The functional expression of a pontine pneumotaxic centre in neonatal rats

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1. Our purpose was to determine whether a pneumotaxic centre could be localized to the rostral pons in newborn rats. We recorded efferent activity of the phrenic nerve in decerebrate, paralysed, vagotomized and ventilated rats, whose age varied from the day of birth to 22 days.

2. The rostral pontine tegmentum was ablated by aspiration and electrolytic lesions. Neuronal activities were blocked by microinjections of the glutamate antagonist MK-801 and were destroyed by the neurotoxins kainic acid and domoic acid.

3. Unilateral ablation or lesions of the pontine tegmentum caused a significant prolongation of the duration of the phrenic burst in animals of all ages. This duration increased further following contralateral destruction and apneusis was established. The period between phrenic bursts increased in most rats whereas peak phrenic height was not consistently altered.

4. Similar changes to those following physical ablations or lesions were recorded after microinjections of MK-801 or neurotoxins.

5. A common region of ablation, lesion and microinjection was the parabrachialis and Köllicker–Fuse nucleus.

6. Exposure to anoxia resulted in an alteration from apnoeusis to gasping.

7. We conclude that from the day of birth, rostral pontine pneumotaxic mechanisms play a significant role in the definition of eupnoea. Moreover, from the day of birth, rats can exhibit the classical ventilatory patterns of eupnoea, apneusis and gasping.

The importance of a rostral pontine pneumotaxic centre for the control of automatic ventilatory activity in adult mammals is well established. In every species examined, the ventilatory pattern of vagotomized preparations is altered from eupnoea to apneusis following ablations of the dorsolateral pontine tegmentum (see Wang, Fung & St John, 1993 for review). Recent work has established that the critical elements removed by these ablations are neurons and not fibres from other regions. Following the injections of neurotoxins or blockers of synaptic transmission into the rostral pons, pronounced prolongations of the duration of inspiration are obtained (Denavit-Saubie, Riche, Champagnat & Velluti, 1980; Fung & St John, 1994*a*; Fung, Wang & St John, 1994*a*).

In newborn animals, there is evidence that the rostral pons may play a role in the control of eupnoeic ventilatory activity. Hence, microinjections of excitatory neurotransmitters into the rostral pons of newborn opossums markedly alters the respiratory rhythm (Farber, 1990). Contrariwise, brainstem transections produce a slowing of the respiratory frequency in vagotomized kittens; however, pauses in the inspiratory position, typical of apneusis, were not observed (Duron & Marlot, 1980). Paradoxically, removal of the pons of the *in vitro* brainstem–spinal cord preparation of the neonatal rat causes an augmentation of the frequency of its rhythmic phrenic bursts (Monteau, Errchidi, Gauthier, Hilaire & Rega, 1989; Smith, Greer, Liu & Feldman, 1990).

Concerning these brainstem transections, alterations in ventilatory activity are confounded by two factors. For *in vivo* preparations, caudal pontine transections may simultaneously remove influences from the rostral and caudal pons. In vagotomized adult animals, having discrete lesions of the pneumotaxic centre, inspiratory durations of apneusis are greatly reduced following subsequent lesions of the caudal pontine reticular formation (for review see Wang *et al.* 1993).

For the *in vitro* preparation of the neonatal rat, much of the pons may be non-functional due to extensive tissue anoxia (Brockhaus, Ballanyi, Smith & Richter, 1993; Okada, Muckenhoff, Holtermann, Acker & Scheid, 1994).

Table 1. Number of rats in different experimental groups

Age (days)	Aspiration	Lesions	MK-801	Neurotoxins
0–4	23	12	13	28
5–8	6	11	10	7
9–22	10	13	3	5

The above discussion makes evident that the role, if any, of the rostral pontine pneumotaxic centre in the control of eupnoeic ventilatory activity in neonatal animals is undefined. The present experiments were undertaken to assess the hypothesis that such a role for the pneumotaxic centre is exercised from the day of birth.

A preliminary report of a portion of this work has been published (Fung & St John, 1994*b*).

METHODS

General preparation

Experiments were performed on Sprague–Dawley rats of either sex from the day of birth (day 0) to 22 days thereafter. The animals were initially anaesthetized with 2% halothane in oxygen. The trachea was cannulated and the vagi were sectioned at a mid-cervical level. The brainstem was transected at a precollicular level. Halothane anaesthesia was then discontinued.

The animals were paralysed with gallamine triethiodide (40 μg g^{-1} I.P.) and artificially ventilated with 100% O_2. The inspired fractional concentrations of oxygen were continuously monitored. Skin temperature on the abdominal surface was monitored and maintained at 37–39 °C by a heating pad. We have observed that such skin temperatures are 0–2 °C higher than temperatures within the abdominal cavity; thus, skin temperature is considered as a reasonable representation of body temperature.

The phrenic nerve was exposed by a dorsolateral approach. The nerve was sectioned and drawn up into a suction electrode. The activity was amplified, filtered (1–500 Hz), viewed on an oscilloscope and recorded on videotape. The activity was also electronically integrated (time constants: 'on', 60 ms and 'off', 100 ms).

Ablation of the rostral pons

The animal was placed prone and the head rigidly held. An occipital craniotomy enabled the inferior colliculi to be visualized. Three different procedures were performed: aspiration of the pontine tegmentum, electrolytic lesions and microinjections.

Aspiration was performed with a pipette, placed just caudal to the inferior colliculus. Phrenic activity was continually monitored and aspiration was continued until a clear prolongation of the phrenic burst was evident. A minimum of 15 min elapsed before aspiration of the contralateral tegmentum was performed.

For electrolytic lesions, a concentric bipolar electrode (diameter, 0·2 mm) was inserted into the brainstem just caudal to the inferior colliculus. In various animals, the electrode was inserted from 1·0 to 3·0 mm lateral to the mid-line and 2–6 mm below the surface of the inferior colliculus. An initial lesion was made by passing 0·3 mA of current for 9 s. Current was gradually increased, up to a maximum of 2 mA, until the phrenic burst was prolonged. If no prolongation was produced, the electrode was moved to a different ipsilateral position. As with aspiration, 15 min were allowed before contralateral lesions were made.

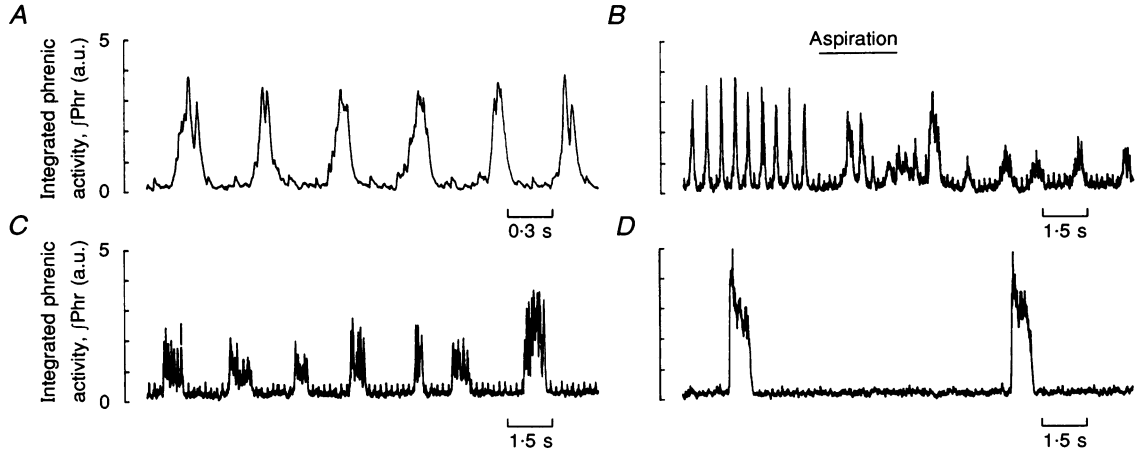


Figure 1. Alteration of the ventilatory pattern from eupnoea to apneusis following ablation, by aspiration, of the dorsolateral pontine tegmentum in a 1-day-old rat

A, integrated activity of the phrenic nerve (∫Phr) during eupnoea. *B*, record obtained during and immediately following a unilateral ablation. Note the immediate increases of t_i and t_e following the ablation. Apneusis was established 10 min after the ablation (*C*). This animal became apnoeic after the bilateral ablation; however, gasping was induced in anoxia (*D*). a.u., arbitrary units.

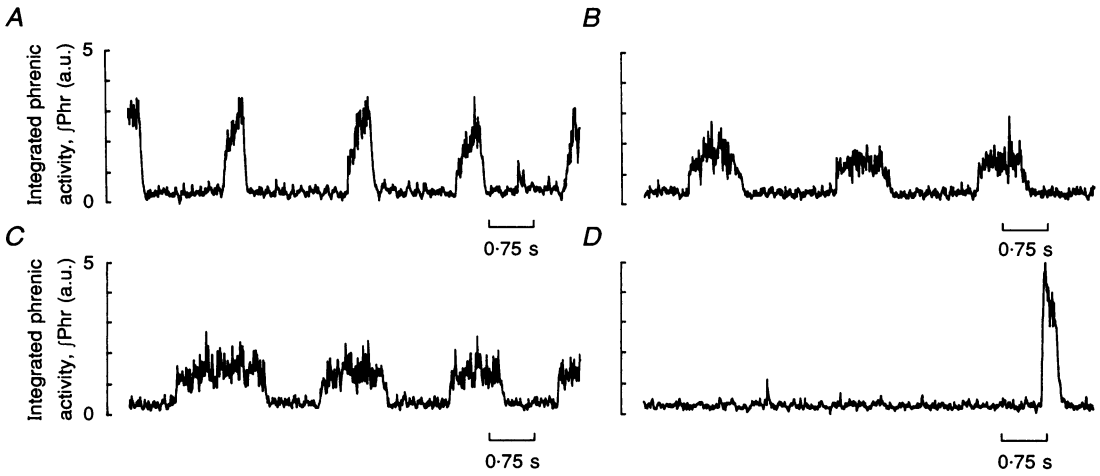


Figure 2. Alteration of the ventilatory pattern from eupnoea to apneusis following ablation, by aspiration, of the dorsolateral pontine tegmentum in a 5-day-old rat

A, integrated activity of the phrenic nerve during eupnoea. *B* and *C* are records obtained following unilateral and bilateral ablation, respectively. The pattern of phrenic activity was altered to gasping in anoxia (*D*).

Micropipettes were inserted into the pons, as was the electrode. Either single- or double-barrelled pipettes, with a total tip diameter of 10 μm, were used. In various experiments, the barrels contained the following: MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclopenten-5,10-imine hydrogen maleate), an antagonist of the NMDA class of glutamate receptors, at 20 and 50 mM (pH 7·4); neurotoxins kainic (4·7 mM, pH 7·4) and domoic acid (20 mM, pH 7·4); saline for control injections (150 mM NaCl, pH 7·4); Fast Green FCF (10%) to localize sites of injection. Microinjections were made by pressurizing the pipette (10–14 lbf in^{-2}) for variable periods. In order to define the volume of injection, calibration curves were constructed for micropipettes

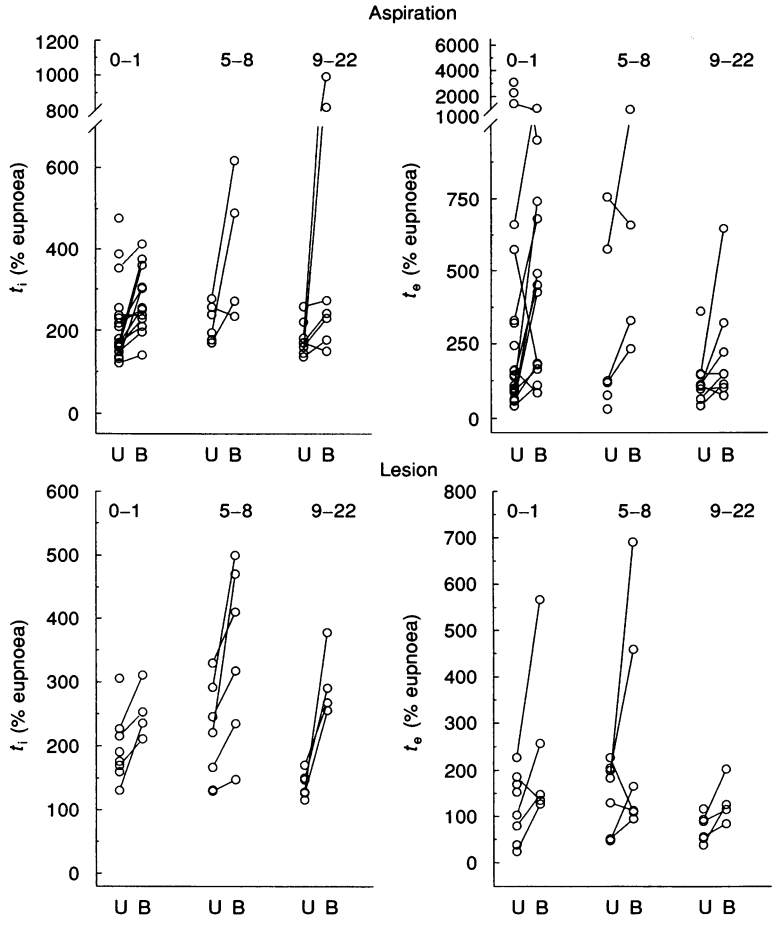


Figure 3. Alterations in the duration of neural inspiration (t_i) and expiration (t_e) following ablation of the pontine tegmentum by aspiration or electrolytic lesions

Each symbol represents data from a single rat following unilateral (U) and bilateral (B) ablations. Lines connecting points are data from a single animal. The presence of data for only a unilateral ablation indicates that the animal became apnoeic following the bilateral ablation. Ages (in days) of rats in various groups appear at the top of each panel.

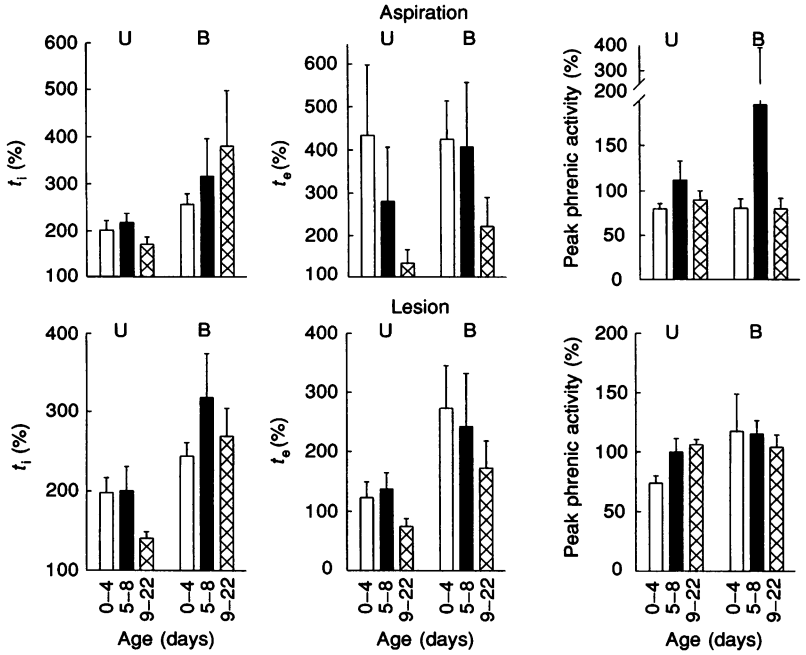


Figure 4. Mean changes in variables of ventilatory activity following unilateral (U) and bilateral (B) ablation of the rostral pontine tegmentum by aspiration or electrolytic lesions

Means and standard errors of the means for the durations of neural inspiration (t_i), expiration (t_e), and peak phrenic activity have been calculated for animals of the various age groups. Data for individual animals appear in Fig. 3.

with known impedances. The volume of injection was defined by measurement of the sphere at the tip of the pipette with the calibrated reticule of a microscope. Volumes ejected at a range of pressures and times were measured.

We found previously that microinjections of MK-801 in the dorsolateral pontine tegmentum reversibly prolonged the duration of the phrenic burst (Fung *et al.* 1994*a*). In some experiments, injections of 10 nl of 20 or 50 mM MK-801 were performed initially to define regions in which this blockade resulted in prolongation of the duration of the phrenic burst by at least 20%. Phrenic activity recovered to pre-injection durations within 15–25 min. Additional injections of MK-801 were either made in different regions or, more commonly, injections of saline, kainic acid or domoic acid were made through the second barrel. In other experiments neurotoxins alone were injected, without any previous injection of MK-801 (Table 1). After neurotoxin injections, a minimum of 15 min was allowed before any other injections were made. A maximum of two regions in which injections caused prolongation of the phrenic burst were examined in any animal.

Following completion of any of the above procedures, animals were maintained for a minimum of 60 min, unless rhythmic phrenic activity ceased in the interim. Experiments were concluded by ventilating the animals with 100% nitrogen in order to induce gasping.

Histology

Brainstems were removed and stored in formalin. Sections of 50–70 μm in the transverse plane were obtained and stained with Cresyl Violet. Regions of ablation or injections were determined. Since no atlas of the brainstem of the neonatal rat is available, regions were defined in reference to the adult brain (Paxinos & Watson, 1986). The locations of injection sites were determined from labelling with Fast Green. The region of distribution of Fast Green varied from a diameter of 0·5 to 1 mm.

Data analysis

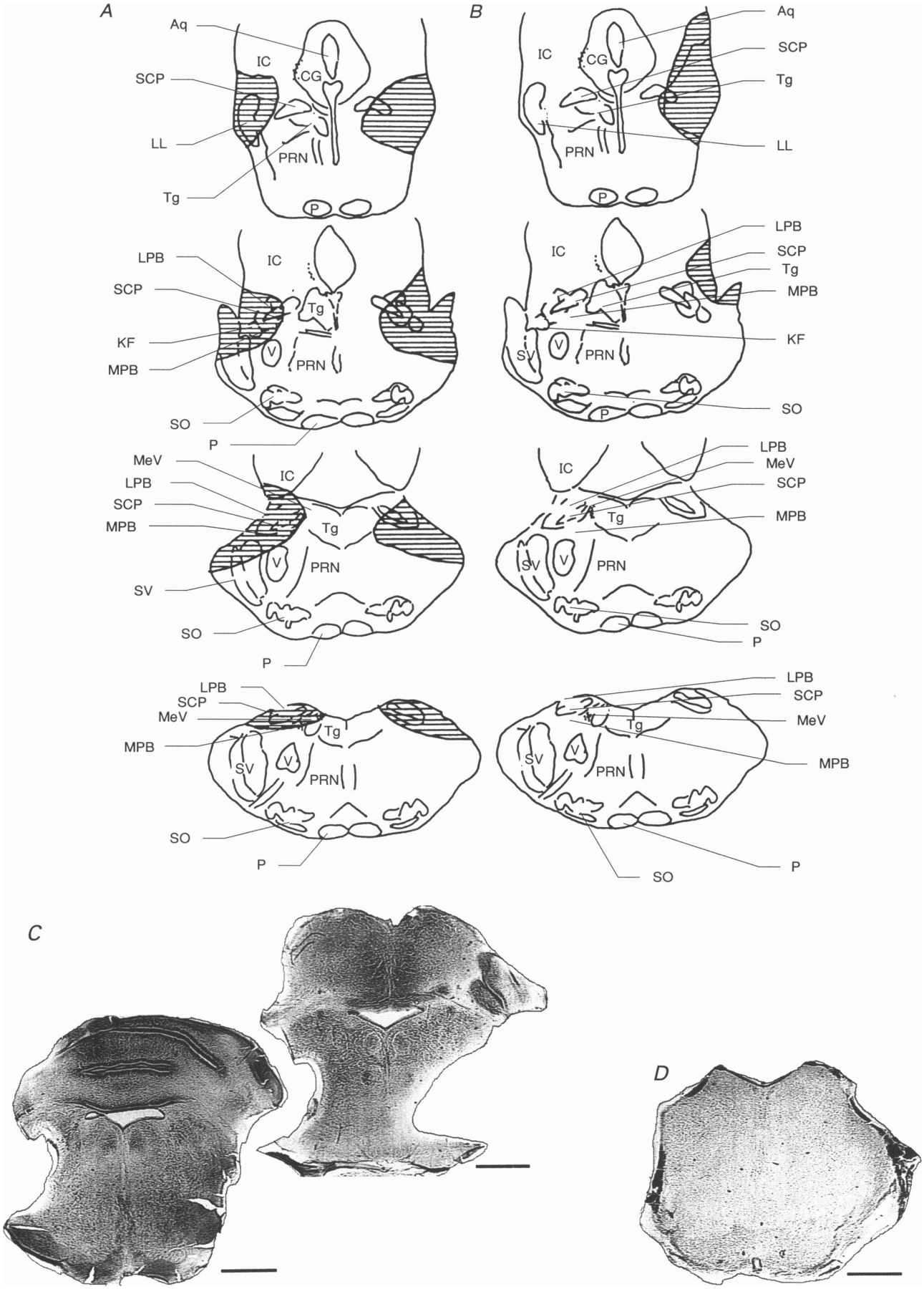
Integrated activity of the phrenic nerve was analysed to determine the duration of neural inspiration (t_i) and expiration (t_e), and peak height (PNA). Neural inspiration was defined as the period from onset to the rapid decline of the phrenic burst. Neural expiration constituted the remainder of the cycle until the commencement of the next phrenic burst.

Values of variables were compared before and after experimental perturbations. Data of twenty to thirty ventilatory cycles under each of the various conditions were averaged. For determination of time-related changes, data were collected at various intervals after a procedure.

Statistical significance was determined by repeated measures, one-way ANOVA for multiple comparisons and, for single comparisons, by Student's paired t test. Probabilities less than

Figure 5. Regions of ablation by aspiration which resulted in prolongation of neural inspiration and apneusis

A, hatching represents a compilation from animals in which prolongation of t_i resulted. *B*, hatching shows regions which were ablated in animals in which t_i was not altered. *C* and *D* are examples of ablations which resulted in apneusis. Calibration bars represent 1 mm. Abbreviations are as follows: Aq, aqueduct; CG, central grey; IC, inferior colliculus; KF, Köllicker–Fuse nucleus; LL, lateral lemniscus; LPB, lateral parabrachialis nucleus; MeV, mesencephalic trigeminal nucleus; MPB, nucleus parabrachialis medialis; P, pyramidal tract; PRN, pontine reticular nucleus; SO, superior olive; SV, trigeminal sensory tract and nucleus; SCP, superior cerebellar penducle; Tg, tegmental nucleus; V, trigeminal motor nucleus.



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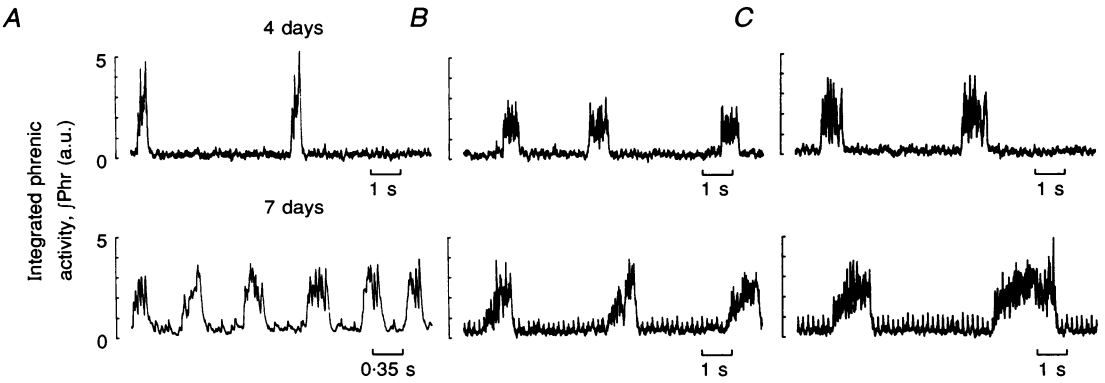


Figure 6. Examples of alterations in integrated activity of the phrenic nerve following electrolytic lesions of the pontine tegmentum

Data are from two rats of ages shown. *A*, before lesions; *B*, unilateral lesion; and *C*, bilateral lesion.

0·05 were considered significant. Data are normalized to the values of pre-lesions and are expressed as means ± S.E.M.

RESULTS

Data were collected from 141 neonatal rats. Animals were grouped by age for statistical evaluations as follows: 0–4, 5–8 and 9–22 days. The number of animals of different ages which were exposed to various procedures is listed in Table 1.

Ablation of the pontine tegmentum

For thirty-five of thirty-nine rats, t_i increased following unilateral ablation, by aspiration, of the tegmentum. Following the contralateral ablation, t_i increased further (Figs 1–3). Note also, in Figs 1 and 2, that the pattern of the phrenic burst changed from the ramp-like rise of eupnoea to a sustained pause, characteristic of apneusis. There was no significant difference between animals of various ages in the augmentations of t_i, following either unilateral or bilateral ablations (Fig. 4). However, following bilateral ablations, phrenic activity was completely eliminated in nine animals aged 0–4 days and two rats in the 5- to 8-day-old group.

There was much variability in the changes of neural expiration following unilateral and bilateral ablations (Fig. 3) and, indeed, changes in t_e were significant only for the youngest animals (Fig. 4). Changes in peak phrenic activity were slight and variable (Figs 3 and 4).

All animals, including those which exhibited apnoea following bilateral ablations, were exposed to anoxia. Gasping was elicited in each rat (Figs 1 and 2).

Histological evaluations of the region of ablation revealed that large portions of the dorsolateral pontine tegmentum had been removed in those animals exhibiting apneusis. This region of ablation included the classical pneumotaxic centre of the parabrachialis nucleus and Köllicker–Fuse nucleus. Ablations were rostral and lateral to these nuclei for the two animals in which t_i did not increase (Fig. 5).

Electrolytic lesions of the rostral pons

In twenty-one of thirty-six animals, t_i increased following unilateral lesions; two animals exhibited apnoea. Bilateral lesions caused a further prolongation of t_i (Figs 3 and 4). Again, eupnoea was altered to apneusis with longer t_i and inspiratory pauses (Fig. 6). As for aspiration of the tegmentum, alterations of t_e and peak phrenic height were variable (Figs 3 and 4). Again, there was no influence of age upon the magnitude of alteration.

Following bilateral lesions, more animals became apnoeic. Hence, four animals of ages 0–4 days, one of the 5–8 day group and three of the oldest animals exhibited no phasic phrenic activity.

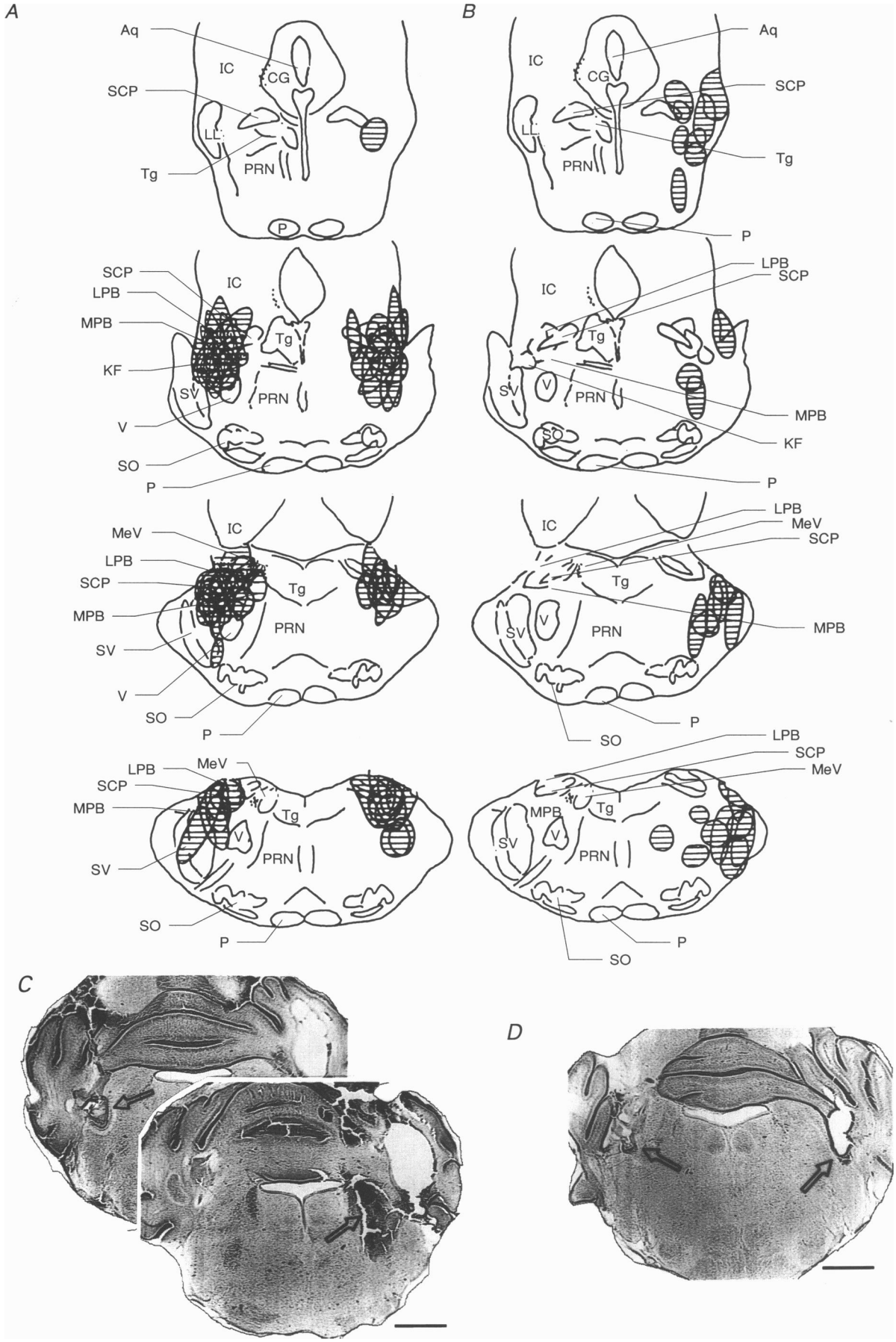
Histological evaluations revealed lesions which encroached upon the parabrachialis and Köllicker–Fuse nucleus for animals in which t_i increased or which exhibited apnoea. Lesions were lateral and/or ventral to these nuclei for animals in which t_i was not altered (Fig. 7). These thirteen animals were of ages 3–15 days; neither t_e nor peak phrenic height was altered in these rats.

Microinjections into the rostral pons

In nineteen of twenty-six animals, regions were found in which injections of MK-801 (20 mM, $n = 18$; 50 mM,

Figure 7. Regions in which electrolytic lesions resulted in prolongations of neural inspiration and apneusis

A, hatching represents a compilation from animals in which prolongation of t_i resulted. *B*, hatching shows regions which were lesioned in animals in which t_i was not altered. *C* and *D* are examples of lesions (arrows) which resulted in apneusis. Abbreviations as for Fig. 5. Calibration bar, 1 mm.



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$n = 11$) increased t_i (Figs 8–10). Increases of t_i were greater following injection of 50 mM than 20 mM MK-801 for animals of 0–4 days only (Fig. 10). For older animals, injections of 20 and 50 mM caused similar changes (Figs 9 and 10). Following injections, the augmentations in t_i were reversible, with values returning to within 20% of control levels in 12–25 min.

Alterations of t_e were variable and changes were generally greater for the younger groups of animals although there was no significant influence of age upon the magnitude of alteration. Peak phrenic activity was not altered (Figs 9 and 10).

Effective regions of injection of MK-801 were in the parabrachialis and Köllicker–Fuse nucleus. Injections of saline into these nuclei caused no changes in ventilatory activity. Likewise, no changes in ventilatory activity were found for injections of MK-801 outside these nuclei in seven rats (Fig. 11).

The neurotoxins kainic and domoic acid were injected into those regions of the pons in which injection of MK-801 had caused prolongations of t_i. In addition, neurotoxins alone were injected into the rostral pons of a different group of rats. Both neurotoxins elicited similar changes and so data for both have been combined.

For rats of all ages, augmentations of t_i were observed within 2–10 min following injections of neurotoxins. Augmentations were generally greater following bilateral injections (Figs 9 and 10). t_e changed significantly only for the youngest group of animals and, for five of these, phrenic activity ceased following the bilateral injections (Fig. 9). Three animals of the 5–8 day age group also exhibited apnoea following bilateral injections. No apnoea was observed following unilateral injections in the oldest rats or after bilateral injections. For animals in which rhythmic phrenic activity persisted, peak activity was not systematically altered (Figs 9 and 10).

In six rats, injections of neurotoxins were found to have been made lateral and/or ventral to the parabrachialis nucleus and Köllicker–Fuse nucleus. Such injections caused no alterations of t_i, t_e or peak integrated phrenic activity (Fig. 11).

As animals having physical lesions of the rostral pons, rats which received microinjections were exposed to anoxia and in each, gasping was induced.

DISCUSSION

The major conclusion of this study is that a pneumotaxic centre is functional in the rostral pons from the day of birth in the rat. This pneumotaxic centre has the same anatomical location and physiological function as has been detailed in adult mammals.

The concept of a 'pneumotaxic centre' in the dorsolateral pontine tegmentum was established by Lumsden in his classic studies in 1923. Based upon a production of apneusis following small lesions of the nucleus parabrachialis medialis and Köllicker–Fuse nucleus in vagotomized cats, these nuclei were considered as the pneumotaxic centre (Bertrand & Hugelin, 1971; Cohen, 1971). These nuclei contain high concentrations of neurons having respiration-modulated discharge patterns (Bertrand, Hugelin & Vibert, 1974; Bianchi & St John, 1982; St John, 1987).

In rats, a comparable location to that in cats of the region of the pneumotaxic centre has been reported (Wang *et al.* 1993; Morrison, Cravo & Wilfehrt, 1994), although Monteau *et al.* (1989) did not obtain apneusis following

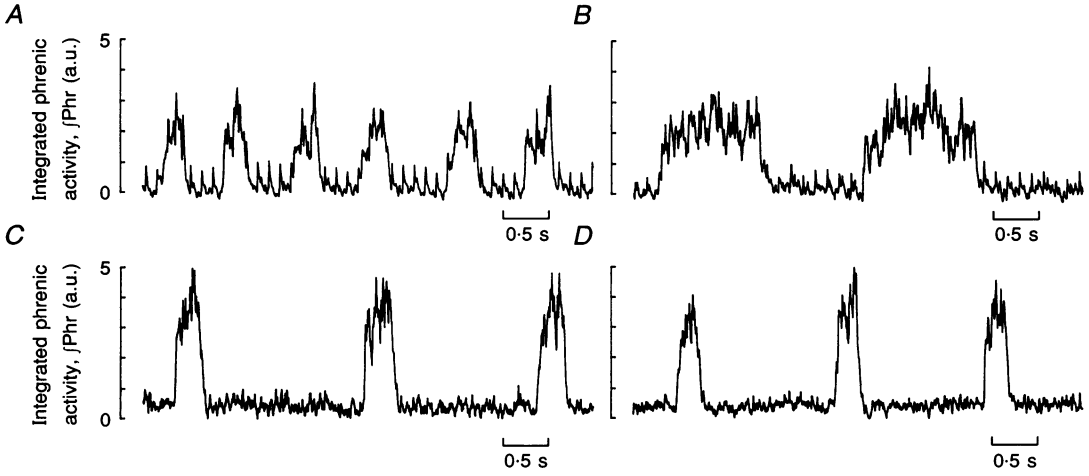

Figure 8. Changes in phrenic activity following injection of MK-801 into the pontine tegmentum of a 7-day-old rat

A and *B* show activity before and after injections. *C*, pattern following recovery. *D*, absence of changes following injections of saline.

pontine transections in vagotomized rats. As we have discussed in our recent reports (Wang *et al.* 1993; Fung *et al.* 1994*a*), the absence of apneusis following pontine transections reflects the simultaneous removal of the pneumotaxic centre and a portion of the caudal pontine reticular formation.

Using techniques from ablations of the brainstem, to small electrolytic lesions and finally, discrete microinjections, the present study establishes that neurons in the region of the parabrachial and Köllicker–Fuse nuclei regulate the duration of inspiration in the newborn rat.

In addition to prolongations of the duration of inspiration, expiratory duration is typically prolonged in adult animals following lesions of the nucleus parabrachialis medialis and Köllicker–Fuse nucleus (e.g. St John, 1979). Changes in expiratory duration were exceedingly variable for the newborn rats in this study, with a systematic increase being seen only in animals from 0–4 days of age. Indeed in

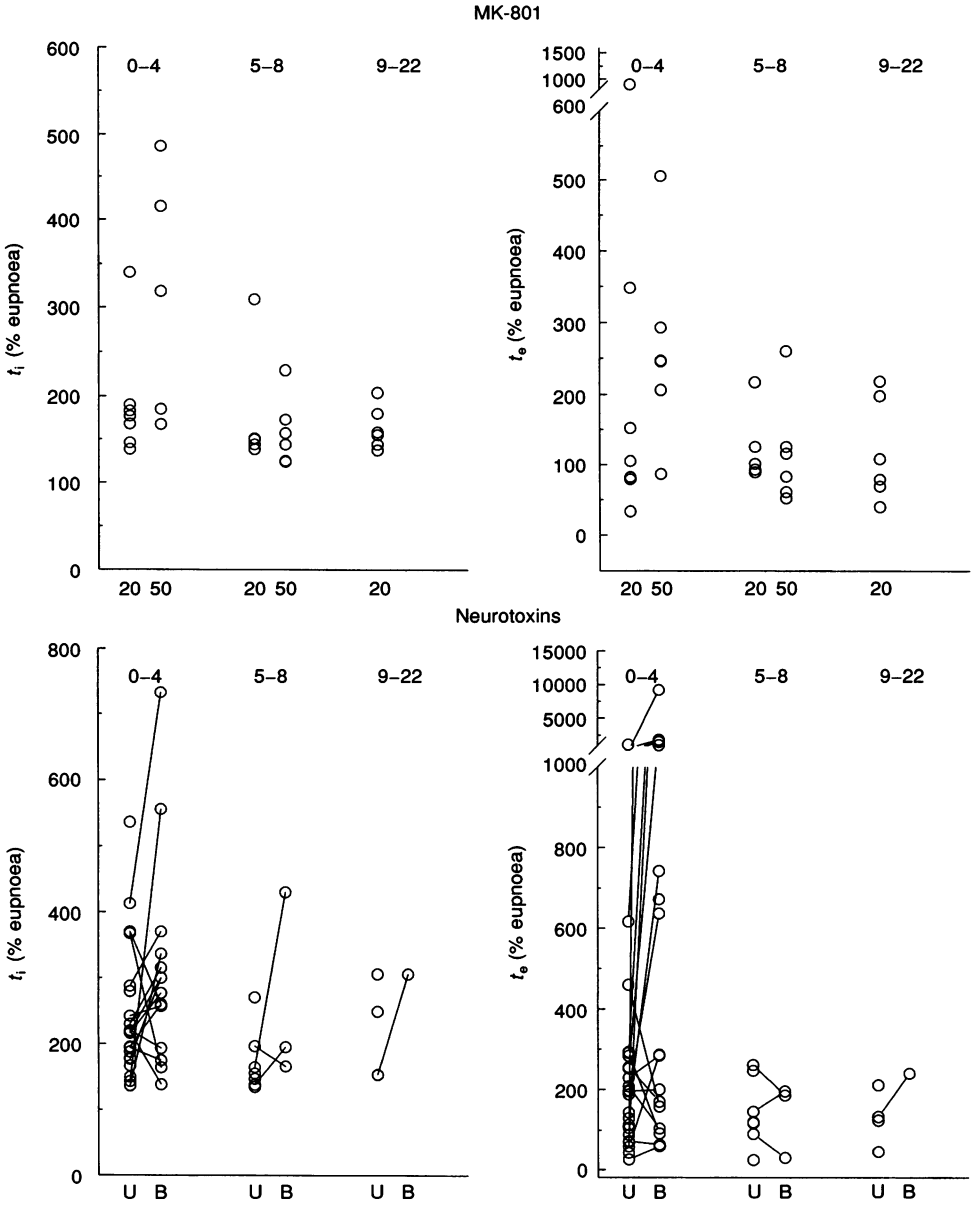

Figure 9. Alterations in t_i and t_e following unilateral injections of MK-801 into the pontine tegmentum and following unilateral and bilateral injections of the neurotoxins

Values on abscissae in the upper panels indicate MK-801 concentration (in mM); each symbol represents data from a single injection. In any single animal, individual regions were injected with one concentration only. For neurotoxins, unilateral (U) and bilateral (B) injections were made. Points connected by lines are data from the same animal. Single points for unilateral injection indicate that the animal became apnoeic following the bilateral injection. Ages (in days) of rats appear at the top of each panel.

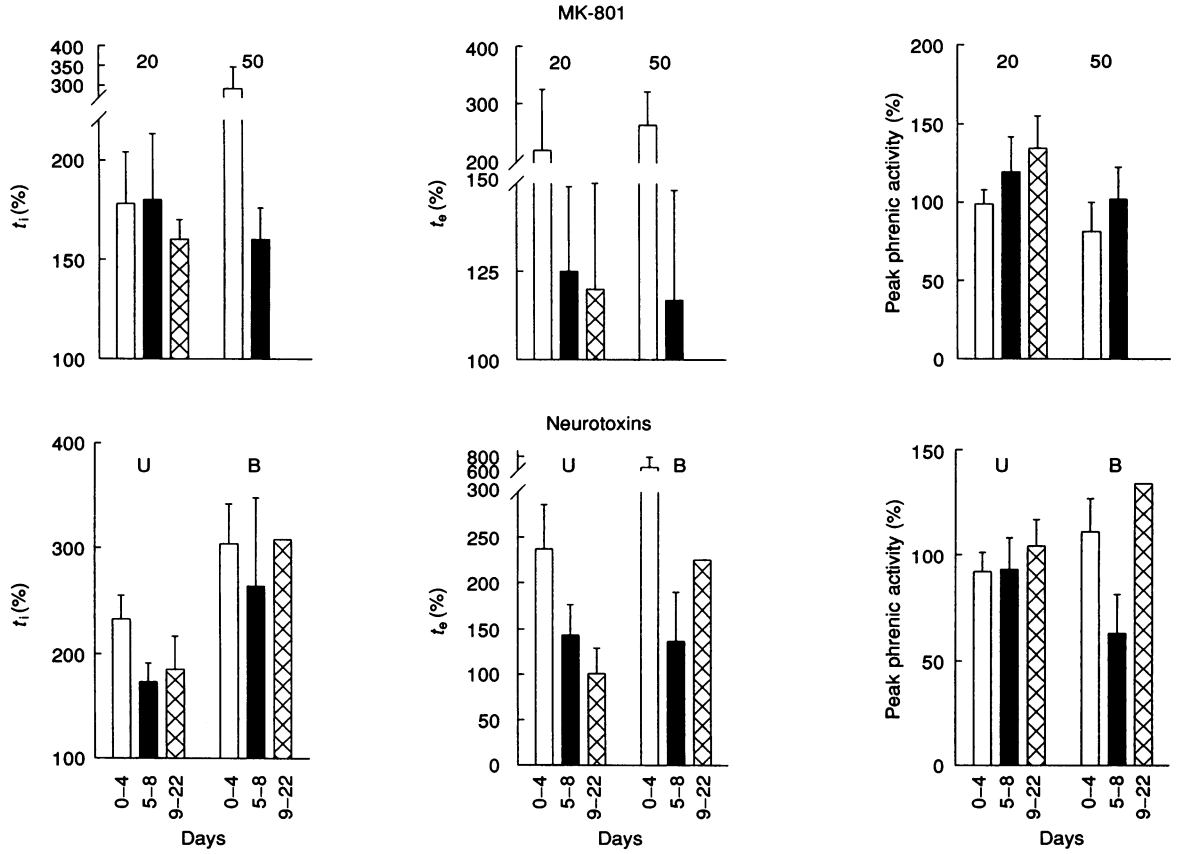

Figure 10. Mean changes in variables of ventilatory activity following injections of MK-801 or neurotoxins into the rostral pontine tegmentum

Numbers above bars in upper panel designate 20 and 50 mM MK-801, which was injected unilaterally. Letters above bars in lower panel designate unilateral (U) or bilateral (B) injections of neurotoxins. Means and standard error of the mean for the durations of neural inspiration (t_i), expiration (t_e), and peak phrenic activity have been calculated for animals of the various age groups. Data for individual animals appear in Fig. 9.

these youngest rats, expiratory apnoea frequently followed bilateral lesions of the pneumotaxic centre. While we have no firm explanation for these variable responses, a number of possibilities do arise.

Our goal in these experiments was to identify a region which regulated the duration of neural inspiration. Physical or chemical lesions were then made in that region. Yet, in a recent report, we have detailed that pontine regions regulating the duration of neural inspiration are more rostral than those regulating the duration of expiration; thus, this regulation is separable anatomically (Fung & St John, 1994*a*). Hence, our lesions, except in the youngest animals, might not have ablated neurons involved in the regulation of both neural inspiration and expiration.

It is probable that a proportionally larger region would be ablated in the youngest animals since the size of the brainstem increases with age. Of course, given the well-described maturation of some respiratory reflexes (e.g. Haddad, Donnelly & Bazzy-Asaad, 1994), a specific age-related factor could account for the more pronounced alterations of neural expiration following pontine lesions. The expiratory apnoea observed in some newborn animals following the pontine lesions has also been found in some adult animals (St John, 1979).

Figure 11. Regions of microinjection of MK-801 and neurotoxins

A, circles represent a compilation from animals in which prolongations of neural inspiration were obtained. *B*, circles show regions of injection for animals in which t_i was not altered. Circles in *C* and *D* are examples of regions in which injections of MK-801 resulted in increases in t_i. Square in *C* is a region of injection of domoic acid which did not result in a prolongation of t_i. Abbreviations as for Fig. 5. Calibration bar, 1 mm.

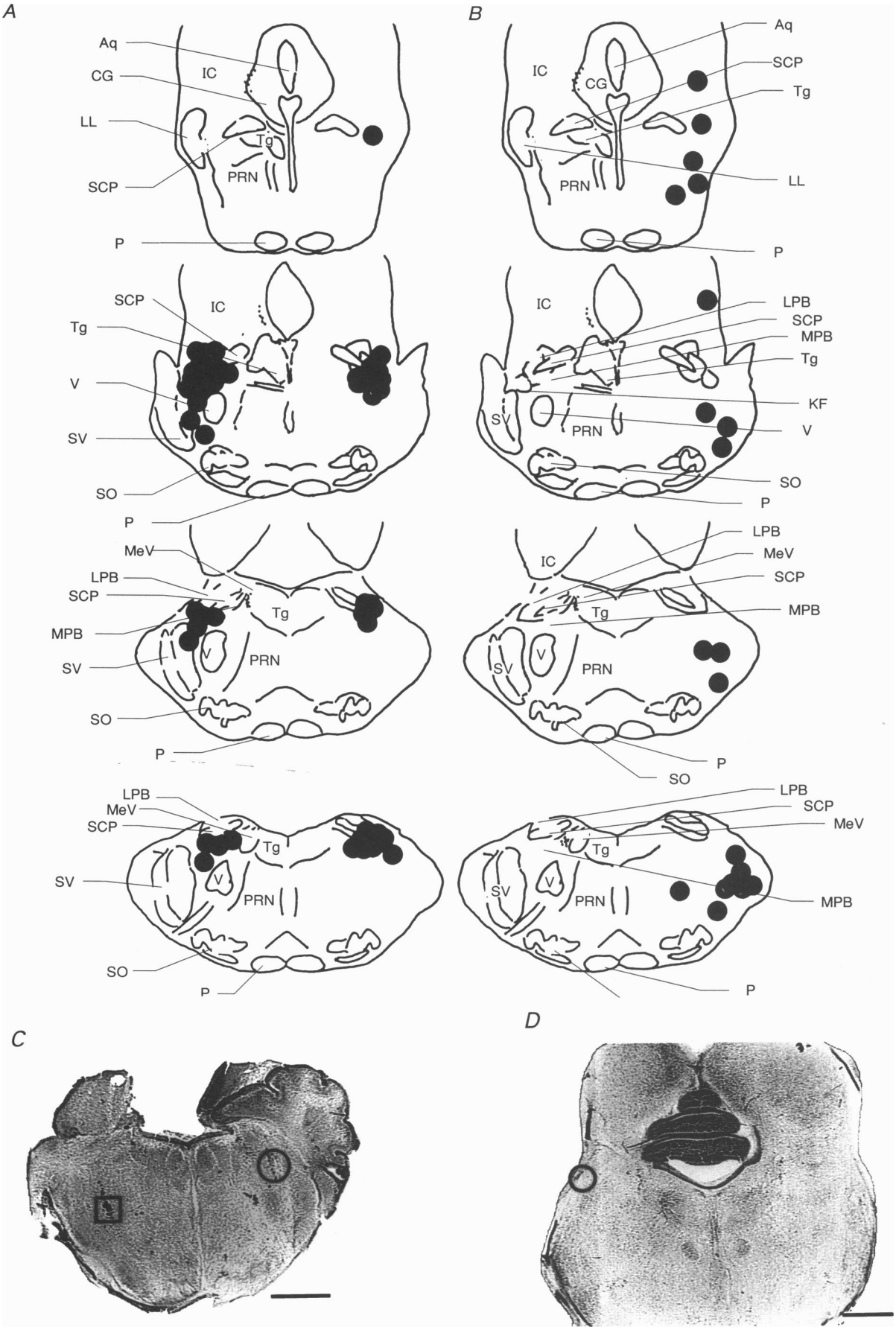

Figure 11. For legend see facing page.

In addition, lesions of the rostral pontine tegmentum have been reported to elevate the threshold level of arterial P_{CO_2} required for automatic ventilatory activity to be manifested (St John, 1975). Since we did not measure end-tidal or arterial P_{CO_2} in the present experiments, it is possible that these levels were lower in rats exhibiting apnoea following pontine lesions. The return of rhythmic ventilatory activity, albeit gasping, in anoxia demonstrates that the apnoea following pontine lesions did not represent a non-specific depression of brainstem function.

Results of the present study establish that, from the day of birth, neurons in a rostral pontine pneumotaxic centre are an integral portion of the mechanism defining the eupnoeic ventilatory cycle. Moreover, as in the adult, the neurons within the pneumotaxic centre are dependent upon the synaptic mechanisms for their discharge. Such dependency has been documented by the prolongations of inspiratory duration following injections of MK-801, a glutamate antagonist, in adult animals (Fung *et al.* 1994*a*; Ling, Karius & Speck, 1994). As discussed in detail previously for findings in adults (Fung *et al.* 1994*a*), these changes following pontine injections demonstrate that the apneusis which follows systemic administrations of MK-801 is due, in fact, to its actions upon pontine neuronal activities. Interestingly, apneusis also follows administrations of MK-801 to newborn animals, again suggesting that a pneumotaxic centre is functional in neonates (Schweitzer, Pierrefiche, Foutz & Denavit-Saubie, 1990; Sica, Siddiqi & Pisana, 1992).

In summary, results of the present and previous studies from this laboratory establish that rats, from the day of birth, can exhibit the classic respiratory patterns of eupnoea, apneusis and gasping. Moreover, exactly as in the adult, the alteration from eupnoea to apneusis follows ablation of neurons in the rostral pontine pneumotaxic centre of vagotomized animals. The alterations from eupnoea or apneusis to gasping can be induced by anoxia.

The three respiratory patterns *in vivo* are in distinct contrast to the single respiratory pattern of the *in vitro* brainstem spinal cord preparations of the newborn rat (e.g. Smith *et al.* 1990). Also, as opposed to findings for both neonatal and adult animals *in vivo*, administration of MK-801 causes no change in the rhythmic activities of the *in vitro* preparation (Greer, Smith & Feldman, 1991). This lack of response reflects the removal of a portion or all of pons in this preparation and, again, the probability that this preparation can only exhibit gasping (see discussion in Fung, Wang & St John, 1994*b*).

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Bertrand, F., Hugelin, A. & Vibert, J. F. (1974). A stereologic model of pneumotaxic oscillator based on spatial and temporal distribution of neuronal bursts. *Journal of Neurophysiology* **37**, 91–107.

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