Long-term facilitation of inspiratory intercostal nerve activity following carotid sinus nerve stimulation in cats

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1. Repeated carotid sinus nerve (CSN) stimulation evokes a serotonin-dependent long-term facilitation (LTF) of phrenic nerve activity in cats. To determine whether CSN stimulation-evoked LTF is a general property of spinal inspiratory motoneurones, phrenic and inspiratory internal intercostal (IIC) nerve activities were recorded in nine cats (eight anaesthetized; one decerebrate), which were vagotomized, paralysed, thoracotomized and ventilated with O_2; airway CO_2 was controlled by means of a servo-respirator. Baseline conditions were established by setting the arterial CO_2 pressure (P_{a,CO_2}) at approximately 2 mmHg above the threshold for IIC activity. One CSN was stimulated (3 times threshold, 25 Hz, 0·5 ms duration) with five (2 min) trains, each separated by 5 min.

2. The peak integrated phrenic activity was elevated by 33 % whereas IIC activity was elevated by 226 % above baseline, 90 min post-stimulation ($P < 0·05$). The results were similar when expressed as a percentage of the maximal neural activities (elicited by combined hypercapnia and CSN stimulation), although differences between the nerves were less pronounced. The burst frequency was not changed following stimulation.

3. In five additional cats that were pretreated with the serotonin receptor antagonist, methysergide maleate (0·5–1 mg kg^{-1}, I.V.), the CO_2 thresholds of the phrenic (12 mmHg) and IIC nerves (22 mmHg) were increased ($P < 0·05$), and LTF could not be elicited in either neurogram.

4. Successive CSN stimulation episodes evoked a previously undescribed phenomenon. Although the peak integrated phrenic activity was unchanged (90–95 % of maximal), IIC activity increased progressively during successive stimulus episodes (66–90 % of maximal; $P < 0·05$). However, after methysergide treatment, the initial stimulus-evoked phrenic response decreased to 58 % of maximal and both neurograms exhibited progressive augmentation of the stimulus-evoked response. As stimulus-evoked augmentation does not require serotonin, it is independent of LTF.

5. We conclude that CSN stimulation-evoked LTF of IIC activity exceeds that of phrenic activity. Since LTF requires the neuromodulator serotonin and is expressed predominantly by changes in burst pattern formation *versus* rhythm generation, serotonin may exert a greater influence on IIC relative to phrenic respiratory motor output. A unique mechanism is described whereby successive CSN stimulus episodes cause progressively increasing responses in both neurograms.

Chemical or electrical stimulation of the peripheral chemoreceptors evokes an abrupt increase in ventilatory activity. However, when the stimulation ends, the ventilatory output remains elevated in both anaesthetized (see Eldridge & Millhorn, 1986) and conscious animal models (Engwall, Daristotle, Niu, Dempsey & Bisgard, 1991; Cao, Zwillich, Berthon-Jones & Sullivan, 1992), and in human subjects (Fregosi, 1991). The decay of ventilatory activity following stimulation reflects at least two distinct mechanisms, with time domains of minutes (short-term potentiation, STP; see Wagner & Eldridge, 1991) to hours (long-term facilitation, LTF; see Eldridge & Millhorn, 1986; Hayashi, Coles, Bach, Mitchell & McCrimmon, 1993). Short-term potentiation (formerly 'after-discharge') is elicited by a wider range of afferent inputs and does not require monoamine neurotransmitters (see Eldridge & Millhorn, 1986; Wagner

& Eldridge, 1991). In contrast, LTF appears to be elicited uniquely by peripheral chemoreceptor stimulation and cannot be evoked following serotonin receptor antagonism (Millhorn, Eldridge & Waldrop, 1980*a*, *b*).

Although stimulation of peripheral chemoreceptor inputs elicits LTF of phrenic nerve activity (Millhorn *et al.* 1980*a*), a recent study (Jiang, Mitchell & Lipski, 1991) failed to detect LTF in respiration-related hypoglossal motor output. The apparent lack of LTF in the hypoglossal motoneurone pool raises an important question: is LTF a general property of inspiratory motor nerve activities, or is it restricted to phrenic motoneurones? The primary objective of this study was to determine whether carotid sinus nerve (CSN) stimulation-induced LTF is a general property of spinal inspiratory motoneurones. Accordingly, we investigated whether CSN stimulation evokes LTF in the spinal inspiratory motoneurones innervating the parasternal intercostal muscles (Fregosi & Bartlett, 1989). We also wished to reaffirm the existence of LTF in phrenic nerve activity in cats because this has not been confirmed since the original reports of Millhorn *et al.* (1980*a*, *b*), and has proved somewhat difficult to demonstrate under slightly different experimental conditions (see Jiang *et al.* 1991). Finally, to determine whether the LTF of inspiratory intercostal nerve activity is serotonin dependent, the protocol was repeated in separate animals after administration of the broad-spectrum serotonin receptor antagonist, methysergide maleate.

METHODS

Experimental preparation

Experiments were conducted on fourteen cats of either sex (2·5–5·3 kg). The animals were anaesthetized initially with halothane in oxygen, placed in a supine position and the trachea was intubated. Rectal temperature was monitored and maintained between 37 and 39 °C with a servo-controlled heating lamp. The femoral artery and vein were both cannulated in order to monitor blood pressure, to withdraw arterial blood samples and to administer drugs and intravenous fluids. The left phrenic nerve and a parasternal branch of an ipsilateral internal intercostal nerve (IIC) from the fourth or fifth thoracic interspace were dissected and prepared for recording (Fregosi & Bartlett, 1989). The cut central ends of both nerves were placed across bipolar platinum electrodes and their activities amplified, filtered (30 Hz–10 kHz), rectified and integrated using a resistance–capacitance (RC) circuit (time constant 100 ms). The ipsilateral CSN was isolated and prepared for stimulation by placing it across bipolar stainless-steel electrodes that were positioned as far from the glossopharyngeal nerve as possible, and crushing the distal end with forceps. In all but three studies, the contralateral CSN was left intact; no significant differences between stimulus responses were observed either with or without a crushed contralateral CSN.

Halothane anaesthesia was slowly discontinued as the animals were anaesthetized gradually with α-chloralose (50 mg kg^{-1}, I.V.; Aldrich Chemical Co., Milwaukee, WI, USA). As the animals were to be subsequently paralysed (see below), the depth of anaesthesia was at first assessed by applying a deep pressure to the front paws using haemostats. Supplemental doses of α-chloralose (5–10 mg kg^{-1}) were administered if the animals withdrew their paws, or if the blood pressure increased during or immediately after this manoeuvre. Once the depth of anaesthesia was judged to be satisfactory, based on the above criteria, additional doses of anaesthetic were not found to be necessary as none of the animals exhibited a pressor response to noxious stimuli for up to 6 h after the final anaesthetic dose. One animal was studied in an unanaesthetized, decerebrate state (mid-collicular) as described previously (Fregosi & Bartlett, 1989).

The cats were vagotomized bilaterally at the mid-cervical level, paralysed with gallamine triethiodide (4–10 mg kg^{-1}; Aldrich Chemical Co.) and ventilated artificially. Bilateral thoracotomies were created, and the expiratory port of the ventilator placed under 3–4 cmH_2O to minimize alveolar atelectasis. Oxygen was added to the inspired gas to increase the arterial P_{O_2} to above 120 mmHg (mean ± S.E.M., 214 ± 31 mmHg). Arterial blood samples were withdrawn at periodic intervals, and the partial pressures of CO_2, O_2 and the pH determined by means of a blood gas analyser (Instrumentation Laboratories Inc., Lexington, MA, USA; model 513). The blood gas and pH values were all corrected to the animal's rectal temperature. The base deficits were corrected to within 0 ± 3 mequiv l^{-1} prior to the initiation of the experimental protocol via the administration of bicarbonate (I.V.). Blood pressure was monitored with a strain gauge transducer (Gould, Valley View, OH, USA; model P-23id). The fractional concentrations of O_2 and CO_2 were measured at the airway opening using rapidly responding analysers (Ametek, Pittsburgh, PA, USA; models S-3A and CD-3A). During the experimental sessions, the CO_2 analyser output was used in conjunction with a CO_2 servo-controlled ventilator (Charles Ward Enterprises Inc., Ardmore, PA, USA), to maintain a constant end-tidal CO_2. Thus, changes in end-tidal (and arterial) CO_2 were minimized during the experimental protocols despite unavoidable changes in body temperature, metabolic rate, blood pressure, etc.

Experimental protocol

Protocol 1: long-term facilitation. The hypothesis that CSN stimulation-induced LTF is a general property of spinal inspiratory motoneurones was tested in nine cats (eight anaesthetized, one decerebrate) using a modified version of the protocol described by Millhorn *et al.* (1980*a*). Baseline conditions were established by setting the end-tidal CO_2 at a value slightly above (*ca* 2 mmHg) the threshold for spontaneous respiration-related IIC activity; the (lower) CO_2 threshold for phrenic nerve activity was also determined.

The threshold current for CSN stimulation-induced IIC activation was established by applying 5 s trains of constant-current pulses (25 Hz, 0·5 ms duration). During this determination, the stimulus intensity was increased progressively from subthreshold levels until IIC activation was observed. Care was taken to ensure that stimulations above the threshold were kept to a minimum during this procedure. The threshold for IIC activation ranged from 5 to 75 μA.

At baseline conditions, an arterial blood sample was withdrawn and control data recorded for 2–5 min. Five (2 min) trains of stimuli (3 times threshold) were then applied to the CSN, with each stimulation period being separated by 5 min. Data were recorded continuously throughout the stimulation protocol, and also for 30 min after the fifth stimulation. Thereafter, 5 min segments of data were recorded on tape at

15 min intervals for a further 90 min; the data were recorded continuously on a polygraph (Grass Instruments Inc., Quincy, MA, USA). After 90 min, the maximal nerve activities were determined by adding CO_2 to the inspiratory port of the ventilator until the nerve activities failed to increase, despite further increases in end-tidal CO_2. The CSN was then stimulated (3 times threshold, 25 Hz, 0·5 ms duration) to ensure that the neural activities were maximal.

Protocol 2: LTF following methysergide administration. The hypothesis that the LTF of both phrenic and IIC activities is serotonin dependent was tested by repeating Protocol 1 in five cats that were pretreated with the broad-spectrum serotonin receptor antagonist, methysergide maleate (0·5–1·0 mg kg^{-1}, I.V.). The experiments commenced approximately 30 min after the drug administration. The CO_2 thresholds for spontaneous neural activity in the phrenic *versus* IIC nerves were determined before and 30 min after methysergide administration.

Analysis

The analog waveforms recorded on the polygraph were digitized using a digitizing tablet that was interfaced with a microcomputer and driven by commercial software (Flexitrace; Treestar Inc., Santa Barbara, CA, USA). In some instances, the taped data were digitized at 3 kHz per channel and stored for further analysis (Dataq Instruments Inc., Akron, OH, USA). The peak heights of the integrated phrenic and IIC neurograms were normalized in two ways: (1) as a percentage of the pre-stimulation control activity; and (2) as a percentage of the maximal neural activity determined at the end of the protocol. Mean arterial blood pressure was calculated as the diastolic pressure plus one-third of the pulse pressure.

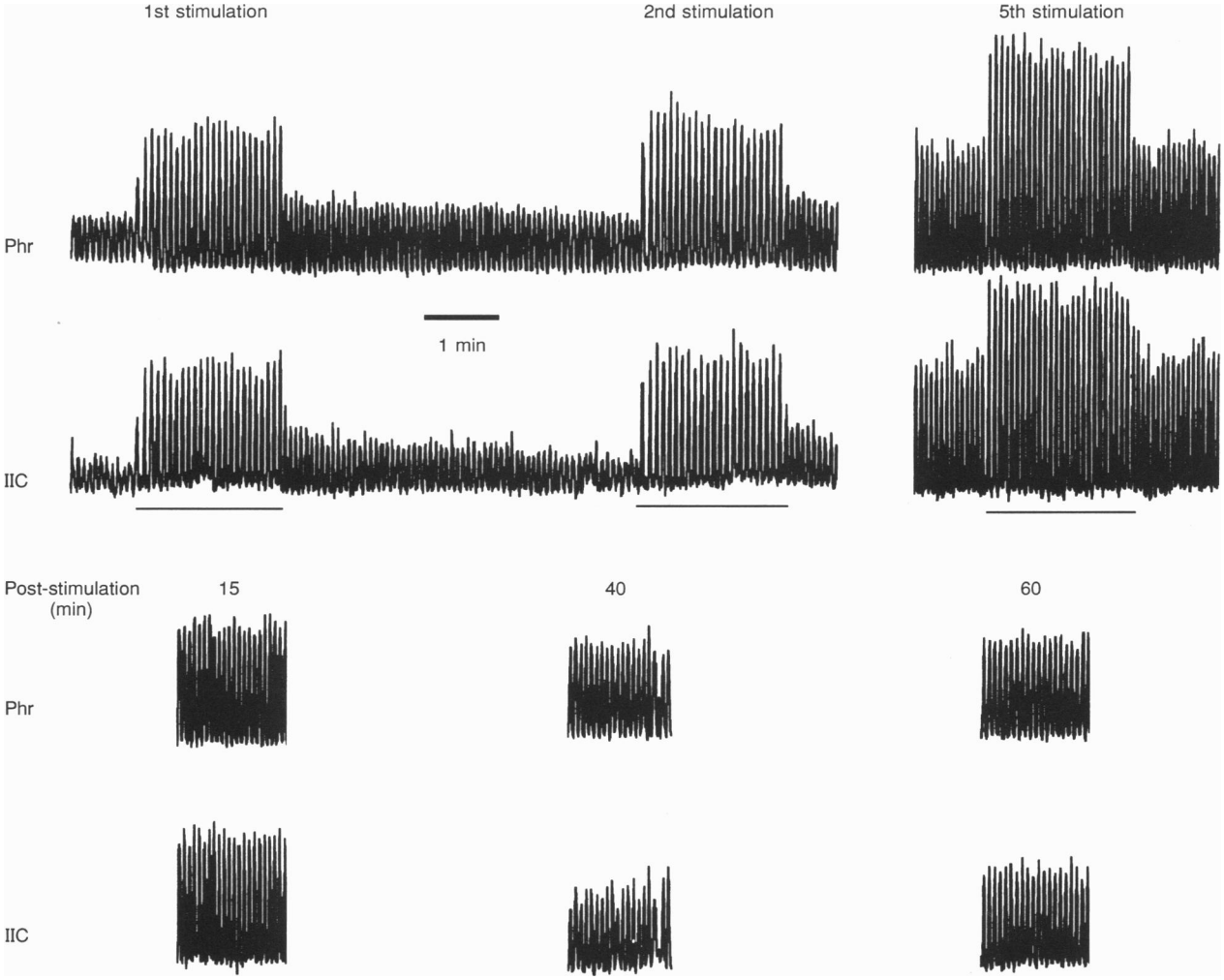

Figure 1. Prolonged increase in phrenic and internal intercostal nerve activities following five episodes of carotid sinus nerve stimulation

Experimental recordings showing the effects of carotid sinus nerve stimulation on integrated phrenic (Phr) and inspiratory intercostal (IIC) nerve activities in one cat. Note that the activity of both nerves remained elevated for over 60 min following the fifth and final stimulation period, and that the effect on IIC activity was relatively greater than that on Phr activity. Both neurograms exhibited augmentation of evoked response in successive CSN stimulus episodes. CSN stimulus episodes are marked by horizontal bars under IIC tracing.

For each variable, the mean of ten respiratory cycles was determined at the following time points: pre-stimulation (control) and at 15, 30, 60 and 90 min post-stimulation. In addition, the amplitude of each integrated phrenic and IIC nerve burst was determined during each stimulus episode, and those of the eight bursts immediately preceding the onset of each stimulus episode (i.e. the interstimulus interval) were also determined. In order to determine significant differences between time points and nerves, a two-way repeated-measures analysis of variance was applied. If statistical significance was established, individual comparisons were made using the Student–Neuman–Keuls *post hoc* procedure. Differences were considered significant if $P < 0.05$. All values in the text and figures are described as means ± S.E.M.

RESULTS

Protocol 1: long-term facilitation

The effects of repeated CSN stimulation on phrenic and IIC nerve activities in one cat are shown in Fig. 1. After the first stimulation, the activity of both nerves decayed slowly (short-term potentiation), and remained slightly above the pre-stimulus control level before the start of the second stimulation period. By the beginning of the fifth and final stimulation period, the activity of both nerves was above the control level, and this facilitation persisted for over 60 min. The magnitude of LTF in IIC activity

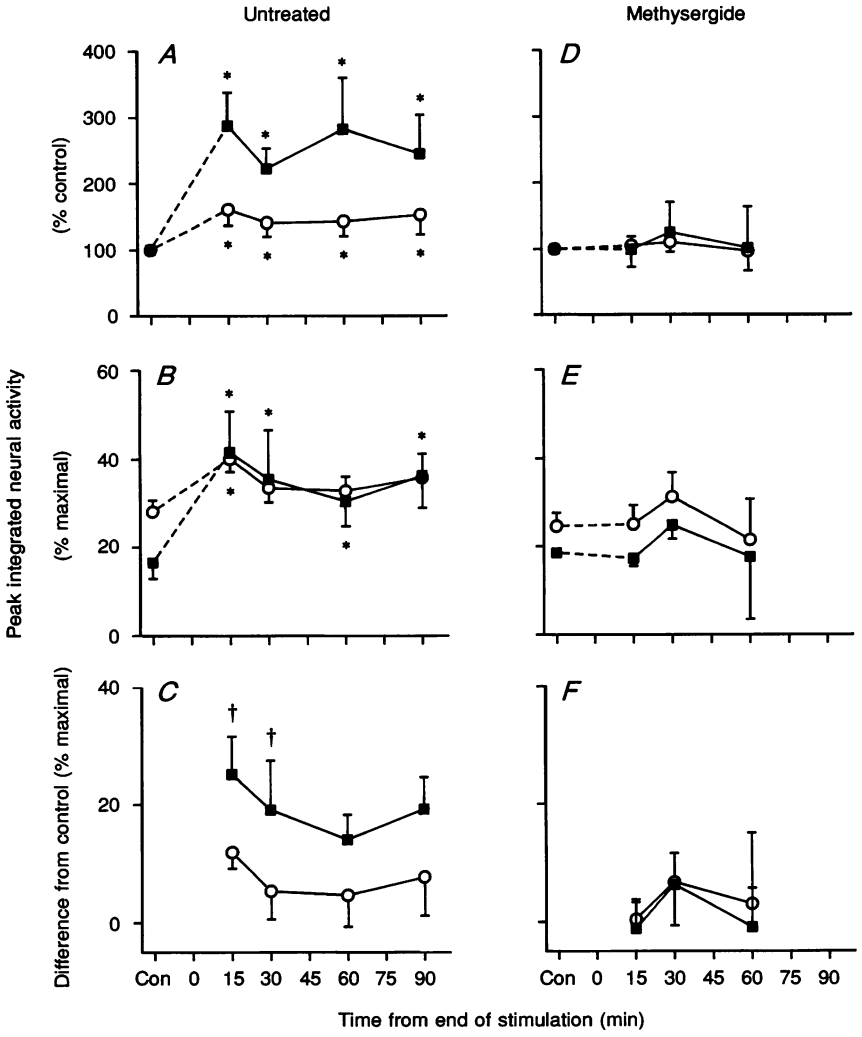

Figure 2. Average phrenic and internal intercostal nerve activities under control conditions and at various times following five episodes of carotid sinus nerve stimulation

Peak integrated phrenic (○) and IIC (■) activities under pre-stimulus control conditions (Con), and at various times after the fifth stimulus episode. Untreated cats (panels *A*, *B* and *C*); methysergide-treated cats (panels *D*, *E* and *F*). Nerve activities are expressed as a percentage of pre-stimulus control activities (top panels), as percentage maximal activities (middle panels), and as the difference between the post-stimulus time point and pre-stimulus control values (expressed as percentage of maximal activity; bottom panels). *Significantly different from control; †significantly different from phrenic activity.

exceeded that observed in phrenic nerve activity. These observations from one animal are consistent with the mean data (Fig. 2). As the single decerebrate cat showed similar responses to the anaesthetized cats, data from this animal are also included in the group mean.

When expressed as a percentage of the pre-stimulus control levels, the peak integrated activities of both nerves remained elevated for at least 90 min following the fifth stimulus episode (Fig. 2*A*; $P < 0{\cdot}05$). The magnitude of LTF at 60 min post-stimulation ranged from 65 to 274 % of control for phrenic activity and from 82 to 525 % of control for IIC activity. The expression of neural activities as a percentage of the control activity is subject to normalization artifacts, as a small change in nerve activity can lead to large percentage changes in the normalized response, due to the low pre-stimulus control level. However, when the data are normalized by expressing the activities as a percentage of the maximal nerve activity, such artifacts are minimized. When normalized in this way, the LTF of IIC activity was still significant at every time point; the phrenic activity, however, was elevated significantly at 15 min only when it was expressed as a percentage of maximal activity (Fig. 2*B*). Absolute levels of phrenic and IIC nerve activities, expressed as a percentage of the maximal activities, were virtually identical following stimulation; the larger LTF observed occurred primarily because the pre-stimulus control value of IIC activity was a lower percentage of the maximal activity (Fig. 2*B*).

The difference in LTF between the phrenic and internal intercostal nerves was evaluated by calculating the change in amplitude (normalized as a percentage of the maximal) between the control and each post-stimulus time point (Fig. 2*C*). The magnitude of the LTF in the IIC nerve was significantly greater than that in the phrenic nerve for 30–60 min post-stimulation ($P < 0{\cdot}05$).

The long-term facilitation was not associated with changes in the mean nerve burst frequency, the partial pressure of end-tidal CO_2 (P_{ET,CO_2}), or mean blood pressure (Fig. 3). The constancy of P_{ET,CO_2} was confirmed by measurements of arterial P_{CO_2} in six cats; arterial P_{CO_2} remained within the range of 3 mmHg for up to 90 min post-stimulation.

The neural activity at the end of each interstimulus interval was also quantified to evaluate the development of

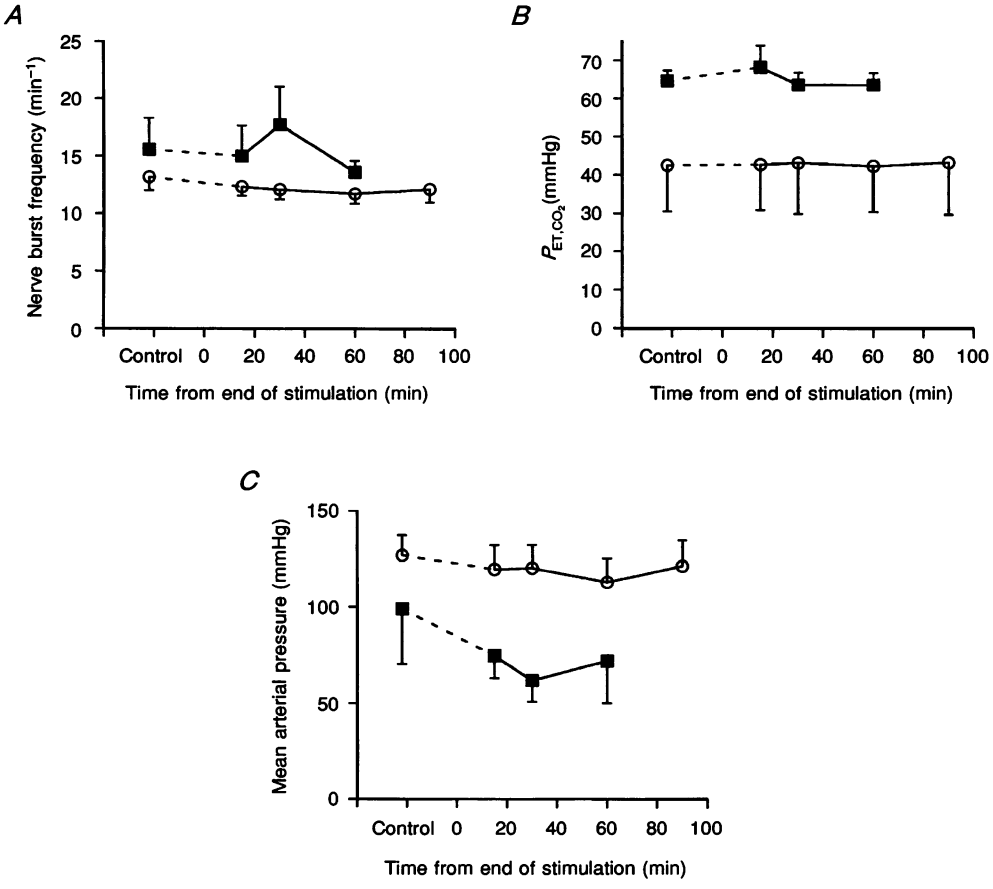

Figure 3. Nerve burst frequency, end-tidal P_{CO_2} and blood pressure under control conditions and at various times following five episodes of carotid sinus nerve stimulation

Mean values for nerve burst frequency (*A*), the partial pressure of end-tidal CO_2 (P_{ET,CO_2}; *B*) and mean arterial blood pressure (*C*) for untreated control (○) and methysergide-pretreated cats (■). There were no significant changes in any of these variables up to 90 min following the final CSN stimulus episode.

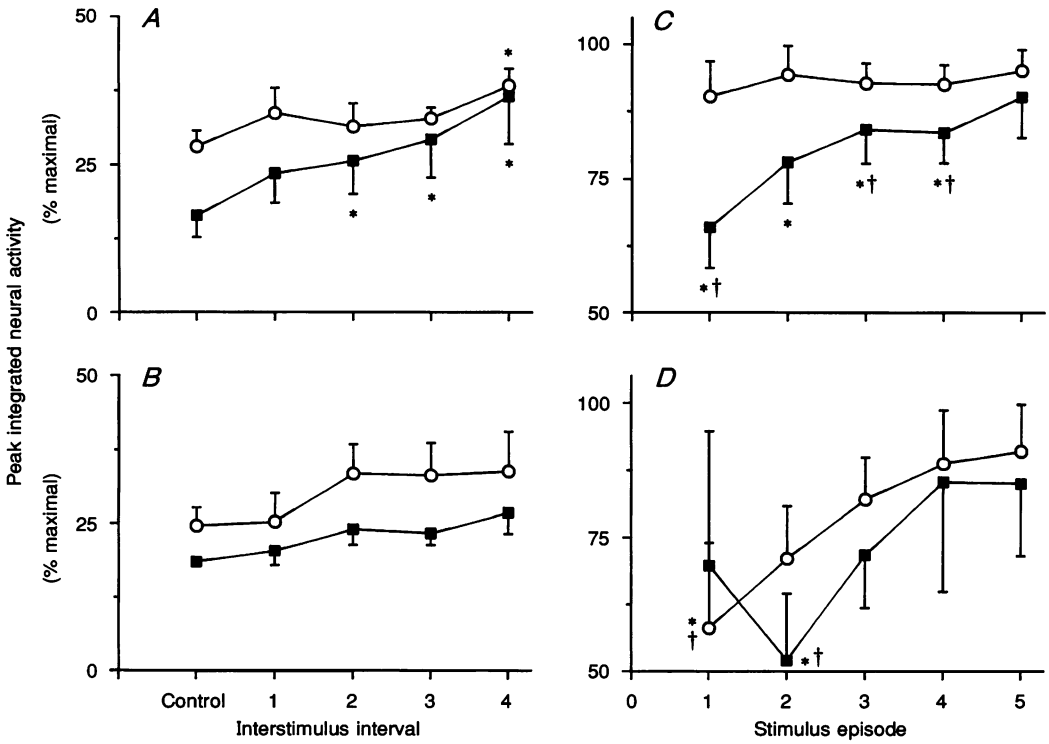

Figure 4. Progressive increase in phrenic and internal intercostal nerve activities during (right) and immediately preceding (left) each episode of carotid sinus nerve stimulation
Mean values of phrenic (○) and IIC (■) nerve activities during successive interstimulus intervals in untreated (*A*) and methysergide-pretreated (*B*) cats; progressive increase in successive intervals indicates LTF development. *Significantly different from first interstimulus interval (*A* and *B*). Mean peak integrated nerve activities during successive CSN stimulus episodes are shown in *C* for untreated and *D* for methysergide-pretreated cats. All values are expressed as a percentage of maximal activities. *Significantly different from 100 %; †significantly different from fifth episode (*C* and *D*).

LTF (Fig. 4*A*). In this analysis, it was assumed that short-term potentiation was no longer in operation, and that any post-stimulus augmentation in neural activity reflected LTF. This assumption was based on reports that the time constant of short-term potentiation is of the order of 100 s in anaesthetized cats (Eldridge & Millhorn, 1986), and will be expressed only minimally 4–5 min post-stimulation. Interstimulus nerve activity increased progressively in both the phrenic (28–42 % of maximal) and the IIC (16–42 % of maximal) neurograms ($P < 0.05$). Apparent increases in the phrenic neurogram occurred following the first and fourth stimulus episodes, although significance was achieved only during the fourth interstimulus interval. In contrast, IIC activity increased progressively

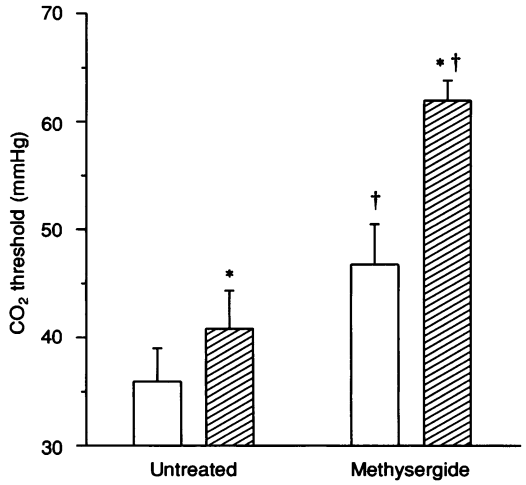

Figure 5. The threshold end-tidal P_{CO_2} associated with the onset of spontaneous phrenic and internal intercostal nerve activities
The CO_2 threshold for spontaneous phrenic (□) and IIC (▨) nerve activities in untreated and methysergide-pretreated cats. Note that methysergide treatment increased the threshold of both nerves, but that the effect on IIC activity was greater. *Significantly different from phrenic; †significantly different from untreated.

with each stimulus episode, indicating that saturation of LTF in the IIC nerve did not occur.

During each CSN stimulation episode, phrenic activity increased to between 90 and 95 % of maximal, and did not differ between successive stimulus episodes (Fig. 4*C*). On the other hand, IIC activity increased to 66 ± 8 % of maximal during the first stimulation, and then increased progressively with each successive episode, reaching 90 ± 8 % of maximal during the fifth episode ($P < 0.05$; Fig. 4*C*). Episodes 1–4 were significantly different from 100 %, and stimulus episodes 1, 3 and 4 were significantly different from episode 5 ($P < 0.05$). Thus, successive stimulus episodes enhanced the evoked response of IIC, but not phrenic nerve activity. The phrenic neurogram appears to be limited in its ability to express this phenomenon as it is bounded by the maximal attainable activity (i.e. saturation).

Protocol 2: methysergide pretreatment

One of the more striking and indeed unexpected findings was the extent to which methysergide pretreatment increased the CO_2 threshold for rhythmic IIC nerve activity. The IIC nerve CO_2 threshold was approximately 5 mmHg larger than the phrenic nerve threshold in pre-stimulus control conditions (Fig. 5; $P < 0.05$). The threshold of both phrenic and IIC nerve activities increased significantly after methysergide administration, but the magnitude of the increase was larger in the IIC (22 mmHg) relative to the phrenic activity (12 mmHg) (Fig. 5; $P < 0.05$). Owing to the fact that the CO_2 threshold was higher after methysergide treatment, it was necessary to conduct the LTF protocol at a higher arterial CO_2 pressure level (P_{a,CO_2}) in order to establish a baseline of rhythmic IIC nerve activity.

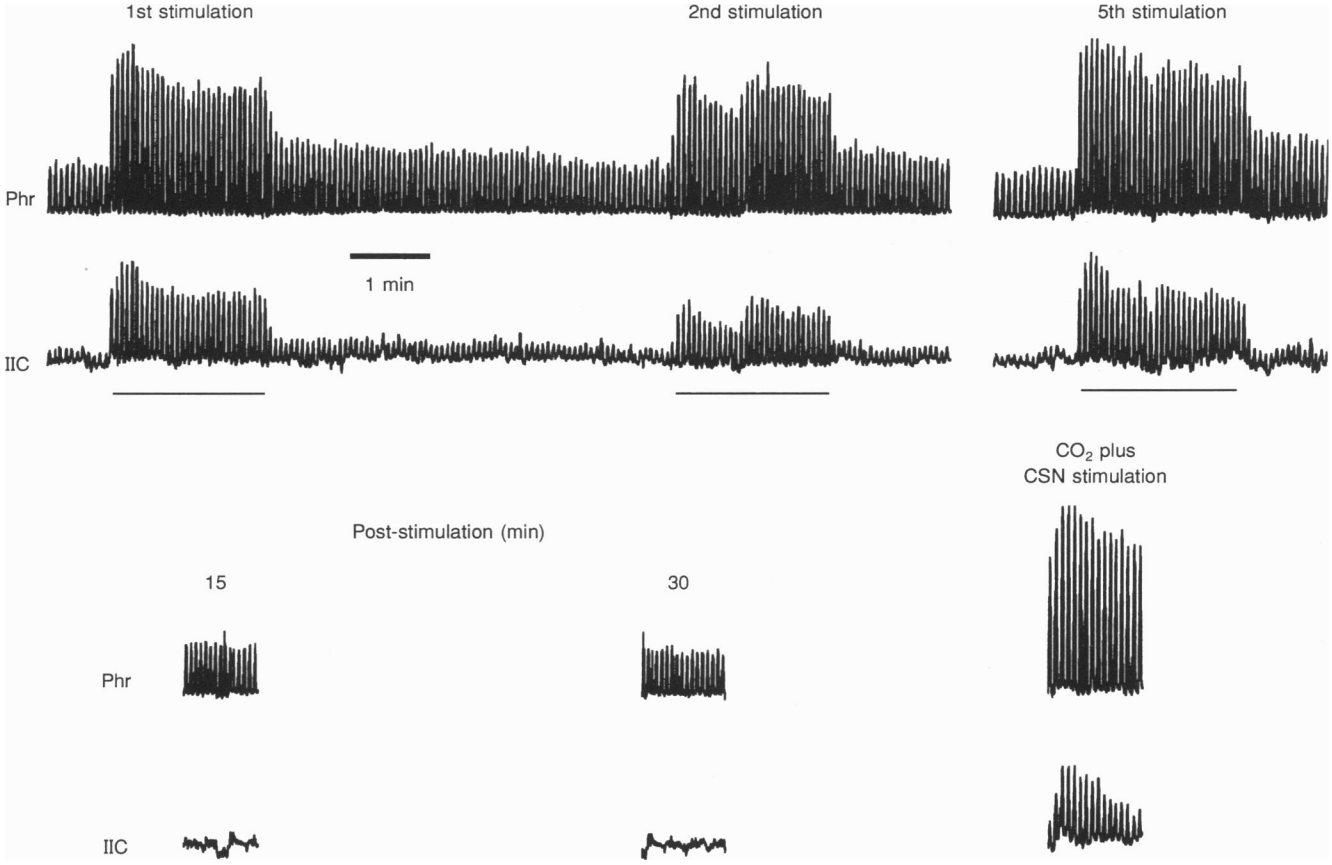

Figure 6. Prolonged increase in phrenic and internal intercostal nerve activities following five episodes of carotid sinus nerve stimulation in a cat pretreated with a serotonin receptor antagonist

Experimental recording showing the effects of CSN stimulus episodes on integrated phrenic (Phr) and IIC nerve activities in one methysergide-pretreated cat (0·5 mg kg^{-1}). Note that CSN stimulus episodes increased the activity of both nerves, but that the activities had returned to pre-stimulus control values by 15 min post-stimulation. IIC activity in this animal was below the pre-stimulus control level after the final stimulation, but elevation of end-tidal CO_2 and/or CSN stimulation increased the activity, indicating that the nerve was not damaged. CSN stimulus episodes are marked by horizontal bars under the IIC trace.

The influence of repetitive CSN stimulation on phrenic and IIC nerve activities in one cat pretreated with methysergide is shown in Fig. 6. No evidence was found for the facilitation of either phrenic or IIC nerve activities. Indeed, in this animal, rhythmic IIC activity was absent 15 min after the fifth and final stimulus. This was not the result of a damaged nerve as hypercapnia and CSN stimulation restored the activity at the end of the observation period (Fig. 6). Long-term facilitation was absent in both nerves after methysergide pretreatment in all five animals (Fig. 2*D*, *E* and *F*). In addition, there were no significant post-stimulus changes in nerve burst frequency, P_{ET,CO_2}, or mean blood pressure (Fig. 3).

Methysergide pretreatment also diminished the development of LTF in both nerves (Fig. 4*B*). However, progressive augmentation of the evoked activity was revealed in both neurograms during successive stimulus episodes (Fig. 4*D*). From the first to fifth episodes of stimulation, CSN stimulation-evoked phrenic activity increased from 58 ± 16 to 91 ± 9 % of maximal ($P < 0{\cdot}04$). The stimulus-evoked IIC activity increased from 70 ± 25 to 85 ± 14 % of maximal ($P < 0{\cdot}03$). Thus, serotonin receptor antagonism brought about by methysergide pretreatment revealed a previously undetected effect of successive CSN stimulus episodes on phrenic activity.

DISCUSSION

Repeated trains of carotid sinus nerve stimulation evoked a methysergide-sensitive long-term facilitation in both phrenic and IIC motoneurone pools. Our findings confirm the earlier report of Millhorn *et al.* (1980*a*) concerning phrenic nerve activity in cats, and also extend observations of LTF to another population of spinal inspiratory motoneurones. The long-term facilitation of IIC motor output exceeded that observed in the phrenic neurogram, although clear differences lasted for only 30–60 min. The observation that respiratory frequency was minimally involved in LTF indicates that the facilitation results primarily from changes in burst pattern formation rather than rhythm generation (see Feldman, Smith, McCrimmon, Ellenberger & Speck, 1988). Serotonin receptor antagonism brought about by pretreatment with methysergide maleate exerted a greater influence on spontaneous IIC than on phrenic nerve activity, although methysergide blocked the LTF in both neurograms. The results suggest that a common mechanism underlies LTF in both neurograms.

A new effect was observed during successive CSN stimulation episodes. The CSN stimulus-evoked IIC (but not phrenic) nerve activity increased progressively with each stimulus episode, indicating an experience-dependent augmentation of IIC nerve burst amplitude. However, following methysergide treatment, both nerves exhibited a progressive augmentation of the CSN stimulus-evoked response. As progressive augmentation of the stimulus-evoked response was either unaffected (IIC) or revealed by methysergide pretreatment (phrenic nerve), the underlying mechanism does not appear to require functional serotonin receptors. Thus, progressive augmentation must occur by a central neural mechanism that is distinct from LTF.

Long-term facilitation

It is doubtful that a decrease in the depth of anaesthesia during the post-stimulation period led to the observed LTF, as the magnitude of the LTF in the one decerebrate animal studied was slightly greater than average. Moreover, we found no evidence of changes in the depth of anaesthesia, as assessed by monitoring the cardiopulmonary responses to noxious stimuli (see Methods). The changes in P_{a,CO_2} observed during the post-stimulus period were not sufficient to explain LTF, although there was a slight trend towards an increase in P_{a,CO_2} at 15 and 60 min post-stimulation; the P_{a,CO_2} at 90 min was within 0·6 mmHg of the pre-stimulus value. Finally, although many of the same factors potentially capable of increasing ventilatory activity with time should have been operative during protocol 2, LTF was not observed. Thus, as concluded previously by Millhorn *et al.* (1980*a*, *b*), it appears that LTF is a unique central mechanism that is not attributable to changes in traditional ventilatory stimuli.

Electrical stimulation of the carotid sinus nerve at 25 Hz has inherent experimental limits. Of particular concern is the fact that baroreceptor fibres are co-activated with chemoreceptor afferent fibres. However, the stimulation protocol used here elicited a pressor response rather than the depressor response expected from baroreceptor activation, indicating that stimulation of chemoreceptor afferent inputs was predominant. Furthermore, it is possible that the spread of current to the adjacent glossopharyngeal nerve may elicit non-specific motor effects. Indeed, although in this study the stimulating electrode was placed as far as possible from the glossopharyngeal nerve, this alone could not rule out such non-specific effects. Nevertheless, when the glossopharyngeal nerve, rather than the carotid sinus nerve, is stimulated directly in rats anaesthetized with urethane (Hayashi *et al.* 1993), there is no evidence of long-term facilitation or other similar motor behaviours. Finally, the stimulus protocol represents a potentially non-physiological neural input to the central nervous system as carotid chemoreceptor afferent fibres are not expected to be activated synchronously at rates as high as 25 Hz. It is reassuring, therefore, that ventilatory effects similar to LTF occur in animals exposed to repeated episodes of hypoxia, which is presumably a more physiological stimulus (Millhorn *et al.* 1980*a*; Cao *et al.* 1992; Hayashi *et al.* 1993).

When expressed as a percentage increase of the control activity, both the phrenic and IIC nerves exhibited a significant LTF for a minimum of 90 min. However, when the activity was expressed as a percentage of the maximal

activity, a more conservative method of normalization, the phrenic nerve exhibited a significant LTF for only 15 min post-stimulation, whereas the IIC nerve exhibited LTF for the entire observation period of 90 min. Although the two forms of normalization employed do not change the fundamental conclusion of this study, the quantitative differences serve to emphasize how important it is to consider the method of data normalization when making comparisons among different respiratory nerve activities, or when comparing the results from different studies.

The reason for the failure to show LTF in the phrenic neurogram for periods longer than 15 min is unclear, but it may relate to the fact that control conditions were established by raising the P_{a,CO_2} to a level nearly 7 mmHg above the CO_2 threshold for spontaneous phrenic nerve activity (Fig. 5; see Methods). As the phrenic neurogram in cats exhibits progressive saturation as respiratory drive is increased (Eldridge, Gill-Kumar & Millhorn, 1981), the relatively high baseline phrenic activity may have obscured the full effect of the LTF; short-term potentiation is similarly obscured at elevated P_{a,CO_2} levels (Eldridge & Millhorn, 1986). This same mechanism may also have led to the finding that IIC LTF was greater than phrenic LTF for a period of at least 30 min post-stimulation. Alternatively, there may be a real difference in the magnitude of LTF in IIC *versus* phrenic motoneurone activities, possibly via a greater serotonergic innervation of IIC motoneurones (see below).

The increase in phrenic and IIC nerve activities between successive CSN stimulus episodes indicates that LTF can be elicited by even one stimulation period. This is in agreement with Millhorn *et al.* (1980*a*) who observed that the magnitude of the LTF in the phrenic nerve reached a maximum after three stimulus episodes. As IIC LTF clearly increases after each stimulus episode, and shows no evidence of saturation, it may be that IIC LTF would be enhanced by additional stimulus episodes.

Effects of methysergide

Serotonin receptor antagonism by methysergide caused a profound depression of the central respiratory drive. Moreover, the depression of IIC activity was greater than that of phrenic nerve activity as assessed by the increase in the CO_2 threshold for spontaneous respiratory activity (Fig. 5). These observations suggest that serotonergic projections make an important contribution to respiratory motoneurone excitability, and that the IIC motoneurone pool is particularly sensitive to serotonergic modulation. Methysergide also abolished the CSN stimulation-induced LTF in both motoneurone pools, indicating that the underlying mechanism requires serotonin. This conclusion must remain tentative, however, due to the possible non-serotonergic effects of methysergide, and the elevated level of P_{a,CO_2} that was necessary to initiate rhythmic IIC nerve activity after drug administration.

Effects of successive CSN stimulus episodes

A new finding is that CSN-evoked IIC activity increases progressively with each stimulus episode until the attainment of near-maximal levels during the fifth period of CSN stimulation. This mechanism must be independent of LTF as it is not affected by methysergide (Fig. 4*D*). The lack of a progressive augmentation of phrenic activity during stimulation is consistent with previous reports (Millhorn *et al.* 1980*a*, *b*). This finding is not unexpected as the first CSN stimulation episode elicited nearly maximal activity and further augmentation of the stimulus-evoked response was not possible (see below). However, in the cats that were pretreated with methysergide, a progressive increase in CSN stimulation-evoked phrenic activity was observed. The parsimonius explanation is that this mechanism was present prior to methysergide treatment, but was obscured by the saturation of phrenic motor output (Eldridge *et al.* 1981). Phrenic motoneurone excitability was diminished after methysergide treatment (Fig. 5) and the progressive augmentation of the phrenic burst amplitude was unmasked as the pathway was no longer saturated. Although not specifically addressed in their paper, the same effect of methysergide on phrenic responses to successive CSN stimulus episodes can be seen in Fig. 5 of Millhorn *et al.* (1980*b*).

Possible mechanisms

Owing to the fact that carotid body afferent fibres coursing through the carotid sinus nerve synapse predominantly in the vicinity of the nucleus of the solitary tract (Housley, Martin-Body, Dawson & Sinclair, 1987), and that the cell bodies of most serotonergic neurones are located in the raphe nuclei of the brainstem (see Skagerberg & Bjorklund, 1985; Jacobs & Azmitia, 1992), LTF must involve brainstem neurones in its underlying mechanism. Activation of carotid chemoreceptors stimulates raphe neurones (Erickson & Millhorn, 1991; Lindsey, Hernandez, Morris & Shannon, 1992), thereby demonstrating the linkage necessary for serotonergic involvement in LTF.

The regions where serotonin exerts its postsynaptic effects during LTF have not been specifically identified. Serotonergic neurones project to many regions of the central nervous system and innervate a number of structures important in ventilatory control. For example, serotonergic raphe neurones innervate the dorsal respiratory group (Voss, DeCastro, Lipski, Pilowsky & Jiang, 1990), the ventral respiratory group (Connelly, Ellenberger & Feldman, 1989) and the ventrolateral surface of the medulla (Erickson & Millhorn, 1991). Furthermore, localized injections of serotonin or serotonin agonists elicit both facilitatory and inhibitory influences on important classifications of medullary respiratory neurones (Sessle & Henry, 1985; Arita & Ochiishi, 1991). However, the net influence of increased supraspinal serotonin is an increase

in respiratory frequency rather than nerve burst amplitude (Monteau, Morin, Hennequin & Hilaire, 1990; Morin, Monteau & Hilaire, 1991; Lindsay & Feldman, 1993).

Millhorn *et al.* (1980*a*) suggested that LTF operates via supraspinal mechanisms, a theory based on the observation that LTF occurs via changes in both phrenic burst amplitude and frequency. However, in their study, the observed changes in frequency were rather small, and subsequent studies on awake dogs and anaesthetized and vagotomized rats or cats indicated that LTF can occur without changes in the mean respiratory or phrenic burst frequency (Cao *et al.* 1992; Hayashi *et al.* 1993; this study). On the other hand, episodic hypoxia elicits LTF in awake goats, largely via effects on respiratory frequency (D. Turner & G. S. Mitchell, unpublished observation). Thus, in a given animal or species, changes in frequency may contribute in a variable manner to the overall facilitatory response. Nevertheless, in anaesthetized vagotomized animals, LTF occurs primarily via effects on burst pattern formation (i.e. amplitude or volume) rather than on rhythm generation, leaving open the possibility that at least some of the serotonergic effects occur at the spinal level.

Serotonin exerts facilitatory effects on phrenic motoneurones (Lindsay & Feldman, 1993) as it does on other motoneurone pools (see Jacobs & Azmitia, 1992). Moreover, when serotonin is injected directly into the phrenic motor nucleus of anaesthetized rabbits, phrenic nerve activity is amplified for a period of hours with no change in burst frequency (Schmid, Bohmer & Merkelbach, 1990), an effect remarkably similar to LTF. Indeed, a direct projection from raphe neurones to the spinal motoneurones may provide an explanation for differences in the magnitude of LTF among various respiratory motoneurone pools as immunoreactive labelling of serotonergic terminals is heterogeneous among these pools. For example, the serotonergic labelling observed in the vicinity of hypoglossal motoneurones filled intracellularly with horseradish peroxidase appears to be less dense (Jiang *et al.* 1991) than the labelling observed near similarly filled phrenic motoneurones (Pilowsky, DeCastro, Llewellyn-Smith, Lipski & Voss, 1990), although serotonin still increases hypoglossal motoneurone excitability in an *in vitro* neonatal rat preparation (Berger, Bayliss & Viana, 1992) and in adult cats (Kubin, Tojima, Davies & Pack, 1992). There also appears to be a greater density of serotonergic terminals near retrogradely labelled nucleus ambiguus (Holtman, 1988) and inspiratory intercostal motoneurones (Jiang & Shen, 1985) relative to phrenic motoneurones (Holtman *et al.* 1984; Zhan, Ellenberger & Feldman, 1989), suggesting the possibility, therefore, that the serotonergic influences on these motor pools are relatively greater.

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