Neurones in the ventrolateral pons are required for post-hypoxic frequency decline in rats

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1. The breathing pattern following acute hypoxia (arterial O_2 pressure (P_{a,O_2}), 27·4 ± 7·7 mmHg) was measured in intact, anaesthetized and spontaneously breathing adult rats ($n = 4$) and in anaesthetized, vagotomized, paralysed and ventilated animals ($n = 14$). Measurements were made both before and after bilateral lesions or chemical inactivation of neurones in the lateral pons. Respiratory motor activity was recorded as an index of the respiratory cycle. We tested the hypothesis that the ventrolateral pons is required for expression of post-hypoxic frequency decline, defined as a decrease in respiratory frequency below steady-state baseline levels following brief exposures to hypoxia.

2. We identified an area in the ventrolateral pons where brief (1 ms) low current ($\leqslant 20$ μA) pulses evoked a short-latency inhibition of phrenic nerve activity. At this site, bilateral electrical or chemical lesions ($n = 3$) were performed, or neural activity was inhibited by focal injections of 10 mM muscimol ($n = 9$). In six control animals, neural activity was inhibited by muscimol injections into the lateral pons, dorsal to the target site.

3. Prior to pontine intervention, respiratory frequency decreased below baseline levels following 20–110 s of 8% O_2. The decrease in frequency resulted from a prolongation of expiration (up to 276%), which gradually returned to baseline levels ($\tau = 45$ s).

4. Following lesions or inhibition of neural activity in the ventrolateral pons, baseline inspiratory (T_I) and expiratory (T_E) durations were altered, albeit minimally, in the animals with intact vagus nerves. Expiratory duration following hypoxia was not different from baseline levels either in vagotomized ($P = 0·18$) or intact ($P > 0·05$) animals. In contrast, injections of muscimol at more dorsal sites did not alter the decrease in frequency normally seen following hypoxia.

5. Histological examination revealed that effective lesion or injection sites were within the lateral pontine tegmental field and included portions of the noradrenergic A_5 cell group.

6. We conclude that the mechanism responsible for post-hypoxic frequency decline involves an active neural process that depends on the integrity of the ventrolateral pons.

In the period following episodes of hypoxia, respiratory rate and amplitude do not immediately return to pre-hypoxic levels (Hayashi, Coles, Bach, Mitchell & McCrimmon, 1993). A neural mechanism may underlie both the sustained increase in phrenic nerve amplitude and the decrease in respiratory frequency that follows hypoxia (Hayashi *et al.* 1993). However, the neural substrate responsible for the decreased respiratory frequency during and following isocapnic hypoxia, referred to as short-term depression (Hayashi *et al.* 1993), is unknown.

Indirect evidence suggests that neurones within the ventrolateral (VL) pons may mediate the decline in respiratory frequency following hypoxia. For example, lesions of the VL pons prolong inspiration, and stimulation of this area decreases respiratory frequency both in anaesthetized adult rats (Jodkowski, Coles & Dick, 1994; Dick, Coles & Jodkowski, 1995) and in the *in vitro* neonatal rat brainstem–spinal cord preparation (Hilaire, Monteau & Errchidi, 1989; Errchidi, Monteau & Hilaire, 1991; Hilaire, Monteau, Errchidi, Morin & Cottet-Emard, 1993; Hamada, Garcia-Rill & Skinner, 1994). Moreover, transection of the rostral pons attenuates the decrease in respiratory frequency associated with prolonged hypoxia in neonatal rabbits (Martin-Body & Johnston, 1988) and, in fetal sheep,

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extensive lesions of the lateral pons reverse the inhibition of breathing movements during hypoxia (Johnston, Gluckman & Parsons, 1985; Gluckman & Johnston, 1987).

In the present study, we observed a sharp decrease in respiratory frequency following brief exposure to severe hypoxia in adult rats. We hypothesized that this response, which we refer to as post-hypoxic frequency decline, is mediated by a mechanism that requires neurones in the VL pons. To address this possibility, we compared respiratory frequency responses to rapid re-oxygenation after a brief exposure to hypoxia both before and after either electrical or chemical lesions, or chemical inhibition, of neuronal activity in the VL pons.

METHODS

General procedures

Eighteen male Sprague–Dawley rats (340–490 g) were anaesthetized with a mixture of 3·0 mg (100 g body wt)$^{-1}$ sodium pentobarbitone and 13·3 mg (100 g body wt)$^{-1}$ chloral hydrate. The adequacy of anaesthesia was assessed by lack of a withdrawal reflex to a noxious paw pinch. The trachea was cannulated and the vagus nerves were transected bilaterally at the cervical level in all but four animals. A femoral artery and vein were cannulated to monitor blood pressure and administer pharmacological agents and a bicarbonate–saline mixture (Na^+, 154 mM; Cl^-, 130 mM; HCO_3^-, 24 mM), respectively. Body temperature was maintained constant at 38·5 ± 0·5 °C. Animals were placed in a stereotaxic frame with the dorsal surface of the cranium horizontal. Bilateral parietal craniotomies were

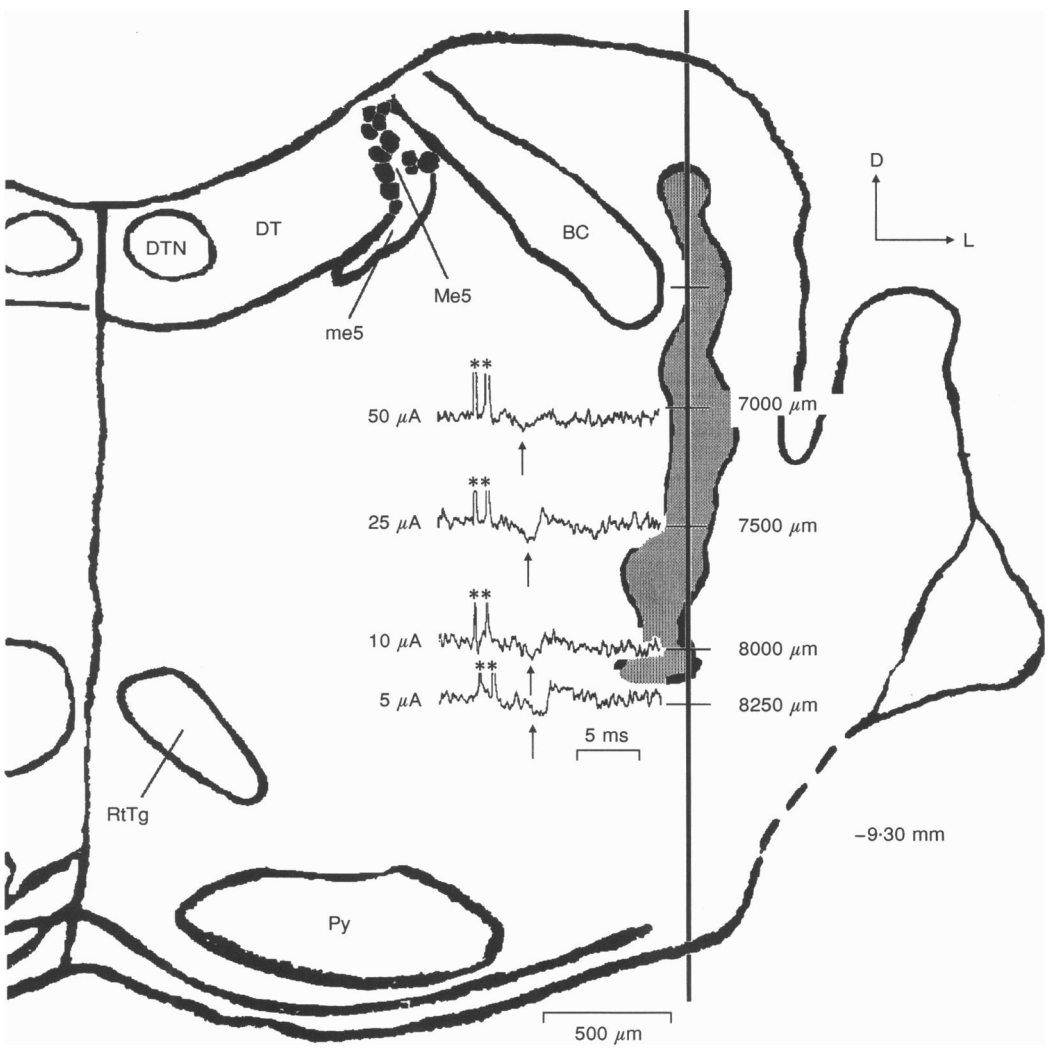

Figure 1. Identification of the target site

Stimulus-triggered averages of rectified phrenic nerve activity (PNA) at different depths from the cerebral surface are displayed on a reconstructed hemisection of the caudal pons, approximately 9·3 mm caudal to bregma. Inhibition of PNA (arrow) followed the stimulus (*) with a short latency (4–5 ms). Threshold stimulus currents are shown to the left of each trace. The optimal stimulation site in this animal was located 8·25 mm ventral to the cerebral surface. Abbreviations: BC, brachium conjunctivum; DT, dorsal tegmentum; DTN, dorsal tegmental nucleus; Py, pyramidal tract; Me5, mesencephalic trigeminal nucleus; me5, mesencephalic trigeminal tract; RtTg, pontine reticulotegmental nucleus. The shaded area indicates tissue damage due to penetration of the electrode.

performed 2·5 mm lateral to the mid-line and 9·0 mm caudal to bregma, and the dura mater and arachnoid membrane were cut and reflected.

Animals with vagus nerves transected. The right phrenic nerve was isolated using a dorsal approach, transected, the central end placed over bipolar silver wire recording electrodes, and the nerve insulated with a mixture of petroleum jelly and mineral oil. The animal was then paralysed (Pavulon, pancuronium bromide, 0·1 mg $(100\ g)^{-1}\ h^{-1}$ I.V.) and ventilated with 100% O_2. The level of anaesthesia was assessed regularly and considered adequate by the absence of cardiorespiratory responses to a noxious paw pinch. Supplemental doses of anaesthetic were infused intravenously as needed.

Phrenic nerve activity (PNA) was amplified and filtered (0·1 Hz to 3 kHz, Grass P511), then rectified and integrated (Paynter filter; time constant, 50 ms), using a moving averager (CWE, Inc., Ardmore, PA, USA). Blood pressure, raw and integrated PNA, airflow, stimulus pulses and event markers were recorded using magnetic tape (Hewlett-Packard) and a chart recorder (Astro-Med Dash 8).

Animals with vagus nerves intact. In four animals, the vagus nerves were left intact to minimize changes in inspiratory and expiratory phase durations following VL pontine intervention. The animals were not paralysed and breathed spontaneously. Bipolar tungsten wire electrodes were inserted into the diaphragm percutaneously to record respiratory muscle activity.

Experimental protocol

Microstimulation and averaging techniques were used to identify sites in the VL pons that produced a decrease in respiratory frequency when stimulated unilaterally using low threshold currents (Fig. 1). The stereotaxic co-ordinates used were the same as those established by Jodkowski *et al.* (1994), where inhibition of VL pontine activity prolonged inspiration. To localize effective sites, a monopolar tungsten electrode (AM Systems, 0·010 inch diameter, 12 MΩ) was initially positioned 4·5 mm ventral to the dorsal cerebral surface, 9·05 mm caudal to bregma and 2·5 mm lateral to the mid-line. Trains of rectangular stimulus pulses (100 μA, 10 or 100 Hz, 1 ms pulse duration) were delivered to elicit a respiratory response. Following each set of test stimuli, the electrode was initially lowered through the brainstem in 500 μm steps until a response was elicited with a 50 μA current. Then the electrode was lowered in progressively smaller increments until the target site was identified. At the effective site, at stimulus currents between 5 and 20 μA, a 10 Hz pulse train produced a transient, short-latency inhibition evident in the stimulus-triggered average of rectified PNA (Fig. 1), while a 100 Hz pulse train elicited a decrease in respiratory frequency. To minimize mechanical damage to the brainstem, this mapping procedure was performed on only one side of the brain. The contralateral target site was located using stereotaxic co-ordinates of the identified target site.

After the target sites were identified, the role of the VL pons in mediating post-hypoxic frequency decline was studied using electrolytic or chemical lesions ($n = 3$) or muscimol injections to inhibit neural activity ($n = 9$ in the target area). Initially, electrolytic lesions ($n = 2$) were used to establish whether tissue damage at the target site abolished the frequency decline typically observed following hypoxia. Electrolytic lesions, however, damage fibres of passage as well as cell bodies. Therefore, an experiment using ibotenic acid, an excitotoxic agent that causes depolarization block of cell bodies, was performed to determine whether differences in the response following electrolytic lesions could have resulted from damage to axons of passage. Since excitotoxic agents may produce lesions distal to the injection site, experiments were also performed using muscimol, a long-lasting $GABA_A$ receptor agonist, to specifically inhibit neuronal activity at the tip of the micropipette ($n = 15$). In the vagally intact rats the airway was occluded momentarily at the end of inspiration to assess the effectiveness of the muscimol injections in the VL pons (see Fig. 2). Control experiments ($n = 6$) consisted of bilateral muscimol injections at sites at least 1 mm dorsal to the optimal stimulation site.

Electrolytic lesions were made using the stimulating microelectrode (5 mA, 10 s, DC pulses). For chemical injections, the electrode was replaced with a glass micropipette filled with either muscimol (10 mM) or ibotenic acid (1 mM). Preliminary studies demonstrated that bilateral injections were required to elicit a change in breathing pattern following hypoxia. Following placement of the microelectrode or micropipettes, but prior to the lesion or microinjection, baseline PNA was recorded while the animal breathed 100% O_2. An initial test response to 8% O_2 (balance N_2) was then recorded. Test gases were presented for a short period (mean, 40 s; range, 20–110 s; see Tables 1 and 2) to minimize metabolic effects of the hypoxic exposure. This hypoxic challenge was sufficient to elicit post-hypoxic frequency decline in all but two animals. In those two animals, the vagi were intact and a more severe stimulus was necessary to elicit post-hypoxic frequency decline (see Table 2). Following the hypoxic episode, the animal was returned to the hyperoxic breathing gas. Electrolytic lesions or bilateral microinjections (10–150 nl) were then made at the target sites and the test was repeated. The volumes (50–500 nl) of control injections dorsal to the target sites were comparable to or greater than the volumes delivered at the target sites. The interval between pre- and post-intervention hypoxic trials ranged from 20 to 150 min. Respiratory parameters were allowed to stabilize (mean, 4 ± 3 min; range, 2–10 min) prior to the second hypoxic exposure. Phrenic nerve activity was recorded for at least 30 min after this final test.

To assess whether reflex cardiorespiratory responses were altered by pontine interventions, and in the absence of effects due to hypoxia, L-lobeline (1 mg kg^{-1}, 0·07 ml per rat, Sigma) and capsaicin (8-methyl-*N*-vanillyl-6-nonenamide, 5 μg kg^{-1}, 0·12 ml per rat, Sigma) were injected intravenously. In vagotomized animals, capsaicin activates sympathetic afferent nerve endings and elicits apnoea (Peterson & Brown, 1971; Lundberg, Brodin & Saria, 1983); lobeline activates carotid chemoreceptor afferents and augments cardiorespiratory output. Only animals with sectioned vagi were tested with these compounds.

Fast Green (2%) was mixed with the injectate in all but one experiment, to mark the sites of pressure injections iontophoretically. The target site was marked on only one side in spontaneously breathing rats. At the end of each experiment the rat was perfused transcardially with heparinized saline followed by 4% paraformaldehyde in 0·2 M phosphate-buffered saline (pH 7·4). The brain was removed, infiltrated with a 30% sucrose-fixative mixture, and the brainstem sectioned (50 or 100 μm) in the coronal plane with a cryostat or freezing microtome. Sections with electrolytic lesions were counterstained (0·1% thionine) and traced using a drawing tube. Sections containing chemical injection sites marked with Fast Green were first traced, then counterstained. The locations of lesion and injection sites from all experiments were consolidated on representative coronal sections of the pons.

Data analysis

Integrated diaphragmatic or phrenic nerve activity was used as an index of the respiratory cycle. The duration of inspiration (T_I) was measured from the beginning of the steepest rise in the slope of

integrated PNA to the beginning of its steepest decline. The duration of expiration (T_E) was measured from the beginning of the steepest decline in the slope of integrated PNA to the beginning of the rise of the next integrated phrenic nerve burst. The duration of the total respiratory cycle (T_{TOT}) was calculated as the sum of T_I and T_E, and breathing frequencies were calculated as the reciprocal of T_{TOT}. In all animals, T_I and T_E were plotted against sequential respiratory cycles before, during and after hypoxia, both before

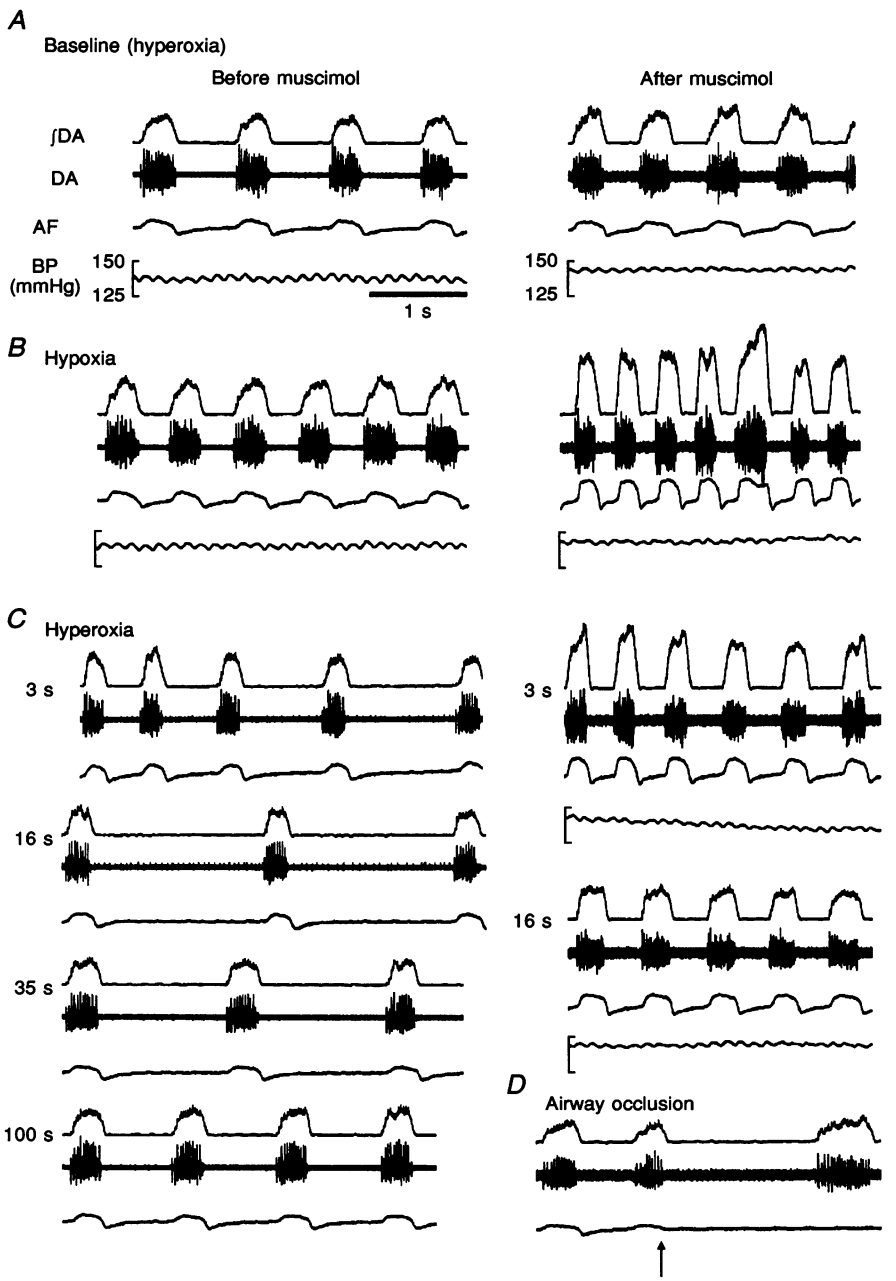

Figure 2. Respiratory response of a spontaneously breathing adult rat with vagus nerves intact before (left-hand panels), and after (right-hand panels), bilateral injections of muscimol in the VL pons

The vagi were left intact to minimize changes in inspiratory and expiratory timing that occur after inhibition of activity in VL pons. *A*, baseline pattern in an animal breathing 100% O_2. *B*, breathing pattern during ventilation with 8% O_2. After muscimol, the peak response during hypoxia was enhanced. *C*, breathing pattern at various times following return to 100% O_2. The post-hypoxic frequency decline that was apparent before the muscimol injection was attenuated after inhibition of neural activity in VL pons. *D*, airway occlusion (at arrow) at the end of inspiration prevented lung deflation and prolonged expiration, indicating functional vagal activity. The prolonged T_I in the following breath indicates that muscimol injections were effective. (Time, 250 s after switching back to hyperoxia.) Data were recorded from rat 1 (Table 2). Traces, from top: integrated diaphragmatic electromyogram (∫DA), raw diaphragmatic activity (DA), airflow (AF) and blood pressure (BP).

and after lesions or microinjections. The time course of the change in frequency following hypoxia was calculated as the time required for recovery of 67% of the original baseline breathing frequency. To quantify the respiratory response before, during and after hypoxia, T_I and T_E were averaged for three consecutive respiratory cycles at the following points: (1) immediately before hypoxia while breathing 100% O_2; (2) at the peak of the hypoxic response, defined as the period of highest phrenic burst frequency during hypoxia; (3) 5–10 s after the peak response but still during hypoxia; (4) 10 s after switching from the hypoxic to the hyperoxic maintenance gas; (5) 30 s following the return to hyperoxia (in the vagally intact animals). We assumed similar 'wash-in' and 'wash-out' dynamics during changes in the breathing gas. Because PNA began to increase within 5–10 s after switching from 100 to 8% O_2, we measured T_I and T_E 10 s after the return to hyperoxia. In vagotomized animals, differences between baseline and post-hypoxic T_E were assessed using Student's paired t tests (two-tailed; $\alpha = 0{\cdot}05$) both before and after VL pontine intervention. In vagally intact animals, the mean T_E after hypoxia both before and after VL pontine intervention was plotted at these five time points. Differences in mean values were evaluated using two-way ANOVA for repeated measures followed by the Bonferroni *post hoc* test ($\alpha = 0{\cdot}05$). In addition, bar graphs were constructed to show the percentage change from baseline in breathing frequency after hypoxia both before and after pontine treatments. Statistical significance of the differences was analysed by the Mann–Whitney U test (two-tailed; $\alpha = 0{\cdot}05$).

RESULTS

Respiratory frequency following brief episodes of hypoxia

Respiratory frequency declined significantly following hypoxia (arterial O_2 pressure (P_{a,O_2}), 27·4 ± 7·7 mmHg) in both intact and spontaneously breathing animals (cf. Fig. 2*A* and *C*, left-hand panels) and in paralysed and ventilated animals with transected vagi (cf. Fig. 3*A a* and *A b*). Post-hypoxic frequency decline occurred consistently (Tables 1 and 2), and resulted from a prolongation of expiration. Expiratory duration increased 113–276% above hyperoxic baseline levels ($P = 0{\cdot}0001$), while inspiratory time was unchanged (Table 1). Respiratory frequency slowly returned to pre-hypoxic values with a time constant of 45 ± 25 s.

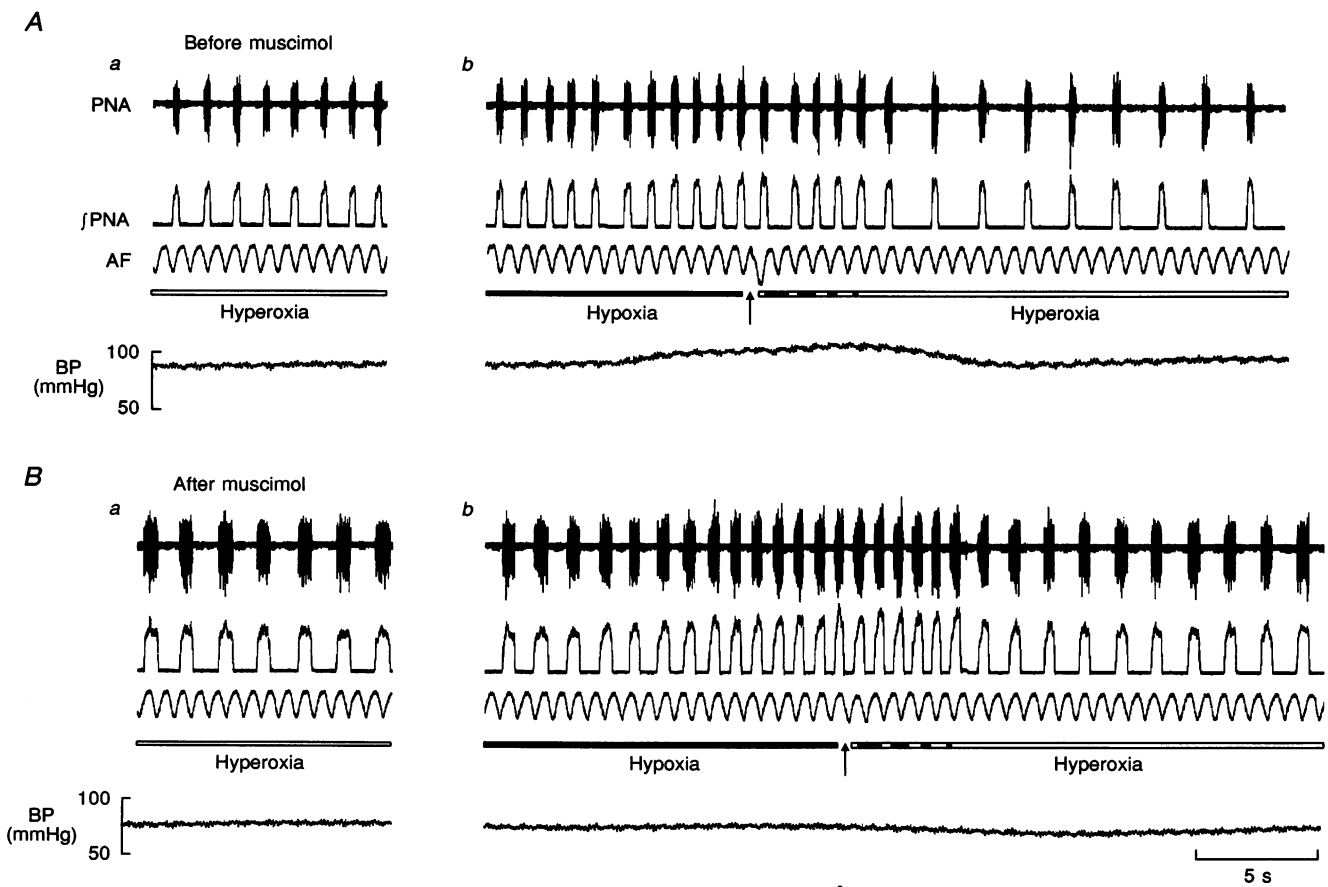

Figure 3. Post-hypoxic frequency decline before (*A*) and after (*B*) bilateral injections of muscimol into VL pons

T_E following hypoxia (arrow in *A b*) was prolonged compared with T_E during baseline hyperoxic conditions (*A a*). After muscimol, T_I increased but T_E remained the same (*B a*). Post-hypoxic frequency decline was attenuated by muscimol (compare *A b* and *B b*), despite the change in baseline timing following injection of the drug. Traces, from top: raw phrenic nerve activity (PNA), integrated phrenic nerve activity (∫PNA), airflow (AF) and blood pressure (BP). Arrows indicate switch from 8 to 100% O_2. Data were recorded from rat 8 (Table 1).

Table 1. Inspiratory (T_I) and expiratory (T_E) time (s) before and after VL pontine intervention

Rat	Duration of hypoxia (s) / Time to peak (s)		Baseline (100% O_2)	Peak hypoxic response (8% O_2)	5–10 s after peak hypoxic response (8% O_2)	10 s post-hypoxic exposure (100% O_2)
		Before lesions/injections				
		Electrolytic lesion				
1	30	T_I (s)	0·42 ± 0·03	0·34 ± 0·01	0·32 ± 0·00	0·25 ± 0·03
	20	T_E (s)	0·60 ± 0·04	0·39 ± 0·02	0·54 ± 0·02	1·66 ± 0·27
2	90	T_I (s)	0·36 ± 0·02	0·32 ± 0·01	0·31 ± 0·01	0·31 ± 0·00
	30	T_E (s)	0·91 ± 0·01	0·70 ± 0·02	0·81 ± 0·05	2·33 ± 0·15
		Ibotenic acid lesion				
3	50	T_I (s)	0·35 ± 0·01	0·32 ± 0·01	0·25 ± 0·01	0·23 ± 0·02
	10	T_E (s)	0·96 ± 0·05	0·62 ± 0·03	0·76 ± 0·02	2·06 ± 0·03
		Muscimol injection				
4	60	T_I (s)	0·88 ± 0·06	0·80 ± 0·20	0·50 ± 0·03	0·42 ± 0·02
	10	T_E (s)	0·94 ± 0·04	0·49 ± 0·02	0·82 ± 0·14	2·27 ± 0·21
5	60	T_I (s)	0·50 ± 0·01	0·45 ± 0·06	0·26 ± 0·02	0·42 ± 0·01
	10	T_E (s)	0·94 ± 0·03	0·50 ± 0·03	0·92 ± 0·06	2·04 ± 0·17
6	30	T_I (s)	0·42 ± 0·01	0·30 ± 0·01	—	0·36 ± 0·03
	30	T_E (s)	1·09 ± 0·02	0·94 ± 0·03	—	1·58 ± 0·09
7	30	T_I (s)	0·39 ± 0·03	0·39 ± 0·04	0·38 ± 0·03	0·36 ± 0·01
	10	T_E (s)	1·03 ± 0·03	0·60 ± 0·05	1·04 ± 0·32	1·68 ± 0·08
8	20	T_I (s)	0·30 ± 0·00	0·30 ± 0·00	—	0·29 ± 0·00
	20	T_E (s)	1·01 ± 0·04	0·62 ± 0·03	—	1·59 ± 0·03
		After lesions/injections				
		Electrolytic lesion				
1	20	T_I (s)	0·66 ± 0·08	0·38 ± 0·07	0·38 ± 0·02	0·51 ± 0·05
	10	T_E (s)	0·98 ± 0·02	0·32 ± 0·02	0·43 ± 0·06	1·23 ± 0·11
2	110	T_I (s)	4·57 ± 0·70	0·89 ± 0·24	—	0·97 ± 0·28
	85	T_E (s)	11·07 ± 0·98	1·88 ± 0·38	—	4·94 ± 1·16
		Ibotenic acid lesion				
3	90	T_I (s)	1·16 ± 0·15	0·37 ± 0·05	0·29 ± 0·04	0·70 ± 0·12
	30	T_E (s)	1·97 ± 0·09	0·80 ± 0·07	0·88 ± 0·02	1·53 ± 0·16
		Muscimol injection				
4	70	T_I (s)	3·00 ± 0·15	0·88 ± 0·49	1·74 ± 0·49	0·61 ± 0·11
	70	T_E (s)	10·47 ± 2·73	0·61 ± 0·13	0·85 ± 0·05	1·62 ± 0·16
5	60	T_I (s)	1·26 ± 0·39	0·32 ± 0·01	0·32 ± 0·01	0·70 ± 0·01
	20	T_E (s)	0·83 ± 0·00	0·35 ± 0·00	0·58 ± 0·04	1·14 ± 0·07
6	30	T_I (s)	1·30 ± 0·03	0·54 ± 0·08	—	1·32 ± 0·12
	30	T_E (s)	1·63 ± 0·25	1·34 ± 0·17	—	1·36 ± 0·06
7	30	T_I (s)	2·02 ± 0·13	0·56 ± 0·14	0·81 ± 0·20	1·73 ± 0·04
	15	T_E (s)	2·13 ± 0·47	0·82 ± 0·13	0·78 ± 0·10	2·14 ± 0·43
8	20	T_I (s)	0·58 ± 0·02	0·40 ± 0·02	—	0·45 ± 0·01
	17	T_E (s)	1·01 ± 0·03	0·43 ± 0·03	—	1·06 ± 0·02

Values presented are means ± S.D. By definition, T_I and T_E occurring 5–10 s after the peak response to hypoxia could not be measured in experiments where the peak coincided with or near the end of the hypoxic challenge.

Table 2. Inspiratory (T_I) and expiratory (T_E) time (s) of intact rats before and after muscimol injections in the ventrolateral pons

Rat	Duration of hypoxia (s) / Time to peak (s)		Baseline (100% O_2)	Peak hypoxic response (8% O_2)	5–10 s after peak hypoxic response (8% O_2)	10 s post-hypoxic exposure (100% O_2)
			Before muscimol injections			
1	*	T_I (s)	0·36 ± 0·01	0·32 ± 0·01	—	0·24 ± 0·02
		T_E (s)	0·56 ± 0·04	0·35 ± 0·00	—	1·76 ± 0·04
2	22 / 14	T_I (s)	0·15 ± 0·01	0·15 ± 0·01	0·16 ± 0·00	0·17 ± 0·01
		T_E (s)	0·39 ± 0·01	0·19 ± 0·00	0·26 ± 0·05	0·68 ± 0·14
3	62 / 16	T_I (s)	0·22 ± 0·00	0·14 ± 0·01	0·20 ± 0·05	0·23 ± 0·01
		T_E (s)	0·46 ± 0·00	0·30 ± 0·02	0·36 ± 0·04	0·68 ± 0·04
4	28 / 16	T_I (s)	0·24 ± 0·00	0·22 ± 0·01	0·25 ± 0·06	0·27 ± 0·00
		T_E (s)	0·49 ± 0·00	0·44 ± 0·01	0·53 ± 0·06	0·65 ± 0·01
			After muscimol injections			
1	*	T_I (s)	0·35 ± 0·05	0·25 ± 0·02	—	0·28 ± 0·01
		T_E (s)	0·52 ± 0·03	0·18 ± 0·02	—	0·36 ± 0·03
2	50 / 10	T_I (s)	0·23 ± 0·02	0·17 ± 0·01	—	0·23 ± 0·01
		T_E (s)	0·32 ± 0·00	0·18 ± 0·02	—	0·28 ± 0·01
3	36 / 28	T_I (s)	0·73 ± 0·00	0·47 ± 0·01	0·48 ± 0·06	0·72 ± 0·01
		T_E (s)	0·31 ± 0·00	0·26 ± 0·01	0·35 ± 0·04	0·30 ± 0·01
4	23 / 22	T_I (s)	0·33 ± 0·01	0·21 ± 0·02	0·17 ± 0·01	0·35 ± 0·00
		T_E (s)	0·73 ± 0·02	0·38 ± 0·05	0·37 ± 0·04	0·67 ± 0·02

Rat 2 was exposed to 100% N_2 and the remaining rats were exposed to 8% O_2. Rat 1 was exposed for 10 min (*); the post-peak hypoxic measurement did not apply in this instance.

Effects of VL pontine intervention on baseline breathing pattern

Chemical ($n = 1$) or electrolytic ($n = 2$) lesions of the VL pons decreased respiratory frequency (Table 1) by increasing both T_I and T_E. Muscimol injections ($n = 5$) into the target area altered baseline respiratory timing in a manner similar to lesions (Table 1). Inspiration was prolonged in all of these animals, and expiration either increased ($n = 3$) or did not change ($n = 2$). Leaving the vagal nerves intact minimized or attenuated changes in baseline phase duration following muscimol injections (Table 2). The effectiveness of the injections was confirmed by observing a prolonged T_I with tracheal occlusion at end inspiration (Fig. 2*D*).

Effects of VL pontine intervention on post-hypoxic breathing frequencies

Lesions or inhibition of neural activity in the VL pons, attenuated or abolished post-hypoxic frequency decline (Figs 2–6; Tables 1 and 2). Post-hypoxic breathing frequencies were significantly increased ($P = 0·0001$) compared with pre-hypoxic baseline frequencies following lesions or microinjections in all animals studied (Fig. 6; Tables 1 and 2). In these animals expiratory duration following hypoxia was not significantly different from that observed under hyperoxic baseline conditions ($P = 0·17$ in the vagotomized animals and $P > 0·05$ in the non-vagotomized animals). Disruption of neural activity in the VL pons attenuated post-hypoxic frequency decline both in the presence of active vagal input and in its absence (cf. Tables 1 and 2), and in vagotomized animals where T_E did not change (Figs 3 and 4).

Control studies

In contrast, post-hypoxic frequency decline remained in all control animals where muscimol injections were made within the lateral pons but dorsal to the target area (Fig. 6*C*; Fig. 7; Table 3). In these animals T_I and T_E changed after muscimol injections (Fig. 7). Inspiratory durations were either prolonged ($n = 2$) or shortened ($n = 4$), and expiratory durations were shortened ($n = 3$), prolonged ($n = 2$), and unchanged ($n = 1$). Despite changes in baseline breathing pattern, post-hypoxic frequency decline remained, because post-hypoxic T_E was significantly longer ($P = 0·01$) than baseline T_E.

Table 3. Inspiratory (T_I) and expiratory (T_E) time (s) in control experiments before and after injections of muscimol in the lateral pons

Rat	Duration of hypoxia (s) / Time to peak (s)		Baseline (100% O_2)	Peak hypoxic response (8% O_2)	5–10 s after peak hypoxic response (8% O_2)	10 s post-hypoxic exposure (100% O_2)
			Before muscimol injections			
1	60 / 10	T_I (s)	0·45 ± 0·01	0·40 ± 0·02	0·36 ± 0·01	0·33 ± 0·00
		T_E (s)	1·06 ± 0·05	0·58 ± 0·00	0·71 ± 0·16	1·51 ± 0·03
2	20 / 10	T_I (s)	0·42 ± 0·00	0·43 ± 0·01	0·43 ± 0·01	0·48 ± 0·02
		T_E (s)	0·98 ± 0·04	0·64 ± 0·07	2·67 ± 0·64	2·25 ± 0·29
3	20 / 12	T_I (s)	0·36 ± 0·01	0·29 ± 0·02	0·30 ± 0·01	0·29 ± 0·00
		T_E (s)	1·46 ± 0·04	0·79 ± 0·08	1·35 ± 0·48	2·97 ± 0·30
4	20 / 10	T_I (s)	0·35 ± 0·01	0·35 ± 0·02	0·30 ± 0·01	0·30 ± 0·03
		T_E (s)	0·78 ± 0·06	0·49 ± 0·04	2·35 ± 1·67	1·65 ± 0·14
5	30 / 5	T_I (s)	0·40 ± 0·01	0·43 ± 0·02	0·38 ± 0·04	0·38 ± 0·01
		T_E (s)	1·28 ± 0·02	0·96 ± 0·17	2·08 ± 0·10	1·71 ± 0·03
6	30 / 13	T_I (s)	0·32 ± 0·02	0·39 ± 0·01	0·42 ± 0·01	0·36 ± 0·01
		T_E (s)	1·40 ± 0·10	0·68 ± 0·05	1·04 ± 0·31	1·58 ± 0·02
			After muscimol injections			
1	60 / 15	T_I (s)	0·37 ± 0·00	0·31 ± 0·01	0·31 ± 0·03	0·42 ± 0·01
		T_E (s)	0·70 ± 0·02	0·46 ± 0·02	0·81 ± 0·12	2·41 ± 0·58
2	20 / 8	T_I (s)	0·66 ± 0·01	0·59 ± 0·03	0·54 ± 0·04	0·66 ± 0·02
		T_E (s)	0·71 ± 0·07	0·60 ± 0·04	0·85 ± 0·07	1·81 ± 0·55
3	20 / 10	T_I (s)	0·32 ± 0·00	0·32 ± 0·01	0·32 ± 0·01	0·34 ± 0·01
		T_E (s)	1·24 ± 0·07	0·89 ± 0·25	2·37 ± 0·61	3·49 ± 0·52
4	20 / 13	T_I (s)	0·61 ± 0·03	0·39 ± 0·04	0·35 ± 0·00	0·46 ± 0·05
		T_E (s)	1·25 ± 0·12	0·75 ± 0·16	0·97 ± 0·04	2·39 ± 0·74
5	20 / 10	T_I (s)	0·40 ± 0·02	0·39 ± 0·02	0·36 ± 0·02	0·38 ± 0·02
		T_E (s)	1·45 ± 0·04	1·13 ± 0·26	1·88 ± 0·13	1·86 ± 0·05
6	30 / 25	T_I (s)	0·38 ± 0·02	0·36 ± 0·01	—	0·39 ± 0·02
		T_E (s)	1·37 ± 0·00	0·32 ± 0·04	—	1·54 ± 0·09

Note that in rat 6 the peak respiratory response occurred at the end of the hypoxic exposure.

Effects of hypoxia, lobeline and capsaicin on respiratory frequency

The initial response to hypoxia remained intact. Respiratory rate and amplitude, and arterial blood pressure increased in response to hypoxia (Fig. 2*B*, left-hand panel; Fig. 3*A b*). These changes remained following VL pontine intervention (Fig. 2*B*, right-hand panel; Fig. 3*B b*).

To determine whether either lesions or muscimol injections altered the respiratory response to selective stimulation of afferent nerves, the effects of intravenous bolus injections of lobeline (Fig. 8*A*) and capsaicin (Fig. 8*B*) were studied. Respiratory responses to either agent were qualitatively similar before (Fig. 8*A a* and *B a*) and after (Fig. 8*A b* and *B b*) either type of VL pontine intervention. Lobeline elicited transient increases in the frequency and amplitude of phrenic discharges and produced a slight increase in arterial blood pressure (Fig. 8*A a*), similar to that observed during hypoxia. Lobeline decreased inspiratory and expiratory times to a similar extent in animals either with or without VL pontine intervention (Fig. 8*A b*). The increases in respiratory rate evoked by lobeline dissipated gradually, in contrast to the abrupt decrease in frequency observed following hypoxia.

Capsaicin evoked a transient (5 s) inhibition of PNA (Fig. 8*B a*). This inhibition preceded a sustained pressor response that developed during the apnoea and remained during the initial few breaths following the apnoeic period. When PNA resumed, the amplitude of the phrenic discharge was initially elevated and diminished gradually, but T_I and T_E remained essentially unchanged. The same

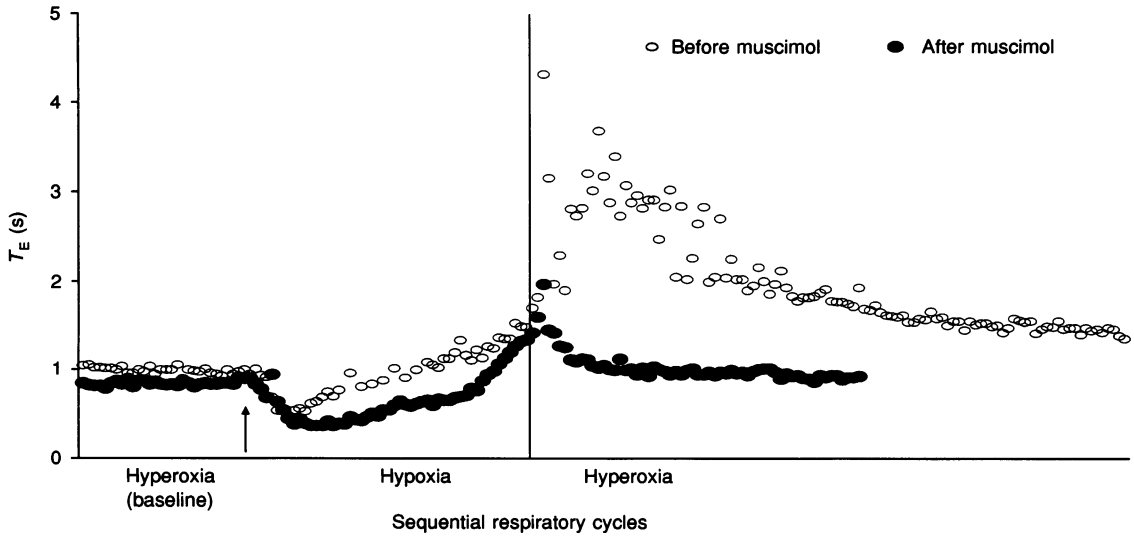

Figure 4. Absolute times (s) of T_E plotted sequentially for each respiratory cycle during hyperoxia, hypoxia (arrow) and after return to hyperoxia (continuous line) both before and after muscimol injections into the VL pons

Comparable durations of T_E prior to hypoxia both before and after pontine injections permitted a direct comparison of post-hypoxic frequency decline. Following muscimol injections, post-hypoxic frequency decline was markedly attenuated. Data were recorded from rat 5 (Table 1).

response to capsaicin was observed following disruption of VL or lateral pontine function (Fig. 8*Bb*), despite changes in baseline timing.

Histological verification of injection and lesion sites

The majority of effective injection or lesion sites was located in the VL quadrant of the caudal pons (Fig. 9*A*). Specifically, sites were located in the lateral pontine tegmental field (approximately 9 mm caudal to bregma) and included portions of an area defined by Dahlström & Fuxe (1964) as the A_5 cell group. The correlation between lesion or injection sites and the A_5 region was based upon anatomical landmarks only (Paxinos & Watson, 1986). The most caudal sites (10 mm caudal to bregma) were located at the level of the facial nerve, and included the caudal most part of the A_5 cell column. The most rostral site (8 mm caudal to bregma) was located in a region containing the rostralmost part of the A_5 cell column. Effective sites were bilaterally asymmetrical in two animals, but included the VL pons on at least one side. Post-hypoxic frequency decline was eliminated in these two animals (Table 1).

Control injections were located in the dorsolateral pons (Fig. 9*B*) and included the Kölliker–Fuse nuclei and the

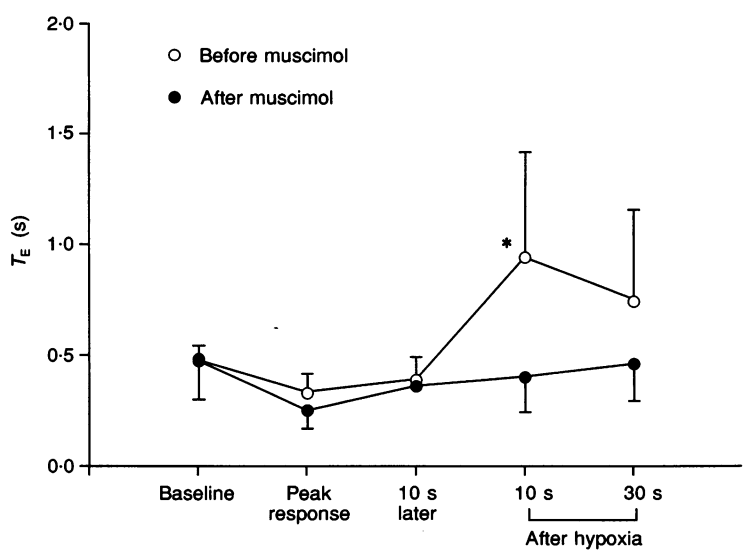

Figure 5. Muscimol injections into the ventrolateral pons eliminate post-hypoxic frequency decline in rats with vagus nerves intact

The mean (± S.D.) of T_E was calculated for three breaths occurring before hypoxia (Baseline), during the peak response to hypoxia (Peak response), 10 s later in hypoxia, and at 10 and 30 s after hypoxia, respectively. Baseline breathing pattern did not change after muscimol in these animals. Mean T_E 10 s after hypoxia was greater before than after muscimol (* $P < 0{\cdot}01$).

area between the sensory and motor nuclei of the trigeminal nerve from which apnoeas have been elicited following chemical stimulation (Chamberlin & Saper, 1994). Two control injections extended more ventrally to overlap the exit of the trigeminal motor nerve, while two other sites included the inferior colliculi, the cerebellum and the middle cerebellar peduncle. The rostrocaudal extent of these lesions ranged between approximately 8 and 10 mm caudal to bregma.

DISCUSSION

Since the studies of Lumsden (1923), it has been recognized that phase duration and breathing frequency are influenced by both vagal afferent and pontine neural activity. For example, in adult rats inspiratory and expiratory durations are altered dramatically if lesions in the dorsolateral pons are combined with vagotomy (Wang, Fung & St John, 1993; Morrison, Cravo & Wilfehrt, 1994). Recently, we demonstrated in adult, vagotomized rats that inhibition of neural activity in the VL pons also alters inspiratory and expiratory timing (Jodkowski *et al.* 1994), and that expiration is prolonged by activation of neurones in the VL pons following microinjections of the excitatory amino acid, glutamate (Dick *et al.* 1995). From these studies, we proposed that the pneumotaxic centre in adult rats be extended to include the VL pons (Dick *et al.* 1995). Here we report that post-hypoxic frequency decline requires the functional integrity of the VL pons in anaesthetized adult rats. Lesions or chemical inhibition of neuronal activity in an area that includes the A_5 cell group resulted in the attenuation or elimination of the frequency decline observed following hypoxia, while responses to carotid body and sympathetic afferent stimulation were unaffected. These data support our hypothesis that the VL pons modulates phase duration and breathing frequency (Jodkowski *et al.* 1994) and demonstrate that the VL pons plays an important role in shaping the breathing pattern immediately after hypoxia. Moreover, our data indicate a neuronal component to the short-term depression of respiratory frequency following hypoxia.

Post-hypoxic frequency decline

Electrical stimulation of the carotid sinus nerve during hyperoxia evokes an increase in T_E and a decrease in respiratory frequency that are similar to the response elicited by isocapnic hypoxia (Hayashi *et al.* 1993), suggesting that carotid body afferent input activates a neural network that prolongs expiration during and after hypoxia. Our study demonstrates that the VL pons is required for this response. Elimination of neuronal activity at specific sites in the VL pons through lesions, or muscimol injections, abolished the post-hypoxic frequency decline normally seen following brief episodes of hypoxia.

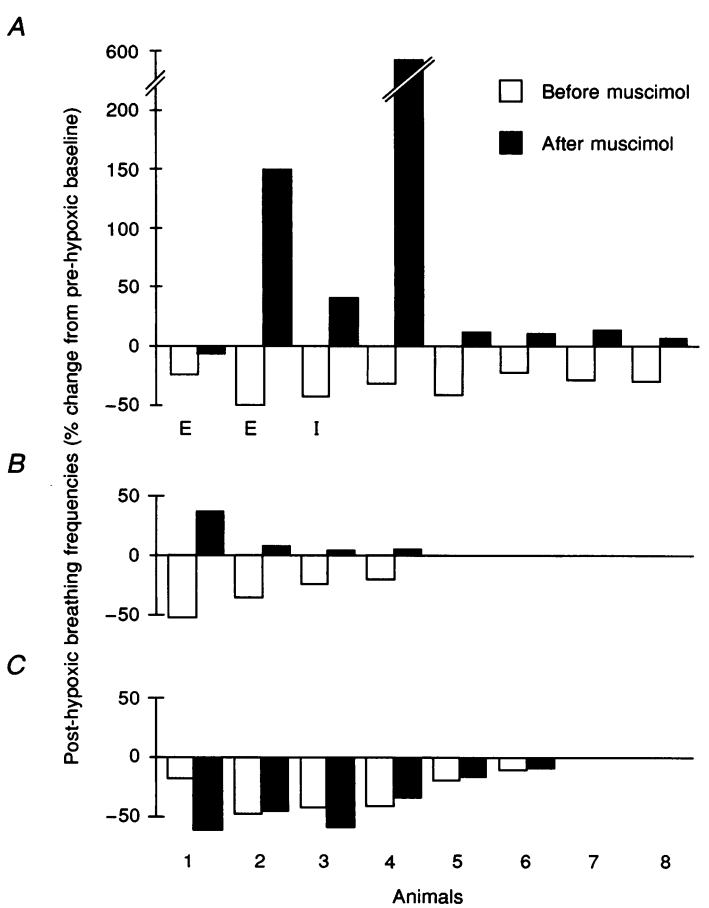

Figure 6. Post-hypoxic breathing frequency (expressed as percentage change from pre-hypoxic baseline) in individual animals, both before and after either electrolytic or chemical lesions or muscimol injections

Interventions in *A* (vagotomized animals) and *B* (animals with vagi intact) were localized to the ventrolateral pons. Control injections were made in the dorsolateral pons of vagotomized animals (*C*). Respiratory frequency following hypoxia decreased in all animals with the ventrolateral pons intact (□). In all cases, the post- hypoxic frequency increased ($P = 0{\cdot}0001$) following ventrolateral pontine intervention (*A* and *B*, ■), while control injections into the dorsolateral pons had no effect (*C*, ■). E, electrolytic lesion; I, ibotenic acid lesion.

In addition to activating central neural mechanisms via carotid body afferent stimulation, hypoxia also exerts metabolic effects at both systemic and cellular levels. For example, an increase in cerebral blood flow during hypoxia could lead to a decrease in P_{CO_2} in the cerebrospinal fluid and decreased respiratory drive from the central chemoreceptors (Neubauer, Melton & Edelman, 1990). However, metabolic factors alone could not account for the observed decline in respiratory frequency during hypoxia in unanaesthetized goats (Gershan *et al.* 1994). At the cellular level, prolonged anoxia depletes phosphocreatine and adenosine triphosphate (ATP) stores, ultimately leading to failure of synaptic transmission (Whittingham & Lipton, 1981). However, direct measurement of these compounds in the brainstem revealed that only phosphocreatine levels were decreased after 2 min of 12% O_2 (Piérard *et al.* 1995). Although respiratory frequency was correlated to intracellular phosphocreatine in this study, the appropriate temporal relationship was not observed. The decrease in respiratory frequency preceded the fall in phosphocreatine levels, the nadir in respiratory frequency preceded the nadir in phosphocreatine, and the depression of respiratory rate persisted after phosphocreatine levels returned to baseline.

In the present study a similar depletion of intracellular phosphocreatine stores might be expected, although rats were exposed to shorter but more severe hypoxic episodes. However, blocking neural activity in the VL pons should not affect the overall decrease in phosphocreatine, since these neurones constitute only approximately 5% of the total volume of the brainstem. Therefore, any reduction in phosphocreatine levels that occurred during hypoxia in our experiments was probably similar before and after muscimol injections. Our finding that post-hypoxic frequency decline was abolished after, but not before muscimol treatment, therefore, argues against a generalized metabolic effect within the brainstem to account for the frequency depression. Taken together, the findings that (a) frequency decline following stimulation of the carotid sinus nerve occurs in the absence of central hypoxia (Hayashi *et al.* 1993), (b) post-hypoxic frequency decline occurs before the appearance of changes in metabolites (Piérard *et al.* 1995), and (c) post-hypoxic frequency decline is reversed after intervention at a specific site in the VL pons, support the hypothesis that post-hypoxic frequency decline occurs primarily through a neurally, rather than a metabolically, mediated mechanism.

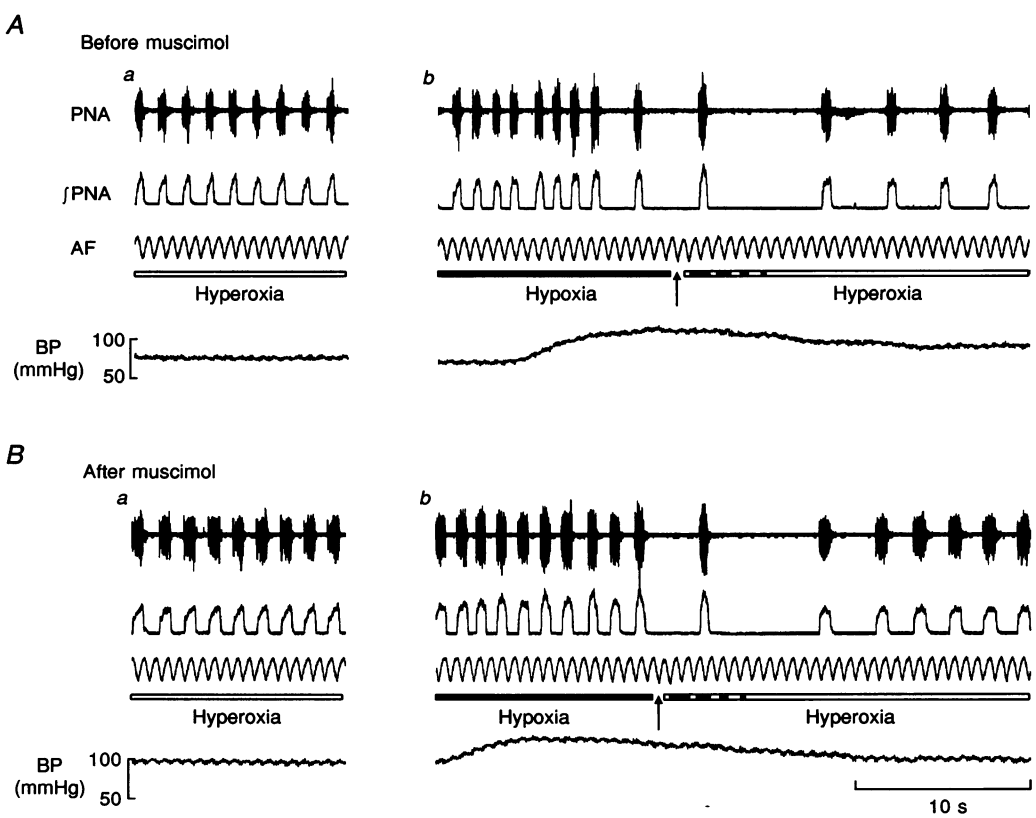

Figure 7. Post-hypoxic frequency decline before (*A*) and after (*B*) bilateral muscimol injections in the lateral pons dorsal to the target region

Post-hypoxic frequency decline was present despite slight changes in breathing pattern following muscimol injections. Arrows indicate switch from 8 to 100% O_2. T_E following hypoxia (arrow in *A b* and *B b*) was prolonged compared with T_E during baseline hyperoxic conditions (*A a* and *B a*). Data were recorded from rat 2 (Table 3). Traces, from top: raw phrenic nerve activity (PNA), integrated phrenic nerve activity (∫PNA), airflow (AF) and blood pressure (BP).

Potential mechanisms activating VL pontine neurones

Neurones in the VL pons could be activated through a number of different mechanisms. For example, the finding that electrical stimulation of the peripheral chemoreceptors produces a response similar to post-hypoxic frequency decline (Hayashi *et al.* 1993) suggests that peripheral chemoafferent input can activate VL pontine neurones, but this reflex pathway may not be obligatory. It is also possible that neurones in the VL pons are themselves chemosensitive and may be activated directly by low O_2, similar to neurones in the rostral ventrolateral medulla described by Sun & Reis (1994). Other possible mechanisms for activating neurones in the VL pons include afferent input from other putative central O_2 sensors, efference copy from the respiratory pattern generator, or disinhibition due to depression of suprapontine inhibitory influences. Indeed, there may be multiple mechanisms, and different mechanisms may be dominant in different situations (Robbins, 1995).

Post-hypoxic frequency decline may be mediated by A_5 neurones

The location of effective interventions included the A_5 noradrenergic cell group in the VL pons. These neurones may play a role in the respiratory response to hypoxia based on the following: (1) A_5 neurones may have reciprocal connections with medullary respiratory groups; (2) A_5 neurones may modulate expiratory duration; and (3) A_5 neurones are activated during hypoxia. In an earlier study, muscimol injections in the A_5 cell group reduced the sympathetic nerve response without affecting the respiratory response to hypoxia, but the duration of anoxia was short

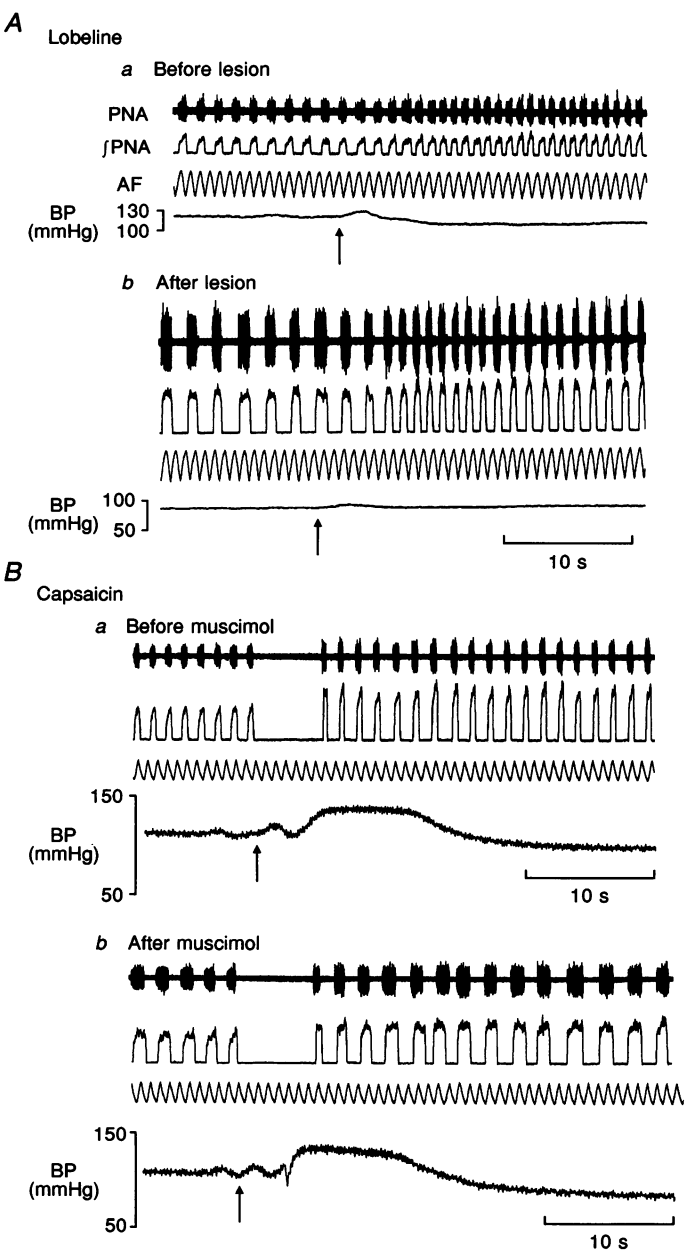

Figure 8. Cardiopulmonary responses to lobeline (*A*) and capsaicin (*B*) before and after VL pontine interventions

Although T_I and T_E increased following either electrolytic lesion (*A b*) or neuronal inhibition with muscimol (*B b*), similar changes in phrenic nerve activity and blood pressure were observed in intact and affected animals following intravenous injections of either agent (arrows). Records are from rat 1 (*A*) and rat 5 (*B*) in Table 1. Traces from top: raw phrenic nerve activity (PNA), integrated phrenic nerve activity (∫PNA), airflow (AF) and blood pressure (BP).

(5–10 s), and post-hypoxic frequency decline was not observed prior to the muscimol injections (Koshiya & Guyenet, 1994; see their Fig. 5). In our study, the initial respiratory response to carotid chemoreceptor activation with hypoxia or lobeline remained intact also. It was the delayed response, especially evident following brief hypoxia (~30 s), that was eliminated after muscimol injections in the VL pons.

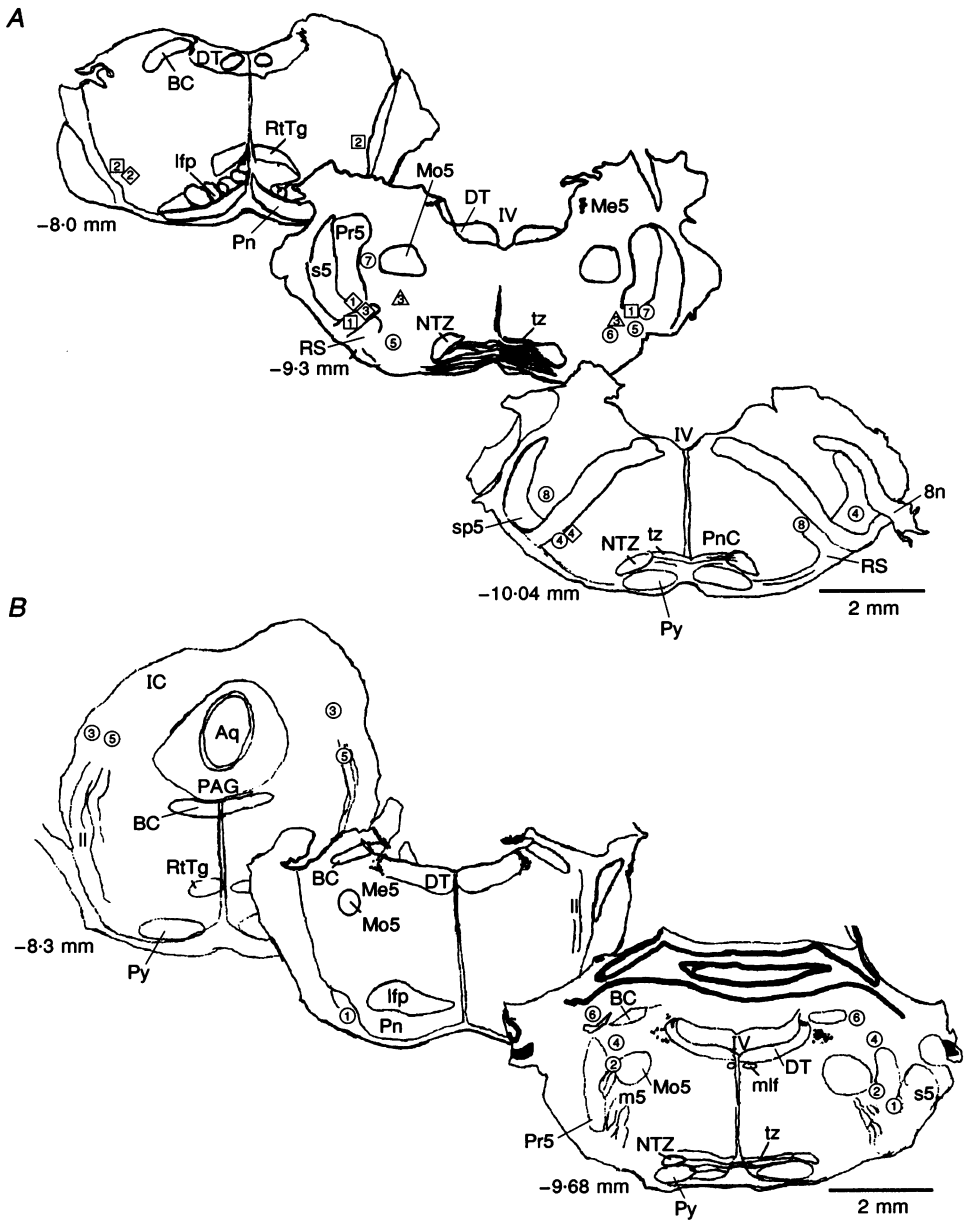

Figure 9. Locations of target (*A*) and control (*B*) microinjections and/or lesions

Sections are arranged from rostral (top) to caudal (bottom); the approximate distances indicated are referenced caudal to bregma. Ibotenic acid injections are indicated with a triangle, muscimol injections with a circle, and electrolytic lesions with a square. Each set of paired injections or lesions are labelled with animal numbers from Tables 1, 2 and 3. Only one site was found for rat 6 in Table 1 and only one side was marked in the spontaneously breathing animals in Table 2 (open diamonds). Abbreviations: 7n, facial nerve; 8n, vestibulocochlear nerve; Aq, Sylvian aqueduct; BC, brachium conjunctivum; DT, dorsal tegmentum; IC, inferior colliculus; IV, fourth ventricle; lfp, longitudinal fasiculus pontis; ll, lateral lemniscus; m5, motor root of V; mlf, medial longitudinal fasciculus; Me5, mesencephalic nucleus of V; Mo5, motor nucleus of V; NTZ, nucleus of the trapezoid body; PAG, periaqueductal gray; Pn, pontine nuclei; PnC, caudal pontine reticular nucleus; Pr5, principal sensory trigeminal nucleus; Py, pyramidal tract; RS, rubrospinal tract; RtTg, reticulotegmental nucleus; s5, sensory root of V; sp5, spinal trigeminal tract; tz, trapezoid body.

Clearly, the brainstem regions innervated by A_5 noradrenergic neurones are involved in cardiovascular regulation. However, recent anatomical studies indicate that neurones in the VL pons project to respiratory-related areas, but it is as yet unclear whether these projections are noradrenergic (Ellenberger & Feldman, 1990; Núñez-Abades, Morillo & Pásaro, 1993; Dobbins & Feldman, 1994). However, A_5 neurones receive afferent input from the caudal ventrolateral medulla, which contains both sympathoinhibitory and respiratory neurones (Byrum & Guyenet, 1987; Li, Wesselingh & Blessing, 1992). Thus noradrenergic neurones can potentially influence the respiratory regulatory system via reciprocal pathways between medullary respiratory neurones and the A_5 cell group.

A tonic noradrenergic inhibition of respiratory frequency was attributed to A_5 neurones in a series of transection, lesion, stimulation and pharmacological experiments in the *in vitro* neonatal rat brainstem–cervical cord preparation (Hilaire *et al.* 1989; Monteau, Errchidi, Gauthier, Hilaire & Rega, 1989; Errchidi *et al.* 1991; Hilaire *et al.* 1993). Pontomedullary transection, selective separation and lesioning of the ventral pons as well as bath application of α_2-antagonists increased respiratory frequency, indicating the presence of a descending tonic inhibition to medullary respiratory rhythm generating areas (Hilaire *et al.* 1989; Monteau *et al.* 1989; Errchidi *et al.* 1991; Hilaire *et al.* 1993). Electrical stimulation of the VL pons decreased respiratory frequency, which was attenuated by bath application of α_2-antagonists (Hilaire *et al.* 1989, 1993). These results from the *in vitro* experiments are supported by recent findings *in vivo*. Systemic injections of the α_2-agonists clonidine and guanabenz prolonged T_E in awake goats (Hedrick, Ryan, Pizarro & Bisgard, 1994), possibly through activation of postsynaptic α_2-receptors (U'Prichard, Bechtel, Rouot & Snyder, 1979). In addition, glutaminergic activation of neurones in the VL pons in anaesthetized adult rats also prolonged expiration (Jodkowski *et al.* 1994; Dick *et al.* 1995), and electrical stimulation of the VL pons in rabbit inhibited inspiration (Schmid, Böhmer & Fallert, 1988). Therefore, A_5 neurones may modulate respiratory timing by prolonging expiration.

A_5 neurones are activated by hypoxia and electrical stimulation of the carotid sinus nerve (Guyenet, Koshiya, Huangfu, Verberne & Riley, 1993; Erickson & Millhorn, 1994). In a double-labelling study, a high degree of colocalization of tyrosine hydroxylase/Fos label was identified in the VL pons after prolonged hypoxia, indicating that A_5 catecholaminergic neurones were activated during hypoxia (Erickson & Millhorn, 1994). Moreover, electrophysiologically identified A_5 neurones with expiratory-modulated activity increased their activity during hypoxia (Guyenet *et al.* 1993). Further pharmacological studies would define the role of catecholaminergic neurotransmitters and receptors in post-hypoxic frequency decline.

Methodological considerations

The centres of muscimol injections were identified histologically from iontophoretic marks. Theoretical calculations indicate that 10–100 nl microinjections diffuse approximately 100–300 μm from the injection site in brain tissue, and that the concentration of the injectate never exceeds approximately 20% of that injected at the perimeter (Nicholson, 1985). The actual spread of injectate was not measured; however, the lowest volume of effective bilateral injections was 10 nl. Injectate from such a discrete microinjection is unlikely to have spread vertically along the electrode shank to the dorsolateral (DL) pons. Moreover, injections of comparable or greater volumes 1 mm dorsal to target sites did not attenuate post-hypoxic frequency decline. In fact, in two animals bilateral injections included the classically defined pneumotaxic centre in the DL pons. In these animals baseline respiratory timing changed, but post-hypoxic frequency decline remained. The largest injection volume at a dorsal control site was 500 nl. None of the control injections eliminated post-hypoxic frequency decline, suggesting that diffusion of injectate from large injections was not extensive. Finally, changes in respiration developed simultaneously with pressure injection, indicating that cells located at or near the electrode tip were affected. In summary, while we cannot rule out the possibility that the DL pons plays a role in changing the respiratory pattern after hypoxia, our present data implicate a role for the VL pons in mediating post-hypoxic frequency decline.

Physiological significance of post-hypoxic frequency decline

The respiratory response to hypoxia varies with the developmental stage of the organism. In the fetus, where gas exchange does not depend on ventilation, fetal breathing movements are suppressed during hypoxia (Boddy, Dawes, Fisher, Pinter & Robinson, 1974). The neonate responds to hypoxia by increasing, then decreasing, its breathing (Martin-Body & Johnston, 1988). In these immature preparations, the neural substrate mediating the inhibitory component of these responses was localized to the rostral pons (Dawes, Gardner, Johnston & Walker, 1983; Johnston *et al.* 1985; Gluckman & Johnston, 1987; Martin-Body & Johnston, 1988). A similar depression of ventilation may occur in adults. We speculate that decrease in breathing frequency that begins during hypoxia results from a neural mechanism that is maximally revealed in the post-hypoxic period. The VL pons appears to play a crucial role in this response, since inhibition of neural activity in this area attenuates or abolishes post-hypoxic frequency decline. Since the high frequency and amplitude of breathing prevalent at the beginning of a hypoxic exposure consumes more oxygen than the slower pattern that develops later, a frequency depression during and after hypoxia may serve to minimize the work of breathing. Post-hypoxic frequency decline may be a component of an adaptive mechanism aimed at minimizing the energy expenditure required to provide sufficient O_2 supplies during sustained hypoxia.

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