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Serotonin and NMDA receptors in respiratory long-term facilitation

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Abstract

Some have postulated that long-term facilitation (LTF), a persistent augmentation of respiratory activity after episodic hypoxia, may play a beneficial role in helping stabilize upper airway patency in obstructive sleep apnea (OSA) patients. However, the neuronal and cellular mechanisms underlying this plasticity of respiratory motor behavior are still poorly understood. The main purpose of this review is to summarize recent findings about serotonin and NMDA receptors involved in both LTF and its enhancement after chronic intermittent hypoxia (CIH). The potential roles of these receptors in the initiation, formation and/or maintenance of LTF, as well as the CIH effect on LTF, will be discussed. As background, different paradigms for the stimulus protocol, different patterns of LTF expression and their mechanistic implications in LTF will also be discussed.

Keywords

Respiratory control; Plasticity; Long-term facilitation, Intermittent hypoxia; Serotonin receptors; NMDA receptors

1. Introduction

Plasticity of neural pathways and networks is the basis for many physiological functions (*e.g.*, learning and memory), and has profound impacts on behavior of various complex motor systems (*cf.* Kandel et al., 2000). Considerable evidence suggests that the respiratory control system also exhibits impressive plasticity similar to that of many other neural networks (Eldridge and Millhorn, 1986; Mitchell et al., 2001a; Mitchell and Johnson, 2003). For example, episodic activation of carotid chemoafferents, either by repeated electrical stimulation of the carotid sinus nerve (CSN) or exposure to acute intermittent hypoxia (AIH, 3–10 episodes of short-term hypoxia), induces a sustained increase in respiratory activity that persists well after the direct effects of the stimulation, which is termed long-term facilitation (LTF). LTF has been studied for many years and has been identified in several animal species, including cats, dogs, rats, goats, birds and mice (Millhorn et al., 1980; Cao et al., 1992; Hayashi et al., 1993; Turner and Mitchell, 1997; Mitchell et al., 2001b; Terada et al., 2008), as well as humans during sleep (Aboubakr et al., 2001; Chowdhuri et al., 2008). In fact, LTF has become

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Invited Review for Special Issues on Neurochemistry of Respiratory Control

one of the most extensively studied forms of respiratory plasticity. LTF was once studied as a model for respiratory plasticity without clear physiological significance (*cf.* Eldridge and Millhorn, 1986). However, in the last decade, people have begun to appreciate its potential clinical implications in diseases, such as obstructive sleep apnea (OSA).

There have been several comprehensive reviews of LTF, covering almost all aspects of this respiratory plasticity (Eldridge and Millhorn, 1986; Mitchell et al., 2001a; Mitchell and Johnson, 2003). The main purpose of this review, however, is to summarize recent findings regarding serotonin (5-Hydroxytryptamine, 5-HT) and NMDA (*N*-Methyl-D-Aspartate) receptors that are involved in both LTF and its enhancement after chronic intermittent hypoxia (CIH), including their anatomy and neuronal roles, and the temporal sequence of their activation during the initiation, formation and/or maintenance of LTF. To date, investigators have employed many different stimulation paradigms and observed very different patterns of LTF expression in different preparations, which are potentially confusing. Thus the impact of the different paradigms/patterns on LTF as well as their mechanistic implications in LTF will be addressed in the beginning of this review.

2. Long-term facilitation (LTF)

About 3 decades ago, Millhorn, Eldridge and Waldrop (1980) first reported respiratory LTF, observing that 5 episodes of 2-min CSN stimulation, separated by 5-min intervals, elicited LTF of phrenic nerve activity (pLTF) in anesthetized, vagotomized, paralyzed and artificially ventilated cats. That pLTF persisted for more than 90 min without clear attenuation. Repeated CSN stimulation later elicited pLTF in rats (Hayashi et al., 1993) and intercostal LTF in cats (Fregosi and Mitchell, 1994) under the same conditions. Exposure to AIH, a more physiological stimulation, has also evoked pLTF (Hayashi et al., 1993; Bach and Mitchell, 1996) and hypoglossal LTF (Bach and Mitchell, 1996) in anesthetized rats, and ventilatory LTF (vLTF) in conscious, spontaneously breathing dogs, goats, ducks, rats and mice (Cao et al., 1992; Turner and Mitchell, 1997; Mitchell et al., 2001b; Olson et al., 2001; McGuire et al., 2002a; Terada et al., 2008).

LTF of ventilation or genioglossal muscle electromyogram (EMG) activity could not be elicited in normal humans during wakefulness (Jordan et al., 2002), but was observed during non-rapid eye movement (NREM) sleep (Chowdhuri et al., 2008). LTF has also been elicited during NREM sleep in humans with inspiratory flow limitation (*e.g.*, snorers and OSA patients) (Aboubakr et al., 2001). Thus it has been hypothesized that LTF may help stabilize upper airway patency in OSA patients after repeated exposure to apnea-induced hypoxia (Bach and Mitchell, 1996; Aboubakr et al., 2001; Ling et al., 2001; Bocchiario and Feldman, 2004; Mahamed and Mitchell, 2007, 2008a).

2.1. Paradigm of stimulation protocol

The stimulation paradigm is important for LTF. For example, intermittent hypoxia, but not sustained hypoxia, induces LTF in both anesthetized and awake animals (Baker and Mitchell, 2000; Olson et al., 2001; McGuire and Ling, 2006a). This notion was further supported by the results obtained from reduced preparations. In an *in vitro* brainstem slice preparation, episodic but not sustained activation of 5-HT₂ receptors on hypoglossal motoneurons induced hypoglossal LTF (Bocchiario and Feldman, 2004). Also, in an *ex vivo* carotid body preparation, intermittent but not mass application of serotonin elicited sensory nerve activity LTF (Peng et al., 2006). These results indicate that episodic vs. sustained stimulation can exert different effects on the hypoxic ventilatory control system. Although the neuronal and cellular mechanisms mediating these different effects are still unclear, Wilkerson et al. (2008) recently reported that sustained hypoxia induced pLTF after serine/threonine protein phosphatase activity inhibition in the cervical spinal cord, suggesting that the very different effects of

intermittent vs. sustained hypoxia on LTF may reflect their differential regulation of certain protein phosphatases.

The stimulation paradigms also have large effects on expression of plasticity in other neural pathways. The best-known example is probably the synaptic plasticity long-term potentiation (LTP), a putative cellular mechanism for learning and memory (Bliss and Collingridge, 1993). Many synapses exhibit a long-lasting increase in their efficacy after they are heavily used, which is termed LTP. LTP has been identified in the hippocampus and other areas of the brain (Bliss and Collingridge, 1993). It has been shown that the frequency of synaptic stimulation determines both the magnitude and direction of the change in synaptic efficacy. High-frequency stimulation (~100 Hz) elicits hippocampal LTP, whereas low-frequency (1–5 Hz) stimulation leads to long-term depression of this synaptic transmission (*cf.* Kandel et al., 2000).

The LTF-related stimulation protocols reported in the literature employ a range of hypoxic episodes at different O₂ levels. Some protocols were successful in inducing LTF while others were not. However, whether the episodic hypoxia selected in different protocols is sufficient to induce LTF or the varying results relate more directly to differences in species, genetics, age or gender was unclear. As a result, the different effects of hypoxic episode number and severity on vLTF were systematically examined in conscious, spontaneously breathing, freely behaving rats (McGuire et al., 2002a). Compared to anesthetized preparations, this awake preparation provides a more physiologically relevant way to study LTF, as possible confounding issues related to anesthesia, surgery and restraint can be eliminated. On the other hand, this preparation also has its own limitations, such as potential stress responses, poikilocapnia and a relatively smaller magnitude in LTF.

Minute ventilation (V_E) was measured by plethymography and vLTF was induced by poikilocapnic AIH. Two series of stimulation protocols were used: 1) different O₂ levels in 5 episodes of hypoxia, and 2) different episode number all at 12% O₂ hypoxia. In the first series of experiments, vLTF was elicited by 5 episodes of 10% O₂, but not by 5 episodes of 12% or 8% O₂. In the second series of experiments, vLTF was elicited by 10, 20 or 72 episodes of 12% O₂, but was not elicited by 3 or 5 episodes of 12% O₂. In addition, vLTF duration increased (from 45 to 75 min) with an increasing number of hypoxic episodes, but vLTF magnitude (see their definitions in the legend to Fig. 2) remained the same (~20% above baseline).

These results suggest that when LTF is induced with AIH, both hypoxic severity and episode number could affect the elicitation, magnitude and duration of vLTF in awake rats. These results also suggest that there is a certain range of (poikilocapnic) hypoxic severity (probably near 10% O₂) that is needed to successfully elicit vLTF in awake rats. Either too mild or too severe hypoxia would reduce or lose the effectiveness in eliciting LTF. Eldridge and Millhorn (1986) reported that pLTF saturates after first several stimuli, as increasing the number of stimulation episodes did not further increase the size of pLTF. Our results suggest that vLTF in awake rats also saturates and that more hypoxic episodes in a stimulus protocol usually increase the effectiveness in eliciting LTF. However, LTF magnitude reaches the saturated level more quickly than LTF duration when increasing episodes of hypoxia.

Most AIH stimulus protocols used hypoxic episodes of 3–5 min duration (CSN stimulation also lasts 2 min in each episode), while the hypoxic episodes experienced by OSA patients are usually much shorter and accompanied with intermittent hypercapnia. As a result, there have been several studies intended to address the discrepancies. Peng and Prabhakar (2003) reported that AIH (10 episodes of 15-s isocapnic 12% O₂ with 5-min intervals), which mimicked OSA events, elicited pLTF in anesthetized rats. Mahamed and Mitchell (2008b) reported that 3 or 6 episodes of 25-s asphyxia with 5-min intervals induced serotonin-dependent pLTF and

hypoglossal LTF in anesthetized rats. We also demonstrated that intermittent but not sustained hypercapnic hypoxia (10 episodes of 2-min 10% O₂ plus 5% CO₂ with 3-min intervals) induced vLTF in awake rats (McGuire and Ling, 2006a).

2.2. Pattern of LTF expression

The pattern of LTF expression is very different in different preparations. Some differences can be observed between animal species. For example, LTF of respiratory nerve and muscle activities always display a progressively augmenting pattern during the first hour after stimulation in anesthetized rats (Fig. 1A, 1B), while LTF displays a “flat” pattern in anesthetized cats (Millhorn et al., 1980; Fregosi and Mitchell, 1994). Other pattern differences can be observed between awake and anesthetized preparations. For example, AIH-induced LTF displays a progressively augmenting pattern in anesthetized rats but a progressively decrementing pattern in awake rats (Fig. 1B, 1C). The CSN stimulus-induced pLTF was once thought to have a decrementing pattern, but later was also found to have an augmenting pattern when post-hypoxia phrenic activity recording was extended from 30 min to 60 min (Zhang et al., 2003). The progressively decrementing pattern is not unique to awake rats, as it is also observed in awake dogs, goats, ducks and mice (Cao et al., 1992; Turner and Mitchell, 1997; Mitchell et al., 2001b; Terada et al., 2008).

Millhorn et al. (1980) reported that a 2-min CSN stimulation caused a sustained increase in phrenic nerve activity and the stimulation termination was followed by a slowly decaying respiratory afterdischarge. However, the respiratory activity remained elevated above the baseline level. Successive stimulations led to a progressive increase in this elevated level of respiratory activity, which could last more than 90 min after the last CSN stimulation (*i.e.*, a pLTF). Actually, the AIH-induced vLTF has been induced in very similar ways in all awake animals to date. Namely, the ventilation also increases during hypoxia and decreases after hypoxia, but ventilation between hypoxic episodes progressively increases during the stimulation protocol. After AIH, the elevated ventilation also begins to gradually decrease and return to baseline level. In our awake rat experiments, it appears that ventilation between hypoxic episodes and shortly after AIH does not progressively increase but stays at levels close to the baseline (Fig. 1C). We argue that it is unlikely an animal species difference, but may well be because we used poikilocapnic AIH while others used isocapnic AIH. This argument is supported by the results from a rat study using isocapnic AIH (Olson et al., 2001) and a more recent study in mice using poikilocapnic AIH (Terada et al., 2008).

The fact that LTF is induced in such a progressively increasing way (between hypoxic episodes) suggests that: 1) LTF is fully developed/formed in the first several hypoxic episodes before AIH ends, and 2) LTF is merely a lengthened residual hypoxic response or an extended respiratory afterdischarge. Furthermore, since all forms of LTF have been induced by episodic inspiratory-excitatory stimulation, it has been assumed by many that: 1) induction of LTF depends on episodic inspiratory augmentation, 2) the size of inspiratory enhancement determines the magnitude of LTF, and 3) episodic inspiratory-inhibitory stimulation cannot elicit LTF. Some even believed that LTF, like LTP, relies on the activity-dependent Hebbian mechanism (*i.e.*, synapses are strengthened when the pre- and post-synaptic elements are synchronously active). Indeed, LTP of the Schaffer collateral pathways is induced as a consequence of coincident pre- and post-synaptic activity, and can no longer be induced if the post-synaptic depolarisation is suppressed (Bliss and Collingridge, 1993).

In reality, none of these assumptions are correct. Exposure to intermittent hypercapnia or repeated calf muscle stimulation (both stimulate inspiration and lead to a typical afterdischarge) failed to induce LTF; intermittent severe hypercapnia even induced a phrenic long-term depression involving α_2 -adrenoreceptors (Eldridge and Millhorn, 1986; *cf.* Kinkead et al., 2001). Moreover, in an *in vitro* brainstem slice preparation, LTF was induced by episodic

neuronal activation with a 5-HT₂ receptor agonist or a α_1 -adrenoreceptor agonist, but not by episodic non-specific activation with an elevation of K⁺ concentration (Neverova et al., 2007). These data indicate that episodic inspiratory augmentation alone is not sufficient to induce LTF. However, the need for inspiratory augmentation in inducing LTF is more difficult to confirm or disprove. To directly address the aforementioned issues, one needs to dissociate LTF induction from inspiratory augmentation, and vagus nerve stimulation (VNS) provides such a unique opportunity.

The long-term effect of repeated VNS on phrenic nerve activity was examined in anesthetized, vagotomized, paralyzed and ventilated rats. Although VNS totally eliminated phrenic nerve rhythmic activity during stimulation, 3 episodes of 5-min phrenic-inhibitory VNS, separated by 5-min intervals, paradoxically induced pLTF (Zhang et al., 2003). This pLTF was very similar in size, pattern and serotonin dependency (see Transmitters and receptors below) to that induced by repeated phrenic-excitatory CSN stimulation. These data suggest that LTF induction can be completely dissociated from inspiratory enhancement and that the Hebbian activity-dependent plasticity plays no role in this pLTF at least at the synapses on the phrenic motoneuron, as those postsynaptic neurons are completely suppressed during VNS.

The dissociation of LTF induction from inspiratory enhancement may be extended to other forms of LTF as well. In a recent study, 3 episodes of 5-min hypoxia (10% O₂) with 5-min intervals successfully induced pLTF in an *in situ* working-heart brainstem preparation of immature rats, despite the fact that phrenic nerve activity was suppressed during the individual hypoxia episodes (Tadjalli et al., 2007).

Taken together, these results suggest that LTF is not just a residual elevation (during induction) of respiratory activity. On the contrary, inspiratory enhancement during hypoxia or CSN stimulation may be the “by product” of LTF induction. Indeed, not all LTF is induced and expressed in such a gradual increase (during induction) and gradual decrease (after the stimuli) way. In fact, as mentioned above, all the AIH-induced LTF in anesthetized rats displays a progressively augmenting pattern (Fig. 1) despite an isocapnic condition being maintained throughout the experiment. The reason for this augmenting pattern is currently unknown. It was thought that interaction between opposing mechanisms might play a role. For example, AIH may activate both excitatory and inhibitory mechanisms in the respiratory control system, and the observed post-hypoxia respiratory activity might be the integrated outcome of both inhibitory and excitatory inputs. Thus the augmenting pattern of LTF may result from the excitatory mechanisms that gradually surpass and outlast the inhibitory ones.

Alternatively, this augmenting pattern of LTF might be simply an intrinsic property of motoneurons, reflecting the time course of development of a series of intracellular signaling events (including protein synthesis and phosphorylation) triggered by AIH. Peng et al. (2006) recently reported that 3 episodes of 15-s serotonin application with 5-min intervals elicited a 5-HT₂ receptor-dependent sensory LTF in an *ex vivo* carotid body preparation. This single-unit sensory LTF clearly displays a progressively augmenting pattern for 60 min after serotonin application, suggesting that this progressive activity increase is an intrinsic property of the type I (glomus) cells in the carotid body. Although there are still peripheral neural structures, there are definitely no central components [*e.g.*, pre-Bötzinger complex, raphe serotonin neurons, and dorsal (DRG), ventral (VRG) and pontine (PRG) respiratory group neurons] present in this *ex vivo* carotid body preparation. Even the cell body of the petrosal ganglion neurons is absent. In *in vivo* preparations, however, both mechanisms (the excitatory-inhibitory interaction and the neuronal intrinsic property) probably play a role in LTF.

Different patterns of LTF have also been observed in different age groups. The effect of young age on vLTF was examined in immature male awake rats (McGuire and Ling, 2005). Two

stimulation protocols, one effective (10 episodes of 5-min poikilocapnic 12% O₂ with 5-min intervals) and the other ineffective in adult rats (5 episodes), were used to evoke vLTF. vLTF was measured twice by plethysmography in the same rats when they were 1 month and 2 months old. We found that the “ineffective” 5-episode protocol, as predicted, failed to elicit vLTF in 2-month-old rats, but elicited vLTF when they were 1 month old. The effective 10-episode protocol also elicited a greater vLTF in 1- vs. 2-month-old rats. Although baseline and post-hypoxia V_E's were all greater in 1- vs. 2-month-old rats, the shift in the vLTF curve (Fig. 1C) did not yield parallel lines, as the curve was steeper in 1- vs. 2-month-old rats (McGuire and Ling, 2005). These results indicate that vLTF is greater in male rats shortly before sexual maturity and suggest that the threshold of inducing LTF is lower in 1- vs. 2-month-old rats.

3. Transmitters and receptors

Since the discovery of the importance of serotonin in LTF, several additional neurotransmitters, neurochemicals and receptors have been identified. These include brain-derived neurotrophic factor (BDNF) and the TrkB receptors (Baker-Herman et al., 2004), protein kinase C (McGuire and Ling, 2004), NMDA receptors (McGuire et al., 2005), α_1 -adrenergic receptors (Neverova et al., 2007) and orexin (Terada et al., 2008). This review, however, focuses on the modulatory transmitter serotonin, the excitatory transmitter glutamate, and their receptors in both LTF and the CIH-enhanced LTF.

3.1. Serotonin and NMDA receptors

In the brain, serotonin is primarily synthesized in and released from the serotonergic neurons in the raphe nuclei, which project to numerous areas in the central nervous system. Serotonin plays an important role in many higher brain functions and mental health/states as well as breathing control (*cf.* Kinkead et al., 2001). Serotonin receptors are divided into seven different types (5-HT₁ to 5-HT₇) and more subtypes as well. All these receptors are G-protein-coupled receptors except 5-HT₃ receptors, which are ligand-gated ion channels. Glutamate receptors are located on neuronal membranes and bind the neurotransmitter glutamate, which is the most prominent one in the brain and whole body. Glutamate receptors are divided into two basic types: metabotropic and ionotropic. Both have many specific subtypes. The metabotropic glutamate receptors are G-protein-coupled receptors and divided into groups I, II and III. Each group also has several subtypes. The ionotropic glutamate receptors include NMDA and non-NMDA (*e.g.*, kainate and AMPA) receptors.

NMDA receptors play key roles in synaptic plasticity, *e.g.*, the LTP of the Schaffer collateral pathways can no longer be elicited after NMDA receptor antagonism (Bliss and Collingridge, 1993). Numerous forms of learning and memory are also abolished or impaired by blockade or alteration of NMDA receptors (*cf.* Kandel et al., 2000). NMDA receptors are also involved in several important respiratory control mechanisms, such as the phase-switching mechanism and the central processing of carotid chemoafferent inputs in the nucleus tractus solitarius (*cf.* Waters and Machaalani, 2005). In addition, the synaptic transmissions of inspiratory drive from premotor neurons to the major inspiratory (*e.g.*, phrenic, hypoglossal and intercostal) motoneurons all use glutamate as a neurotransmitter and are mediated through both NMDA and non-NMDA receptors (McCrimmon et al., 1989; Steenland et al., 2006). These ionotropic glutamate receptors and their receptor-associated currents may be modified to produce plasticity of the synapses, as they do in the induction and maintenance of LTP (*cf.* Kandel et al., 2000).

3.2. 5-HT receptors in LTF

Shortly after identifying the first respiratory LTF, Millhorn et al. also reported the dependency of LTF on serotonin (*cf.* Eldridge and Millhorn, 1986). They found that pLTF could no longer

be elicited by the same repeated CSN stimuli in anesthetized cats after serotonin receptor antagonism by systemic injection of methysergide (a broad-spectrum 5-HT receptor antagonist). pLTF was also impaired following application of the tryptophan hydroxylase (the rate limiting enzyme in serotonin biosynthesis) inhibitor parachlorophenylalanine or the serotonergic neurotoxin 5,7-dihydroxytryptamine. On the other hand, since pre-treatment with α -methyltyrosine (an inhibitor of dopamine and norepinephrine synthesis) did not block pLTF, they concluded that dopamine and norepinephrine are not essential to the CSN stimulus-induced pLTF.

The AIH-induced (phrenic and hypoglossal) LTF in anesthetized rats is also serotonin-dependent (Bach and Mitchell, 1996). More specifically, the AIH-induced pLTF requires activation of 5-HT₂ receptors, since systemic injection of the specific 5-HT₂ receptor antagonist ketanserin eliminated pLTF (Kinkead et al., 2001; Ling et al., 2001). These results, however, may not lead to the same conclusion for vLTF in awake animals, as vLTF is expressed as an integrative function involving muscles (e.g., intercostals) besides the phrenic nerve-innervated diaphragm and the hypoglossal nerve-innervated tongue muscles. Thus the effect of serotonin receptor antagonism on vLTF was examined in awake rats. We observed that AIH-induced vLTF was prevented by systemic injection of the serotonin antagonist methysergide, ketanserin or clozapine (McGuire et al., 2004). These results indicate that vLTF also requires activation of 5-HT₂ receptors and suggest that the motor output LTF of all major inspiratory muscles depend on serotonergic mechanisms.

The studies described above all used systemic injection of drugs. Therefore, the exact location (s) of the receptors was unclear. Baker-Herman and Mitchell (2002) injected the related drugs intrathecally into the cervical spinal cord of anesthetized rats and observed that AIH-induced pLTF was attenuated or abolished (hypoglossal LTF was unaffected) by antagonism of serotonin receptors or inhibition of protein synthesis in the cervical spinal cord. Using the same injection method, Baker-Herman et al. (2004) further demonstrated that synthesis of BDNF (a protein that plays important roles in numerous forms of synaptic plasticity, including LTP) in the cervical spinal cord is necessary and sufficient for AIH-induced pLTF. These data strongly suggest that activation of serotonin receptors and synthesis of new protein in the phrenic motoneurons are necessary for the AIH-induced LTF of these neurons.

Although serotonin has long been known to be necessary for LTF, the underlying mechanism was poorly understood. Serotonin was previously thought to have an overall excitatory effect on the medullary neural networks subserving respiratory control during the entire period of LTF, as pre-treatment with methysergide prevented pLTF and methysergide injected 90 min post-CSN stimuli led to a dose dependent decrease in the still elevated phrenic nerve activity (Eldridge and Millhorn, 1986). However, new evidence suggests that serotonin may play a trigger role, acting only during the LTF induction period. Fuller et al. (2001) demonstrated that systemic injection of ketanserin before AIH eliminated pLTF in anesthetized rats, whereas the injection immediately or 45 min after AIH had little effect (*i.e.*, LTF was normal), suggesting that activation of serotonin receptors is required for induction but not maintenance of pLTF.

Thus far, all forms of LTF, either induced by AIH or evoked by CSN stimuli, are serotonin dependent. More specifically, all forms of LTF require 5-HT₂ receptors with the sole exception of repeated VNS-evoked pLTF (Zhang et al., 2004). We found that that pLTF could no longer be evoked by repeated VNS in anesthetized rats pre-treated with methysergide (a serotonin 5-HT_{1,2,5,6,7} receptor antagonist) or clozapine (5-HT_{2,6,7} antagonist). However, pLTF was similar, if not greater, in animals pre-treated with ketanserin (a specific 5-HT₂ antagonist). These data suggest that unlike AIH-induced pLTF, which is 5-HT₂ receptors dependent, the VNS-induced pLTF requires non-5-HT₂ serotonin receptors, perhaps 5-HT₆ and/or 5-HT₇.

subtype(s). However, whether it is unique to this VNS-evoked pLTF or it is a general property of all sensory nerve stimulus-induced LTF is unknown at this time.

3.3. Chronic intermittent hypoxia (CIH)

Before reviewing the role of 5-HT receptors in the CIH effect on LTF, by way of background, CIH treatment and the CIH-induced enhancement of LTF will be discussed. Plasticity itself can be modulated (*e.g.*, synapse's history affects the present plasticity) and this plasticity of synaptic plasticity is often referred to as metaplasticity. Respiratory LTF can also be modulated by previous experience (*cf.* Mitchell and Johnson, 2003). Our previous results (McGuire et al., 2002a) showed that a prolonged AIH protocol (72 episodes of 5-min 12% O₂) increased vLTF duration from 45 min to 75 min, but had little effect on vLTF magnitude (~20%), indicating saturation of the plasticity. However, it was unclear what the effects would be if a longer intermittent stimulation protocol is used. In many forms of neural plasticity, the “dose” effect of stimulation plays an important role. For example, a single train of stimuli, given for 1 s at 100 Hz, elicits an early LTP of the Schaffer collateral pathway in the CA1 region of the hippocampus, which persists for 2–3 h, while 4 such trains, separated by 10-min intervals, elicit the late phase of LTP, which lasts for more than 24 h (*cf.* Kandel et al., 2000).

The effect of 1-week CIH on the hypoxic ventilatory control was first examined in anesthetized rats (Ling et al., 2001). The CIH treatment was achieved by exposing rats to quasi-square-wave intermittent hypoxia (5-min 11–12% O₂/5-min normoxia), and was repeated for 12 h/night for 7 nights. Following this CIH treatment, the usual AIH protocol (3 episodes of 5-min isocapnic hypoxia with 5-min intervals) was used to induce pLTF. It was found that the size of pLTF was enhanced and the phrenic response to severe hypoxia was augmented. Additionally, the phrenic amplitude (and frequency) responses to CSN stimulation were enhanced, suggesting a central component to this plasticity. Thus, the CIH effect on LTF is a good example of mataplasticity in respiratory motor control. The pre-treatment with CIH establishes a new background condition, which modulates a subsequent LTF when it is similarly elicited.

However, several questions remained that could not be easily answered in this anesthetized preparation. Thus the effect of 1-week CIH treatment on vLTF was later examined in awake, freely behaving rats (McGuire et al., 2003). We found that resting ventilation was not affected by the CIH, *i.e.*, CIH persistently changed post-AIH ventilation while having little effect on ventilation *per se*. The post-hypoxia vLTF curve (Fig. 1C) was shifted upwards after CIH in parallel with the original curve (McGuire et al., 2003, 2004), reflecting an augmented vLTF magnitude (from ~20% to ~30%) and prolonged vLTF duration (from 45 min to 75 min). In addition, the CIH treatment reduced the stimulation threshold for eliciting LTF, converting an originally ineffective protocol to an effective one. This persisted for at least 3 days (McGuire et al., 2003).

It appears that the CIH effect can be induced in all age groups. For example, CIH enhanced pLTF in geriatric female rats (Zabka et al., 2003). We observed that if 2-day old rats were treated with 1-week CIH, vLTF were still enhanced when they were 1 month old, but the CIH-induced enhancement was lost when they were 2 months old (McGuire and Ling, 2005). These data suggest that: 1) neonatal CIH also enhances LTF, 2) the CIH enhancing effect on LTF lasts even longer than that induced by usual (adult) CIH (~3 weeks vs. ~3 days), and 3) this LTF enhancement does not last to adulthood. The CIH effect on LTF also appears to saturate once certain levels of stimulation intensity have been reached. For example, we found that 1-week CIH induced similar enhancement of vLTF (additional ~10%), whether CIH was performed during nighttime or daytime (rats' sleep time), and whether 5-min/5-min, 2-min/2-min or 1-min/1-min intermittent hypoxia was used in the stimulation paradigm (McGuire and Ling, unpublished data).

The CIH treatment (15-s 5% O₂ with 5-min interval, 9 episodes/h, 8 h/day for 10 days), which mimics hypoxic episodes of sleep apnea, similarly enhanced the AIH-induced pLTF in anesthetized rats (Peng and Prabhakar, 2003). This CIH treatment also induced at least 2 unique forms of plasticity in carotid body function: 1) CIH enhances the carotid afferent response to short-term hypoxia, and 2) AIH elicits carotid sensory activity LTF in the CIH-treated rats but not in control rats (Prabhakar, 2001).

3.4. 5-HT receptors in the CIH effect

The effect of serotonin receptor antagonism on the CIH-enhanced pLTF was first examined in anesthetized rats (Ling et al., 2001). Pre-treatment with methysergide (5-HT_{1,2,5,6,7} antagonist) and ketanserin (5-HT₂ antagonist) reversed the CIH-induced augmentation of the short-term hypoxic phrenic response. Methysergide also totally eliminated the CIH-enhanced pLTF, but ketanserin only partially blocked it. These results suggest that the CIH effect on LTF is also serotonin dependent but may involve an upregulation of a non-5-HT₂ serotonin receptor subtype(s).

The effect of serotonin receptor antagonism on the CIH-enhanced vLTF was also examined in awake rats (McGuire et al., 2004). The effects of methysergide, ketanserin or clozapine (5-HT_{2,6,7} antagonist) on both vLTF and the CIH-enhanced vLTF were examined to determine which specific receptor subtype(s) is involved. Each drug was tested in 4 related rat groups (*i.e.*, control, CIH, control+drug, CIH+drug). We found the resting ventilation, metabolic rate and hypoxic ventilatory response (HVR) were all minimally changed (HVR was reduced by clozapine in some rats), but vLTF was eliminated by each of the drugs. The CIH-enhanced vLTF was completely abolished by methysergide and clozapine but was only attenuated by ketanserin. Collectively, these data suggest that vLTF requires 5-HT₂ receptors and that the CIH effect on vLTF requires non-5-HT₂ serotonin receptors, probably 5-HT₆ and/or 5-HT₇ subtype(s).

3.5. NMDA receptors in LTF

The effect of NMDA receptor antagonism on LTF was first examined in anesthetized rats (McGuire et al., 2005). The usual AIH stimulation protocol (3 × 5-min hypoxia) was used to elicit pLTF. We found that pre-treatment (systemic injection) of the NMDA receptor antagonist MK-801 eliminated pLTF, while having little effect on both the CO₂-apneic threshold and the hypoxic phrenic response. To more precisely locate these NMDA receptors, we microinjected MK-801 directly into the phrenic motoneuron region, which avoided possible drug diffusion problems caused by intrathecal injection and eliminated other confounding issues caused by systemic injection of MK-801. We found that the ipsilateral pLTF could no longer be elicited after the microinjection, while the hypoxic phrenic response was minimally affected (McGuire et al., 2005). These results suggest that activation of NMDA receptors on the phrenic motoneuron is required for the AIH-induced pLTF in anesthetized rats.

These results, however, cannot be automatically extended to vLTF, as the role of NMDA receptors in other major inspiratory motor output LTF, *e.g.*, intercostal and hypoglossal LTF, is unknown. It is also desirable to confirm the conclusion (LTF requires NMDA receptors) in an un-anesthetized preparation. Moreover, whether those NMDA receptors are required for induction or maintenance of LTF is unknown. In addition, although non-NMDA ionotropic glutamate receptors (*e.g.*, AMPA and kainate receptors) play important roles in many forms of neural plasticity (*e.g.*, LTP), the role of these non-NMDA receptors has not been explored previously in any form of respiratory LTF.

Thus the effects of NMDA or non-NMDA receptor antagonism on the formation and/or maintenance of vLTF were examined in awake rats (McGuire et al., 2008). vLTF, induced by

5 episodes of 5-min poikilocapnic hypoxia (10% O₂) with 5-min normoxia intervals, was measured with plethysmography. Vehicle or APV (a NMDA receptor antagonist) was systemically injected before, immediately after and 20 min after AIH in different groups. We found that the AIH elicited vLTF in all vehicle injected rats but failed to elicit vLTF in rats with APV injection before or immediately after the AIH. Baseline V_E and HVR were not significantly changed after the APV administration. When APV was injected 20 min after AIH, AIH elicited a normal LTF at 15 min post-AIH, but the individual LTF at 30 and 45 min points were significantly decreased or eliminated, indicating that even a fully developed vLTF can be eliminated by NMDA receptor antagonism. In contrast, systemic injection before AIH of the non-NMDA receptor antagonist CNQX even at very high doses did not block vLTF (vLTF was actually enhanced). On the other hand, baseline V_E was substantially reduced, indicating the drug efficacy. Taken together, these data suggest that activation of NMDA but not non-NMDA ionotropic glutamate receptors is required for both formation and maintenance of the AIH-induced vLTF in awake rats (McGuire et al., 2008).

The involvement of metabotropic glutamate receptors in some form of LTF has also been reported recently. In an *in vitro* brainstem slice preparation from the neonatal rat, episodic activation of 5-HT₂ receptors on hypoglossal motoneurons: 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 motoneurons, which could be blocked by antagonism of group-I metabotropic glutamate receptors (Bocchiaro and Feldman, 2004).

3.6. NMDA receptors in the CIH effect

The role of NMDA receptors in the CIH effect on vLTF was examined in awake rats in one of our early studies (McGuire et al., 2002b). The NMDA receptor antagonist used was MK-801. To avoid behavioral problems during ventilatory measurement, we used a 0.2 mg/kg drug dose. Although MK-801 at this dose did not completely block vLTF, the results obtained still showed clear effects of NMDA receptor antagonism (Fig. 2). vLTF, elicited by 5 episodes of 5-min poikilocapnic hypoxia (10% O₂) with 5-min normoxia intervals, was measured twice in each rat, before and after MK-801, CIH or CIH+MK801. In the first group, MK-801 decreased vLTF magnitude and duration. In the second group, CIH enhanced vLTF as usual. In the third group, MK-801 substantially decreased both the magnitude and duration of the “CIH-enhanced” vLTF. After combining the 3 control groups as one, a 2-way ANOVA revealed a significant interactive effect ($F_{1,32}=14.59$; $P=0.00058$) between the CIH factor and the drug factor in vLTF magnitude (Fig. 2). The reduction in magnitude after drug was also greater ($P<0.05$) in the CIH-enhanced vLTF [$14.7 \pm 2.3\%$; Column 4 (28.1%) vs. Column 6 (13.4%)] vs. vLTF *per se* [$8.7 \pm 0.6\%$; Columns 1 (18.1%) vs. Column 2 (9.3%)], suggesting that MK-801 has a direct action on the CIH effect on vLTF besides its action on vLTF *per se*. Using an antagonist (*e.g.*, APV) that can completely block vLTF or an anesthetized preparation (without behavioral problem) should generate more conclusive data.

4. Potential mechanism

LTF was thought to be a neuronal rather than a humoral mechanism from the beginning (Millhorn et al., 1980; Eldridge and Millhorn, 1986). The majority of LTF studies were conducted in anesthetized, vagotomized, paralyzed and mechanically ventilated animals, in which arterial blood gases and body temperature were carefully controlled, thus ruling out the possibility that LTF is secondary to changes in chemical (PaCO₂, PaO₂ and pH) and/or mechanical feedbacks. This notion was further supported by successful induction of neuronal LTF in more reduced preparations (Bocchiaro and Feldman, 2004; Peng et al., 2006; Neverova et al., 2007). The fact that CSN stimuli can elicit pLTF, which is not abolished by decerebration or spinal transection at C7-T1 level, suggests that pLTF requires neural mechanisms located

in the brainstem and/or the cervical spinal cord, and that the carotid body, respiratory mechanics, systemic hypoxia, the forebrain, and the lower spinal cord are not necessary for its basic expression (Eldridge and Millhorn, 1986). Recent studies further demonstrated that activation of serotonin receptors and synthesis of BDNF in the cervical spinal cord and activation of NMDA receptors in the phrenic motonucleus area are necessary for pLTF (Baker-Herman and Mitchell, 2002; Baker-Herman et al., 2004; McGuire et al., 2005).

4.1. A hypothetical model

We use pLTF to explain where and how the serotonin and NMDA mechanism are involved in the initiation, formation and/or maintenance of LTF, as the phrenic nerve innervates the major inspiratory muscle (diaphragm) and pLTF is the most studied form of LTF. Additionally, certain potential neuronal and cellular mechanisms underlying this pLTF have been well described in hypothetical models (Mitchell et al., 2001a; Feldman et al., 2003; Mahamed and Mitchell, 2007). Very briefly, the carotid chemoafferent inputs, activated by AIH or repeated CSN stimulation, excite the raphe nuclei. Released serotonin from the raphe serotonin neuron terminals activates 5-HT₂ receptors on the phrenic motoneuron. This initiates a series of intracellular signaling events, including an increase in protein kinase(s) activity, which phosphorylates the subunit(s) of the NMDA receptors and augments the inward NMDA-current, thereby leading to an elevated motoneuronal response to the descending inspiratory drive (*i.e.*, a pLTF).

The observation that NMDA receptor antagonism can eliminate both pLTF (McGuire et al., 2005) and vLTF (McGuire et al., 2008) suggests that NMDA receptors are also required for intercostal and hypoglossal LTF. If NMDA antagonism eliminates pLTF but not intercostal LTF, vLTF is unlikely to be totally eliminated, as the remaining intercostal muscle activity LTF, which increase respiratory efforts, would still contribute to ventilation. The remaining LTF of the hypoglossal nerve-innervated tongue muscles is also likely to contribute to overall ventilation by reducing upper airway resistance. Thus, the NMDA mechanism, like the serotonin one, is probably another common feature shared by the LTF of different major inspiratory motoneurons.

The results that serotonin receptor antagonism immediately after AIH had minimal effect on pLTF (Fuller et al., 2001), while NMDA receptor antagonism at a similar time eliminated vLTF (McGuire et al., 2008) suggests that activation of 5-HT₂ receptors initiates the intracellular process, whereas modification of NMDA receptors is the “end product” of this process. In other words, the formation and maintenance of LTF result mainly from phosphorylation of NMDA receptors on the associated motoneurons, whereas the gradual reduction with time and eventual disappearance of LTF result primarily from de-phosphorylation of those NMDA receptors. Thus, although both NMDA and non-NMDA receptors are present on the motoneurons of several major inspiratory muscles and mediate respiratory drive, only NMDA receptor activation is necessary for the formation and maintenance of the AIH-induced vLTF. On the other hand, based on the above discussion, we believe that activation of NMDA receptors is not required for LTF induction during the AIH period. However, to convincingly test this hypothesis, one would need to block NMDA receptors only during but not after AIH. APV and MK-801 are not suitable for such studies, as their effects on NMDA receptors persist much longer than the AIH duration (45 min).

This hypothetical model, however, cannot satisfactorily explain the respiratory frequency portion of LTF. Although LTF in anesthetized animals mainly manifests as an amplitude augmentation, many studies also show a frequency LTF (Bach and Mitchell, 1996). In awake animal studies, dogs and ducks mainly show a tidal volume LTF (Cao et al., 1992; Mitchell et al., 2001b), mice show a frequency LTF (Terada et al., 2008), while goats and most rats show a mixed LTF, with the frequency augmentation usually being the major contributor (Turner

and Mitchell, 1997; McGuire et al., 2008). Actually, one could argue that LTF occurs not only in motoneurons but also in many respiratory-related neurons located in the brainstem, such as the raphe serotonergic neurons, DRG/VRG premotor neurons, and the pre-Bötzinger pacemaker neurons (Morris et al., 2001; Blitz and Ramirez, 2002), which are the major determinants of respiratory rhythm. Numerous DRG/VRG neurons and pre-Bötzinger complex neurons (Blitz and Ramirez, 2002; Morgado-Valle and Feldman, 2007) express NMDA receptors and receive projection from the raphe serotonin neurons. The respiratory rhythm generated in the pre-Bötzinger complex can also be modulated by NMDA receptor antagonism (Morgado-Valle and Feldman, 2007). Therefore, it is conceivable that AIH can induce LTF in the medullary neural circuitry that generates respiratory rhythm, which in turn produces frequency LTF at motor neuron levels.

4.2. The CIH effect

How CIH enhances LTF is unknown. Where and how the serotonin and NMDA mechanisms are involved is also unclear. The CIH effect on LTF is unlikely a humoral mechanism, as most data were collected 8 h or even 3 days after CIH, and this CIH effect can be readily eliminated by serotonin receptor antagonism or attenuated by NMDA receptor antagonism. LTF *per se* and the CIH effect on LTF are similar in several aspects, *e.g.*, both are forms of intermittent hypoxia-induced respiratory plasticity, which are serotonin-dependent and constitute a central mechanism (carotid body may also play a role, *cf.* Prabhakar, 2001). However, they differ in at least 3 aspects: 1) elicitation protocol (AIH vs. CIH), 2) the role of 5-HT₂ receptors (required for LTF but not the CIH effect), and 3) their persistence (LTF lasting minutes/hours vs. the CIH effect lasting days). We speculate that CIH repeatedly activates the neural networks, leading to an activity-dependent up-regulation of serotonin receptors on (phrenic) motoneurons. These newly synthesized serotonin receptors (mainly of 5-HT₆ and/or 5-HT₇ subtype) increase the overall efficacy of serotonergic synaptic transmission. This leads to an additional increase in the BDNF synthesis, protein kinase(s) activity and the number of modified NMDA channels when LTF is subsequently induced, thus enhancing this LTF.

4.3. LTF vs. LTP

It is interesting to compare respiratory LTF with the well-known synaptic plasticity LTP. LTF and most LTP share some common features. For example, both are elicited by episodic but not sustained stimulation (Baker and Mitchell, 2000; *cf.* Kandel et al., 2000), and expressed as a long lasting enhanced efficacy of the key synapse, which can lead to behavioral changes. In addition, both require activation of protein kinase(s) and ionotropic glutamate receptors (*cf.* Kandel et al., 2000; Mitchell et al., 2001a; McGuire and Ling, 2004). However, they are different in at least 4 aspects. First, induction of LTP requires activation of NMDA receptors (Bliss and Collingridge, 1993; *cf.* Kandel et al., 2000), whereas induction of LTF probably does not require NMDA receptors but depends on repeated activation of 5-HT₂ receptors. Second, the maintenance of LTF requires NMDA receptors, whereas the maintenance of LTP does not require NMDA receptors but depends on non-NMDA receptors, despite both being present on the postsynaptic pyramidal neurons in the CA1 region in the case of the hippocampal LTP of the Schaffer collateral pathways (*cf.* Kandel et al., 2000). Third, LTP persists much longer in awake animals (days) vs. *in vitro* slice preparation (hours), whereas LTF lasts longer in anesthetized (hours) vs. awake animals (usually ≤60 min) (Bliss and Collingridge, 1993; Mitchell et al., 2001a; McGuire et al., 2008). Finally, LTP is a typical activity-dependent plasticity in conformity with Hebb's rule (Bliss and Collingridge, 1993). In contrast, LTF is not activity-dependent but modulatory input-dependent plasticity, which probably does not require the Hebbian mechanism at least at key synapses on the motoneuron (Zhang et al., 2003).

5. LTF and obstructive sleep apnea (OSA)

LTF was pursued early on mainly as a model for long-term respiratory plasticity; its physiological significance was unclear. However, accumulative evidence suggests that LTF may play a role in OSA syndrome. Intermittent hypoxia occurs under some pathophysiological conditions. For example, OSA is associated with recurrent pharyngeal airway collapse due to decreases in upper airway dilator muscle activity during sleep (*cf.* Bradford et al., 2005). OSA patients thus experience intermittent hypoxia throughout the night. Cao et al. (1992) reported the first LTF case in an awake animal and proposed that intermittent hypoxia-induced hyperexcitability of the respiratory control system might contribute to the onset of sleep apnea in OSA patients, thus implicating LTF in OSA as a potential pathophysiological factor. On the other hand, Bach and Mitchell (1996) postulated that LTF might be a beneficial factor for maintaining respiratory homeostasis during sleep, *e.g.*, LTF might increase upper airway muscles activity, thus stabilizing upper airway patency.

Although LTF of ventilation or genioglossal muscle activity was not elicited in normal humans during wakefulness (Jordan et al., 2002), it was elicited during NREM sleep (Chowdhuri et al., 2008). LTF was also elicited in individuals with inspiratory flow limitation (*e.g.*, snorers and OSA patients) during NREM sleep (Aboubakr et al., 2001), mainly as a persistent decrease in upper airway resistance, an indication of upper airway dilatation due to dilator muscles LTF. Furthermore, our preliminary data showed that in anesthetized rats, the usual AIH protocol induced a progressive decrease in pharyngeal airway collapsibility, which inversely correlated with genioglossal activity LTF (McGuire and Ling, 2006b). Collectively, these data suggest that LTF might be an adaptive mechanism, helping stabilize upper airway patency in OSA patients after repeated exposure to apneas and/or hypopneas.

Although AIH induces LTF, and (1-week) CIH enhances the AIH-induced LTF and the hypoxic chemoresponsiveness (Ling et al., 2001; McGuire et al., 2003), more extensive (4–5 weeks) CIH impairs pharyngeal dilator responses to hypoxia/asphyxia (Bradford et al., 2005) and perhaps LTF as well (*cf.* Reeves et al., 2006). It was also reported that 3-week CIH reduced hypoglossal nerve responsiveness to microinjections of serotonin or NMDA into the hypoglossal motonucleus region (Veasey et al., 2004). In addition, OSA patients in the early stages of the disease tend to have an augmented HVR, while those with a long OSA history usually have depressed HVR.

How can one reconcile those obviously contradictory observations? We believe that exposure to intermittent hypoxia can be both beneficial and pathological, depending on which physiological function is evaluated and what paradigm (*e.g.*, severity of hypoxia, length of hypoxic/interval episodes and the duration of the whole treatment) is applied. Intermittent hypoxia protocols with severe hypoxia usually cause detrimental effects. For example, 5 episodes of 5-min 10% O₂ successfully induced vLTF, but 5 episodes of 5-min 8% O₂ failed to induce vLTF (McGuire et al., 2002a) (10 episodes even induced ventilatory long-term depression). The overall duration of intermittent hypoxia is also an important factor. For example, 1-week CIH enhances the AIH-induced LTF and hypoxic chemoresponsiveness, whereas 4–5 weeks of CIH impair them (Bradford et al., 2005; *cf.* Reeves et al., 2006). Thus intermittent hypoxia can be classified into at least 3 categories: AIH, 1-week CIH and ~1-month CIH, which have been shown to cause distinct, sometimes even opposite, effects on respiratory control mechanisms. We thus speculate that LTF and proper CIH may be among the early defensive and/or compensatory mechanisms against OSA pathogenesis/progression, but their beneficial roles are probably relevant only in the early stages of OSA.

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Fig. 1.

Different expression pattern of respiratory long-term facilitation (LTF) induced by acute intermittent hypoxia in anesthetized and awake rats. **A:** Integrated phrenic neurogram recorded in one anesthetized rat, before (baseline), during (hypoxia) and up to 60 min after 3 episodes of 5-min isocapnic 12% O₂, separated by 5-min intervals of 50% O₂. The phrenic responses to hypercapnia (CO₂) are also presented but only one hypoxic episode data are presented. **B:** Group data of changes from baseline in phrenic burst frequency (f), peak phrenic amplitude (Phr) and minute phrenic activity (f × Phr). LTF at each time point is defined as an increase from baseline in post-hypoxia value at that specific time point, normalized as a percentage of the baseline. **C:** Minute ventilation (V_E) measured by plethysmography in one group of awake rats, before (baseline, BL), during (hypoxia) and up to 90 min after 5 episodes of 5-min

poikilocapnic 10% O₂ (black bars), separated by 5-min intervals of normoxia (white bars). The V_E data, expressed as means $\pm$ SE in the right panel, are the same as those in the left panel excluding the hypoxic data. * indicates a significant difference from baseline ($P<0.05$).

Fig. 2.

The effect of NMDA receptor antagonist MK-801 (0.2 mg/kg, i.p.) on ventilatory long-term facilitation (vLTF) and its enhancement after chronic intermittent hypoxia (CIH) in awake rats. vLTF is numerically expressed as a magnitude and duration. The vLTF magnitude is calculated by averaging the first 3 individual LTF values at 15, 30 and 45 min post-hypoxia by using the equation: $100 \times [(V_{E15} + V_{E30} + V_{E45})/3 - \text{baseline}]/\text{baseline}$. The time (min) within each column represents the LTF duration, defined by the last post-hypoxia time point, at which V_E is significantly higher than baseline (see Fig. 1B). In all 3 groups (each $n=6$), LTF was measured twice in the same rats, before (as control, C) and after MK-801 (C+M), CIH or a combination of CIH and MK-801 (CIH+M). Data are means $\pm$ SE. * indicates significant differences from baseline; † indicates significant differences from all other groups ($P<0.05$).