded from XII rootlets which, however, were either irregular or disappeared after a period of approximately 30 min. Thus, we routinely raised the potassium concentration of the ACSF to 8 mM over a period of another 30 min in order to obtain regular rhythmic activity lasting for up to 13 h. The use of elevated K^+ concentration to maintain oscillatory activity has previously been reported for neonatal slice preparations (e.g. Smith *et al.* 1991).

There was no obvious difference noted in the viability of the preparations at different ages. However, at ages older than P20 it got increasingly difficult to obtain rhythmic activity in XII rootlets. This may be due to the difficulty to capture the PBC, the hypoglossus nucleus and the connections from PBC to the XII nucleus and from the XII nucleus to the rootlets in one 700 μm slice. In the slices from adult mice (older than 6 weeks) that did not exhibit rhythmic activity in XII rootlets we were still able to record stable rhythmic activity in the PBC, indicating that a limited O_2 diffusion or increased oxygen demands are most probably not the reason for a lack of motoneurone population activity in XII rootlets in adult mice. Due to the missing motoneurone population activity in XII rootlets of adult mice, it was not possible to determine phase relationships of neurones recorded in the PBC. Thus, slices without rhythmic XII rootlet

activity were not further evaluated. Consequently, all 150 rhythmic slices that were included in this study generated rhythmic activity in the XII rootlets. Each slice was used for only one set of experiments.

Recording and data analysis

Activity from the peripheral end of cut XII rootlets was recorded with suction electrodes (Fig. 1*B*, lower trace), amplified (2000 times) and filtered (low pass, 1·5 kHz; high pass, 250 Hz). XII activity was also rectified, low-pass filtered and integrated (Fig. 1*B*, middle trace) using an electronic filter (Paynter filter, set at a time constant, $\tau = 20$–30 ms). Intracellular recordings were obtained from either PBC neurones (Fig. 1*B*, upper trace) or hypoglossal neurones (Fig. 5*D*). Electrodes were positioned in the hypoglossal nucleus which was anatomically visible under a Zeiss binocular microscope (location, see Fig. 1*A*). Similarly the PBC was recognized using anatomical landmarks, such as the Sp5 region and the nucleus ambiguus which were visible under the binocular microscope (Fig. 1*A*). Somatic recordings were clearly distinguished from intra-axonal recordings by the shape of the action potential and presence of synaptic activity. In several instances, correct anatomical location was verified by staining neurones with Lucifer Yellow through the pipette. The recordings were obtained with either fine-tipped or patch electrodes manufactured from filamented borosilicate glass (Clarke GC150F). Fine-tipped electrodes (resistance, 60–100 MΩ) were filled with 2 M potassium acetate and patch electrodes (resistance, 7–8 MΩ) were filled with a solution containing (mM): 140 D-gluconic acid (potassium salt), 1 $CaCl_2$, 10 EGTA, 2 $MgCl_2$, 4 Na_2ATP and 10 Hepes (pH 7·3–7·4).

Patch electrodes with an internal pressure of 30–50 mm Hg were advanced into the PBC or hypoglossal nucleus using a nanostepper. Electrode resistance was continuously measured by applying negative current pulses (−1 nA, 50 ms) in current clamp recordings or negative voltage steps (10 mV, 50 ms) in voltage clamp recordings. Respiratory neurones were identified by recording extracellularly their rhythmic action potential discharges which were synchronized with rhythmic XII nerve activity. The electrode was further advanced in 10 μM steps until the electrode resistance increased by approximately 60%. For seal formation, positive pressure in the pipette was released and gigaseals (1–8 GΩ) were formed by slight negative pressure. The membrane was ruptured to achieve whole-cell configuration by applying brief negative pressure pulses. Membrane potential and currents were measured using an intracellular amplifier (NPI SEC-10L). An Axopatch 200 amplifier (Axon Instruments) was used for voltage clamp recordings. In either case series resistance was completely compensated.

Electrical signals were stored on VCR tape (VR 100A, Instrutech, Great Neck, NY, USA). Data were analysed off-line with two software programs written for IBM-compatible personal computers. For one program raw data were digitized using a DT 2821 interface (Scientific Solutions, Inc., Solon, OH, USA). This program was used to determine average burst duration, cycle length and rate of rise of integrated XII nerve recordings. It was possible to evaluate hundreds of bursts per slice. However, to avoid mistakes in the computer analysing process, only those integrated recordings from XII rootlets were used which were characterized by very good signal-to-noise ratios. All quantitative data are given as means ± standard errors of the mean. Significances were determined by Student's t test. In another computer program, data were digitized using a labmaster interface (Scientic Solutions, Inc.) in order to evaluate the average shape of integrated XII rootlet activity or rhythmic membrane deflections in respiratory neurones. It was possible to obtain the average shape of up to twenty-five events sampled from different slice preparations.

Solution and drugs

ACSF contained (mM): 128 NaCl, 3 (or 8) KCl, 1·5 $CaCl_2$, 1 $MgSO_4$, 24 $NaHCO_3$, 0·5 NaH_2PO_4 and 30 D-glucose equilibrated with Carbogen at 27 °C (pH 7·4). Strychnine was obtained from Sigma.

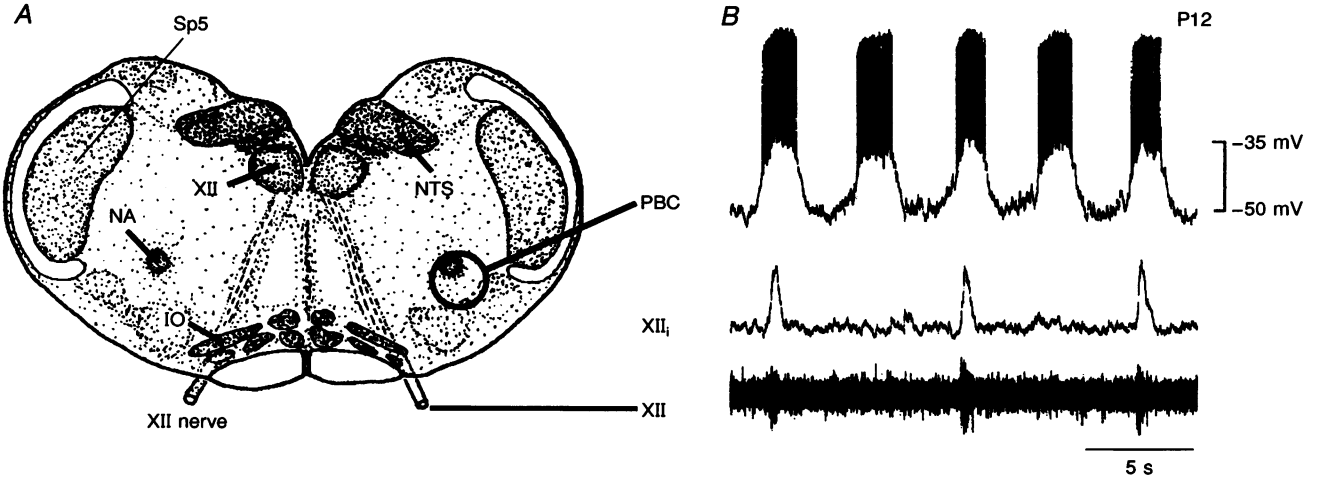

Figure 1. Schematic representation of the transverse brainstem slice obtained from mice (*A*) together with a recording from a rhythmically active neurone (*B*)

A, transverse slice; the location of the pre-Bötzinger complex is indicated schematically by the circle. IO, inferior olive; NA, nucleus ambiguus; NTS, nucleus of the solitary tract; PBC, pre-Bötzinger complex; Sp5, spinal trigeminal nucleus; XII, hypoglossal motor nucleus; XII nerve, hypoglossal rootlet. *B*, whole-cell patch recording from a neurone in the PBC of a P12 mouse (upper trace) obtained simultaneously with a XII rootlet recording using suction electrodes (lower trace). Middle trace shows the integrated XII (XII_i) activity.

RESULTS

Changes in XII rootlet activity

Developmental changes occurring in XII rootlet activity were evaluated by averaging burst duration, cycle length, rate of rise and shape of XII bursts (Fig. 2 for definition). The data were assessed for two age groups: (1) slices obtained during the first postnatal week (P0–7) and (2) those obtained in mice older than one week (P8–18). Between both age groups, there was no statistically significant change in cycle length (Fig. 3*B*, $P = 0{\cdot}21$) and burst duration (Fig. 3*C*, $P = 0{\cdot}37$). However, as also seen in these figures, mean values for cycle length tended to decrease while those for burst duration tended to increase during postnatal development. The tendency towards longer bursts was even more obvious when comparing mean burst duration values of 0- to 3-day-old mice ($0{\cdot}44 \pm 0{\cdot}22$ s, $n = 10$) with those of 12- to 22-day-old mice ($0{\cdot}62 \pm 0{\cdot}2$ s, $n = 10$). This tendency is also seen in the average of twenty-five hypoglossal bursts obtained from five slices from the age groups P0–7 and P8–18.

A more pronounced developmental change occurred in the pattern of XII rootlet discharge. In slices obtained from animals younger than 7 days (P0–7) XII bursts were decrementing with the highest frequency of discharge occurring within the initial 50 ms (Fig. 2*B*). In slices obtained from mice older than 7 days, integrated nerve activity was characterized by a bell-shaped pattern (Fig. 2*D*, upper trace). This change in the XII pattern was documented by the average shape of twenty-five hypoglossal bursts from five slices per age group (Fig. 3*A*). The developmental change was also quantitatively reflected in a significant ($P = 0{\cdot}008$) decrease in the rate of rise of integrated XII bursts by 43·4% (Fig. 3*D*).

Changes in the activity of neurones within the PBC

We evaluated two types of rhythmically active neurones that were intracellularly recorded in the PBC: (1) the so-called inspiratory neurones which were rhythmically active in phase with hypoglossal activity (Fig. 4) and (2) the so-called expiratory neurones which discharged out of the hypoglossal phase (Fig. 5*C*). Although we did not discriminate between different subtypes of inspiratory and expiratory neurones in this study, some obvious developmental changes were seen in the activity of these neurones and their coupling to the XII rootlet activity. In slice preparations from P0–3 mice, inspiratory neurones were characterized by a rapid onset of activity, steep depolarizing and decrementing drive potentials (Fig. 4*A*). Although synaptic input occurred also during the interburst intervals it did not summate to generate long-lasting waves of membrane potential changes (Fig. 5*A*). In our recordings the activity of inspiratory neurones was always coupled in a 1 : 1 manner with the activity recorded

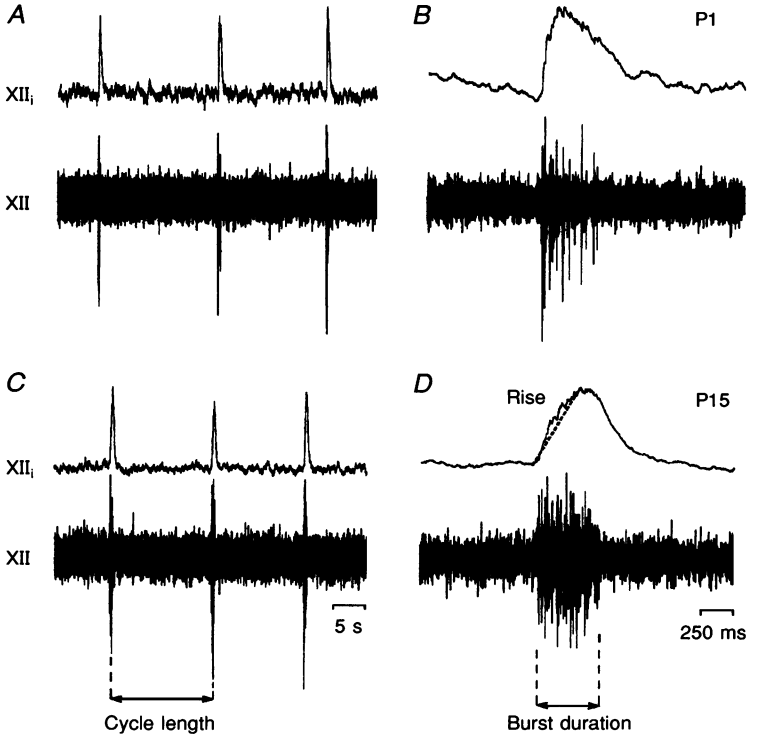

Figure 2. Qualitative changes in rhythmic XII activity

A–D, XII rootlet activity (lower traces) and the integrated signal (upper traces) obtained from slices of a neonatal (P1, *A* and *B*) and older mouse (P15, *C* and *D*). Note that the cycle length (*A* and *C*) and burst duration (*B* and *D*) are qualitatively not different, but the pattern of the XII burst changed, decrementing in the neonate (*B*) and being bell shaped in the older mouse (*D*).

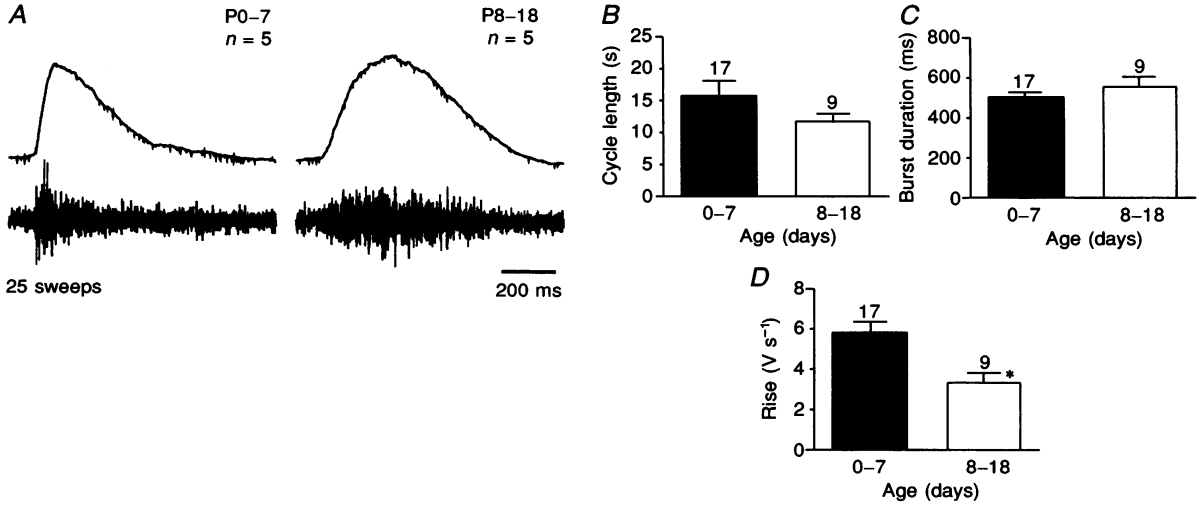

Figure 3. Quantitative changes in the properties of XII activity

A, average bursts for two age groups. Each burst is the average of 25 bursts obtained from 5 slices per age group. *B–D*, histograms demonstrating the absence of an age-dependent effect on cycle length (*B*) and burst duration (*C*), and the developmental change in the rate of rise of the XII burst (*D*). *n* values shown above bars in *B–D*. Mean values were evaluated by averaging 10 cycles per slice. Bars indicate S.E.M., * $P = 0{\cdot}008$.

from XII rootlets: each burst in the PBC corresponded to one burst in XII rootlets (Figs 4 and 5*A*). A 1 : 1 coupling occurred even during long interburst intervals exceeding, 20 s in some slices (Fig. 6 shows data quantitatively evaluated for 6 inspiratory neurones of this age group, ○).

In older mice (P5–18), the onset of activity in inspiratory neurones and the underlying depolarizing drive potentials were less steep compared with the situation in the younger age group (Fig. 4*B*). During interburst intervals, waves of membrane potential changes due to synaptic summation were typical for inspiratory neurones in this older age group (Fig. 5*B*) which in 84% of the neurones ($n = 25$) led to rhythmic oscillations at a relatively short cycle length (Fig. 5*B*). The average cycle length quantitatively evaluated for eleven of these neurones was $3{\cdot}46 \pm 0{\cdot}25$ s. Membrane potentials of PBC neurones oscillated at this relatively high frequency, even if the cycle length of XII rootlet activity was long (Fig. 6, ●, $n = 11$). Thus, although still synchronized, the coupling between rhythmic activity in inspiratory neurones and XII rootlets was less pronounced in slices obtained from mice older than P5. On average, a burst in XII rootlets occurred every 3·5 bursts in inspiratory neurones ($n = 11$). The membrane depolarizations occurring in phase with XII bursts were larger in amplitude than those occurring during the interburst intervals (Fig. 5*B*). All bursts were followed by brief hyperpolarizations but those coinciding with XII rootlet activity were more pronounced than those occurring during the interburst interval (Fig. 5*B*). The developmental changes in rhythmic activity occurred between P4 and P5. A 1 : 1 coupling

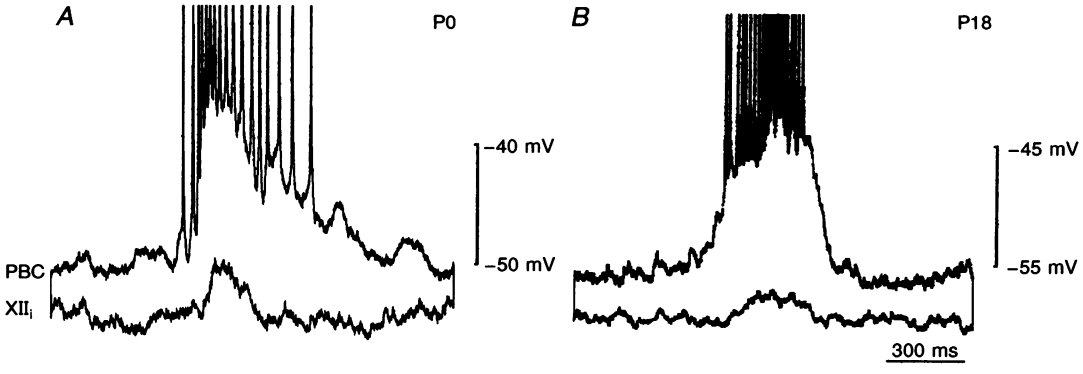

Figure 4. Developmental change in the pattern of activity exemplified by two pre-Bötzinger complex neurones recorded from P0 (*A*) and P18 mice (*B*)

Whole-cell patch recordings from inspiratory neurones (upper traces) were obtained together with integrated XII nerve recordings (lower traces).

occurred in 75% of the neurones ($n = 4$) at P4 but only in 20% of the neurones ($n = 5$) at P5.

Changes in inspiratory neurones were paralleled by similar changes in expiratory neurones. In slices obtained from neonatal mice, expiratory neurones also exhibited a 1 : 1 coupling ($n = 3$) while those neurones recorded from animals older than P5 ($n = 4$) exhibited typically a 3 : 1 coupling. As exemplified in the original recording (Fig. 5*C*), rhythmic hyperpolarizations coinciding with XII bursts were also more pronounced than those occurring in the interburst intervals.

The enhanced depolarizations and/or hyperpolarizations in membrane potentials of inspiratory and expiratory neurones coinciding with XII bursts, seem to be due to an increased number of active neurones in the respiratory network that discharged in phase with XII bursts. We found that 16% of the inspiratory neurones which were recorded in older mice (P8–18) were coupled to hypoglossal activity in a 1 : 1 manner. These neurones might contribute to the increased activity coinciding with XII bursts.

Thus, an important issue arising from these findings is whether increased activity within the respiratory network leads to a suprathreshold activation of hypoglossal neurones and the generation of a burst, while decreased activity in the respiratory network results in a subthreshold activation of the XII neurones which fails to generate a burst in XII rootlets. As shown in the voltage clamp recording in Fig. 5*D* this is indeed the case. This hypoglossal neurone received weak synaptic drive (low amplitude inward current, upper trace) during cycles where no burst was generated in the integrated XII rootlet recording (lower trace) and a suprathreshold synaptic drive which coincided with a burst recorded in the XII rootlet.

Effect of strychnine on rhythmic XII activity and neurones in the PBC

Rhythmic activity was maintained when superfusing transverse slices with ACSF containing strychnine at increasing concentrations (0·5–50 μM). In none of the examined slices which were obtained from mice during the first postnatal week (P0–7, $n = 6$) and from mice between 1 and > 3 weeks of age (P8–22, $n = 10$) were rhythmic XII discharges blocked in the presence of strychnine (Fig. 7*A*). The effect of increasing strychnine concentrations up to 10 μM is shown for various parameters of XII rhythmic activity in the sequential event histograms in Fig. 7*B* for P1 and P12 slices. Nine of these experiments, in which we

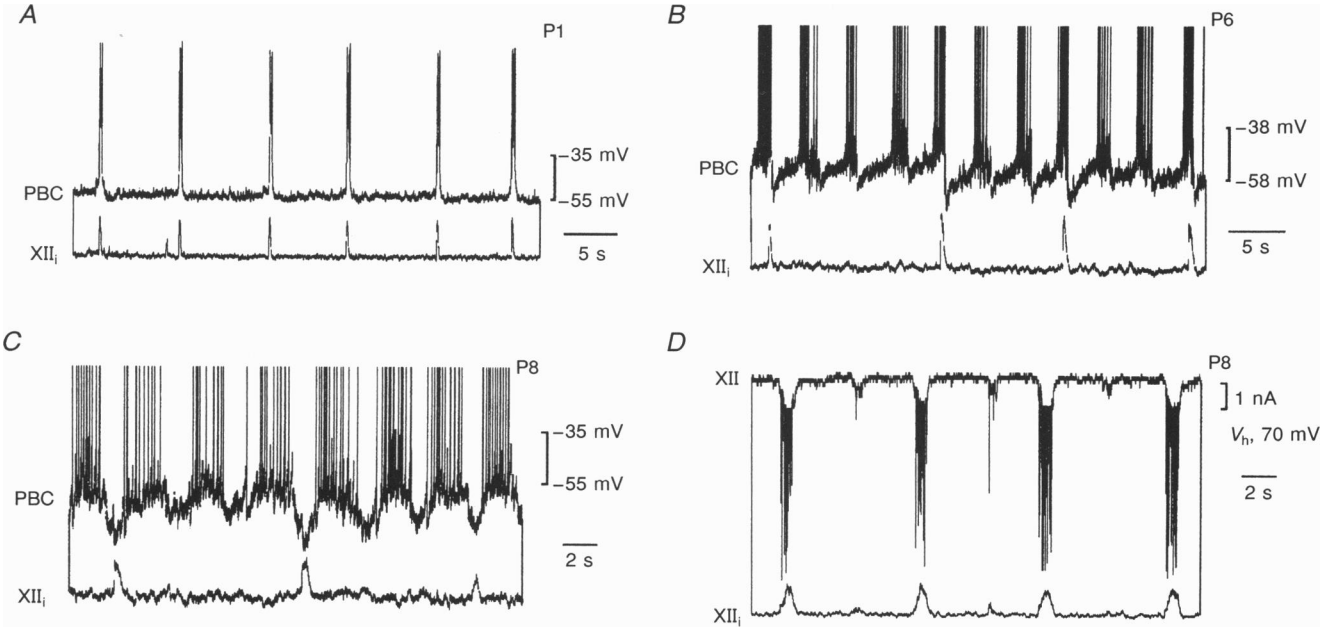

Figure 5. Rhythmic synaptic activation of neurones in the pre-Bötzinger complex and hypoglossal nucleus in relation to integrated XII nerve activity

Whole-cell patch recordings in the bridge-balance mode were obtained from pre-Bötzinger complex (PBC) neurones (upper traces) together with integrated XII nerve recordings (lower traces) in transverse slices from P1 (*A*), P6 (*B*) and P8 (*C*) mice. Note that the coupling between the activity in the PBC and the hypoglossal rootlet changes during postnatal development being 1 : 1 in the neonate (*A*) and variable in older animals (*B* and *C*). *D*, whole-cell patch recording under voltage clamp conditions was obtained from a XII neurone (upper trace) together with integrated XII nerve recordings (lower trace) in a transverse slice from a P8 mouse. Note that the hypoglossal neurone received a weak amplitude synaptic input in the interphase between two bursts of integrated XII nerve activity and a large amplitude synaptic input coinciding with bursts in the hypoglossal rootlets.

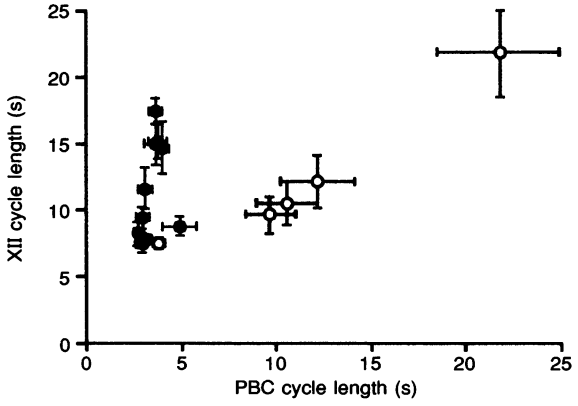

Figure 6. The coupling between the activity in pre-Bötzinger complex neurones and the XII rootlet changes during postnatal development

Comparison of the cycle length in XII rootlets (ordinate) and pre-Bötzinger complex neurones (abscissa) in two different developmental age groups (P0–4, $n = 6$, ○; P5–18, $n = 11$, ●). Note that the coupling between XII and PBC activity was 1 : 1 in the younger age group and variable in the older age group.

examined 1 μM strychnine only, revealed that the frequency of rhythmic XII activity increased by 64 ± 22% in younger mice (P0–7, $n = 3$), and by 16·1 ± 5% in older mice (P8–22, $n = 6$). We observed changes in the duration, amplitude and rate of rise of integrated XII bursts in several slices as shown in Fig. 7. However, these effects were inconsistent and a quantitative evaluation of nine experiments revealed that 1 μM strychnine did not produce a significant effect at either developmental stage.

As expected from the effects described for XII activity, strychnine also induced a decrease in the cycle length of rhythmic activity in PBC neurones that was recorded in slices at the age of P0–4 (Fig. 8*A*). We examined the effect of strychnine at this developmental stage in four inspiratory neurones and observed no obvious change in the shape of rhythmic oscillations (e.g. Fig. 8*B*). The resting membrane potentials in these neurones also revealed no obvious changes.

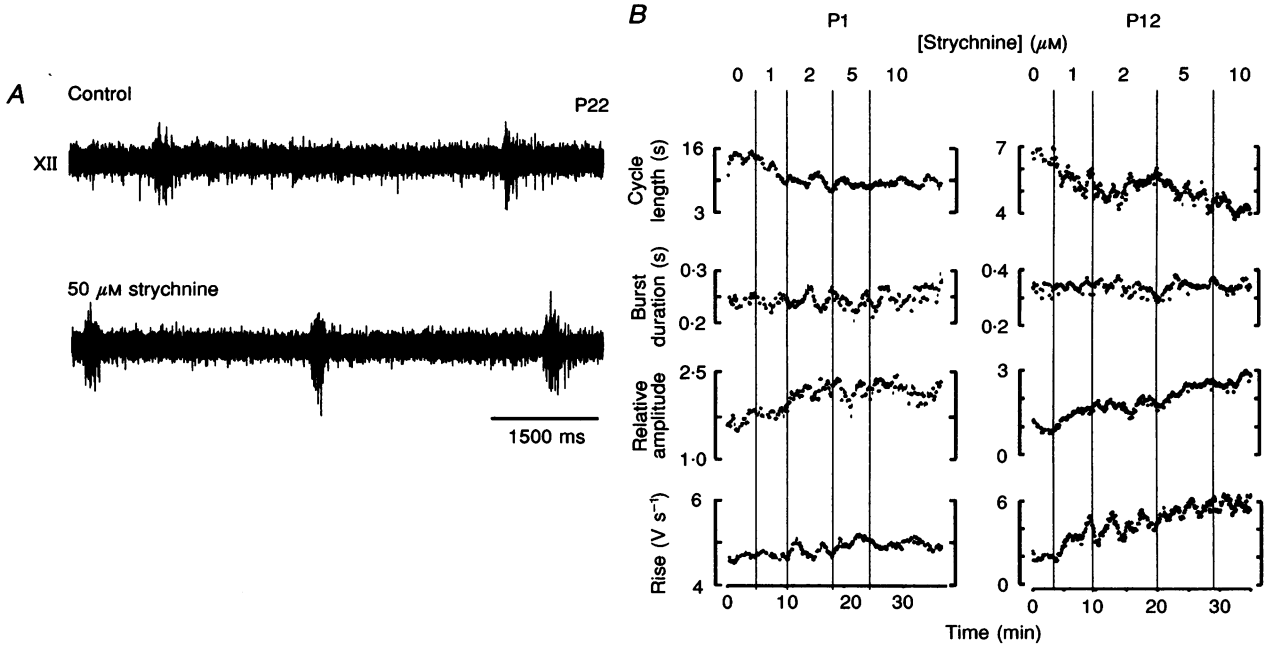

Figure 7. Effect of strychnine on the rhythmic activity in XII rootlets of P22 (*A*), P1 and P12 (*B*) mice

A, the frequency of rhythmic XII activity recorded with suction electrodes in a P22 mouse was increased in the presence of 50 μM strychnine (lower trace) compared with control conditions (upper trace). *B*, sequential event histograms demonstrating changes in cycle length, relative burst duration, relative amplitude and rate of rise over time at increasing concentrations of strychnine (1–10 μM). Amplitude measurements were obtained relative to a threshold set by a window discriminator. Burst duration of integrated XII activity was also measured at a certain threshold which was above randomly occurring activity deflections. Thus values are smaller than the burst duration values when measured at the basis of a burst of XII integrated activity. Note that rhythmic activity in the P1 and P12 mouse was maintained in the presence of strychnine without obvious effects in the cycle–cycle variation of the shown parameters of rhythmic XII activity.

There was a decrease in the cycle length of rhythmic discharges in PBC neurones recorded in older slices (P8–22, Fig. 9). In four inspiratory neurones that were examined, the cycle length decreased on average from 3·7 ± 0·3 s in control to 3·08 ± 0·25 s in 5 μM strychnine. As seen in Fig. 9*A* the coupling between rhythmic activity in the inspiratory neurone and the XII rootlet was not affected by strychnine. For four inspiratory neurones, the coupling was, on average, 1 : 2·93 ± 0·54 during control and 1 : 2·76 ± 0·51 ($n = 25$ cycles) in 10 μM strychnine.The blockade of glycinergic inhibition always caused an increase in the rate of rise and amplitude of rhythmic drive potentials of inspiratory neurones (Fig. 9*B*).

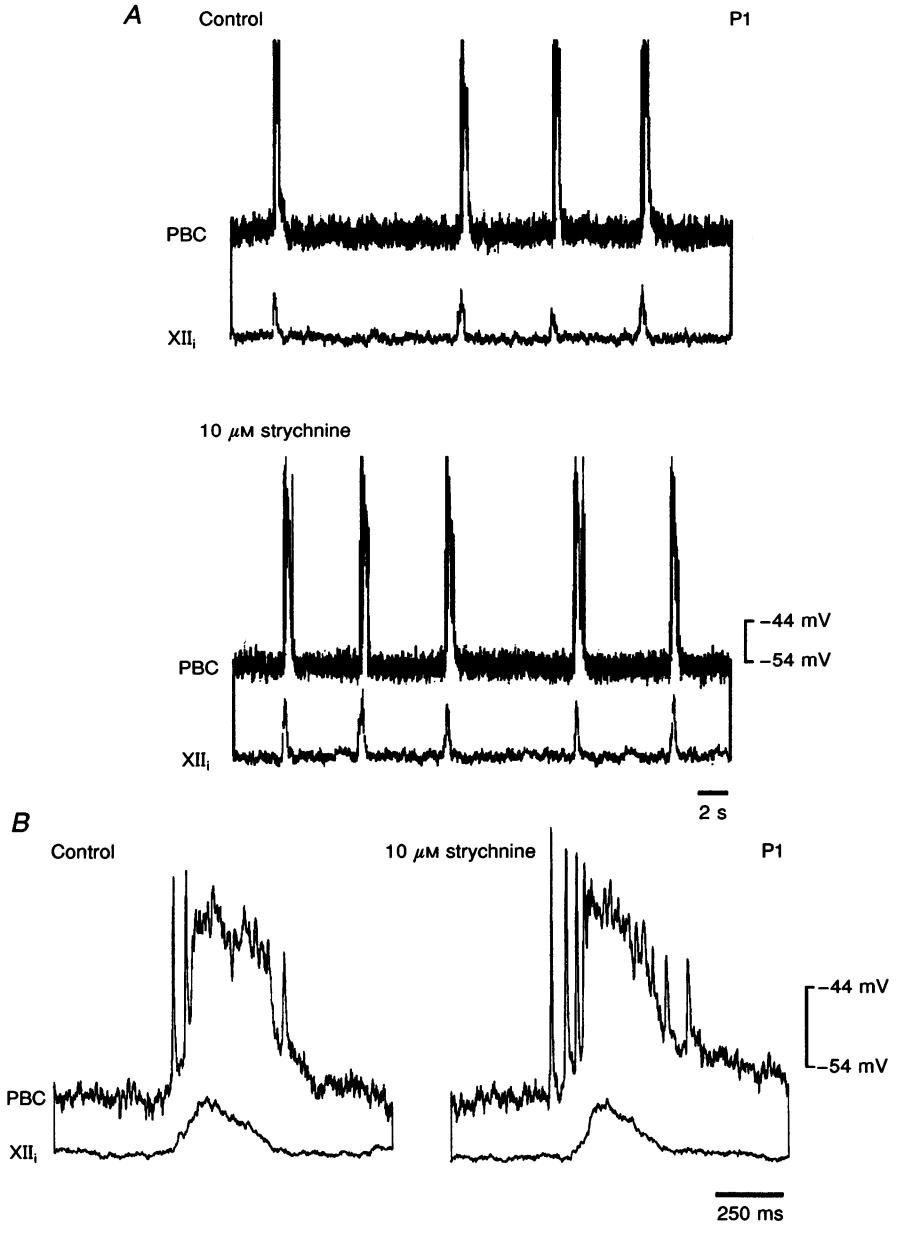

Figure 8. Effect of strychnine on the rhythmic activity in XII rootlets and PBC neurones of a P1 mouse

A, the frequency of rhythmic discharges in the whole-cell patch recording from a PBC neurone (upper traces) and the suction electrode recording from XII rootlets (lower traces) was, in comparison with the controls (upper panels), increased in the presence of 10 μM strychnine (lower panels). The recording was obtained 5 min following the strychnine application. *B*, the shape of rhythmic depolarization in the whole-cell patch recording from a P1 neurone (left panel) was not changed in the presence of 10 μM strychnine (right panel, 5 min after strychnine application). To better reveal the drive potentials Na^+ spike inactivation was induced by overdepolarizing the neurone.

As seen for the inspiratory neurones, strychnine did not have an obvious effect on the resting membrane potential of the expiratory neurones ($n = 4$). However, strychnine greatly reduced the amplitude of rhythmic hyperpolarizations as exemplified in Figs 10 and 11 for neurones from P12 and P8 mice. The hyperpolarizations occurring in the interphase between hypoglossal bursts were more sensitive to strychnine than the hypopolarizations coinciding with the hypoglossal bursts (Fig. 10). By using the onset of the hypoglossal burst as a trigger for averaging the shape of fifteen rhythmic hyperpolarizations, it is possible to demonstrate a dose-dependent strychnine effect for these hyperpolarizations (Fig. 11*B*). The average reveals that the hyperpolarizations consist of two components: (1) a slow initial ('pre-inspiratory') hyperpolarization followed by (2) a fast hyperpolarization that coincided with the occurrence of the XII burst ('inspiratory'). The slow pre-inspiratory component was already blocked at 2 μM strychnine, while higher concentrations of strychnine were also needed to reduce the hyperpolarizations coinciding with the XII burst.

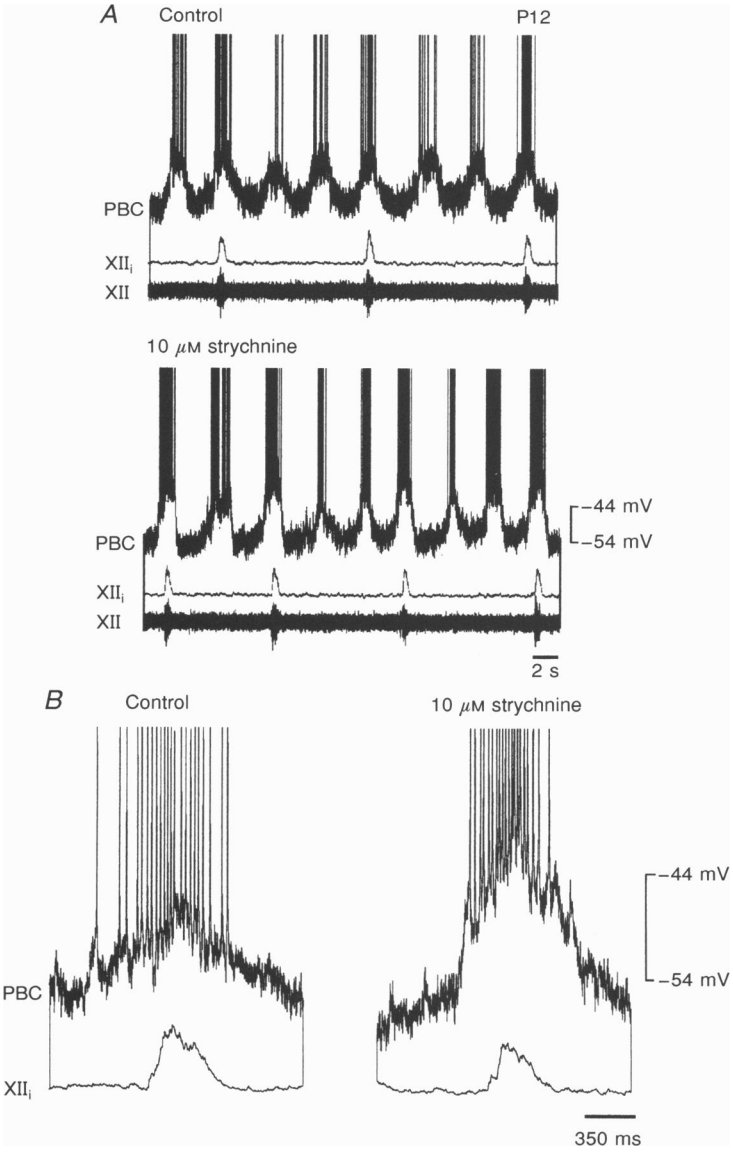

Figure 9. Effect of strychnine on the rhythmic activity in XII rootlets and PBC neurones of a P12 mouse

A, the frequency of rhythmic discharges in the whole-cell patch recording from a PBC neurone (upper traces) and the suction electrode recording from XII rootlets (lower traces) was, in comparison with the controls (upper panels), increased in the presence of 10 μM strychnine (lower panels). The recording was obtained 5 min following the strychnine application. Note that the intensity of bursting increased in the presence of strychnine. *B*, the amplitude of the inspiratory burst in the whole-cell patch recording (left panel) was increased in the precence of 10 μM strychnine (right panel, 5 min after strychnine application).

This component was never completely abolished, suggesting that GABAergic mechanisms are also involved in the generation of this hyperpolarization (P. Telgkamp, J. M. Ramirez, U. J. A. Quellmalz, F. P. Elsen & D. W. Richter, unpublished observation).

DISCUSSION

The transverse slice containing the PBC has initially been developed for neonatal rats (Smith *et al.* 1991; Funk *et al.* 1993). More recently this preparation has been adapted in parallel to this study for neonatal and older mice (Funk *et al.* 1994) which generates rhythmic activity up to an age of 22 days (Ramirez *et al.* 1995). The data presented in this report further indicate that this preparation can serve as a model to study the early postnatal development of the respiratory system. Age-specific patterns of rhythmic XII activity and the responses to strychnine described in this study, are similar to those observed in neonatal and adult *in vitro* and *in vivo* preparations. These aspects will be discussed separately.

Developmental changes in the motor pattern

In this study we observed that the cycle length and the duration of individual XII bursts were not statistically different when comparing P0–7 animals with those of the age of P8–18. There was, however, a tendency towards longer bursts in the older age group which was not only reflected in higher mean values (49% increase when comparing mean burst duration values of P0–3 mice with those of P12–22 mice), but also seen in a comparison between the average shapes of integrated XII bursts obtained from two different age groups, P0–7 and P8–18 (Fig. 3*A*). A relatively small increase of 45% in the duration of inspiratory time has also been described for *in vivo* animals (Paton & Richter, 1995*a*). The relatively small developmental change in burst duration as seen in the transverse slice is certainly closer to the *in vivo* situation than the 625% increase as described for the tilted-sagittal slice preparation (Paton & Richter, 1995*a*). One reason for this discrepancy, might be that developmental changes in the tilted-sagittal preparation were not assessed from the XII motor output, but based on a comparison of single hypoglossal neuronal discharges which are known to exhibit great variability in their activation pattern in adult cats (e.g. Hwang, Bartlett & St John, 1983*a*).

A pronounced developmental alteration was found in the pattern of XII bursts which changed significantly from decrementing (P0–7) to bell shaped (P8–18). Similar changes in the pattern of XII activity have also been observed under *in vivo* conditions, where the rate of rise in neonates was steeper than the rate of rise in older mice (Paton & Richter, 1995*a*). Decrementing XII bursts as described in this study for neonatal mice have previously been observed in rats using different *in vitro* preparations (Smith *et al.* 1990; Smith *et al.* 1991; Funk *et al.* 1993). A bell-shaped pattern of the XII burst as seen in older transverse slice preparations is also typical for adult *in vivo* cats (Sumi, 1963; Hwang *et al.* 1983*a*; Hwang, St John & Bartlett, 1983*b*; Withington-Wray *et al.* 1988). A developmental change seems not to be restricted to the XII motor output, since comparable developmental differences have also been reported for the C4 motoneuronal discharge of vagotomized neonatal and adult *in vivo* rats and mice (Smith *et al.* 1991; Paton & Richter, 1995*a*). Thus, our findings indicate that the developmental changes observed *in vivo* can also been seen in the isolated respiratory

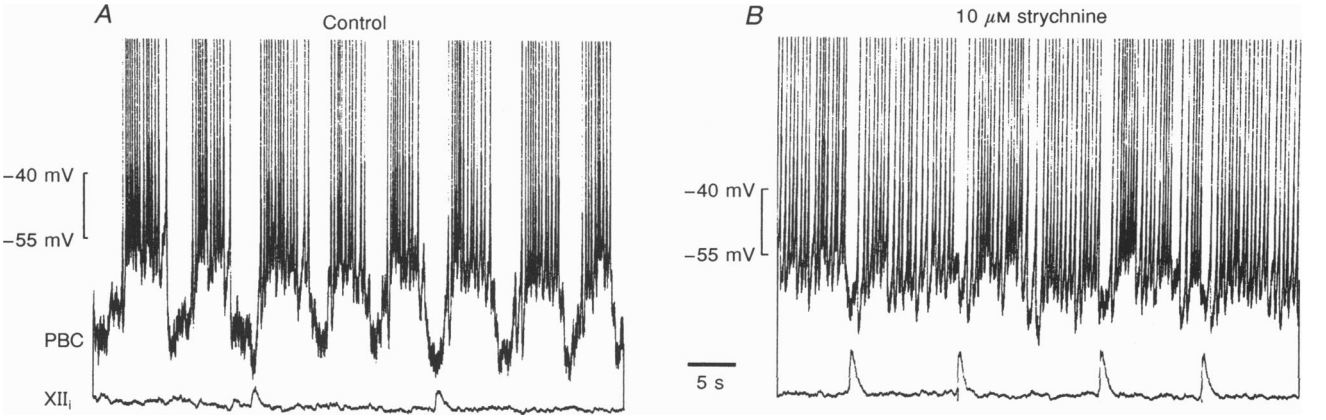

Figure 10. Effect of strychnine on rhythmic activity in an expiratory PBC neurone of a P12 mouse

The phasic hyperpolarization in the PBC neurone (upper traces, whole-cell patch recording) occurring in phase with integrated hypoglossal activity (lower traces) was greatly reduced in the presence of 10 μM strychnine (*B*) compared with the control conditions (*A*). Note that the phasic hyperpolarizations occurring in the interphase between two bursts are more reduced by strychnine than those occuring in phase with hypoglossal activity.

network. The underlying mechanisms are as yet only poorly understood and may involve age-dependent differences in the role of afferent mechanosensory inputs as has previously been discussed by Smith *et al.* (1990). It is also known that various membrane properties of hypoglossal motoneurones change during postnatal development (Viana *et al.* 1994). These changes may contribute to the changes seen in the hypoglossal motor output of the rhythmically active transverse slice. However, the developmental changes seen in the hypoglossal output must also reflect changes in the respiratory network. Our data indicate that changes in the hypoglossal motor output were paralleled by changes in the activity of neurones within the PBC. Although not quantitatively evaluated, we found qualitatively that the rate of rise of rhythmic drive potentials was steeper in neonatal inspiratory neurones (e.g. Figs 4, 5 and 8) than in neurones of older mice (Figs 1, 4, 5 and 9). However, it must be emphasized that further, more quantitative, studies of the drive potentials will be necessary in order to investigate whether this qualitatively seen difference does indeed reflect a developmental difference in the organization of synaptic input to inspiratory neurones. Another qualitative difference observed in this study was that the membrane oscillations of inspiratory neurones recorded in the PBC of older mice (P6–18) were in general more complex compared with those of neonatal mice (P0–5). This

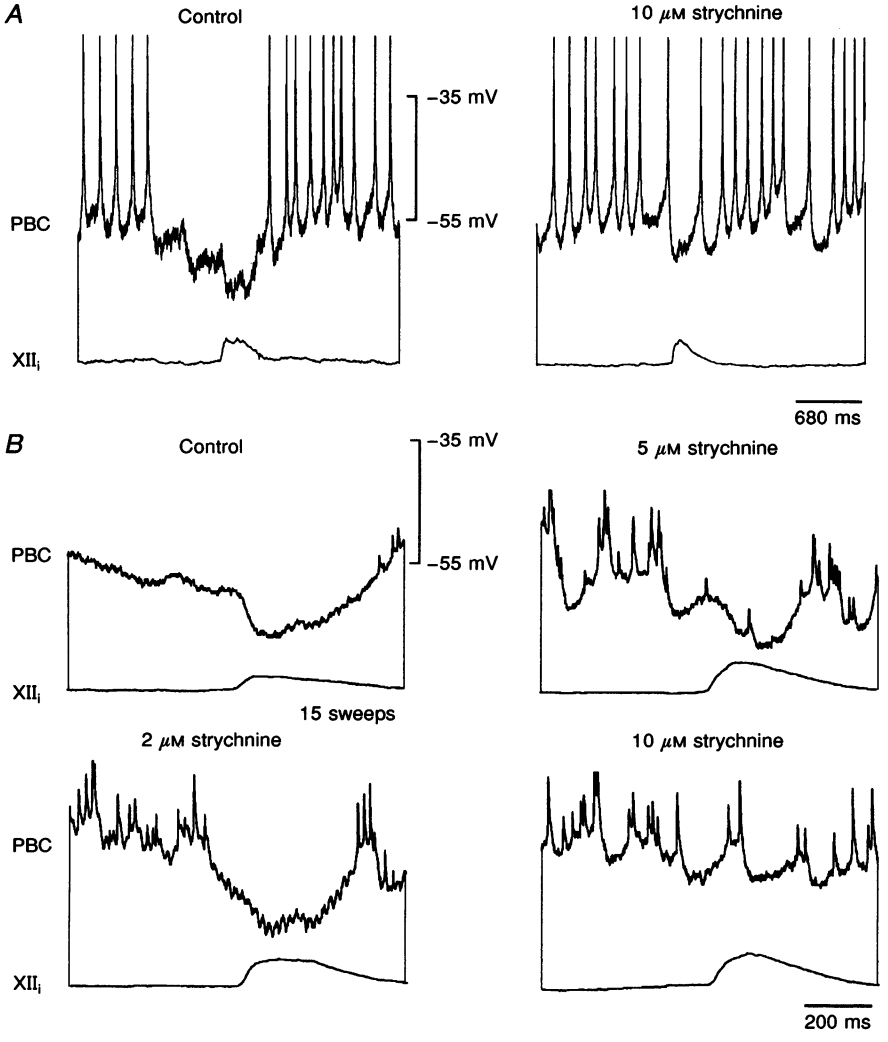

Figure 11. Effect of strychnine on the phasic hyperpolarizations coinciding with the hypoglossal burst shown for an expiratory PBC neurone of a P8 mouse

A, the hyperpolarization in the PBC neurone (upper traces, whole-cell patch recording) occurring in phase with integrated hypoglossal activity (lower traces) was greatly reduced in the presence of 10 μM strychnine (right panel) compared with the control conditions (left panel). *B*, the hyperpolarization consists of two components as revealed by averaging 15 sweeps triggered by the onset of integrated hypoglossal activity (lower traces). The 2 components are affected differently by strychnine. A pre-inspiratory, early component was already abolished at 2 μM strychnine (lower left panel) but the later, inspiratory, component was not completely abolished even at high concentrations of strychnine (5 μM and 10 μM, lower right panels).

was most obvious during the interburst intervals when membrane potentials of neurones recorded from older mice were characterized by waves of potential changes (e.g. Figs 1, 5 and 9) which were most probably due to the summation of complex synaptic input. In contrast, only brief membrane fluctuations were seen in neonatal inspiratory neurones during interburst intervals (e.g. Figs 5 and 8), although synaptic events occurred also during this time (e.g. Fig. 4*A*). This suggests that synaptic input in neonatal neurones is either more homogenously distributed over this period due to a simpler neuronal network or that synaptic potentials do not summate in neonates to the same extent that neurones in older mice do. It is indeed known that the composition and physiology of transmitter receptors change during postnatal development (e.g. Ben-Ari *et al.* 1988, 1989; Betz, 1991; Smart *et al.* 1994). However, as already emphasized, more detailed investigations of the synaptic input to these neurones at different developmental stages will be necessary to unravel the mechanisms leading to the changes in the activity of respiratory neurones.

Another interesting developmental change occurred in the coupling of XII rootlet activity and the activity of neurones recorded intracellularly in the PBC. While rhythmic activity in XII rootlets and the PBC were coupled in a 1 : 1 manner in neonates, this coupling was weaker in animals older than 4–5 days. The reason for this developmental change is as yet unknown and open for speculation. It may reflect an increased dependency on complex synaptic interactions within the respiratory network of older animals which results in a failure to synchronize at every cycle all respiratory neurones under these *in vitro* conditions. This failure to synchronize all respiratory neurones may be responsible for cycle–cycle variations in the amplitude of inward currents as observed in hypoglossal neurones. An alternative explanation is that the cycle–cycle variations in the hypoglossal motor output may reflect a weaker coupling of the XII nucleus to the respiratory network. The hypoglossal nerve innervates 'accessory' respiratory muscles and thus is not such an essential 'respiratory nerve' as the phrenic nerve; and a weaker coupling of the XII nerve to the respiratory network can sometimes also been seen in adult anaesthetized cats under *in vivo* conditions (D. W. Richter, O. Pierrefiche & J. M. Ramirez, unpublished observations). Similarly, expiratory neurones in the hypoglossal nucleus, can sometimes discharge only every second cycle (Hwang *et al.* 1983*a*). The developmental change in the coupling may reflect a functional change of the hypoglossal nerve in controlling both respiratory and swallowing movements of the tongue. It is conceivable that a relatively stronger respiratory coupling of the hypoglossal nerve is more important in a 'suckling' than in an older animal. Indeed a developmental difference in the coupling between the swallowing and respiratory motor pattern has been previously reported in the hypoglossal motor output of cats. In some hypoglossal motor fibres asphyxia produced an activity of spontaneous bursts of swallowing without locking to the respiratory rhythm (Sumi, 1963). In contrast in the kitten, the majority of fibres of the kitten locked onto the respiratory rhythm and irregular bursts that were not locked to the respiratory rhythm were only seen in the minority of neurones (Sumi, 1967). While none of the hypoglossal efferent fibres to 'swallowing muscles' were active during quiet breathing in the adult cat (Sumi, 1963), some were active in the kitten (Sumi, 1967).

Effect of strychnine on the respiratory rhythm

We also examined possible developmental changes in the role of glycinergic mechanisms in the isolated respiratory network. It is well established that glycine modulates respiratory rhythm generation, since a selective blockade of glycine receptors by strychnine increases respiratory frequency in various *in vivo* and *in vitro* neonatal and mature preparations: in anaesthetized *in vivo* rabbits (Schmid, Böhmer & Gebauer, 1991) and mice (Paton & Richter, 1995*b*) as well as in the *in vitro* neonatal rat spinal cord brainstem preparation (Onimaru, Arata & Homma, 1990) and perfused brainstem of adult rats (Hayashi & Lipski, 1992). In contrast to the tilted-sagittal slice preparation, where strychnine had no effect on rhythmic activity in neonatal mice and abolished rhythmic activity in older mice (Paton & Richter, 1995*b*), we confirmed in the transverse slice these *in vivo* and *in vitro* findings. Strychnine applied at concentrations between 1–50 μM significantly decreased cycle length of rhythmic XII activity in the transverse slice of neonatal (P0–7) and older mice (P8–22). The effect on the isolated respiratory network of the transverse slice was never as strong and did not disturb the rhythm as described when strychnine was applied topically to the surface of the ventrolateral medulla in the intact *in vivo* preparation of the adult mouse (Paton & Richter, 1995*b*). This difference may be due to the disturbance of glycinergic mechanisms in regions that are not included in the transverse slice preparation.

As previously described for neonatal rats (Smith & Feldman, 1987; Feldman & Smith, 1989) our findings indicate that glycinergic mechanisms modulate rhythm generation also in the isolated medullary respiratory network of older mice (P8–22). Blockade of glycinergic receptors enhanced the slope and amplitude of drive potentials and the discharge frequency of inspiratory neurones in older animals (e.g. P12, Fig. 9). A more dramatic strychnine effect was seen in expiratory neurones of older mice. The rhythmic synaptic inhibition in these neurones was mainly due to glycinergic inhibition and following application of 10 μM strychnine only a very weak phasic inhibition was left which was probably due to GABAergic inhibition (P. Telgkamp, F. P. Elsen, J. M. Ramirez & D. W. Richter, unpublished observation). While

glycinergic mechanisms clearly modulate respiratory rhythm generation, they do not seem to be essential for generating rhythmic inspiratory activity in the isolated medullary respiratory network. We never observed that rhythmic activity in XII rootlets and inspiratory neurones of the PBC was blocked by strychnine at any developmental stage. Following strychnine application expiratory neurones became almost tonic (Figs 10 and 11, upper right panel), suggesting that these neurones are not essential for generating rhythmic inspiratory activity. Due to similar findings in neonatal rats, Smith & Feldman (1987) suggested that in neonates respiratory rhythmic activity is based on pacemaker properties. Such properties have indeed been described for some neurones in the VRG (Smith *et al.* 1991; Johnson, Smith, Funk & Feldman, 1994), but it remains uncertain whether these neurones are actually involved in respiratory rhythm generation, what function they constitute in an intact network and whether their functions change during development.

This study has demonstrated that the transverse slice manufactured from mice undergoes developmental changes typical for the respiratory system. The developmental changes in the motor pattern are consistent with findings obtained in different other *in vitro* and *in vivo* preparations, thus indicating that it is a functionally relevant model system. In our study we found that not all changes occurred at the same time, the change in the coupling between PBC neurones and XII activity occurred on average between P4 and 5, while the typical bell-shaped pattern of XII rootlet activity became significant in animals older than 1 week (P8–18). These examples indicate that the age window captured by the transverse slice of mice (P0–22) includes an interesting period of the postnatal development of the respiratory system and for the near future we expect that this preparation will provide further insights into development-dependent transitions of various aspects of respiratory control and their underlying cellular mechanisms.

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