Adaptation to Hypobaric Hypoxia Involves GABA<sub>A</sub> Receptors in the Pons

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Abbreviated title: GABA<sub>A</sub> receptors participate in hypoxic adaptation
Number of pages: 28
Number of figures: 7
Keywords: hypoxia, GABA<sub>A</sub> receptor, plasticity, pons, adaptation, sympato-respiratory network

Acknowledgements:
This work was supported by grants from the National Institutes of Health HL25830, HL080318 and NS34317. We gratefully acknowledge the technical assistance of Ning Wang, Rishi Dingra and Mallika Padival and critical review of the manuscript by Drs. Jeffery Tatro and Martin Snider.
Survival in low oxygen environments requires adaptation of sympatho-respiratory control networks located in the brainstem. The molecular mechanisms underlying adaptation are unclear. In naïve animals, acute hypoxia evokes increases in phrenic (respiratory) and splanchnic (sympathetic) nerve activities that persist after repeated challenges (long-term facilitation, LTF). In contrast, our studies show that conditioning rats to chronic hypobaric hypoxia (CHH), an environment characteristic of living at high altitude, diminishes the response to hypoxia and attenuates LTF in a time dependent manner. Phrenic LTF decreases following 7 d of CHH, and both sympathetic and phrenic LTF disappear following 14 d of CHH. Previous studies demonstrated that γ-aminobutyric acid (GABA) is released in the brainstem during hypoxia and depresses respiratory activity. Furthermore, the sensitivity of brainstem neurons to GABA is increased following prolonged hypoxia. In this study we demonstrate that GABA<sub>A</sub> receptor expression changes along with the CHH-induced physiologic changes. Expression of the GABA<sub>A</sub> receptor α4 subunit mRNA increases 2 fold in animals conditioned to CHH for 7 d. In addition, de novo expression of δ and α6, a subunit normally found exclusively in the cerebellum, is observed after 14 d. Consistent with these changes, diazepam-insensitive binding sites, characteristic of GABA<sub>A</sub> receptors containing α4 and α6 subunits, increase in the pons. Immunohistochemistry revealed that CHH-induced GABA<sub>A</sub> receptor subunit expression is localized in regions of sympatho-respiratory control within the pons. Our findings suggest that a GABA<sub>A</sub> receptor mediated-mechanism participates in adaptation of the sympatho-respiratory system to hypobaric hypoxia.
INTRODUCTION

Neural networks that control homeostasis are plastic. Adaptation to reduced oxygen at high altitude requires days-to-weeks of continuous exposure (Weil, 1994; Beall et al., 2002; Lahiri et al., 2002; Ramirez et al., 2006, 2007). This adaptation involves both peripheral and central control mechanisms that maintain homeostasis. Central sympatho-respiratory control networks express short-term plasticities elicited by brief (45 s) hypoxic challenges. These plasticities result in different sympatho-respiratory motor output after challenges are terminated. Two types of behaviors are evoked depending on the pattern of acute hypoxic challenges. A single challenge elicits a transient (min) decrease in respiratory frequency termed Post-Hypoxic Frequency Decline (PHFD) and increases respiratory effects on sympathetic nerve activity (Coles and Dick, 1996; Dick et al., 2004). Multiple challenges elicit a sustained (h) increase in sympatho-respiratory motor output termed Long-Term Facilitation (LTF) (Baker and Mitchell, 2000; Ilyinsky et al., 2003; Dick et al., 2006). Exposure to chronic hypoxia (days) attenuates PHFD, whereas its effect on LTF is unknown (Ilyinsky et al., 2003; Hsieh et al., 2004).

Prolonged hypoxic exposures also increase tyrosine hydroxylase expression in brainstem catecholaminergic cells (Schmitt et al., 1994; Dumas et al., 1996; Roux et al., 2000; Pascual et al., 2001). Although these changes in sympatho-respiratory motor patterns are mediated by brainstem nuclei, the neurotransmitters involved and the interplay among these nuclei are not fully identified.

The pons, a brainstem region, contains nuclei that participate in the expression of PHFD and LTF. We previously demonstrated that cells in the ventrolateral (vl) pons mediate PHFD. Disrupting activity in this region blocked PHFD without affecting the
respiratory response during acute hypoxia (Coles and Dick, 1996; Jodkowski et al., 1997). Furthermore, stimulating the A5 neuronal population increased splanchnic sympathetic nerve discharge (Huangfu et al., 1992). Cells in the ventromedial pons (caudal raphé) project to sympatho-respiratory motor nuclei and mediate LTF (Veasey et al., 1995; Morris et al., 2000; Morris et al., 2003). The contribution of these pontine nuclei to the adaptation to chronic hypobaric hypoxia (CHH) has not been investigated.

Many findings suggest that the response and adaptation to hypoxia depend on the GABAergic system. During hypoxia, GABA is released in brainstem regions involved in the control of homeostasis (Richter et al., 1999). Furthermore, bilateral microinjections of a GABA\textsubscript{A} receptor agonist, muscimol, in the vl pons blocks PHFD (Coles and Dick, 1996). In contrast, injection of a GABA\textsubscript{A} receptor antagonist, bicuculline, alters the hypoxic response (Hsieh et al., 2004). Finally, we previously found that the mRNAs encoding the GABA\textsubscript{A} receptor \(\alpha6\) and \(\delta\) subunits are expressed \textit{de novo} and exclusively in the pons after two weeks of CHH (Hsieh et al., 2004).

To delineate the role of GABAergic system in the physiologic adaptation to CHH, we compared the temporal relationship between changes in activity-dependent plasticity (LTF) and GABA\textsubscript{A} receptor expression. We report that CHH-elicited attenuation of LTF is associated with induction of the expression of select GABA\textsubscript{A} receptor subunit mRNAs in pontine nuclei involved in sympatho-respiratory control. These changes include an increase in the \(\alpha4\) subunit and \textit{de novo} expression of the \(\alpha6\) and \(\delta\) subunits. The upregulation of subunit expression is associated with an increase in GABA\textsubscript{A} receptor number in the pons. In addition, the \(\alpha6\) subunit polypeptide was expressed only by cells
in the raphé and vl pons. These findings suggest that GABA<sub>A</sub> receptor plasticity contributes to adaptation to hypobaric hypoxia.

**Materials and Methods**

**Animals**

Adult male Sprague-Dawley (Zivic Miller, Zelienople, Pa) rats (280-320 g) were maintained in hypoxic (n = 56), normoxic conditions (n = 12), or left in naïve state (n = 12). The hypoxic group was placed in a hypobaric chamber simulating hypoxia at high altitudes (0.5 atm) for 3, 7 or 14 d. Normoxic animals were placed in the chamber without hypoxic exposure for similar times. Animals were placed on a circadian light/dark cycle, had free access to food and water, and were inspected daily. The hypobaric chamber was opened 2-3 times a week to clean cages and to replenish food and water; during this period of about 15 min, the animals were at the normal barometric pressure (approximately 1 atm). Naïve animals were never placed in the chamber.

**Electrophysiology**

The hypoxic response (HR) and long term facilitation (LTF) in the anesthetized *in vivo* rodent preparation were assessed as previously described (Coles and Dick, 1996; Hsieh et al., 2004). All surgical and experimental protocols followed NIH guidelines and were approved by the Institutional Animal Care and Use Committee. In brief, immediately after hypobaric hypoxic conditioning, animals were anesthetized with equithesin (30 and 133 mg/kg sodium pentobarbital and chloral hydrate, respectively) and anesthetic level was evaluated by assessing the withdrawal reflex before neuromuscular blockade and by assessing the cardiorespiratory response after neuromuscular blockade.
(pancuronium bromide -0.1 mg / 100 g of body weight). If responses were evoked by
nociceptive stimuli, then anesthesia was supplemented by intravenously administering a
ten-th of the initial dose. Neuromuscular blockade was supplemented hourly also by
intravenously administering a tenth of the initial dose. The femoral artery and vein were
cannulated to monitor blood pressure and administer pharmacologic agents, and the
trachea was cannulated to ventilate the animal. The cervical vagi were isolated and
transected bilaterally to prevent afferent feedback. The left phrenic and splanchnic
sympathetic nerves were isolated, transected, and mounted on bipolar electrodes for
recording. Animals were ventilated with 100% O₂ before and after the hypoxic challenge.
Elevation in splanchnic sympathetic and respiratory activities was increased by exposure
to ten hypoxic challenges (8% O₂ for 45 s with a 5 min recovery period). The exposures
were poikilocapnic and end-tidal PCO₂ decreased less than 2 mm Hg during this brief
hypoxic exposure.

Quantitative analysis of respiratory and sympathetic responses to hypoxia

Breathing pattern was measured by assessing phrenic nerve activity (PNA). Onset
of PNA was the point on the positive slope of the integrated PNA signal that was 10%
above the inter-burst value. Offset of PNA was identified on the negative slope of the
signal that was 25% below the peak value. The end of the cycle was identified as the
onset of the next breath. The peak activity of PNA was averaged for 10 cycles
immediately before starting the 10 cycles of intermittent hypoxia, in the 5-min recovery
period between the 9th and 10th acute hypoxic stimuli and 1 h after the 10th acute hypoxic
stimulus.
Quantitative analysis of splanchnic sympathetic nerve activity (sSNA) was based on cycle-triggered averages (CTA) constructed to compare the coupling patterns between PNA and sSNA. Averaging increased the signal-to-noise ratio of sSNA that was time-locked to the respiratory cycle (Dick et al., 2004). For averaging, the analog signal of sSNA was rectified, integrated (CWE, Inc., Wood Dale, IL; Paynter Filter, 50-ms time constant), sampled at 200 Hz and summed (National Instruments, Analog-to-Digital board, Austin, TX). The reference point (time zero) for CTAs was the phase transition between inspiration (I) and expiration (E).

The average amplitude under the curves was calculated for PNA and sSNA. The inspiratory and expiratory portions of the sSNA cycle-triggered averages were divided in half thereby allowing direct comparison of the magnitude of sSNA. The significance of observed differences in values proceeding and following repeated acute hypoxic exposures were determined by 2-way ANOVA for repeated measures. The factors for the two-way repeated measures ANOVA were: 1) subject (the animal), 2) days of conditioning, and 3) time point (before the acute hypoxic exposure, 5 min between the 9th and 10th exposures, and 60 min after the 10th acute hypoxic exposure). The variables, area of integrated PNA, and average amplitude of integrated sSNA in the first and second halves of inspiration and of expiration were ranked transformed to pass the normality test. Significant differences within variables were identified by the Student-Newman-Keuls test. To identify significant correlations among sSNA and PNA, linear regression analysis during the first and second halves of each phase were performed and values of \( p \leq 0.05 \) were taken as significant. Data are presented as means ± SEM as indicated.

*RNA isolation and RT-PCR*
Relative levels of GABA<sub>A</sub> receptor subunit mRNAs in normoxic and hypoxia-exposed animals were determined using a semi-quantitative RT-PCR protocol essentially as previously described (Beattie and Siegel, 1993; Behringer et al., 1996; Rieff et al., 1999). In brief, the pons, medulla, thalamus, and cerebellum were dissected from brains of euthanized animals after the indicated exposure to CHH (naïve rats were housed in the chamber with airflow but not hypobaric pressure). Pontine tissue was dissected using the following landmarks: rostral - caudal to the inferior colliculi (dorsal) and rostral to the pontine gray (ventral); caudal - adjacent to origins of cranial nerves V, VI, VII and VIII (the inferior border of the pontine prominence). RNA was extracted from all samples using Trizol™ according to manufacturer’s (Invitrogen, Carlsbad, CA) protocol, reverse transcribed, and processed for PCR using GABA<sub>A</sub> receptor subunit-specific primers in buffer containing [α-<sup>32</sup>P] dCTP. The expression of GAPDH and 18S RNAs were also quantified as internal controls. All experiments were performed at least six times using tissue prepared from different animals.

The PCR products were separated on 8% nondenaturing polyacrylamide gels, which were dried and detected with a Molecular Dynamics Phosphorimager. The band intensities were quantified using ImageQuant Software (Molecular Dynamics, Sunnyvale, CA). To compare mRNA expression in the different experimental conditions, the intensities of the receptor subunit mRNAs were normalized to the intensity of either the GAPDH or 18S bands in the samples. Significant differences in receptor subunit mRNA levels in naïve vs. hypoxia-conditioned animals were assessed by applying a 2-way Analysis of Variance (ANOVA) (Sigma Stat 2.03). Specific differences were then identified using Student-Newman-Keuls Post-Hoc Test. Primer sequences are shown in
the following Table.

<table>
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<td>GTTTTCCTCCAGGAGGCATGT</td>
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**Ligand binding assay**

Membranes from the pons, medulla, thalamus, and cerebellum of hypoxia-conditioned and normoxic rats were harvested and binding assays were performed using the benzodiazepine partial inverse agonist [³H]Ro-15-4513 (Perkin-Elmer, Waltham, MA), a ligand that recognizes diazepam-insensitive sites characteristic of GABA<sub>A</sub> receptors containing α6, α4, and ε subunits. Naïve and CHH-conditioned rats were euthanized by exposure to hypercapnic hypoxia. Protein extraction was performed as
previously described (Zhao et al., 2003). Samples (50-300 µg of protein) were incubated with saturating concentrations of 10 µM diazepam and the indicated concentrations of \(^{3}\text{H}\)Ro15-4513 for 1 h at 4°C and harvested on nitrocellulose membranes using 50mM Tris HCl, pH 6.9 and counted. Graphpad Prism (3.0) software was used to fit the data and to a one site binding hyperbola. The data represent values obtained from 3 assays in 5 different animals from each hypoxic exposure period.

**Immunohistochemistry**

For immunostaining, control and hypoxia-exposed rats were euthanized and immediately perfused via cannulation through the left ventricle with 100 ml heparinized (20 units ml\(^{-1}\)) 10 mM saline followed by 200 ml 4% paraformaldehyde in 0.1 M phosphate buffer (PBS), pH 7.4. The brains were removed, postfixed at 4°C overnight, transferred to 30% sucrose in PBS at 4°C, and then stored at -80°C until use.

Coronal sections (15 µm) were cut through the entire brainstem and mounted on poly-L-lysine slides. The sections were permeabilized in PBS containing 0.3% Triton X-100 and 5% bovine serum albumin for 1 h. Sections were then incubated in dilution buffer containing the antibodies against the α6 subunit at 1:200 (Chemicon) and tryptophan hydroxylase at 1:1000 (Chemicon) for 3 h at room temperature. Western blots demonstrated that the α6 antibody recognizes a single band of the appropriate molecular weight in the cerebellum of naïve rats, supporting its specificity. After incubation, the sections were rinsed three times in PBS and incubated with species-specific secondary antibodies at 1:500 (Jackson Immunoresearch) for 1.5 h at room temperature. After washing three times for 10 min in PBS, sections were mounted and examined with a Nikon FX microscope. Background staining was determined by incubating adjacent
sections with secondary antibody alone. Images were collected from experimental and control samples prepared from at least 4 different animals.

RESULTS

CHH induces alterations in the sympatho-respiratory response to acute hypoxia

Exposure to brief hypoxic stimuli elicited changes in sympatho-respiratory activity. In naïve rats, repeated acute hypoxic challenges (n = 10) augmented both sSNA and PNA during the stimulus period (Fig. 1A, compare left to middle panel). The elevation in sSNA and PNA remained 1 h after cessation of the hypoxic challenges, indicative of LTF (Fig. 1A, right panel).

Conditioning to CHH altered the sympatho-respiratory response to acute hypoxic challenges in a time-dependent manner. After 3 d of conditioning, acute hypoxia increased sSNA as in naïve animals (Fig. 1B, middle panel); in contrast, PNA did not increase. Both motor outputs, however, augmented progressively following cessation of the hypoxic challenges (Fig. 1B, right panel) and thus exhibited LTF. In contrast, following 7 d of CHH, sSNA and PNA were affected differentially by acute hypoxic stimuli; LTF persisted in sSNA, but was absent in PNA (Fig. 1C). After 14 d of CHH, the repetitive acute hypoxic stimuli failed to augment either sSNA or PNA, and as a result, LTF of both activities (evident 1 h after cessation of acute hypoxic challenges) was not evoked (Fig. 1D).

Sympathetic and respiratory hypoxic responses were quantified by examining the effects of CHH on the average amplitude of integrated sympathetic activity and the peak amplitude of integrated phrenic nerve activity. Analysis of sSNA was based on the
Hsieh et al. respiratory pattern. In naïve animals, the amplitude of sSNA was elevated most notably during the first half of expiration (sE-1), and this increase persisted 1 h after cessation of repetitive acute hypoxic challenges (Fig. 2A, top panel). The rise in sSNA during sE-1 roughly paralleled increased PNA (Fig. 2B, top panel). After 3 d of CHH conditioning, sympathetic activity increased uniformly across the respiratory cycle and exhibited an enhanced LTF (Fig. 2A, second panel). In contrast to the individual animal shown in Fig. 1, group data showed that PNA did increase immediately after the acute hypoxic challenges after 3-d of CHH conditioning. Similar to the individual animal’s tracing, group data showed robust LTF after 1 h of recovery (Fig. 2B, second panel). After conditioning for 7 d, sSNA was recruited primarily in the second half of inspiration (sI-2) during hypoxic challenge (Fig. 2A, third panel). Further, expression of LTF within PNA was abrogated completely (Fig. 2B, third panel). After 14 d of CHH, acute hypoxic challenges failed to elicit increases in sympathetic and phrenic activities, either during the challenges or after 1 h of recovery (Fig. 2A&B bottom panels), at which time LTF was clearly evident in naïve rats. This analysis clearly demonstrates that CHH conditioning differentially affects the temporal changes in the patterns of sSNA and PNA. In addition, 14 d of CHH conditioning was required to abolish LTF completely in both classes of motor activity.

Altered pontine GABA<sub>A</sub> receptor subunit expression accompanies CHH-induced changes in the sympatho-respiratory response

To determine if GABA<sub>A</sub> receptor expression in the brainstem could contribute to the CHH-induced plasticity of sympatho-respiratory responses, we measured receptor subunit mRNAs in regions involved in their control. GAPDH mRNA expression in the
brainstem tissue remained consistent throughout the hypoxic period. Within 3 d of CHH, and parallel with the onset of the delay of LTF in PNA, the level mRNA expression for the GABA\textsubscript{A} receptor \(\alpha4\) subunit rose 2-fold in the pons; its level remained elevated throughout the experimental period (Fig. 3A). Following 14 d of CHH, and coinciding with attenuation of the sympathetic response to hypoxia (Figs. 1&2), robust de novo expression of the mRNAs encoding the \(\alpha6\) and \(\delta\) subunits in the pons; neither subunits were detectable in naïve or normoxic (not shown) animals. In contrast, expression of the \(\beta2\) subunit mRNA, which is abundant in this brain region, did not increase either during or after CHH. In addition, CHH fail to induce the expression of the \(\varepsilon\) subunit, a subunit that confers properties similar to those of the \(\alpha4\) and \(\alpha6\) subunits. These observed changes in GABA\textsubscript{A} receptor subunit mRNA were not observed in the medulla (Fig. 3B).

To show that elevated expression of subunit mRNAs was associated with increased GABA\textsubscript{A} receptor number, receptor levels were measured by radioligand binding. Because the \(\alpha4\) and \(\alpha6\) subunits contribute to receptors that are insensitive to diazepam, binding was assessed with \(^{3}\text{H}\) Ro 15-4513, a ligand selective for these sites. These studies demonstrated that the diazepam-insensitive sites rose progressively within the pons during CHH. \(^{3}\text{H}\)Ro 15-4513 binding was unaltered after 3 d of hypoxia but then increased by 250\% and 500\% over the control level after 7 and 14 d of CHH (Fig. 4A). The K\text{d} of Ro 15-4513 binding was the same in animals exposed to all periods of CHH (Fig. 4B & Table 4.1), indicating that receptor affinity is unaltered.
The CHH-induced increase in receptor concentration was restricted to the pons. No changes in diazepam-insensitive binding were seen in the medulla, in agreement with the lack of detectable changes in subunit mRNA expression in this region (data not shown). In addition, CHH did not alter receptor binding in the cerebellum, the only brain region in which the α6 subunit is expressed in naïve animals. The concentrations of diazepam-sensitive receptors in the pons, medulla and cerebellum were likewise unaffected by CHH (data not shown), underscoring the selectivity of the CHH-induced elevations of pontine Ro 15-4513 binding.

Finding GABA<sub>A</sub> receptor α6 subunit mRNA expression in the pons is novel and surprising. To localize expression of this subunit, α6 polypeptide distribution was examined immunohistochemically. As expected, α6 subunit staining was not detected in the pons of naïve animals (Fig. 5B) or in animals exposed 3 and 7 d to CHH (data not shown). In contrast, subunit-expressing cells were found after 14 d CHH when the subunit mRNA was first detected (Fig. 3). At this time, α6-subunit positive cells were found in an area in the vl pons containing A5 adrenergic neurons (Fig. 5C&D), cells involved in sympatho-respiratory control. In contrast, staining was absent from the pontine reticular formation and the medulla (not shown). As expected, α6 subunit expression in cerebellar granule neurons was unaltered by CHH (Fig. 5E&F).

Additional studies demonstrated that the α6 subunit was expressed in ventromedial pons. Following 14 d of CHH, positive cells were detected in the raphé pallidus (RPa, Fig. 6A). The staining was colocalized with serotonergic cells containing tryptophan hydroxylase. Previous studies have shown that this region contains elements of the respiratory neural control network. Together, these findings suggest that GABA<sub>A</sub>
receptors containing the α6 subunit participate in reshaping LTF in response to repetitive hypoxic challenges.

DISCUSSION

The association of de novo GABA_A receptor subunit expression and the absence of plasticity in the motor activity is consistent with the GABAergic system partially mediating the physiologic adaptation of adult rats to CHH. These studies demonstrate that the sympatho-respiratory plasticity evoked by acute intermittent hypoxia depends on the number of days of conditioning in hypobaric hypoxia. Concurrent with these physiologic changes, GABA_A receptor expression is upregulated in pontine nuclei that participate in sympatho-respiratory control.

Our physiological results reveal that CHH attenuates sympatho-respiratory LTF in a time-dependent manner. The loss of respiratory LTF (PNA) occurred progressively. Acute hypoxic challenges triggered an increases in PNA in naïve and 3-d CHH-conditioned animals, however the onset of this change could be delayed in animals exposed 3 d to CHH (comparing Figs 1 and 2). By 7 d, respiratory LTF was abolished, a change that remained at 14 d. In contrast, the change in sympathetic LTF was much slower, with no attenuation detected until 14 d of CHH. These temporal disparities in the effects of CHH suggest that sympathetic and respiratory LTF are differentially regulated.

In support of the role of GABAergic system in the adaptation to CHH, our studies demonstrate that expression of a subset of the GABA_A receptor subunits is upregulated or induced in pontine nuclei by this stress. Our findings suggest that GABA_A receptors containing the α4, α6, and δ subunits are critical for adaptation due to their
temporal and spatial patterns of expression. At 3 d, the increase in $\alpha_4$ subunit mRNA expression coincides with the decrease in respiratory LTF. By 14 d, the *de novo* expression of the $\alpha_6$ and $\delta$ subunits mRNAs in the pons coincides with the absence of activity-evoked plasticities. Cells expressing the $\alpha_6$ polypeptide appeared exclusively in the raphé and vl pons, regions of sympatho-respiratory control. This finding is novel because the $\alpha_6$ subunit is restricted to cerebellar granule neurons in naïve adult rodents. Finally, it is striking that no changes in subunit expression were observed in the medulla, the region containing the central respiratory pattern generator.

In agreement with changes in GABA$_A$ receptor subunit expression, GABA$_A$ receptor number was upregulated in the pons. Most importantly, the number of diazepam-insensitive GABA$_A$ receptors increased. This is consistent with the fact that the $\alpha_4$ and $\alpha_6$ subunits confer diazepam insensitivity. It is of interest that expression of the $\alpha_6$ and $\delta$ subunits was induced simultaneously. These two subunits have been shown to coexist in one GABA$_A$ receptor type (Jones et al., 1997; Nusser et al., 1999). The fact that the number of diazepam-sensitive sites did not change suggests that CHH induces the expression of a select type of GABA$_A$ receptor.

Both our physiologic and molecular findings implicate the importance of cells in the vl pons and raphé in mediating adaptation to CHH. Moreover, along with the anatomical and physiologic findings of other investigators (Stamp and Semba, 1995; Morris et al., 2003; Cao et al., 2006), our results support the importance of GABA and GABA$_A$ receptors in these regions for adaptation. In previous physiologic studies we demonstrated that bilaterally lesioning the vl pons and/or inhibiting activity in this region with injections of the GABA$_A$ agonist, muscimol, block PHFD (Coles and Dick, 1996)
and attenuate the sympathetic response to hypoxia (Koshiya and Guyenet, 1994a, b). Conversely, stimulating the vl pons or blocking the GABA<sub>A</sub> receptor with the antagonist, bicuculline, increased the duration of expiration during hypoxia (Hsieh et al., 2004). Finally, recordings of vl pontine activity identified neurons that are activated during hypoxia and remain activated when the hypoxic stimulus is terminated (Dick and Coles, 2000).

It is surprising that a prolonged period (14 d) of CHH was required to induce α6 and δ subunit expression. We speculate that the delay in the expression of these subunits depends on the vital contribution of the peripheral mechanisms, such as the vascularization of carotid body and changes in circulating cerebrospinal fluid bicarbonate (Powell et al., 1998). Furthermore, the delay may reflect the complex interactions between neurotransmitters (glycine, nitric oxide, glutamate, substance P, and GABA) that mediate the hypoxic response (Bianchi et al., 1995; Bonham, 1995; Richter et al., 1997; Burton and Kazemi, 2000).

In conjunction with the findings of other investigators, our findings raise the possibility that the GABAergic and serotinergic systems interact in the raphé to regulate LTF. Previous studies have shown that LTF is dependent on serotonin receptor activation and that systemic or intrathecal methysergide, a receptor antagonist, block LTF (Morris et al., 2000; Fuller et al., 2001; Dick, 2003; Dick et al., 2007). Moreover, the caudal raphé is the sole source of serotonin in the spinal cord, and serotinergic neurons in the raphé magnus project directly to the phrenic motor nucleus. Recent work has shown that the serotinergic and GABAergic systems interact in the raphé (Cao et al., 2006), a possibility supported by our immunohistochemical findings. It is possible that the signaling in the
raphé provides a central, rather than peripheral, control of sympatho-respiratory LTF motor activity. Our findings suggest that increased GABA$_A$ receptors in CHH animals decrease phrenic and sympathetic LTF by inhibiting activity in the caudal raphé.

Based on these findings, we present a model of the pathways involved in regulating LTF in naïve and CHH-conditioned animals (Fig. 7). In this model, pontine GABA$_A$ receptors play a key role in mediating the plasticity manifested by the sympatho-respiratory hypoxic response. In naïve animals, hypoxic conditions detected by the carotid body cause signaling to the nuclei of the solitary tract and this is relayed to both the pons and ventrolateral medulla. These sensory and initial transmission phases of the response to hypoxia are mediated by glutamate (Richter et al., 1999; Jin et al., 2004). The sensory input returns to baseline following acute hypoxic challenges and ceases to drive respiratory and sympathetic activities. In contrast, pontine neurons in the raphé and A5 areas that are activated during hypoxia remain active after the challenge and mediate the expression of LTF (Fig. 7A, solid arrows). The extent of raphé and A5 activation is regulated by GABA (Fig. 7A, solid broken arrow). Consequently, during long hypoxic exposures, GABA is released after the initial response and acts to decrease ventilation partially by inhibiting these modulatory nuclei.

In contrast, exposure to CHH leads to upregulation of the expression of select GABA$_A$ receptor subunits within the raphé and A5 areas. This may be due to either a prolonged release of GABA or the feedback inhibition to the excitatory glutameric input from the nTS. In any case, the change in GABA$_A$ receptor subunit expression alters the interplay between the GABAergic, serotonergic (raphé), and adrenergic (A5) neurons within this region. Thus, while these pontine nuclei excite the sympatho-respiratory
system following acute hypoxia in naïve animals, the enhanced GABA-mediated inhibition of these pontine subnuclei in animals exposed to CHH precludes the activation of PNA and sSNA, thus preventing LTF.

The model predicts that blocking expression of select GABA_A receptor subunits within the pons prevents adaptation to CHH. Our study raises the possibility that the observed plasticity of GABA_A receptor subunit expression within the pons represents a molecular basis of the neural mechanism for CHH-induced attenuation of both sympathetic and respiratory activities (Fig. 7B), an issue to be addressed in future studies using subunit-deficient animals. Furthermore, it is possible that with a return to normoxia, GABA_A receptor subunit will be downregulated and the hypoxic response will return to that found in naïve animals. In the proposed model, the de novo expression of GABA_A receptor subunits diminishes activation of neurons within the raphé and A5 area. We view this as an extension of the GABA-gain hypothesis, wherein both the neurophysiologic properties and the plasticity of respiratory bursts is attenuated by increases in GABA-induced activity (Zuperku and McCrimmon, 2002). Finally, we speculate that in pathophysiologic states, including heart failure and obstructive sleep apnea, the accompanying sympatho-respiratory dysfunction may be due in part to concomitant alterations in the expression of GABA_A receptor subtypes within the pons.

PERSPECTIVES AND SIGNIFICANCE

Our studies provide new insights into the neural mechanisms mediating the plasticity of the sympatho-respiratory neural network. Using both molecular and physiologic approaches, we demonstrate that CHH exposure selectively alters GABA_A
receptor subunit expression in the pons and attenuates sympato-respiratory LTF. Based on these findings, we present a conceptual model that defines the role of GABA\(_A\) receptor in the blunted LTF found in CHH exposed animals. Future studies need to identify the relationship between LTF and the expression of specific GABA\(_A\) receptor subunits in the raphé, a ponto-medullary regions known to evoke LTF. For example, does blocking expression of specific GABA\(_A\) receptor subunits rescue LTF and re-establish sympato-respiratory plasticity in CHH conditioned animals? Furthermore, in CHH conditioned animals, does a return to normoxia restore LTF and plasticity in the sympato-respiratory network? If so, is this due to the downregulation of GABA\(_A\) receptor subunits? Answers to these questions will provide further information concerning the role of GABA\(_A\) receptor plasticity in mediating adaptation of the sympato-respiratory system to chronic hypoxia.
References


Figure 1
Conditioning with chronic hypobaric hypoxia (CHH) reshaped the sympatho-respiratory response to acute hypoxia in a time-dependent manner. A-D) Representative tracings of integrated splanchnic sympathetic (sSNA) and phrenic nerve (PNA) activities are shown before (left), during (middle), and following a 1 h recovery period after acute repetitive hypoxic challenges (right). Recruitment of sSNA and PNA by repetitive hypoxic exposures (10 challenges of 8% O₂, 45 s, separated by 5 min of recovery) increased after 3 days of conditioning (B) whereas PNA was attenuated by 7 days (C). By 14 days, both sSNA and PNA LTF were blunted (D). Thus, CHH affects both sympathetic and respiratory response to hypoxia over different time courses.

Figure 2
Quantitative analysis showed that CHH differentially alternates the respiratory modulated sympathetic and inspiratory activities. A) The amplitude of sympathetic nerve activity (% of baseline) throughout the respiratory phases in naïve, 3 and 7 days CHH rats increased in response to hypoxia. This increase was maintained following 1 h after the termination of the hypoxic challenge. Exposure to 14 days of CHH blunted both initial and sustained increase in average amplitude of sympathetic activity. B) The increase in the amplitude of peak phrenic activity in response to hypoxia was blocked after 7 days of CHH. Mean ± SEM (n = 10). Abbr.: sI-1, first half of inspiration; sI-2, second half of inspiration; sE-1, first half of expiration; sE-2, second half of expiration; and I, inspiration. *p < 0.005.
Figure 3

CHH induces the expression of select GABA<sub>A</sub> receptor subunit mRNAs within the pons. A) Autoradiograph of a representative RT-PCR experiment shows that the mRNAs encoding the GABA<sub>A</sub> receptor α<sub>6</sub> and δ were induced in the pons after 14 days CHH. β<sub>2</sub> subunit expression was constant and ε subunit expression was absent. In contrast, expression of the α<sub>4</sub> subunit was upregulated at least 2 fold to plateau levels by 3 days of CHH. Similar patterns were observed in 6 independent experiments. B) CHH failed to elicit similar changes in GABA<sub>A</sub> receptor subunit expression in the medulla. GAPDH, which remained at a constant level, was used to normalize data. Abbr.: Cb, cerebellum; Th, Thalamus.

Figure 4

CHH increases the concentrations of GABA<sub>A</sub> receptors recognized by Ro 15-4513 in pontine tissue. A) Saturation binding assays show that binding levels are very low in naïve (σ) and 3 d CHH (λ) rats but increase 250% after 7 d (√) and 500% after 14 d CHH (○). B) Scatchard analysis indicates that Ro 15-4513 binding affinities (K<sub>d</sub>) are unaltered by CHH conditioning.

Table 4.1. Pharmacologic properties of Ro 15-4513 in pons of naïve and conditioned animals

*Values represent Means ± SEM (n = 3 in independent experiments). Data for equilibrium binding [dissociation constant (K<sub>d</sub>) and maximum binding (B<sub>max</sub>)] were measured as described in the Methods.
Figure 5

The GABA<sub>A</sub> receptor α<sub>6</sub> subunit is expressed in the vl pons after 14 d CHH. A) Cresyl violet staining of the vl pons shown for orientation. B-F) Fluorescence micrographs of cells stained with an α<sub>6</sub> subunit-specific antibody. B) The expression of the α<sub>6</sub> subunit is absent from the vl pontine neurons of naïve animals. C) Cells positive for α<sub>6</sub> subunit are prominent within the A5 region after 14 d of CHH. D) Higher magnification of boxed area shown in C. E) GABA<sub>A</sub> receptor α<sub>6</sub> subunit staining is present in the cerebellar granule cell neurons but not in the Purkinje cell layer in naïve rats, as expected. F) Similar levels of expression in the cerebellum were observed after 14 d of CHH. Abbr.: SO, superior olive; 7n, facial nerve; tz, trapezoid body; rs, rubrospinal tract; G, cerebellar granule cell layer; and P, Purkinje cell layer. Scale bar = 100 µm.

Figure 6

GABA<sub>A</sub> receptor α<sub>6</sub> subunit expression was induced in rostral regions of the raphé pallidus nucleus after 14 d CHH. A) Fluorescence photomicrograph of cells stained with an α<sub>6</sub> subunit-specific antibody. B) Cells in the same region positive for tryptophan hydroxylase (TrpOH) an enzyme identifying serotinergic cells. C) Overlay of the α<sub>6</sub> and TrpOH staining demonstrating colocalization within cells in the raphé pallidus. The arrow indicates a neuron in the raphé pallidus expressing both α<sub>6</sub> and TrpOH staining. Abbr.: py, pyramidal tracts; tz, trapezoid body; RPa, Raphé Pallidus; Scale bar = 50 µm.
Figure 7

Model of neural interactions after repetitive hypoxic exposures. A) In naïve animals, the effects of afferent inputs from the carotid body (open arrows) increases pontine activation of spinal circuits (solid arrows) and pontine input to the medulla (solid arrows). This activation elicits a sustained increase in sympathetic and phrenic activity (LTF). B) After 14 d of CHH, LTF is ablated although afferent inputs from the carotid are present. After This reflects an increased GABA$_A$ receptor number (Υ) within the raphé and A5 nuclei. Activation of these GABA$_A$ receptors attenuates the input of the raphé and A5 and thus prevents LTF. Abbr.: LTF, long term facilitation; DI, diazepam-insensitive GABA$_A$ receptors; DS, diazepam-sensitive GABA$_A$ receptors; open arrows, baseline activity; closed arrows, increased activity.
Table 4.1

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<th>Condition</th>
<th>Kd (nM)</th>
<th>Bmax (fmol)</th>
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<td>340 ± 25*</td>
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<tr>
<td>3 d</td>
<td>3.60 ± 0.4</td>
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<td>7 d</td>
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<td>14 d</td>
<td>3.90 ± 0.3</td>
<td>1500 ± 160</td>
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</table>
Before Challenges | After Challenges (5 m) | After Challenges (1 h)

A. Naïve

\[ \text{j}_{sSNA} \]

\[ \text{j}_{PNA} \]

\[ \text{j}_{PNA} \]

2 s

B. 3 Days

\[ \text{j}_{sSNA} \]

\[ \text{j}_{PNA} \]

C. 7 Days

\[ \text{j}_{sSNA} \]

\[ \text{j}_{PNA} \]

D. 14 Days

\[ \text{j}_{sSNA} \]

\[ \text{j}_{PNA} \]
A. Pons

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B. Medulla

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A. Naïve

B. 14d Hypobaric Hypoxia

115x62mm (300 x 300 DPI)