Phrenic long-term facilitation requires NMDA receptors in the phrenic motonucleus in rats

Michelle McGuire, Yi Zhang, David P. White and Liming Ling

Division of Sleep Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

Exposure to episodic hypoxia induces a persistent augmentation of respiratory activity, known as long-term facilitation (LTF). LTF of phrenic nerve activity has been reported to require serotonin receptor activation and protein syntheses. However, the underlying cellular mechanism still remains poorly understood. NMDA receptors play key roles in synaptic plasticity (e.g. some forms of hippocampal long-term potentiation). The present study was designed to examine the role of NMDA receptors in phrenic LTF and test if the relevant receptors are located in the phrenic motonucleus. Integrated phrenic nerve activity was measured in anaesthetized, vagotomized, neuromuscularly blocked and artificially ventilated rats before, during and after three episodes of 5 min isocapnic hypoxia ($P_{a,O_2} = 30$–45 mmHg), separated by 5 min hyperoxia (50% O_2). Either saline (as control) or the NMDA receptor antagonist MK-801 (0.2 mg kg^{-1}, I.P.) was systemically injected ∼1 h before hypoxia. Phrenic LTF was eliminated by the MK-801 injection (vehicle, 32.8 ± 3.7% above baseline in phrenic amplitude at 60 min post-hypoxia; MK-801, −0.5 ± 4.1%, means ± S.E.M.), with little change in both the CO_2-apnoeic threshold and the hypoxic phrenic response (HPR). Vehicle (saline, 5 × 100 nl) or MK-801 (10 μM; 5 × 100 nl) was also microinjected into the phrenic motonucleus region in other groups. Phrenic LTF was eliminated by the MK-801 microinjection (vehicle, 34.2 ± 3.4%; MK-801, −2.5 ± 2.8%), with minimal change in HPR. Collectively, these results suggest that the activation of NMDA receptors in the phrenic motonucleus is required for the episodic hypoxia-induced phrenic LTF.

(Resubmitted 29 March 2005; accepted after revision 1 June 2005; first published online 2 June 2005)
Corresponding author L. Ling: Division of Sleep Medicine @ BIDMC, Brigham and Women's Hospital, Harvard Medical School, 75 Francis Street, Boston, MA 02115, USA. Email: lling@partners.org

In several animal species, exposure to a series of short-term hypoxia episodes induces an augmentation of respiratory activity that lasts for many minutes to hours after the episodic hypoxia has ended, which is known as long-term facilitation (LTF). There is LTF of phrenic (Hayashi *et al.* 1993; Bach & Mitchell, 1996) and hypoglossal (Bach & Mitchell, 1996) nerve activity in anaesthetized rats and ventilatory LTF in awake animals (Cao *et al.* 1992; Turner & Mitchell, 1997; Olson *et al.* 2001; McGuire *et al.* 2002). Historically, respiratory LTF (manifested as phrenic LTF) was first elicited by repeated carotid sinus nerve (CSN) stimulation in anaesthetized cats (Millhorn *et al.* 1980), and later in rats (Hayashi *et al.* 1993; Ling *et al.* 1997) or in forms of intercostal nerve (Fregosi & Mitchell, 1994) and genioglossus muscle activities (Mateika & Fregosi, 1997). LTF can be modified by previous experience (cf. Mitchell *et al.* 2001). For example, pretreatment with chronic intermittent hypoxia enhances LTF (Ling *et al.* 2001; McGuire *et al.* 2003) and reduces the stimulus intensity threshold for eliciting LTF (cf. McGuire *et al.* 2003). Although common LTF-inducing protocols employ episodic hypoxia with 3–5 min duration, other stimulus protocols, whose hypoxic episodes mimic apnoea events in obstructive sleep apnoea (OSA) patients, also induce similar phrenic LTF and its enhancement after chronic intermittent hypoxia (Peng & Prabhakar, 2003).

Respiratory LTF requires serotonergic mechanisms. Both CSN stimulation- and episodic hypoxia-induced LTF can no longer be elicited following blockade of serotonin receptors with systemic administration of methysergide, a broad-spectrum serotoninergic antagonist (Eldridge & Millhorn, 1986; Bach & Mitchell, 1996). More specifically, hypoxia-induced phrenic (and ventilatory) LTF is eliminated by 5-HT_2 receptor antagonism with ketanserin (Ling *et al.* 2001; McGuire *et al.* 2004). The chronic intermittent hypoxia effect on LTF also depends on serotonin receptors (Ling *et al.* 2001; McGuire *et al.* 2004). The serotonin receptors crucial for phrenic LTF are located in the cervical spinal cord (Baker-Herman & Mitchell, 2002). Protein syntheses in the cervical spinal cord are also required for phrenic LTF (Baker-Herman *et al.* 2004). In a recent study using a medullary slice preparation

 DOI: 10.1113/jphysiol.2005.087650

from neonatal rats, episodic activation of 5-HT_2 receptors on hypoglossal motoneurones induces LTF of hypoglossal nerve motor output (Bocchiaro & Feldman, 2004). Nitric oxide has also been reported to play an important role in ventilatory LTF (Kline *et al.* 2002). However, neither noradrenaline (norepinephrine) nor dopamine antagonism abolishes phrenic LTF (Eldridge & Millhorn, 1986). Despite these findings, the cellular mechanisms underlying phrenic LTF remain poorly understood.

NMDA (*N*-methyl-D-aspartate)-subtype glutamate receptors play important roles in synaptic plasticity, such as long-term potentiation (LTP), which is thought to serve as a model for cellular mechanisms of learning and memory (Nicoll & Malenka, 1999; Kandel, 2000). Activation of NMDA receptors is essential for LTP in most cases. For example, LTP in the Schaffer collateral pathways of the hippocampus can no longer be elicited after NMDA receptor antagonism (Nicoll & Malenka, 1999). Various forms of learning and memory are also blocked/impaired by NMDA receptor antagonism (Castellano *et al.* 2001) or in mice with genetically modified NMDA receptors (Tonegawa, 1995). In the respiratory control system, the synaptic transmission of respiratory drive from bulbospinal respiratory neurones to phrenic motoneurones uses glutamate as a neurotransmitter and is mediated through both NMDA (minor) and non-NMDA (main) glutamatergic receptors (Liu *et al.* 1990; Chitravanshi & Sapru, 1996). Since NMDA receptors are important in synaptic plasticity and respiratory LTF resembles LTP in some ways, we hypothesized that activation of NMDA receptors is required for phrenic LTF and that these NMDA receptors are located on the phrenic motoneurones.

Methods

The Harvard Medical Area Standing Committee on Animals approved all experimental procedures described here. Experiments were conducted on adult male Sprague-Dawley rats (250–350 g, colony 236, Harlan, Madison, WI, USA). The present study mainly involved two series of experiments. The first series explored the role of NMDA receptors in phrenic LTF by examining phrenic LTF following systemic injection of the NMDA receptor antagonist MK-801 (vehicle, $n = 14$; MK-801, $n = 7$). The second series was designed to localize the NMDA receptors by examining phrenic LTF following microinjection of MK-801 into the phrenic motonucleus region (vehicle, $n = 8$; MK-801, $n = 6$). MK-801 (dizocilpine, Sigma, USA) is a widely used non-competitive NMDA receptor-associated channel blocker, which easily dissolves in saline and crosses the blood–brain barrier.

Experimental preparation

The rats were anaesthetized initially with isoflurane in a closed chamber and the anaesthesia was then maintained by gas mixtures (2.5–3.0% isoflurane; 50% O_2; balanced N_2) delivered via a facemask. The trachea was cannulated, and the rats were mechanically ventilated (Harvard Apparatus Inc, Holliston, MA, USA) while maintaining the inspired isoflurane concentration. Bilateral vagotomy was performed in the mid-cervical region. Two femoral venous catheters were inserted bilaterally for anaesthetic and other fluid administration. A femoral arterial catheter was also placed to allow blood pressure measurement and blood sample withdrawal for arterial blood gases and pH analysis. The anaesthetic was then slowly converted to urethane (1.6 g kg^{-1} in distilled water, I.V.) and the isoflurane was withdrawn. The adequacy of anaesthesia was tested before neuromuscular blockade by the suppression of the corneal reflex, and after neuromuscular blockade by the suppression of the blood pressure response to toe pinch. Pancuronium bromide (2.5 mg kg^{-1}, followed by additional doses as needed) was then given to obtain neuromuscular blockade. Shortly after this pancuronium injection, a supplementary dose of urethane (0.16 g kg^{-1}) was given to provide stable anaesthesia for the remainder of the experiment. A slow infusion of sodium bicarbonate (5%) and lactated Ringer solution (50 : 50, $\sim$1.7 ml kg^{-1} h^{-1}) was initiated $\sim$1 h after induction of anaesthesia to maintain fluid and acid–base balance. Rectal temperature was maintained near 37.5°C with a servo-controlled heat blanket.

End-tidal CO_2 partial pressure (P_{ET,CO_2}) was monitored in the expired line of the ventilator circuit using a flow-through capnograph (Novametrix; Wallingford, CT, USA) with sufficient response time (< 75 ms) to measure P_{ET,CO_2} in rats. P_{ET,CO_2} values obtained with this method closely approximate CO_2 partial pressure in arterial blood (P_{a,CO_2}) in most rats (usually within 1–2 mmHg). Inspired gases were 50% O_2 (balance N_2) during baseline and other non-hypoxia periods to improve the rat's tolerance of experimental stresses and prolong the viability of the preparation. At the end of the experiments, rats were killed with an overdose of urethane (3.2 g kg^{-1}, I.V.) followed by dislocation of the cervical spinal cord.

Phrenic nerve recording. The left phrenic nerve was dissected via a dorsal approach, cut distally, desheathed and prepared for recording with a bipolar silver wire electrode. The phrenic nerve activity was filtered (300–10 000 Hz) and amplified ($\times$2000, BMA-200 AC/DC Bioamplifier, CWE Inc., Ardmore, PA, USA). The amplified signal was full-wave rectified and integrated (Paynter Filter, BAK Electronics, Inc., USA; time constant = 100 ms). The integrated phrenic signals were digitized and acquired with computer software (LabView 5.0, National Instruments Corporation, USA), and analysed with a program developed in our laboratory. This program determines the amplitude and timing of

integrated phrenic nerve bursts, from which the minute phrenic activity can be calculated.

Microinjection of MK-801 into the phrenic motonucleus. In the microinjection experiments, after phrenic nerve isolation, a laminectomy was also performed over cervical vertebrae C3–C5 and the exposed dura was cut and retracted. Using a micromanipulator, a glass micropipette (30–80 μm outer diameter at the tip), connected to a 1 μl (1000 nl) Hamilton syringe, was inserted into the left phrenic motonucleus region. The phrenic motonucleus is shaped like a column and located in the middle of the ventral horn along the C3–C5 cervical spinal cord (cf. Furicchia & Goshgarian, 1987). Hence, five injections were made at spots distributed rostrocaudally and evenly between the middle points of the C3 and C5 dorsal root entries (~6 mm apart), 1.0–1.2 mm lateral to the midline and 1.5–1.8 mm below the dorsal surface (Fig. 1). A 100 nl volume of saline (in vehicle rats) or MK-801 solution (10 μM in saline in MK-801 rats) was slowly injected into each spot, to give a total volume of 500 nl and ~10 min in time for the entire injection (5 × 100 nl). These relatively large injections were aimed to cover most of the phrenic motoneurones (through diffusion).

Experimental protocols

The experimental protocols began at least 1 h after completion of the surgical preparation. The experiments using systemic injection began with an MK-801 injection (0.2 mg kg^{-1}, I.P.) in MK-801 rats or the same amount of saline (~1 ml) in vehicle rats. About 20 min after the injection, the rat's CO_2-apnoeic threshold was determined. The CO_2-apnoeic threshold is defined as the P_{ET,CO_2} at which respiratory rhythmic activity resumes from previous, hypocapnic silence in phrenic nerve recording. Baseline phrenic nerve activity was then stabilized for at least 30 min in a hyperoxic (50% O_2) and normocapnic condition (3 mmHg above the CO_2-apnoeic threshold), by manipulating the inspired CO_2 and respiratory pump rate and/or volume while monitoring P_{ET,CO_2} levels. One or two arterial blood samples (~0.3 ml in a 1 ml heparinized syringe; unused blood was returned to the animal) were drawn for blood gases and pH analysis (ABL-700; Radiometer, Copenhagen, Denmark) with correction for rectal body temperature. All subsequent blood samples were compared to this initial baseline value.

The experiments using microinjection began with the determination of the CO_2-apnoeic threshold and measurement of the first baseline phrenic activity (B1). MK-801 (10 μM in saline, 5 × 100 nl) was microinjected into the left phrenic motonucleus in MK-801 rats and saline (5 × 100 nl) was microinjected in vehicle rats. A second, stable baseline (B2) was measured at ~10 min after the microinjection. The baseline reduction was calculated by the equation: $100 \times (B2 - B1)/B1$. Both hypoxic phrenic response (HPR) and LTF values were calculated based on the B2. HPR was defined as an increase from B2 in phrenic activity during hypoxia, normalized as a percentage of the B2. Phrenic LTF was defined as an increase from B2 in post-hypoxia time points, normalized as a percentage of the B2.

In all these experiments, following the baseline measurement, integrated phrenic nerve activity was measured during and up to 60 min after three episodes of 5 min isocapnic hypoxia (P_{a,O_2} = 30–45 mmHg), separated by 5 min intervals of hyperoxia (50% O_2). Two sets of data were taken from these phrenic recordings: (1) during three hypoxia episodes to determine the short-term HPR; and (2) at 15, 30, 45 and 60 min post-hypoxia time points to determine LTF of phrenic nerve activity. Blood samples were also taken at all of these points (except the 45 min post-hypoxia point) to ensure an isocapnic condition. If any deviations in P_{ET,CO_2} (or P_{a,CO_2}) from isocapnic conditions were noted, corrections were made by adjusting the respiratory pump rate, thus ensuring that P_{a,CO_2} was generally within 1 mmHg of the baseline value. At the end of the protocol, the phrenic nerve responses to hypercapnia (P_{ET,CO_2} = 90–95 mmHg) were recorded to obtain a measure of approximately maximal phrenic activity. Since baseline is artificially set (see above), it occasionally becomes disproportionately too low or too high, which may overestimate or underestimate HPR and LTF values (cf. McGuire *et al.* 2003), as both are relative to baseline. Thus in the present study, the ratio of baseline phrenic activity to hypercapnic phrenic response was used

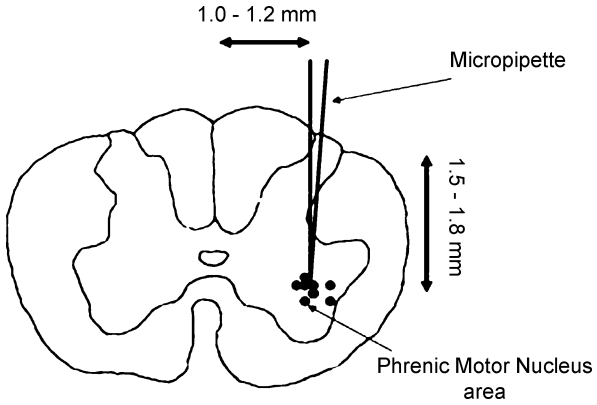

Figure 1. Superimposed schematic drawing of the spinal microinjection sites in one vehicle and one MK 801 rat
This diagram depicts a cross-section of the spinal cord at C3–C5 segments. Eight black dots in the lower right portion of the section represent the tip sites of the micropipettes as seen under a light microscope. Note that actual volumes and diffusion areas of the injected fluid are much larger than those spots. The dimensions shown here are similar to those reported by other laboratories (Furicchia & Goshgarian, 1987; Chitravanshi & Sapru, 1996).

as a criterion to exclude potentially extreme cases, and the ratio was within a 22–42% range for all included rats.

Methods for other supportive studies (with incomplete rat groups)

Time control. The time course of the MK-801 effect on phrenic activity, independent of episodic hypoxia, was examined in two separate rats. In these experiments, the same MK-801 microinjection procedure and dose (10 μM, 5 × 100 nl) as well as the same phrenic activity measurement (see above) were used, except that the episodic hypoxia protocol (to elicit LTF) was not employed.

Dose effect. Before the fixed dose (10 μM, 5 × 100 nl) was selected for the MK-801 group, the dose effect of MK-801 was assessed in three separate rats. In these experiments, the same microinjection procedure and phrenic measurement were used, except that one of three different doses of MK-801 (5 mM, 5 × 2 μl; 300 μM, 5 × 2 μl; and 10 μM, 5 × 0.5 μl) was chosen for each rat. The criterion for a proper dose was to block phrenic LTF while having as little effect as possible on baseline and HPR.

Receptor specificity. NMDA receptor specificity was tested in three separate rats by using the competitive NMDA receptor antagonist AP-5 (DL-2-amino-5-phosphonovaleric acid, Sigma, USA). In these experiments, the same microinjection procedure and phrenic measurement were used except that instead of MK-801, AP-5 was microinjected at three different doses (20 mM, 1 mM and 10 μM; 5 × 100 nl).

NMDA microinjection. The effect of NMDA (an NMDA receptor agonist, Sigma, USA) on phrenic nerve activity was examined in two rats using modified microinjection and phrenic measurement procedures. In these experiments, NMDA solution (1 mM in saline; 50 nl) was microinjected into the phrenic motonucleus at only one spot at C4 level, and phrenic nerve responses were measured before, during and after this single microinjection. Subsequently, phrenic responses to the NMDA microinjection were re-examined ∼20 min after systemic injection (I.P.) of MK-801 (0.2 mg kg^{-1} in one rat and 0.4 mg kg^{-1} in another rat).

Intrathecal injection. Before the microinjection method was chosen, intrathecal injection was attempted in seven rats. This intrathecal injection method has been successfully used in another laboratory (Baker-Herman & Mitchell, 2002). Briefly, a laminectomy was performed over C2–C3, and a small hole was cut through the dura at the edge of C3. A small plastic catheter was inserted into the hole such that the tip lay over C4. Thus, artificial cerebrospinal fluid (aCSF; ∼10 μl; $n = 3$) or MK-801 in aCSF (∼10 μl; $n = 4$; 25, 12, 10 or 6 μg kg^{-1}) was slowly injected onto the spinal cord around C4 level. The episodic hypoxia was initiated 20–30 min after the injection.

Bilateral recording. Both phrenic nerves were isolated and bilateral phrenic activities were measured in several rats to address the potential drug diffusion problem and to provide additional within-subject no-drug control data. In these experiments, the same unilateral microinjection procedure and MK-801 dose (10 μM; 5 × 100 nl) as well as the same phrenic measurement procedure (except for the bilateral phrenic recording) were used, intending to block the left-side LTF while keeping the right-side LTF intact.

Histological examination. In two microinjection experiments, the injected MK-801 solution (10 μM in saline, 5 × 100 nl; $n = 1$) and vehicle (saline, 5 × 100 nl; $n = 1$) contained 2% Pontamine Sky Blue. At the end of the experiments, the spinal cord, cut at the C2 and C6 levels, was removed and immersed in 4% formalin. To assess the exact sites of the microinjections, the spinal cords were frozen and sectioned (40 μm thickness) using a cryostat microtome. These sections were mounted on a glass slide and viewed under a light microscope (see Fig. 1).

Data analysis

Results included in the analysis were collected from successful experiments, in which the arterial blood pressure was always > 80 mmHg, P_{a,O_2} was > 140 mmHg (except during hypoxia) and P_{a,CO_2} was within 1.5 mmHg of the baseline value throughout an experiment. Integrated phrenic nerve activity was averaged in approx. 1 min bins at 8 time points (baseline, 3 hypoxia episodes and 4 post-hypoxia time points). Variables determined include: the peak amplitude of integrated phrenic activity (arbitrary units), phrenic nerve burst frequency (bursts min^{-1}) and minute phrenic activity (peak amplitude × burst frequency). For phrenic LTF and HPR, increases above baseline in phrenic amplitude and minute phrenic activity were normalized as a percentage of the baseline (%baseline). Changes from baseline in burst frequency used absolute units (bursts min^{-1}). Data on hypoxic responses were recorded during the last 2 min of hypoxia when phrenic amplitude reached a plateau, and averaged over three hypoxia episodes since there was little difference among these episodes.

For LTF, both the increases from baseline and the differences between groups in the post-hypoxia phrenic amplitude (also burst frequency and minute phrenic activity) were statistically analysed using a two-way

Table 1. Arterial blood gases in control and MK-801 rats

				Post-hypoxia time		
		Baseline	Hypoxia	15 min	30 min	60 min
Systemic Injection						
P_{a,CO_2}	Vehicle ($n = 12$)	43.1 ± 0.7	44.7 ± 0.9	43.8 ± 0.8	43.8 ± 0.6	43.4 ± 0.7
	MK-801 ($n = 7$)	43.6 ± 0.7	44.4 ± 1.3	44.9 ± 0.4	44.6 ± 0.7	44.6 ± 0.4
P_{a,O_2}	Vehicle ($n = 12$)	204 ± 5	33.7 ± 0.8*	159 ± 7*	167 ± 9*	168 ± 10*
	MK-801 ($n = 7$)	184 ± 7†	37.2 ± 2.3*	145 ± 10*	147 ± 12*	142 ± 11*
Microinjection						
P_{a,CO_2}	Vehicle ($n = 4$)	40.1 ± 1.3	40.2 ± 1.1	39.5 ± 2.1	40.1 ± 1.1	39.9 ± 1.7
	MK-801 ($n = 4$)	40.7 ± 1.9	41.7 ± 1.0	40.6 ± 2.2	41.0 ± 1.9	39.9 ± 1.9
P_{a,O_2}	Vehicle ($n = 4$)	202 ± 14	35.7 ± 0.8*	174 ± 11	187 ± 3	192 ± 4
	MK-801 ($n = 4$)	199 ± 6	37.4 ± 1.6*	176 ± 6	195 ± 8	196 ± 6

Values are means ± S.E.M. (in mmHg). There was no significant difference between groups in P_{a,CO_2} or P_{a,O_2} values at any point, except the baseline P_{a,O_2} between the systemic injection groups. * Significant difference from baseline; † significant difference from vehicle value.

ANOVA with repeated measures, followed by Tukey's *post hoc* tests (SigmaStat version 2.0, Jandel Corporation, San Rafael, CA, USA). Only baseline and post-hypoxia data were included in this two-way ANOVA; data recorded during hypoxia were not included. These data were percentage increases above baseline (amplitude and minute activity) or absolute changes from baseline (burst frequency). Thus, the baseline values were numerically set at zero for each rat in the data analysis (not shown in the figures). For blood gas values and HPR, a one-way ANOVA was used to statistically analyse the differences between and within groups. $P < 0.05$ was considered significant. All values are expressed as a mean ± S.E.M.

Results

Systemic injection of MK-801

Blood gases. The CO_2-apnoeic threshold for phrenic activity was not significantly different ($P = 0.91$) between vehicle (39.1 ± 0.5 mmHg, $n = 14$) and MK-801 rats (39.0 ± 1.0, $n = 7$), neither were baseline P_{a,CO_2} values ($P = 0.64$; Table 1) or the ratio of baseline phrenic activity to the hypercapnic phrenic response (vehicle, 33.1 ± 2.2%; MK-801, 34.5 ± 5.4%; $P = 0.77$). In both groups, the average P_{a,CO_2} values at 15, 30 and 60 min post-hypoxia were not significantly different from baseline (all $P > 0.23$; Table 1), indicating a consistent isocapnic condition throughout the experiment. The average P_{a,O_2} at all post-hypoxia points decreased from baseline (all $P < 0.05$) but still remained above 140 mmHg (Table 1), indicating a consistent hyperoxic condition after hypoxia. The post-hypoxia P_{a,O_2} values were not significantly different between groups (all $P > 0.1$; Table 1) although P_{a,O_2} was lower in MK-801 rats at baseline ($P < 0.05$).

Hypoxic phrenic responses (HPRs). The phrenic responses (including phrenic amplitude, burst frequency and minute phrenic activity) to short-term isocapnic hypoxia were not different among three hypoxia episodes and were therefore averaged. The average HPR in phrenic amplitude was not significantly different ($P = 0.58$; Fig. 2) between vehicle (189 ± 15% above baseline) and MK-801 rats (209 ± 42%). The average HPR in burst frequency (vehicle, 8.7 ± 1.2 breaths min^{-1} above baseline; MK-801, 9.7 ± 2.4 breaths min^{-1}) and HPR in minute phrenic activity (vehicle, 245 ± 20% above baseline; MK-801, 259 ± 54%) were also not significantly different between groups (both $P > 0.68$). These data indicated that systemic injection of MK-801 at a dose of 0.2 mg kg^{-1} had a minimal effect on the HPR.

Long-term facilitation (LTF). In the 2-way ANOVA analysis, there was a significant interaction effect ($F_{4,69} = 5.35$; $P < 0.0008$) between drug factor (2 levels: vehicle and MK-801 rats) and time factor (5 levels: baseline, 15, 30, 45 and 60 min post-hypoxia) in phrenic amplitude data. The post-hypoxia phrenic amplitude value was significantly lower in MK-801 *versus* vehicle rats at each time point (all $P < 0.05$; Fig. 3*A*). In vehicle rats, the episodic hypoxia protocol induced LTF of phrenic amplitude, as phrenic amplitudes at post-hypoxia 15, 30, 45 and 60 min time points were all significantly increased from baseline (all $P < 0.05$; Fig. 3*A*). In MK-801 rats, the phrenic amplitude was not significantly different from baseline at any post-hypoxia point (all $P > 0.276$; Fig. 3*A*). Finally, LTF of minute phrenic activity was similar to its component parts (Fig. 3*C*). These results suggest that phrenic LTF was abolished by pretreatment with MK-801.

In vehicle rats, the episodic hypoxia protocol also induced LTF of phrenic burst frequency, as post-hypoxia burst frequency was significantly increased from baseline at all post-hypoxia time points (all $P < 0.05$; Fig. 3*B*). However, in MK-801 rats, burst frequency was

significantly decreased from baseline at all post-hypoxia points (all $P < 0.05$; Fig. 3*B*). Average change from baseline in frequency was also significantly lower in MK-801 *versus* vehicle rats at each post-hypoxia time point (all $P < 0.05$; Fig. 3*B*). Baseline frequency in MK-801 rats (57.0 ± 3.3 breaths min^{-1}) was significantly higher ($P < 0.05$) than vehicle value (45.5 ± 1.0 breaths min^{-1}), suggesting that MK-801 at dose of 0.2 mg kg^{-1} (I.P.) increased baseline frequency about one hour after MK-801 injection.

Microinjection of MK-801 into phrenic motonucleus

Blood gases. The CO_2-apnoeic threshold for phrenic activity was not significantly different ($P = 0.437$) between vehicle (35.9 ± 0.6 mmHg, $n = 8$) and MK-801 rats (35.2 ± 0.6 mmHg, $n = 6$), neither were baseline P_{a,CO_2} values ($P = 0.803$; Table 1) nor the ratio of baseline phrenic activity to the hypercapnic phrenic response (vehicle, $33.4 \pm 3.4\%$; MK-801, $37.6 \pm 2.5\%$; $P = 0.37$). In both groups, the average P_{a,CO_2} values at 15, 30 and 60 min post-hypoxia were not significantly different from baseline (all $P > 0.981$), indicating a consistent isocapnic condition throughout the experiment. The average P_{a,O_2} values at all post-hypoxia time points were also not significantly different from baseline (all $P > 0.109$), indicating a consistent hyperoxic condition after hypoxia. The post-hypoxia P_{a,O_2} values were not significantly different between groups (all $P > 0.43$; Table 1). Vehicle and MK-801 rats were exposed to similar hypoxic severity during the stimulus protocol since average hypoxic P_{a,O_2} values were not significantly different between groups ($P = 0.805$; Table 1). Due to technical problems, blood gas data were not collected in all rats in either group (Table 1). However, a constant P_{ET,CO_2} was very carefully maintained throughout each experiment and the results (average LTF magnitude and pattern) were very similar between the groups with blood gas data (vehicle, $n = 4$; MK-801, $n = 4$) and their full groups (vehicle, $n = 8$; MK-801, $n = 6$).

Baseline and hypoxic phrenic responses (HPR). Microinjection of saline (5×100 nl) into the left phrenic motonucleus caused little baseline reduction in phrenic amplitude ($-4.5 \pm 3.9\%$; $P = 0.273$). Microinjection of MK-801 (10 μM, 5×100 nl) caused a small but significant baseline reduction in phrenic amplitude ($-14.1 \pm 5.4\%$; $P = 0.026$). The phrenic responses to short-term isocapnic hypoxia were similar among three hypoxia episodes and were averaged. The average HPR in phrenic amplitude was not significantly different ($P = 0.668$) between the vehicle ($182 \pm 22\%$ above

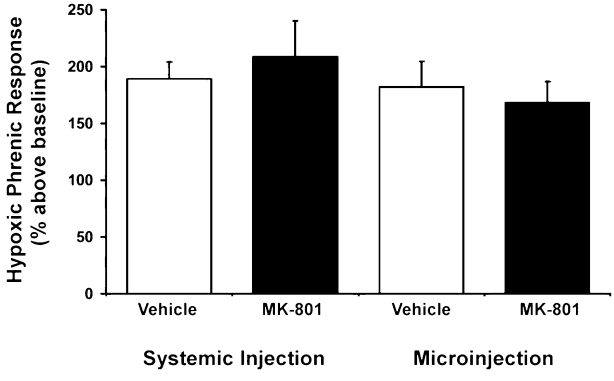

Figure 2. The effect of NMDA receptor antagonism on hypoxic phrenic response (HPR) in the peak amplitude of integrated phrenic nerve activity
This HPR is the average of 3 episodes of hypoxia and expressed as a percentage increase above baseline. Saline or MK-801 was systemically injected and microinjected (into the phrenic motonucleus region). Data are presented as means ± S.E.M. There is no significant difference between these groups (all $P > 0.7$).

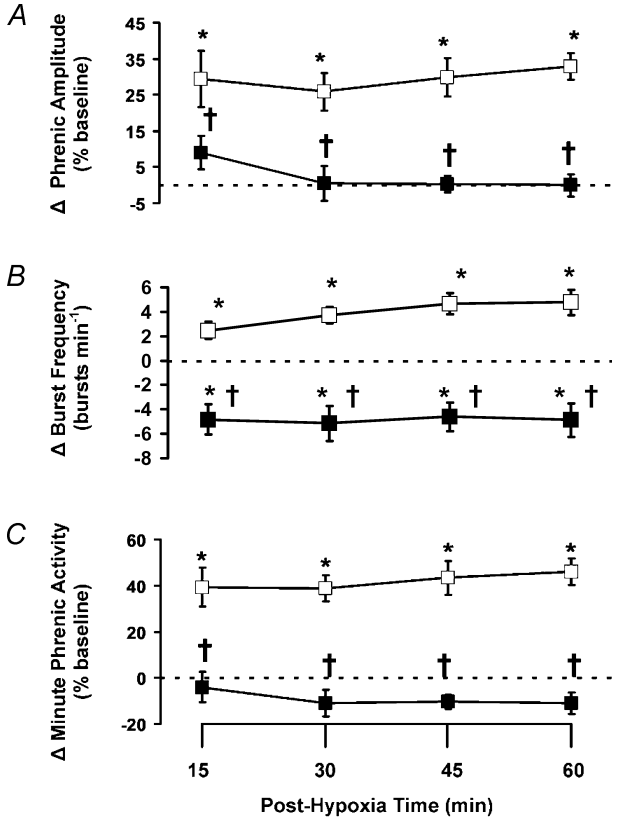

Figure 3. The effect of MK-801 (systemic injection) on phrenic long-term facilitation (LTF)
A, average changes from baseline in peak amplitude of integrated phrenic nerve activity, normalized as a percentage of the baseline (%baseline). *B*, changes from baseline in phrenic burst frequency (bursts min^{-1}). *C*, changes from baseline in minute phrenic nerve activity, normalized as a percentage of the baseline (%baseline). These were obtained following 3 episodes of 5 min isocapnic hypoxia in vehicle ($n = 14$, □) and MK-801 ($n = 7$, ■) rats. Data are expressed as means ± S.E.M. * Significant difference from baseline; † significant difference from vehicle group ($P < 0.05$).

baseline) and MK-801 groups (169 ± 18%), neither were HPR in frequency (vehicle, 2.0 ± 3.2 breaths min^{-1} above baseline; MK-801, 7.2 ± 4.3 breaths min^{-1}; $P = 0.345$) and HPR in minute phrenic activity (vehicle, 197 ± 30% above baseline; MK-801, 225 ± 42%; $P = 0.575$). These data suggest that the microinjection of MK-801 slightly reduces baseline phrenic activity but does not significantly change the HPR.

Long-term facilitation (LTF). There was a significant interaction effect between drug and time factors in phrenic amplitude data ($F_{4,47} = 13.89$; $P < 10^{-6}$). The phrenic amplitude at 45 and 60 min post-hypoxia was significantly lower in MK-801 *versus* vehicle values (both $P < 0.05$; Figs 4 and 5*A*). In vehicle rats, the episodic hypoxia protocol induced LTF of phrenic amplitude, as phrenic amplitude at 45 and 60 min post-hypoxia was significantly increased from baseline (both $P < 0.05$; Figs 4 and 5*A*). In MK-801 rats, the phrenic amplitude was not significantly different from baseline at any post-hypoxia time point (all $P > 0.623$; Figs 4 and 5*A*). There was no difference in frequency between groups at all post-hypoxia time points, although both frequencies at 15 min post-hypoxia (Fig. 5*B*) were significantly lower than baseline (vehicle, 49.4 ± 1.9 breaths min^{-1}; MK-801, 48.0 ± 1.7 breaths min^{-1}). Finally, LTF of minute phrenic activity was very similar to the LTF of phrenic amplitude (Fig. 5*C*). Collectively, these results suggest that phrenic LTF was abolished by microinjection of MK-801 into the phrenic motonucleus.

Other supportive studies (with incomplete rat groups)

Time control. The phrenic activity fluctuation from baseline at all time points was less than 6.0% of the baseline values, suggesting that other than a slight baseline reduction, MK-801 microinjection causes a minimal hypoxia-independent effect on integrated phrenic nerve activity in about 2 h.

Dose effect. There appeared to be a MK-801 dose effect on baseline, HPR and LTF. In one rat, microinjection with a high dose (5 mM, 5 × 2 μl) caused a gradual decline and an eventual disappearance of phrenic activity. A lower dose (300 μM, 5 × 2 μl) caused a large baseline reduction (−87%) with HPR being 970% above baseline and LTF of phrenic amplitude at the 60 min post-hypoxia time point (LTF_{60}) being −43%. In the third rat, a still lower dose (10 μM, 5 × 0.5 μl) abolished phrenic LTF (LTF_{60}, −8%) with less of an effect on baseline reduction (−31%) and a normal HPR (192%). These data suggest that with certain doses, it is possible to eliminate phrenic LTF with much less effect on baseline or HPR.

Receptor specificity. There also appeared to be an AP-5 dose effect on baseline, HPR and LTF. Microinjection of AP-5 (20 mM, 5 × 100 nl) caused a large baseline reduction (−75%) with HPR being 141% and LTF_{60} being −43%. A lower dose (1 mM, 5 × 100 nl) caused less baseline reduction (−47%) with HPR being 208% and LTF_{60} −39%. A still lower dose (10 μM, 5 × 100 nl) blocked LTF (LTF_{60}, −15.9%) with minimal effect on baseline (−15.6%) and a normal HPR (187%). These data indicate

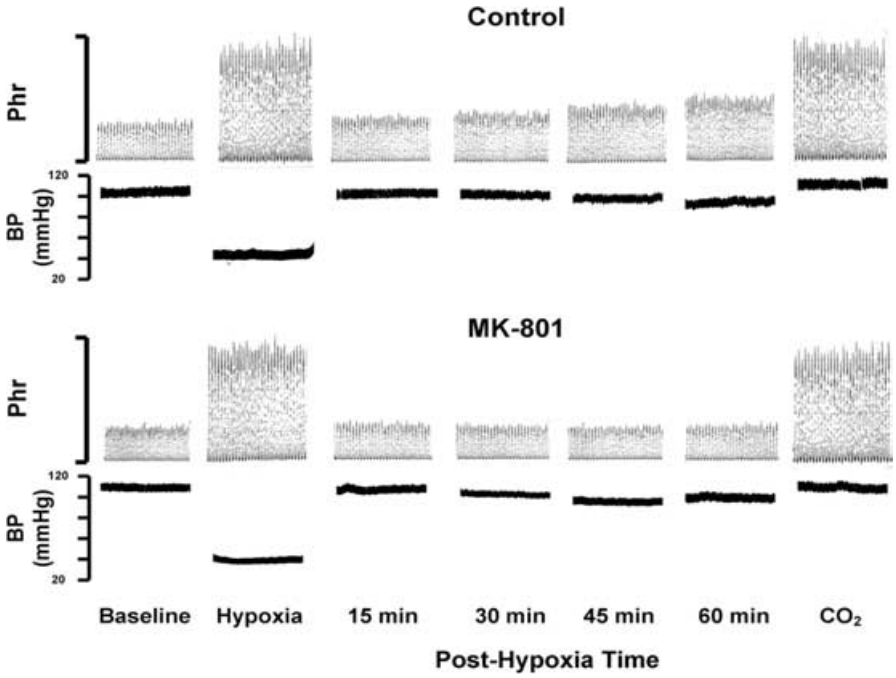

Figure 4. Representative tracings of the integrated phrenic neurogram (Phr) and mean arterial blood pressure (BP)
These were recorded before (baseline), during (hypoxia) and after 3 episodes of isocapnic hypoxia in one control (vehicle) and one MK-801 rat in the microinjection (into the phrenic motonucleus region) study. Note that the phrenic responses to hypercapnia (CO_2) are also presented but the data for only one hypoxic episode are presented.

that NMDA receptors are indeed crucial for phrenic LTF and suggest that AP-5 at a proper dose can also eliminate phrenic LTF while having a minimal effect on baseline and HPR.

NMDA microinjection. The first microinjection of NMDA (1 mM; 50 nl) into the phrenic motonucleus area at C4 level produced an increase in both tonic phrenic activity (∼66% baseline shift) and rhythmic phrenic activity (∼55% amplitude increase) in the two rats tested. All responses disappeared within 5 min. After systemic injection of MK-801 (0.2–0.4 mg kg^{-1}, I.P.), the second microinjection of NMDA (1 mM, 50 nl) produced little effect on both tonic and rhythmic phrenic activities (0% and 3%, respectively). However, in both rats, the third NMDA injection with a larger dose (1 mM, 200 nl) still produced a noticeable increase in both tonic and rhythmic phrenic activities (∼10% and ∼16%, respectively) although much less than those without prior MK-801 injection.

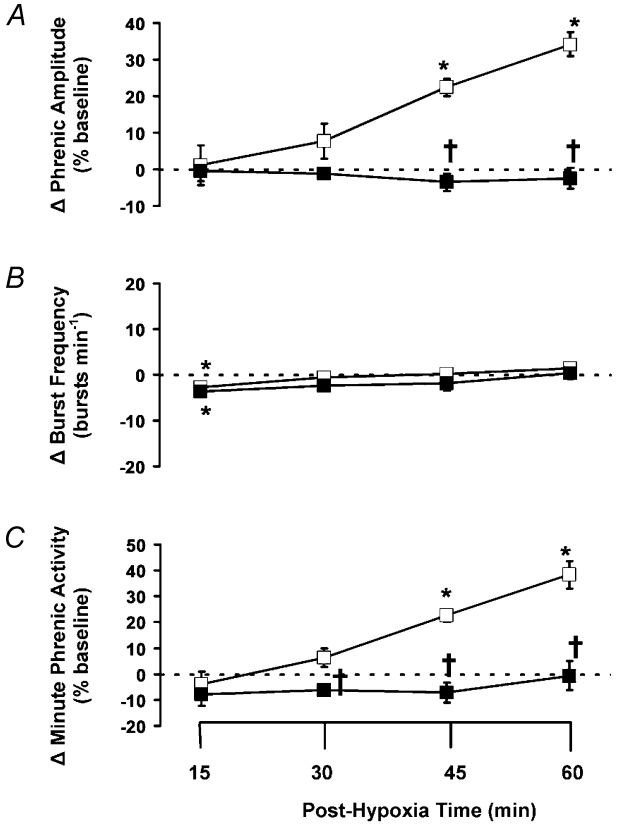

Figure 5. The effect of MK-801 microinjection (into the phrenic motonucleus region) on phrenic long-term facilitation (LTF)
A, average changes from baseline in peak amplitude of integrated phrenic nerve activity, normalized as a percentage of the baseline (%baseline). *B*, changes from baseline in phrenic burst frequency (bursts min^{-1}). *C*, changes from baseline in minute phrenic nerve activity, normalized as a percentage of the baseline (%baseline). These were obtained following 3 episodes of 5 min isocapnic hypoxia in vehicle ($n = 8$, □) and MK-801 ($n = 6$, ■) rats. Data are expressed as means ± S.E.M. * Significant difference from baseline; † significant difference from vehicle group ($P < 0.05$).

Intrathecal injection. There appeared to be a dose effect on baseline, HPR and LTF with intrathecal injection. MK-801 injection at dose of 25, 12, 10 and 6 μg kg^{-1} all eliminated phrenic LTF (LTF_{60}: −13%, 2.5%, 4.0% and −7%, respectively) but with different effects on baseline reduction (−45%, −25%, −53% and −15%, respectively) and HPR (605%, 178%, 230% and 242%, respectively). On the other hand, in three vehicle rats, baseline phrenic amplitude was minimally changed (all < 6%), HPR was 190 ± 65% and LTF_{60} was 35 ± 10%, thus indicating normal HPR and phrenic LTF.

Bilateral phrenic recording. We did not choose this bilateral approach for our major experimental groups because of its low success rate. Appropriate blood pressure and/or body temperature were difficult to maintain in most rats. Phrenic activity also unexpectedly disappeared in some rats during hypoxic challenges. Although we do not know the exact reason, this may somehow relate to more muscle/tissue removal to expose the right phrenic nerve. Nevertheless, we were able to successfully collect data in three out of the seven rats tested (i.e. blood pressure and body temperature were within an acceptable range). In one sham rat (with the same surgery but no microinjection), LTF_{60} was 36.5% on the left side and 44.3% on the right side. In one MK-801 rat, the left LTF_{60} was 1.0% and the right LTF_{60} was 19.4%. In another MK-801 rat, the left LTF_{60} was −30.5% and the right LTF_{60} was 18.3%. For all three rats, HPR magnitude was similar between the two sides and comparable to those reported above. These data suggest that microinjection of MK-801 into the phrenic nucleus can block ipsilateral phrenic LTF while having no or much less effect on the contralateral LTF.

Histological examination. Eight of the ten microinjections from one vehicle rat and one MK-801 rat were successfully located. The positions of micropipette tips during injection in relation to the ventral horn are shown in Fig. 1. Phrenic LTF was elicited by the episodic hypoxia protocol in the vehicle rat (LTF_{60}, 32.9%) but not in the MK-801 rat (LTF_{60}, 5.6%). These data suggest that MK-801 (and other drugs) was indeed microinjected into the middle of the ventral horn at the C3–C5 cervical spinal cord, an area corresponding to the phrenic motonucleus.

Discussion

The present study demonstrated that systemic injection of the NMDA receptor antagonist MK-801 (0.2 mg kg^{-1}, I.P., a subthreshold dose for many other respiratory

effects) eliminated phrenic LTF in anaesthetized rats, while having minimal effect on short-term hypoxic phrenic response (HPR). Microinjection of MK-801 into the phrenic motonucleus region also eliminated phrenic LTF with little change in HPR. These results indicate that activation of NMDA receptors is required for phrenic LTF induced by episodic hypoxia, and suggest that the NMDA receptors crucial for phrenic LTF are located in the phrenic motonucleus.

Methodological considerations

Experiments were conducted in anaesthetized, vagotomized, neuromuscularly blocked and ventilated rats, in which arterial blood gases and body temperature could be controlled, and episodes of isocapnic hypoxia could be easily implemented. The method of microinjecting drugs can more precisely locate the (drug action) sites, avoid the possible drug diffusion problem caused by intrathecal injection and greatly alleviate other possible confounding issues caused by systemic injection of MK-801 (see below).

Our vehicle data in the systemic injection experiments ($n = 14$) were averages of two groups of data. One group ($n = 5$), collected recently, showed phrenic LTF with a progressively augmenting pattern similar to those reported by other laboratories (cf. Mitchell *et al.* 2001) as well as the microinjection data in the present study (Figs 4 and 5*A*). Another group ($n = 9$), collected several months ago, showed LTF with a decrementing (15–30 min post-hypoxia) plus augmenting (30–60 min post-hypoxia) pattern. We are not sure why they were different, but this difference might be related to our earlier experimental setting and/or different experimenter. However, the critical average values at the 60 min post-hypoxia time point were very similar between these two groups ($33.5 \pm 1.8\%$ *versus* $32.3 \pm 9.1\%$).

MK-801 microinjection tended to reduce baseline phrenic activity. However, we are not particularly concerned about this baseline reduction, not only because this reduction is relatively small, but also because the reduced baseline theoretically should have enlarged LTF magnitude since LTF magnitude is expressed as an increase above baseline in phrenic activity normalized to the baseline. In the experiments using bilateral phrenic recording, it is unclear why the right-side LTF_{60} appears to be attenuated in both MK-801 rats. However, this LTF attenuation might result from two factors: (1) some drug diffused to the other side, and (2) these two rats happened to have smaller LTF magnitude. On the other hand, these data strongly suggest that MK-801 molecules, injected at the dose of the present study, cannot easily diffuse to the other side of the phrenic nucleus let alone the brainstem, where the raphe nuclei and the integrative centres responsible for rhythm generation and burst pattern formation are contained.

NMDA receptors and breathing control

NMDA receptors are involved in several central mechanisms of breathing control. For example, the respiratory frequency and/or pattern are greatly changed by blockade of NMDA receptors via either systemic administration of MK-801 or local application to medullary regions associated with breathing control (Foutz *et al.* 1988; Monteau *et al.* 1990; Ling *et al.* 1994). The central processing of carotid chemoafferent inputs also requires the activation of NMDA receptors in the nucleus tractus solitarius (Mizusawa *et al.* 1994; Ohtake *et al.* 1998). In particular, MK-801 injection ($3\ mg\ kg^{-1}$; I.V.) has been reported to abolish the hypoxic ventilatory response (Ohtake *et al.* 1998). Microinjection of MK-801 into the caudal nucleus tractus solitarius also reduced the hypoxic ventilatory response (Mizusawa *et al.* 1994). Therefore, we were surprised that systemic injection of MK-801 did not impair HPR in the present study since the NMDA mechanisms mentioned above all appear to be excitatory. We speculate that this difference might result from different doses, i.e. our dose ($0.2\ mg\ kg^{-1}$) might be too low to impair this crucial synaptic transmission. In support of this argument, a $0.3–1.0\ mg\ kg^{-1}$ dose also did not impair HPR (cf. Coles *et al.* 1998).

The descending, bulbospinal respiratory drive (to phrenic motoneurones) also uses glutamate as a neurotransmitter and is mediated partially through NMDA receptors (Liu *et al.* 1990; Chitravanshi & Sapru, 1996). In an *in vivo* adult rat model, microinjection of glutamate or NMDA into the phrenic motonucleus elicited an increase in phrenic nerve activity with little effect on phrenic burst frequency (Chitravanshi & Sapru, 1996). In contrast, microinjection of an NMDA receptor antagonist (AP-7) significantly reduced phrenic amplitude. Phrenic bursts were virtually eliminated by sequential microinjections of AP-7 and a potent non-NMDA receptor blocker (NBQX), suggesting that both NMDA and non-NMDA receptors located on the phrenic motoneurones play a significant role in the neurotransmission of the inspiratory drive in adult rats (Chitravanshi & Sapru, 1996). In one of our supportive studies, microinjection of NMDA into the phrenic nucleus also produced an increase in phrenic nerve activity, and this increase was blocked by pretreatment with MK-801, thus supporting the above results.

There is a correlation between HPR and LTF magnitude in anaesthetized rats (Fuller *et al.* 2000), suggesting that the size of HPR may determine the magnitude of LTF. However, our data suggest that MK-801 does not abolish phrenic LTF through reducing hypoxic responsiveness since HPR is not impaired by MK-801. In the present study, LTF was more sensitive to the NMDA receptor

antagonism than baseline or HPR. This difference might be due to the fact that the descending bulbospinal respiratory drive is mediated mainly via non-NMDA mechanisms (Liu *et al.* 1990) whereas phrenic LTF is mediated primarily via NMDA mechanisms (see below).

Potential mechanisms

Repeated carotid sinus nerve (CSN) stimulation elicited phrenic LTF, which was not abolished by decerebration or spinal transection at the C7–T1 level, suggesting that phrenic LTF requires neural mechanisms located in the brainstem and/or the cervical spinal cord, and that the carotid body, respiratory mechanics, systemic hypoxia, forebrain and the lower spinal cord are not necessary for its basic expression (Eldridge & Millhorn, 1986). Recent evidence further suggests that activation of serotonin receptors and synthesis of new proteins (e.g. brain-derived neurotrophic factor, BDNF) in the cervical spinal cord are necessary for phrenic LTF (Baker-Herman & Mitchell, 2002; Baker-Herman *et al.* 2004). On the other hand, there is also other evidence suggesting that the carotid body chemoafferents (Peng *et al.* 2003) and medullary pre-motor neurones (Morris *et al.* 2001) also exhibit some LTF. Interestingly, repeated stimulation of other (central or peripheral) hypoxic chemoreceptors, without activating carotid chemo-afferent neurones, also appears to be able to induce phrenic LTF, suggesting that carotid activation is not the only source of generating phrenic LTF (Bavis & Mitchell, 2003; Zhang *et al.* 2003).

Although substantial progress has been made in recent years on where and how LTF is generated, the exact location(s) and cellular/neuronal mechanisms underlying this neural plasticity of respiratory motor control still remain poorly understood (cf. Eldridge & Millhorn, 1986; Mitchell *et al.* 2001; Feldman *et al.* 2003). The hypothetical mechanisms of phrenic LTF have been proposed and refined many times (Eldridge & Millhorn, 1986; Fuller *et al.* 2000; Mitchell *et al.* 2001; Feldman *et al.* 2003). Briefly, the carotid chemo-afferent inputs, activated by episodic hypoxia or repeated CSN stimulation, stimulate the raphe nuclei. Released serotonin from the raphe serotonergic neurone terminals activates 5-HT_2 receptors on the phrenic motoneurone, which initiate a series of intracellular signalling events, leading eventually to LTF. It has been reported that the serotonergic mechanisms are required for LTF elicitation but not LTF maintenance (Fuller *et al.* 2001). The potential role of NMDA receptors for phrenic LTF has also been postulated in some hypothetical models (Fuller *et al.* 2000; Mitchell *et al.* 2001). Briefly, activation of 5-HT_2 receptors may increase the activity of certain intracellular protein kinase(s), which then phosphorylates the subunit(s) of the NMDA receptor-associated channels and augments the inward NMDA current, thereby producing an elevated response to the descending inspiratory drive (i.e. a phrenic LTF). There have been reports demonstrating that serotonin enhances NMDA current by activating 5-HT_2 receptors (Rahman & Neuman, 1993; Blank *et al.* 1996) and that antagonism of 5-HT_2 receptors by ketanserin or inhibition of protein kinase C (PKC) eliminates this serotonin-dependent increase in NMDA current in some other neurones and motoneurones (Rahman & Neuman, 1993).

Since antagonism of NMDA receptors in the phrenic motonucleus completely blocks phrenic LTF, we speculate that phrenic LTF is expressed primarily as a result of modification of the NMDA channels on the phrenic motoneurones, although LTF elicitation is likely to require other components in the brainstem (e.g. the raphe serotonin neurones). Thus, the NMDA mechanisms, unlike the serotonergic mechanisms, may not be necessary for earlier stages of LTF elicitation but may be required for LTF expression and maintenance. It should be noted, however, that although we believe these NMDA receptors are located on the phrenic motoneurone, based on our results and other published data in the literature (cf. Eldridge & Millhorn, 1986; Mitchell *et al.* 2001; Feldman *et al.* 2003), our experimental approaches cannot rule out the possibility that other NMDA receptors on some interneurones in the cervical spinal cord may also play a role in phrenic LTF.

Glutamate receptors and neural plasticity

The role of NMDA receptors in phrenic LTF is reminiscent of other forms of neural plasticity, such as the previously mentioned LTP and the recently identified LTF of hypoglossal motoneurones, which also require glutamatergic receptors. It is widely accepted that the activation of postsynaptic NMDA receptors causes calcium ion influx, which triggers LTP (Nicoll & Malenka, 1999; Kandel, 2000). The increased intracellular Ca^{2+} activates certain protein kinases (e.g. α-Ca^{2+}–calmodulin-dependent kinase II (CaMKII) and/or PKC), which in turn phosphorylate the target proteins (e.g. subunits of the AMPA receptor-associated ion channels). This channel modification increases neuronal sensitivity to glutamate, thereby expressing and maintaining a LTP (Nicoll & Malenka, 1999; Kandel, 2000). Recently, in an *in vitro* brainstem slice preparation from the neonatal rat, episodic activation of 5-HT_2 receptors on hypoglossal motoneurones (1) induced LTF of hypoglossal nerve activity and LTF of hypoglossal motoneuronal inspiratory-related drive current, and (2) increased the AMPA-mediated current in synaptically isolated hypoglossal motoneurones, which could be blocked by antagonism of group I metabotropic glutamate receptors (Bocchiaro & Feldman, 2004). These data

advance our understanding of the cellular mechanisms underlying respiratory LTF.

These three forms of neural plasticity (phrenic LTF, LTP and hypoglossal LTF) are similar in several ways. For example, they are all elicited by episodic, but not sustained, stimulation (Kandel, 2000; Baker & Mitchell, 2000; Bocchiaro & Feldman, 2004), and expressed as a long-lasting efficacy enhancement of the key synapse, which can bring out behavioural changes. In addition, they all require activation of glutamate receptors and (also likely) protein kinase(s) (Kandel, 2000; Mitchell *et al.* 2001; Bocchiaro & Feldman, 2004). However, they are different at least in three aspects. First, elicitation of LTP requires activation of NMDA receptors (Kandel, 2000), whereas elicitation of both phrenic and hypoglossal LTF does not require NMDA receptors but depends on the activation of 5-HT_2 receptors (Fuller *et al.* 2001; Bocchiaro & Feldman, 2004; see 'Potential mechanisms' above). Second, expression of hypoglossal LTF is blocked by antagonism of metabotropic glutamate receptors (Bocchiaro & Feldman, 2004), whereas expression of phrenic LTF (see 'Potential mechanisms' above) and many forms of LTP (Kandel, 2000) is blocked by the antagonism of NMDA receptors. Finally, LTP is induced as a consequence of pre- and postsynaptic elements being synchronously active (Bliss & Collingridge, 1993; Kandel, 2000), thus conforming with activity-dependent Hebb's rule (coincident pre- and postsynaptic activity strengthens synapses). In contrast, both hypoglossal and phrenic LTF are likely modulatory input-dependent but activity-independent plasticity, which does not require a Hebbian mechanism at least at key synapses on the motoneurone (Zhang *et al.* 2003; Bocchiaro & Feldman, 2004). Our recent study demonstrated that episodic phrenic-inhibitory vagus nerve stimulation paradoxically induced phrenic LTF, suggesting that the Hebbian mechanism plays no role in that LTF at the synapses on phrenic motoneurones as those neurones were totally suppressed during vagus nerve stimulation (Zhang *et al.* 2003). Therefore, despite the previously mentioned apparent similarities, these three forms of neural plasticity obviously have different underlying neurochemical mechanisms.

LTF and obstructive sleep apnoea (OSA)

Normal breathing and sleep require the maintenance of upper airway patency during sleep, which depends on the stability of the ventilatory motor output. OSA occurs when the upper airway dilator muscles become less active during sleep, thus blocking the upper airway, interrupting breathing and causing episodic hypoxaemia and hypercapnia (Malhotra & White, 2002). In normal human subjects, LTF of ventilation and genioglossal electromyographic activity could not be induced during wakefulness (Jordan *et al.* 2002). However, in those with inspiratory flow limitation (e.g. snorers and OSA patients), LTF was elicited during non-rapid eye movement sleep. This LTF was mainly displayed as a persistent decrease in upper airway resistance, an indication of upper airway dilatation due to motor output LTF of dilator muscles (Babcock & Badr, 1998; Aboubakr *et al.* 2001; Babcock *et al.* 2003). Ventilatory LTF was also elicited in some OSA patients (Babcock & Badr, 1998; Babcock *et al.* 2003) but was abolished after elimination of the flow limitation by continuous positive airway pressure (Babcock *et al.* 2003).

It has thus been speculated (Babcock & Badr, 1998; Ling *et al.* 2001; Zabka *et al.* 2001; Bocchiaro & Feldman, 2004; McGuire & Ling, 2005) that respiratory LTF may be an adaptive behaviour, temporarily stabilizing breathing and upper airway patency in OSA patients after repeated apnoeas/hypopnoeas. Also, chronic intermittent hypoxia may somewhat increase and prolong this beneficial effect since chronic intermittent hypoxia enhances LTF and reduces the stimulus intensity threshold for eliciting LTF (Ling *et al.* 2001; McGuire *et al.* 2003). In consistency with this speculation, the age effects on LTF magnitude and prevalence of OSA may be somewhat inversely correlated. Old age is associated with a loss of hypoglossal LTF in rats and a higher OSA prevalence in the old *versus* adult human population (Zabka *et al.* 2001), whereas younger ages (shortly before sexual maturity) are associated with a greater LTF in rats and a relatively lower OSA prevalence in the immature population (McGuire & Ling, 2005).

In conclusion, like hippocampal LTP and hypoglossal LTF, the episodic hypoxia-induced phrenic LTF also requires the activation of glutamatergic receptors. More specifically, this phrenic LTF requires the activation of NMDA receptors in the phrenic motonucleus, since it is completely blocked by either systemic injection or microinjection (into the phrenic nucleus area) of the NMDA receptor antagonist MK-801.

References

Aboubakr SE, Taylor A, Ford R, Siddiqi S & Badr MS (2001). Long-term facilitation in obstructive sleep apnea patients during NREM sleep. *J Appl Physiol* **91**, 2751–2757.

Babcock MA & Badr MS (1998). Long-term facilitation of ventilation in humans during NREM sleep. *Sleep* **21**, 709–716.

Babcock M, Shkoukani M, Aboubakr SE & Badr MS (2003). Determinants of long-term facilitation in humans during NREM sleep. *J Appl Physiol* **94**, 53–59.

Bach KB & Mitchell GS (1996). Hypoxia-induced long-term facilitation of respiratory activity is serotonin dependent. *Respir Physiol* **104**, 251–260.

Baker TL & Mitchell GS (2000). Episodic but not continuous hypoxia elicits long-term facilitation of phrenic motor output in rats. *J Physiol* **529**, 215–219.

Baker-Herman TL, Fuller DD, Bavis RW, Zabka AG, Golder FJ, Doperalski NJ, Johnson RA, Watters JJ & Mitchell GS (2004). BDNF is necessary and sufficient for spinal respiratory plasticity following intermittent hypoxia. *Nat Neurosci* **7**, 48–55.

Baker-Herman TL & Mitchell GS (2002). Phrenic long-term facilitation requires spinal serotonin receptor activation and protein synthesis. *J Neurosci* **22**, 6239–6246.

Bavis RW & Mitchell GS (2003). Intermittent hypoxia induces phrenic long-term facilitation in carotid-denervated rats. *J Appl Physiol* **94**, 399–409.

Blank T, Zwart R, Nijholt I & Spiess J (1996). Serotonin 5-HT_2 receptor activation potentiates N-methyl-D-aspartate receptor-mediated ion currents by a protein kinase C-dependent mechanism. *J Neurosci Res* **45**, 153–160.

Bliss TV & Collingridge GL (1993). A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* **361**, 31–39.

Bocchiaro CM & Feldman JL (2004). Synaptic activity-dependent persistent plasticity in endogenously active mammalian motoneurons. *Proc Natl Acad Sci U S A* **101**, 4292–4295.

Cao K-Y, Zwillich CW, Berthon-Jones M & Sullivan CE (1992). Increased normoxic ventilation induced by repetitive hypoxia in conscious dogs. *J Appl Physiol* **73**, 2083–2088.

Castellano C, Cestari V & Ciamei A (2001). NMDA receptors and learning and memory processes. *Curr Drug Targets* **2**, 273–283.

Chitravanshi VC & Sapru HN (1996). NMDA as well as non-NMDA receptors mediate the neurotransmission of inspiratory drive to phrenic motoneurons in the adult rat. *Brain Res* **715**, 104–112.

Coles SK, Ernsberger P & Dick TE (1998). A role for NMDA receptors in posthypoxic frequency decline in the rat. *Am J Physiol* **274**, R1546–R1555.

Eldridge FL & Millhorn DE (1986). Oscillation, gating, and memory in the respiratory control system. In *Handbook of Physiology*, section 3, *The Respiratory System*, vol. II, *Control of Breathing*, ed. Cherniack NS & Widdicombe JG, pp. 93–114. American Physiological Society, Bethesda, MD, USA.

Feldman JL, Mitchell GS & Nattie EE (2003). Breathing: rhythmicity, plasticity, chemosensitivity. *Annu Rev Neurosci* **26**, 239–266.

Foutz AS, Champagnat J & Denavit-Saubié M (1988). Respiratory effects of the N-methyl-D-aspartate (NMDA) antagonist MK-801 in intact and vagotomized chronic cats. *Eur J Pharmacol* **154**, 179–184.

Fregosi RF & Mitchell GS (1994). Long-term facilitation of inspiratory intercostal nerve activity following carotid sinus nerve stimulation in cats. *J Physiol* **477**, 469–479.

Fuller DD, Bach KB, Baker TL, Kinkead R & Mitchell GS (2000). Long term facilitation of phrenic motor output. *Respir Physiol* **121**, 135–146.

Fuller DD, Zabka AG, Baker TL & Mitchell GS (2001). Phrenic long-term facilitation requires 5-HT activation during but not following episodic hypoxia. *J Appl Physiol* **90**, 2001–2006.

Furicchia JV & Goshgarian HG (1987). Dendritic organization of phrenic motoneurons in the adult rat. *Exp Neurol* **96**, 621–634.

Hayashi F, Coles SK, Bach KB, Mitchell GS & McCrimmon DR (1993). Time-dependent phrenic nerve responses to carotid afferent activation: intact vs. decerebellate rats. *Am J Physiol* **265**, R811–R819.

Jordan AS, Catcheside PG, O'Donoghue FJ & McEvoy RD (2002). Long-term facilitation of ventilation is not present during wakefulness in healthy men or women. *J Appl Physiol* **93**, 2129–2136.

Kandel ER (2000). Cellular mechanisms of learning and the biological basis of individuality. In *Principles of Neural Science*, ed. Kandel ER, Schwartz JH & Jessell TM, pp. 1247–1279. McGraw-Hill, New York.

Kline DD, Overholt JL & Prabhakar NR (2002). Mutant mice deficient in NOS-1 exhibit attenuated long-term facilitation and short-term potentiation in breathing. *J Physiol* **539**, 309–315.

Ling L, Fuller DD, Bach KB, Kinkead R, Olson EB & Mitchell GS (2001). Chronic intermittent hypoxia elicits serotonin-dependent plasticity in the central neural control of breathing. *J Neurosci* **21**, 5381–5388.

Ling L, Karius DR & Speck DF (1994). Role of N-methyl-D-aspartate receptors in the pontine pneumotaxic mechanism in the cat. *J Appl Physiol* **76**, 1138–1143.

Ling L, Olson EB Jr, Vidruk EH & Mitchell GS (1997). Integrated phrenic reponses to carotid afferent stimulation in adult rats following perinatal hyperoxia. *J Physiol* **500**, 789–796.

Liu G, Feldman JL & Smith JC (1990). Excitatory amino acid-mediated transmission of inspiratory drive to phrenic motoneurons. *J Neurophysiol* **64**, 423–436.

McGuire M & Ling L (2005). Ventilatory long-term facilitation is greater in 1-month- versus 2-month-old awake rats. *J Appl Physiol* **98**, 1195–1201.

McGuire M, Zhang Y, White DP & Ling L (2002). Effect of hypoxic episode number and severity on ventilatory long-term facilitation in awake rats. *J Appl Physiol* **93**, 2155–2161.

McGuire M, Zhang Y, White DP & Ling L (2003). Chronic intermittent hypoxia enhances ventilatory long-term facilitation in awake rats. *J Appl Physiol* **95**, 1499–1508.

McGuire M, Zhang Y, White DP & Ling L (2004). Serotonin receptor subtypes required for ventilatory long-term facilitation and its enhancement after chronic intermittent hypoxia in awake rats. *Am J Physiol* **286**, R334–R341.

Malhotra A & White DP (2002). Obstructive sleep apnea. *Lancet* **360**, 237–245.

Mateika JH & Fregosi RF (1997). Long-term facilitation of upper airway muscle activities in vagotomized and vagally intact cats. *J Appl Physiol* **82**, 419–425.

Millhorn DE, Eldridge FL & Waldrop TG (1980). Prolonged stimulation of respiration by a new neural mechanism. *Respir Physiol* **41**, 87–103.

Mitchell GS, Baker TL, Nanda SA, Fuller DD, Zabka AG, Hodgeman BA, Bavis RW, Mack KJ & Olson EB Jr (2001). Invited review: Intermittent hypoxia and respiratory plasticity. *J Appl Physiol* **90**, 2466–2475.

Mizusawa A, Ogawa H, Kikuchi Y, Hida W, Kurosawa H, Okabe S, Takishima T & Shirato K (1994). In vivo release of glutamate in nucleus tractus solitarii of the rat during hypoxia. *J Physiol* **478**, 55–66.

Monteau R, Gauthier P, Rega P & Hilaire G (1990). Effects of N-methyl-D-aspartate (NMDA) antagonist MK-801 on breathing pattern in rats. *Neurosci Lett* **109**, 134–139.

Morris KF, Shannon R & Lindsey BG (2001). Changes in cat medullary neurone firing rates and synchrony following induction of respiratory long-term facilitation. *J Physiol* **532**, 483–497.

Nicoll RA & Malenka RC (1999). Expression mechanisms underlying NMDA receptor-dependent long-term potentiation. *Ann N Y Acad Sci* **868**, 515–525.

Ohtake PJ, Torres JE, Gozal YM, Graff GR & Gozal D (1998). NMDA receptors mediate peripheral chemoreceptor afferent input in the conscious rat. *J Appl Physiol* **84**, 853–861.

Olson EB Jr, Bohne CJ, Dwinell MR, Podolsky A, Vidruk EH, Fuller DD, Powell FL & Mitchel GS (2001). Ventilatory long-term facilitation in unanesthetized rats. *J Appl Physiol* **91**, 709–716.

Peng Y-J, Overholt JL, Kline D, Kumar GK & Prabhakar NR (2003). Induction of sensory long-term facilitation in the carotid body by intermittent hypoxia: Implications for recurrent apneas. *Proc Natl Acad Sci U S A* **100**, 10073–10078.

Peng Y-J & Prabhakar NR (2003). Reactive oxygen species in the plasticity of respiratory behavior elicited by chronic intermittent hypoxia. *J Appl Physiol* **94**, 2342–2349.

Rahman S & Neuman RS (1993). Activation of 5-HT_2 receptors facilitates depolarization of neocortical neurons by N-methyl-D-aspartate. *Eur J Pharmacol* **231**, 347–354.

Tonegawa S (1995). Mammalian learning and memory studied by gene targeting. *Ann N Y Acad Sci* **758**, 213–217.

Turner DL & Mitchell GS (1997). Long-term facilitation of ventilation following repeated hypoxia episodes in awake goats. *J Physiol* **499**, 543–550.

Zabka AG, Behan M & Mitchell GS (2001). Long-term facilitation of respiratory motor output decreases with age in male rats. *J Physiol* **531**, 509–514.

Zhang Y, McGuire M, White DP & Ling L (2003). Episodic phrenic-inhibitory vagus nerve stimulation paradoxically induces phrenic long-term facilitation in rats. *J Physiol* **551**, 981–991.

Acknowledgements

This work was supported by the National Institutes of Health (NIH) grant HL64912.