UNIVERSITY OF CALIFORNIA, SAN DIEGO

Non-NMDA receptors in the nucleus of the solitary tract play a role in ventilatory acclimatization to hypoxia in rats

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

John Austin Carr

Committee in charge:

Professor Frank L. Powell, Co-Chair
Professor Nicholas Spitzer, Co-Chair
Professor Kathleen A. French

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The Thesis of John Austin Carr is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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For my parents,
I love you.

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LIST OF ABBREVIATIONS

aCSF	artificial cerebrospinal fluid
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CH	chronic hypoxia
CSN	carotid sinus nerve
HCVR	hypercapnic ventilatory response
HVR	hypoxic ventilatory response
iGluR	ionotropic glutamate receptor
KA	kainic acid
mGluR	metabotropic glutamate receptor
N	normoxia (sea level controls)
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NMDA receptor	N-methyl-D-aspartate receptor
Non-NMDA receptor	AMPA and kainate receptors
NTS	nucleus of the solitary tract
VAH	ventilatory acclimatization to hypoxia

LIST OF SYMBOLS

CO_2	carbon dioxide
F_{ICO_2}	fraction of inspired CO_2
F_{IO_2}	fraction of inspired O_2
f_R	respiratory frequency
N_2	nitrogen
O_2	oxygen
Pa_{O_2}	arterial partial pressure of O_2
Pa_{CO_2}	arterial partial pressure of CO_2
P_B	barometric pressure
P_{CO_2}	partial pressure of CO_2
P_{O_2}	partial pressure of O_2
P_{IO_2}	partial pressure of inspired O_2
T_E	expiratory time
T_I	inspiratory time
V_I	total inspired minute ventilation
V_T	tidal volume
V_T/T_I	index of the ventilatory drive to breath

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ABSTRACT OF THE THESIS

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Professor Frank L. Powell, Co-Chair
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Ventilatory acclimatization to hypoxia (VAH) increases both (1) the hypoxic ventilatory response (HVR) to acute decreases in arterial P_{O_2}, and (2) normoxic ventilatory drive when inspired O_2 is returned to normal. Previous studies have shown that N-methyl-D-aspartate (NMDA) receptors in the nucleus of the solitary tract (NTS) are involved in the acute HVR of awake rats. We hypothesized that non-NMDA

receptors in the NTS are necessary for the complete manifestation of the VAH. To test this, male Sprague-Dawley rats were randomly divided into two groups: (1) normoxic sea level control (N, n = 13) and (2) chronically hypoxic rats (CH, n = 11) acclimatized in a hypobaric chamber (P_{IO_2} = 70 Torr) for 7 days. We microinjected artificial cerebrospinal fluid (aCSF) as a sham, and a non-NMDA receptor antagonist into the NTS (0.02 mmol NBQX in 50 nL, bilaterally) through a stereotaxically placed guide cannula. We used barometric pressure plethysmography to measure ventilation during acute hypoxia (10% O_2), normoxia (21% O_2) and acute hypercapnia (7% CO_2). We microinjected AMPA into the same location in the NTS of a subset of the same animals to confirm effectiveness of the NBQX. Microinjection sites were identified by colloidal gold or Evan's blue microinjections after the experiment. Non-NMDA receptor blockade with NBQX significantly ($P<0.05$) decreased the normoxic ventilatory drive after chronic hypoxia, but not in the normoxic control rats. In addition, non-NMDA receptor blockade depressed ventilation for both normoxic and chronically hypoxic rats acutely breathing hypoxic and hypercapnic gas. The ventilatory response to NBQX resulted primarily from significant effects on respiratory frequency, but not tidal volume. Hence, non-NMDA receptors in the NTS play a role in VAH, and are important for ventilatory drive during chronic and acute hypoxia.

I. INTRODUCTION

Exposure to low oxygen, or hypoxia, has a range of physiological effects on biological organisms that depend on oxygen for proper function. The physiological response of a living organism to hypoxia depends crucially on the rate and degree of hypoxic exposure. Upon chronic exposure to hypoxia (i.e. hours to months at high altitude or a hypobaric chamber), healthy humans and animals undergo a time-dependent increase in ventilation as a protective response to low P_{O_2} (Bouverot 1985). This hypoxia-induced hyperventilation helps to maintain proper O_2 supply in the blood and minimizes the hypoxic insult to the tissues. This phenomenon has been termed ventilatory acclimatization to hypoxia (VAH, (Weil 1986; Bisgard et al. 1995), and represents one time domain of the hypoxic ventilatory response (HVR, (Powell et al. 1998).

The HVR is an increase in ventilation that is mediated by the arterial chemoreflex and is brought about by exposure to hypoxia. The carotid body responds to a fall in arterial P_{O_2} by increasing action potential input to sites of central integration in the brainstem, specifically the nucleus of the solitary tract (NTS, (Finley et al. 1992). This, in turn, produces a reflex increase in ventilation via the respiratory motor output, which is predominantly the phrenic motor nerve to the diaphragm. In general, the HVR is determined by a complex interplay between several distinct mechanisms that depend on the pattern and intensity of hypoxic exposure and the time course of the response from seconds to years. Cellular and molecular changes brought about by the different degrees of hypoxic exposure are necessary for the complete manifestation of the HVR. Some of

these changes have a long-lasting effect, which indicates a degree of "memory" in the ventilatory control mechanism (Powell et al. 1998; Mitchell et al. 2003).

VAH is one time domain of the HVR that represents the response to long-lasting exposure to hypoxia. The rat has been shown to have a similar time course (7-10 days) in acclimatization to hypoxia to humans (Aaron et al. 1993). This, along with other similar physiological and metabolic adaptations to hypoxia (Olson et al. 1978), has made the rat a good experimental model for VAH in humans. VAH has been characterized as (1) an increase in the O_2-sensitivity of peripheral chemoreceptors in the carotid body (Bisgard et al. 1995) as well as (2) an increase in the CNS responsiveness to the afferent inputs from the carotid body (Dempsey et al. 1982; Powell et al. 2000).

The carotid body is located at the bifurcation of the common carotid artery and is the main sensory organ responsible for detecting changes in arterial P_{O_2} (Housley et al. 1988; Powell 2007). These receptors then transduce changes in arterial P_{O_2} into changes in carotid sinus nerve afferent activity to the brain stem, thereby eliciting the hypoxic chemoreflex. There is abundant literature showing that the carotid body undergoes many different cellular and molecular changes during acclimatization to chronic hypoxia in order to increase its O_2-sensitivity (Prabhakar et al. 2005). Work done on goats (Nielsen et al. 1988), cats (Barnard et al. 1987) and rats (Wang et al. 1995) has shown that the discharge rate of the carotid body afferent fibers to the CNS is enhanced following chronic hypoxia.

Dwinell and Powell (2000) described a second mechanism contributing to the measured HVR in VAH: an increase in the CNS gain of the HVR. When electrically stimulating the carotid sinus nerve of anesthetized, paralyzed and vagotamized rats,

phrenic nerve activity to the diaphragm was increased in chronically hypoxic rats compared to normoxic rats (Dwinell et al. 1999). This indicates a contribution of neural plasticity in the CNS circuits for the hypoxic chemoreflex.

CO_2-sensitivity adds complexity to the acute HVR after chronic hypoxia. A reduced arterial P_{CO_2} along with hypocapnic alkalosis caused by the hypoxic-induced hyperventilation is characteristic of VAH in humans (Rahn et al. 1949) and rats. (Olson et al. 1988). This tends to blunt the HVR by decreasing the effect of CO_2 to stimulate ventilation. However, the net effect is that ventilation increases with VAH. If the isocapnic HVR is measured, which corrects for hypocapnia by maintaining P_{aCO_2} constant, the slope of the HVR is increased in humans (Tansley et al. 1998) and rats (Aaron et al. 1993) following chronic exposure to hypoxia also. The HVR under isocapnia is a more accurate measurement for testing the O_2 responsiveness of peripheral chemoreceptor reflexes (Weil et al. 1976).

Unlike O_2-sensing which is restricted to the peripheral chemoreceptors, there are central and peripheral chemoreceptors that respond to CO_2. CO_2 reflexes are thought to depend predominantly on pathways outside of carotid body afferent input to the NTS (Nattie et al. 2008). There are multiple nuclei within the brainstem that are involved in central chemoreception of CO_2 and pH changes, including the NTS itself with intrinsic CO_2-sensitivity (Dean et al. 1989; Coates et al. 1993). Changes in central CO_2-sensitivity are hypothesized to contribute to VAH, but the exact mechanisms remain unknown (Dempsey et al. 1982).

Afferent input from carotid body chemoreceptors enters the brainstem via the IX cranial nerve (glossopharyngeal) and terminates at the nucleus of the solitary tract (NTS),

(Donoghue et al. 1984; Housley et al. 1987; Cheng et al. 2002). Injections of horseradish peroxidase (HRP) directly into the carotid body of the rat have revealed carotid body input terminates in the caudal region of the NTS (Finley et al. 1992). The NTS is a longitudinal structure in the dorsomedial region of the medulla oblongata. As the primary site of central chemoreceptor integration, the NTS contains bulbospinal neurons that project directly to the phrenic motor nuclei (PMN) and propriobulbar (premotor) neurons that project to the ventral respiratory group to control inspiratory muscles (Chung et al. 2006).

Glutamate (L-glutamate) is the primary excitatory neurotransmitter released from the carotid sinus nerve terminal in the NTS and plays an essential role in the chemoreflex pathway (Miller et al. 1988; McCrimmon et al. 1995). Microdialysis techniques on awake rats exposed to acute hypoxia have demonstrated an increase in the extracellular glutamate concentration in the NTS during peripheral chemoreceptor stimulation (Mizusawa et al. 1994).

Glutamate binds to two general families of amino acid receptors, namely the metabotropic and ionotropic glutamate receptors. The metabotropic glutamate receptors (mGluR) bring about rather slow synaptic and intracellular changes relative to the ionotropic receptors (Nakanishi 1994). They are coupled to GTP-binding proteins (G-proteins) which activate various second messenger cascades. It has been shown that mGluRs are involved in time-dependent changes in neurotransmission primarily by mediating afferent processing in the NTS (Glaum et al. 1992). Li (1995) demonstrated that blockade of mGluRs with (±)-MCPG in the retrotrapezoid nucleus (RTN, involved in respiratory control) during prolonged glutamate injection blocks long lasting increases in

phrenic nerve activity. Hence, mGluRs seem to play a role in sustained activation of the chemoreflex pathways.

The ionotropic glutamate receptors (iGluR) are ligand-gated, cation-selective channels that bring about rapid neuronal excitation via fast ionic currents. Ionotropic glutamate receptors can be further classified according to their preferred exogenous agonists: (1) α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (2) kainic acid (KA), and (3) N-methyl-D-aspartic acid (NMDA). While NMDA receptors have been shown to have unique antagonists that set them apart from the other iGluRs, antagonists have not been identified to clearly distinguish between the AMPA and KA receptors. Therefore, AMPA and KA receptors are usually collectively referred to as non-NMDA receptors in literature. However, compositional and molecular differences exist between the two subgroups (Gasic et al. 1992).

There is abundant literature showing that the different subgroups of iGluRs are highly expressed in the NTS. Quantitative autoradiography with radiolabeled NMDA demonstrated an abundance of NMDA receptors (Monaghan et al. 1985). Kessler (1999) demonstrated the presence of the AMPA receptors in the caudal region of the NTS by immunolabeling specific AMPA receptor subunits. Additionally, kainic acid-induced lesions in the NTS diminished the chemoreceptor response to hypoxia (Housley et al. 1988).

NMDA and non-NMDA receptors have been shown to play a critical role in neurotransmission in the NTS (Reis et al. 1981; Miller et al. 1988; Machado et al. 1992), and their roles in the acute HVR have been widely considered (Bonham 1995; Gozal et al. 2000; Almado et al. 2005). Previous studies have indicated that NMDA receptors in

the NTS of conscious rats (Ohtake et al. 1998), anesthetized dogs (Ang et al. 1992), and unanaesthetized piglets (Lin et al. 1996) are important for the normal HVR. Non-NMDA receptors in the NTS have not been studied in awake, adult animals. They play a critical role in combination with NMDA receptors in anesthetized adult rats (Vardhan et al. 1993), but only a minor role in awake neonatal rats (Whitney et al. 2000). Also, none of these studies considered ventilatory acclimatization to hypoxia. Reid et al. (2005) studied systemic blockade of NMDA receptors with intravenous MK-801 (non-competitive NMDA receptor-specific antagonist) in the rat and described a loss of O_2-senstivity to acute hypoxia before and after acclimatization; however, those results cannot tell us about the role of glutamate receptors specifically in the NTS in VAH.

Taken together, these studies suggest a role for two subtypes of ionotropic glutamate receptors in VAH. NMDA receptors are important for the increased HVR after chronic hypoxia, but the specific role of non-NMDA receptors in VAH is still unknown. The objective of this study was to determine the role of non-NMDA receptors in VAH. We tested the hypothesis that non-NMDA receptors in the NTS are necessary for the complete manifestation of VAH. We predicted that the local effects of a non-NMDA receptor-specific antagonist (NBQX) in the NTS of chronically hypoxic rats would be significantly different from those in control rats maintained under normoxic conditions.

II. MATERIALS AND METHODS

A. Surgery

1. Animals - General

All surgical procedures and protocols were done in accordance with guidelines of The University of California, San Diego Institutional Animal Care and Use Committee. Male Sprague-Dawley Rats (Charles River Farms, Wilmington, MA) weighing 318 ± 7 g at the time of surgery were housed under a 12:12 h light-dark cycle with free access to food and water. All surgeries were done under isoflourane anesthesia (initially 5% isoflourane in 100% O_2 and maintained at 2-3% isoflourane). The rats were randomly divided into two groups: (1) normoxic sea level controls (N, n=13) and (2) chronically hypoxic rats (CH, n=11) acclimatized to a simulated altitude of 5,500 meters in a hypobaric chamber at 380 Torr (P_{IO_2}= 70 Torr) for 7 days. The chamber was returned to sea level for 15 minutes every 3-4 days for general cage maintenance or when it was necessary to remove animals for experimentation. At least 6 days prior to ventilatory measurements, all animals underwent surgery for the implantation of a guide cannulae, arterial catheter, and emitter telemetry probe. The CH group was allowed to recover from surgery overnight at sea level before being transferred into the hypobaric chamber.

2. Guide Cannula

Stereotaxic surgery (Kopf Instruments, Tujunga, CA) was used to implant a stainless steel guide cannula (Plastics One, Roanoke, VA) bilaterally into the NTS (AP -0.3mm (calamus scriptorius), ML -0.7mm, DV -0.5mm) to deliver pharmacological agents. Figure 1 shows the brainstem of the rat and diagrams the coordinates of the NTS. The guide cannula was secured to the skull using acrylic resin that fixed the guide

cannula to two screws drilled firmly into the skull. The microinjection needle was 1 mm longer than the guide cannula and projected into the NTS.

3. Arterial Catheters

Arterial Catheters were inserted through the femoral artery and reached the abdominal aorta of the rat. Polyethylene tubing (PE-50) was heated and stretched to fit the diameter of the artery, and sutured in place. The stretch allowed for a single tube with no joints that was more resistant to the formation of blood clots. The catheter was tunneled beneath the dorsal skin and exited at the back of the neck through a stainless steel headbutton that was sutured in place for easy access. A stainless steel ring was screwed to the headbutton to protect the catheter. The arterial catheter was used for obtaining blood samples for arterial blood gas analysis.

4. Emitter

At the same time, a telemetry thermometer probe (Emitter, Respironics, Bend, OR) was implanted to monitor body temperature. The body temperature is required for an accurate calculation of the tidal volume (V_T, see "The Plethysmograph"). The Emitter was implanted into the abdominal cavity and sutured in place to the interior wall of the abdomen. The Emitters were calibrated by the company before delivery.

B. Plethysmography

1. The Plethysmograph (Ventilatory Measurements)

Ventilation (V_I) was measured using barometric pressure plethysmography modified for continuous flow (Jacky 1978). On the experimental day, individual animals were sealed into a Plexiglas chamber (7 liters). Figure 2 diagrams the plethysmograph and all of its components. An electronic gas mixer (MFC-4, Sable Systems, Las Vegas,

NV) was used to supply the animal with an inflowing gas mixture (3 L/min) of controlled O_2 and CO_2 concentrations with the balance being composed of N_2. Inflowing gas entered the chamber through a tube (7 cm long and 1 cm in diameter) that was filled with smaller PE-50 tubing of similar length. This created a high impedance input to reduce the loss of pressure signals. Continuous flow and proper chamber pressure were achieved by a vacuum pump (Dayton Electric, Chicago, IL) that sucked gas from the chamber through a vacuum valve. Pressure inside the box was referenced to atmospheric pressure using a water manometer. Atmospheric pressure corrected for standard gravity and room temperature was recorded on each experimental day as a reference. To ensure a controlled gas mixture in the chamber, the pressure inside the chamber was maintained slightly inside positive (<0.5 cmH_20) by adjusting the vacuum valve. Chamber gas concentrations were measured using a mass spectrometer (Perkin-Elmer 1100 Medical Gas Analyzer, Pomona, CA) that was calibrated for O_2 and CO_2 on each experimental day. A chamber temperature probe (Thermalert TH-5, Physitemp, Clifton, NJ) was sealed inside the box, and a humidity probe entered the box through a hole that was cut and sealed for this purpose. Inspiration produces humidity-related changes in pressure that can be monitored with a differential pressure transducer (DP45, Validyne, Northridge, CA) referenced to atmosphere. Output from the transducer was recorded on a digital data acquisition system (Labdat, see "Data collection and analysis" below). Respiratory frequency (f_R) was calculated directly from the ventilation-induced pressure swings. Tidal Volume (V_T) was calculated from the ventilation-induced pressure changes using an equation from Drorbaugh and modified for animal plethysmography by Jacky (Drorbaugh et al. 1955; Jacky 1978):

$$V_T = V_{cal} * \frac{P_m}{P_{cal}} * \frac{\left(P_B - P_{CH_2O}\right)}{\left[T_A\left(P_B - P_{CH_2O}\right) - T_C\left(P_B - P_{AH_2O}\right)\right]}$$

where V_{cal} = volume of the calibration pulse (mL); P_m = peak height (A-D units); P_{cal} = peak height of calibration pulse (A-D units); P_B = barometric pressure (mmHg); P_{CH_2O} = chamber vapor pressure (mmHg); P_{AH_2O} = animal vapor pressure (mmHg); T_A = body temperature of the animal (˚K); T_C = chamber temperature (˚K).

Calibration pulses (0.5 and 1.0 mL) were generated by using a gas-tight syringe and injecting air pulses into the chamber at a rate similar to the rats' inspiratory time before and after the experiment.

2. The Protocol

Ventilation was determined under poikilocapnic conditions. The experimental protocol used to take ventilatory measurements is shown in figure 3. The animals were given at least 40 minutes to habituate to the plethysmograph at their chronic inspired O_2 levels before study. Two inspired O_2 levels (F_{IO_2} = 10% and 21%) were used in the study. We used these two stimuli to obtain a two-point poikilocapnic HVR (V_I at 10% O_2 minus V_I at 21% O_2). The 10% O_2 level for the HVR measurements produce the same P_{IO_2} as that in the hypobaric chamber used for acclimatization to chronic hypoxia. V_I was measured at 10-15 minutes after the rats' were exposed to an acute inspired gas concentration. We used a 7% CO_2 concentration in 21% O_2 to measure the hypercapnic ventilatory response (HCVR, V_I at 7% CO_2 minus V_I at 0% CO_2).

3. Arterial Blood Gas Measurements

Single arterial batch samples (0.2 mL) were taken while the rats were breathing 10% or 21% O_2 (n=3 for N group and n=3 for CH group), as well as 7% CO_2 in 21% O_2

(n=2 for N group and n=3 for CH group). Samples were obtained after control and drug microinjections. These samples were corrected for body temperature and analyzed (Instrumentation Laboratory Gem Premier 5000, Lexington, MA) for arterial O_2 pressure (Pa_{O_2}), arterial CO_2 pressure (Pa_{CO_2}), and arterial pH.

4. Microinjections

Drug Preparation. All of the drugs that were used were prepared in artificial cerebrospinal fluid (aCSF). The aCSF was made in stock of (in mM): 115 NaCl, 2.0 KCl, 2.2 KH_2PO_4, 25 $NaHCO_3$, 10 $_D$-glucose, 1.2 $MgSO_4$ and 2.5 $CaCl_2$ (Youssef et al. 2001) and set to a physiological pH (7.4). The aCSF was microinjected as a sham in a subset of the normoxic (n=6) and chronically hypoxic (n=5) rats studied.

NBQX (1,2,3,4-Tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide disodium salt) and AMPA ((±)-α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid) were purchased from Sigma-Aldrich (St. Louis, MO). The NBQX was used at 4 mM and the AMPA at 0.25 mM in aCSF.

NBQX Microinjection Tests. A microinjection needle made to fit the guide cannula was connected to a Hamilton microsyringe through a polyethylene tube. The microinjection needle projected 1 mm beyond the guide cannula into the NTS. The polyethylene tube ran through the lid of the plethysmograph and was sealed airtight. After control ventilatory measurements were made at 10% and 21% O_2 and 7% CO_2, 50 nL of NBQX (0.2 nmol) was injected into the NTS. Ventilatory measurements were again collected in each gas mixture. Ventilatory effects of the NBQX were determined by comparing the data obtained following the control measurements with those collected following the drug microinjection.

AMPA Response Tests. To confirm AMPA receptor blockade by NBQX, AMPA was microinjected into the NTS in the presence and absence of NBQX. The rat was placed in the plethysmograph and allowed to stabilize for 40 min. After baseline ventilatory measurements were made, 50 nL of AMPA (12.5 pmol) were injected bilaterally into the NTS and ventilatory data were collected at 1, 2, 3, 4, 5, 10,15 and 30 minutes post-injection. For ventilatory measurements in the presence of the blocker, the rat was microinjected injected with 50 nL of NBQX (0.2 nmol) before being placed in the plethysmograph, and the same protocol was followed. All measurements were taken during acute normoxia (21%).

C. Histology and Localization

We used colloidal gold or Evan's Blue microinjection to localize the microinjection sites. At the end of the experiment, Colloidal Gold (50 nL) or Evan's Blue (50 nL) was microinjected through the guide cannulae into the NTS at the same site as the drug delivery. The animals were anaesthetized with an overdose of sodium pentobarbital and transcardially perfused with 4% paraformaldehyde. The brainstem was removed and postfixed in paraformaldehyde for 1 day, and then stored in sucrose (30%). The brainstems were frozen in isopentane (-140°C) and sectioned (30-50μm) on a cryostat (Cryocut 1800, Leica Biosystems, Wetzlar, Germany). For animals microinjected with the colloidal gold, the gold was enhanced using silver intensification solution (Ted Pella Inc., Redding, CA) which marked the microinjection sites with black staining. Aqueous Eosin Y was used as a cytoplasmic counter-stain. Only the animals in which the microinjection site was located in the NTS were included in the final analysis.

D. Systems and Analysis

1. Data Collection and Analysis

Ventilatory measurements were recorded on a digital data acquisition system (Labdat model DT-2801-A, National Instruments) sampling at 200-Hz for 60 seconds. Representative periods of at least 10 breaths were selected for analysis. Analysis was done on LabRead 16.0 (modified in-house).

2. Statistical Analysis

General. Statistical analysis was performed using commercial software (SPSS 15.0, SPSS Inc., Chicago, IL). Values are presented as mean ± SEM. $P < 0.05$ was considered to achieve statistical significance.

NBQX Microinjection. A repeated measures analysis of variance (ANOVA) was used to determine if there was a statistically significant difference between the three independent factors considered: (1) the chronic ventilatory condition (N vs. CH), (2) the acute ventilatory condition (F_{IO_2} of 10% O_2 vs. 21% O_2 or 0% CO_2 vs. 7% CO_2) and (3) the treatment (Control vs. NBQX). A post hoc multiple comparisons test was run on each of the dependent variables to compare the single point means of interest. The dependent variables analyzed were: total minute ventilation (V_I), respiratory frequency (f_R), tidal volume (V_T), inspiratory time (T_I), expiratory time (T_E) and the inspiratory slope (V_T/T_I) as an index of the ventilatory drive to breath. A paired-samples t-test was used to compare the effects of NBQX the acute changes in HVR of between the N and CH groups.

AMPA Response. A repeated measures ANOVA was used to determine whether there was a statistically significant difference in the time-dependent response to AMPA in

the presence and absence of NBQX. The between-subject factors (normoxic controls vs. chronic hypoxia, and AMPA in the presence vs. absence of NBQX) were considered against time. A simple contrast referenced the value at each time point to the pre-AMPA control ('CON,' time = 0) value. Sphericity could not be assumed, so the Greenhouse-Geisser test was used because it is the most conservative for a small sample size.

III. RESULTS

Effects of Sham

The effects of aCSF as a sham are shown in figure 4 for a subset of normoxic control (N, n=6) and chronically hypoxic (CH, n=5) animals. The aCSF had no effect on ventilation in 21% O_2 or 10% O_2 in either group compared to the control ventilation (no microinjection treatment). Because the sham had no effect on ventilation, we compared the ventilatory effects of the drug (NBQX) to control measurements (no microinjection).

Responses to AMPA

Figure 5 shows the temporal response to bilateral microinjections of AMPA in the presence and absence of NBQX for normoxic (n=4) and chronically hypoxic (n=6) rats. All measurements were taken in 21% O_2. AMPA significantly increased V_I within the first minute of microinjection in both the CH and N groups. In normoxic rats, V_I increased from 551.0 ± 53.8 to 727.3 ± 33.0 ml/min/kg, and for the chronically hypoxic rats from 902.5 ± 106.5 to 1153.1 ± 155.7 ml/min/kg. The response to AMPA in the normoxic rats was mainly a result of an increase in the frequency (Fig. 6A). Tidal volume (Fig. 6B) remained unchanged for both groups in all time periods. Microinjecting NBQX into the NTS before treatment with AMPA abolished the response to AMPA to both CH and N groups.

Effects of NBQX on Normoxic Animals

NBQX had no effect on V_I in N rats breathing 21% O_2, but it significantly decreased V_I by 20.3% in 10% O_2 (Fig. 8A). As a result, NBQX significantly depressed the slope of the acute HVR in N rats (Fig. 8B).

The decrease in V_I of N animals is due to a significant decrease in f_R (Fig. 9) in both 10% and 21% O_2 while V_T (Fig. 10) tended to increase to compensate. Both T_I (Fig. 11A) and T_E (Fig. 11B) significantly increased with NBQX in both 10% and 21% O_2. The drive to breathe as indexed by V_T/T_I (Fig. 12) shadowed V_I. NBQX significantly reduced V_T/T_I in N animals breathing 10% O_2 while it was unaltered in 21% O_2.

Effects of Chronic Hypoxia

Chronic hypoxia significantly increased V_I (Fig. 8A) in acute 10% and 21% O_2 compared to N rats. Chronic hypoxia caused a parallel increase in V_I such that the slope of the HVR of CH rats was unchanged compared to N rats. This is quantified in figure 6B. The increase in ventilation was due to both significant increases in f_R (Fig. 9) and V_T (Fig. 10) compared to the N controls. T_I (Fig. 11A) and T_E (Fig. 11B) were unchanged after chronic hypoxia when acutely breathing 10% O_2. However, T_E was significantly decreased in chronically hypoxic animals breathing 21% O_2, while the decrease in T_I (Fig 11A) was only approaching significance ($P = 0.056$). Like V_I, the drive to breathe (V_T/T_I) was significantly greater following chronic hypoxia (Fig. 12).

Effects of NBQX on Chronically Hypoxic Animals

NBQX significantly decreased V_I (Fig. 8A) in CH rats breathing both 10% and 21% O_2. The CH rats had a parallel reduction in V_I with NBQX, and as a result, NBQX had no effect on the slope of the HVR (Fig. 8B). The decrease in V_I of CH animals is due to a significant decrease in f_R (Fig. 9) in both 10% and 21% O_2 while V_T (Fig. 10) tended to increase to compensate. T_I (Fig. 11A) significantly increased with NBQX in both 10% and 21% O_2. T_E (Fig. 11B) was only significantly increased with NBQX in

21% O_2. The drive to breathe as indexed by V_T/T_I (Fig. 12) again shadowed V_I. NBQX significantly reduced V_T/T_I in CH animals acutely breathing both 10% and 21% O_2.

Effects of CH and NBQX on Arterial Blood Gases

Blood gases are summarized in figure 7 for a subset of N (n=3) and CH (n=3) rats. Changes in Pa_{O_2} with chronic hypoxia were insignificant, but Pa_{O_2} was generally higher in each CH animal compared to N. Chronic hypoxia generally decreased Pa_{CO_2}, which was significant in 21% O_2. Decreased Pa_{CO_2} in CH animals caused the arterial pH to become alkalotic compared to N rats, although the difference was not significant in this small sample size.

Microinjection of NBQX had no significant effects on arterial blood gases (Fig. 7). However, NBQX tended (P = 0.085) to increase Pa_{CO_2} in CH rats breathing 21% O_2.

Effects of NBQX with Acute Hypercapnia

The ventilatory response to hypercapnia is shown in figure 13A. When the rats were exposed to acute hypercapnia, (7% CO_2), V_I of CH rats (2192.3 ± 764.9 ml/min/kg) was elevated 31.4% compared to N (1668.2 ± 78.8 ml/min/kg). The f_R response to hypercapnia (Fig. 14A) was not significant while the V_T (Fig. 14B) increased significantly (10.11 ± 0.37 to 12.15 ± 1.11 ml/kg, for N and CH rats, respectively). The magnitude of the slope of the HCVR tended to increase (P=0.110) in CH rats (1301.2 ± 145.6 ml/min/kg) compared to N rats (1129.0 ± 80.2 ml/min/kg, Fig. 13B).

The response of both groups to NBQX in hypercapnia was a general depression in V_I based on lower respiratory frequency with no change in tidal volume (Figs. 14A and 14B, respectively). Microinjection of NBQX had no significant effects on the slope of the HCVR for either the CH or the N groups (Fig. 13B).

Localization

Figure 15 shows a coronal section of the brainstem of two different rats, representative of the group. The micrograph shows the sites of successful microinjection in the caudal region of the NTS. Only the animals in which the microinjection site was located in the NTS were included in the final analysis.

IV. DISCUSSION

We tested the hypothesis that non-NMDA receptor plasticity is necessary for the complete manifestation of VAH. Our experiments support the hypothesis by showing that blockade of non-NMDA receptors decreased the normoxic ventilatory drive after chronic hypoxia, but not in the normoxic control rats (Fig. 12). In addition, non-NMDA receptors are necessary for a normal hypoxic and hypercapnic ventilatory response in both normoxic and chronically hypoxic rats (Fig. 8, 13).

Critique of Methods

Ventilatory measurements in the present study compare a control group vs. NBQX when considering the effect of drug on ventilation. The control group represents resting ventilatory data with no microinjection. Because NBQX was dissolved in physiologic aCSF, a representative subset of the rats studied also received microinjection of aCSF as a sham to differentiate between the physiological effects of the NBQX and aCSF. Microinjection of aCSF did not change V_I in the normoxic or chronically hypoxic rats breathing 10% or 21% O_2 when compared to control.

We predicted that the local effects of a non-NMDA receptor-specific antagonist (NBQX) in the NTS of chronically hypoxic rats would be significantly different from the effects in control rats maintained under normoxic conditions. Non-NMDA receptors consist of the AMPA and Kainic Acid (KA), subtypes which can only be distinguished to date by their molecular differences (Gasic et al. 1992). It has been established that both the AMPA and the KA receptors are present in the NTS (Berger et al. 1982; Kessler et al. 1999) and that both have physiological roles in different homeostatic functions mediated by the NTS (Talman et al. 1981; Isenovic et al. 2007). AMPA receptors are the

glutamatergic receptors that are largely responsible for the fast excitatory synaptic transmission, or 'fast throughput,' in the rat brain, as well as the human brain. They are thought to be required for adaptive changes that mediate the expression of synaptic plasticity believed to underlie learning and memory. (Bliss et al. 1993). AMPA receptors have been shown to be associated with respiratory functions by experiments in which the agonist (AMPA) was microinjected into the NTS (Almado et al. 2005). We confirmed this finding by microinjecting AMPA into the same location of the NTS as our NBQX delivery, which elicited a hyperventilatory response. KA receptors have also been linked to the NTS in respiratory and cardiovascular function (Talman et al. 1981).

To date, there is no clear pharmacological antagonist that distinguishes AMPA receptors from KA receptors. The present study uses NBQX, a quinoxalinedione derivative, to competitively block non-NMDA receptor function. NBQX is more selective than the two other non-NMDA receptors antagonists currently available (CNQX and DNQX), which also act as an antagonist of the glycine-binding site of NMDA receptors, and hence potentially alter NMDA receptor function as well (Kessler et al. 1989). It has been suggested that NBQX in low micromolar concentrations may selectively inhibit AMPA receptors over KA receptors (Mulle et al. 2000) and that it may do so with rather high affinity compared to other non-NMDA receptor antagonists (Watkins et al. 1981). However, we microinjected NBQX in millimolar concentrations (4 mM), so it is unlikely that we selectively inhibited AMPA receptors over KA receptors. Locally blocking non-NMDA receptors with NBQX is an initial step in determining the role of non-NMDA receptors in VAH, with the obvious limitation that we cannot functionally distinguish between the two subtypes of non-NMDA receptors.

Interpretation of Results

We observed a differential effect of NBQX in normoxic compared to chronic hypoxic rats indicating a role for non-NMDA receptors in VAH. NBQX decreased V_I in the normoxic control rats breathing 10% O_2, but had no effect in 21%. In 10% O_2, the depression in V_I was primarily mediated by a decrease in f_R while there was no change in the V_T component. In 21% O_2, however, the decrease in f_R resulting from the NBQX led to a compensatory increase in V_T, so V_I did not change. As a result, NBQX decreased the slope of the poikilocapnic HVR in the normoxic animals. The compensation for a blockade of non-NMDA receptors in 21% O_2 indicates that overall ventilation of normoxic control rats is not entirely reliant on non-NMDA receptors under conditions of baseline ventilatory drive, but the pattern of breathing is. Thus, non-NMDA receptors contribute to the acute HVR of normoxic rats by increasing ventilation in acute hypoxia but not in normoxia.

Our results show that V_I was significantly increased when breathing both 10% and 21% O_2 after chronic hypoxia, although the slope of the poikilocapnic HVR was unchanged. These effects are consistent with what has been extensively reported in literature for chronic hypoxia (Bisgard et al. 1995; Reid et al. 2005). The increased ventilatory drive was expressed by an increase in f_R in 10% O_2, and an increase in both f_R and V_T in 21% O_2.

Our second finding was that NBQX reversed the hyperventilation after chronic hypoxia, but there was no significant change in the slope of the poikilocapnic HVR of chronically hypoxic rats after NBQX microinjection. Similar to the responses seen in the normoxic rats, NBQX depressed V_I primarily by decreasing f_R. The frequency-driven

hypoventilation was caused by a mismatch in the V_T response, and is consistent with our blood gas data. Thus, we report for the first time that non-NMDA receptors are necessary for increased ventilation with chronic hypoxia.

Arterial blood gases tend to change in a predictable manner. $Paco_2$ decreases with chronic hypoxia as a result of the hypoxia-induced hyperventilation. This phenomenon has been widely discussed in literature (Smith et al. 2001). In our experiments, $Paco_2$ tended to increase after microinjection of NBQX. Elevated $Paco_2$ after the NBQX can be attributed to the f_R-mediated hypoventilation. Because ventilation is generally lower in rats acutely breathing 21% vs. 10%, a larger decrease in $Paco_2$ is expected for a given decrease in V_I with NBQX in 21%, and that is what we observed. The lack of significance is likely due to the low n values (n=3 for both N and CH).

Changes in V_T/T_I mirror those expected when analyzing V_I in all conditions. V_T/T_I is an index for the ventilatory drive to breathe. For example, in normoxic rats, V_T/T_I increased when they were breathing 10% O_2. Non-NMDA receptors increased the overall ventilatory drive in acute hypoxia and after chronic hypoxia apparently via a significant decrease in T_I. Our results indicate that non-NMDA receptors play a role in mediating the ventilatory drive to breathe in acute hypoxia as well as in chronic exposure to hypoxia.

The ventilatory response to hypercapnia follows the same general trend as seen in hypoxia. Chronically hypoxic animals had an elevated V_I when breathing 7% CO_2 compared to normoxic animals. NBQX significantly decreased V_I in chronically hypoxic and normoxic animals breathing 7% CO_2, but not 0% CO_2. The lack of

significance for the chronically hypoxic animals breathing 0% CO_2 (which is the same as 21% O_2) is because of the low n value (n=5) as this is the same point that was significantly different with the larger n (n=11) used to study the HVR.

It has been established that CO_2 reflexes predominantly depend on pathways outside the NTS (Nattie et al. 2008). While blocking peripheral reflex input in the NTS significantly depressed the acute response to hypercapnia in both chronically hypoxic and normoxic animals, it is not possible to completely separate out the central CO_2 reflex mechanisms. In addition, the NTS also has intrinsic CO_2-sensitivity (Dean et al. 1989; Coates et al. 1993). The slope of the HCVR was not significantly different with NBQX compared to control. This indicates that some other system besides non-NMDA receptors in the NTS is primarily responsible for the HCVR.

Comparison with Literature

Previous studies have established that the acute HVR is critically dependant on NMDA receptors in the NTS (Ohtake et al. 1998; Reid et al. 2005). These studies were conducted by systemically blocking NMDA receptors with MK-801. Data supporting a role of non-NMDA receptors in the acute HVR is more limited. The role of non-NMDA receptors in the acute HVR has only been studied with systemic injection of the antagonist, or in neonatal animals. Borday (1998) systemically injected awake adult mice and cats with NBQX and witnessed no change in ventilation while they were spontaneously breathing room air. In the present study, we demonstrated that microinjection of NBQX directly into the NTS of spontaneously breathing adult rats attenuated the acute HVR of normoxic animals. This result indicates that non-NMDA receptors are involved in the acute HVR of control animals. We found that NBQX did

not alter ventilation for normoxic animals breathing 21% O_2, and that the difference in the HVR was caused by decreased sensitivity to acute hypoxia. This is consistent with Borday's finding that NBQX did not change ventilation in room air. In neonatal rats, systematic injection of NBQX failed to alter the acute HVR (Whitney et al. 2000). The difference between that study and our results probably can be attributed to the fact that the neonate undergoes significant developmental changes in the first two weeks of life with respect to the ventilatory response to hypoxia (Eden et al. 1987).

A previous study (Vardhan et al. 1993) examined the effects of DNQX microinjected into the NTS of anesthetized adult rats on ventilatory responses to carotid body stimulation. In that study, non-NMDA receptors played a critical role only in combination with NMDA receptors in anesthetized adult rats. Neither DNQX nor AP-7 (NMDA receptor antagonist) alone altered the ventilatory response to stimulation of the carotid body. However, when the two were combined, the response to carotid body stimulation was abolished. Our study has shown that non-NMDA receptors alone can effect reflexes from carotid body activation with acute hypoxia. The major difference is that Vardhan used anesthetized rats and we studied awake and spontaneously breathing rats.

Vardhan also discussed the possibility that DNQX was not specific for non-NMDA receptors because it also blocked responses to NMDA at higher doses. Drug specificity might explain the differences between the results without stimulation of the carotid body. It is possible that the differences between the results from this study in acute hypoxia and those reported by Vardhan, et.al. with carotid body stimulation are due to different dosages. Vardhan used a dose of DNQX that was sufficient to block the

response of a 5 pmol dose of AMPA, while our dose of NBQX was sufficient to block the response of a larger 12.5 pmol dose of AMPA.

Potential Mechanisms and Future Directions

Our study supports a differential role of NMDA and non-NMDA receptors in VAH. Together, NMDA and non-NMDA receptors explain different components of chronic hypoxia. Reid (2005) systemically injected MK-801 to block NMDA receptors in the rat and described a loss of O_2-senstivity to acute hypoxia before and after acclimatization. More recently, Nguyen and Reid (unpublished) microinjected MK-801 into the NTS of the rat and found no effect in normoxic ventilatory drive before or after chronic hypoxia. However, ventilation in acute hypoxia (10%) decreased after MK-801 in chronically hypoxic, but not control rats. Reid's studies strongly suggest a role for NMDA receptors in the increased O_2 sensitivity and increase in ventilation while breathing 10% O_2 after chronic hypoxia.

The present study suggests a contribution of non-NMDA receptors to ventilatory drive in hypoxia and in normoxia after chronic hypoxia. MK-801 microinjection in Reid's study did not affect the normoxic ventilatory drive in chronically hypoxic rats, but NBQX microinjection in the present study attenuated the normoxic ventilatory drive in chronically hypoxic rats. In contrast, both MK-801 and NBQX attenuated the ventilatory response to hypoxia in acclimatized rats, but only NBQX attenuated ventilation during acute hypoxia in control rats. Our results also show that non-NMDA receptors are involved in the hypercapnic ventilatory drive. Collectively, NBQX seems to depress V_I whenever V_I is greater than normoxic control (i.e. acute hypoxia, acclimatized normoxia, and acute hypercapnia). Therefore, one possible hypothesis is

that non-NMDA receptors are necessary for increased ventilatory drives under all conditions. NMDA receptors only appear to be important for increased hypoxic ventilation after acclimatization although the effects of MK-801 in the NTS on the HCVR have not been studied yet.

A possible future direction would be to study the interaction of NMDA and non-NMDA receptors by microinjecting a cocktail of MK-801 and NBQX into the NTS of normoxic and chronically hypoxic rats. Vardhan (1993) showed that the cocktail completely blocked the phrenic response to carotid body stimulation in anesthetized animals, but that blocking NMDA or non-NMDA receptors alone does not alter the response. If the cocktail does not completely block the HVR in awake control rats, one could test for changes in the effect of combined blockade with chronic hypoxia.

An alternative mechanism might involve metabotropic glutamate receptors. Activation of mGluRs is associated in neurons with stimulation of phospholipase C, which generally causes a rise in intracellular calcium (Schoepp et al. 1990; Miller 1991), and has been linked to synaptic plasticity (McGuinness et al. 1991). Performing whole-cell recordings in regions of the NTS, Glaum (1992) activated mGluRs with the agonist 1S,3R-ACPD and produced excitatory postsynaptic and inhibitory presynaptic effects. Also, mGluR's have been shown to contribute to increased phrenic nerve activity with sustained activation in the retrotrapezoid nucleus (RTN (Li et al. 1995)). Together, these studies suggest a possible role for mGluRs in the NTS, and one future direction would be to microinject an mGluR antagonist into the NTS of chronically hypoxic rats to determine if mGluRs contribute to VAH.

Another possible future direction would be to consider the differences in the roles of AMPA and KA receptors in VAH. While there are presently no pharmacological antagonists to differentiate the two, one initial approach would be to compare the effects of agonists of the two receptors (AMPA and kainate acid) in the presence of NBQX with the effects of the general excitatory amino acid receptor agonist, $_{L}$-glutamate. This proposed study would verify that both AMPA and KA receptors independently contribute to VAH.

Conclusions

In conclusion, our results demonstrate a critical role for non-NMDA receptor plasticity in the NTS with ventilatory acclimatization to hypoxia. Blockade of glutamate-mediated neurotransmission via non-NMDA receptors at the chemoreceptor projection site partially blocked one component of the long term ventilatory response to hypoxia that is necessary for a complete manifestation of VAH. This finding contributes to a better understanding of mechanisms underlying neural control of breathing, and may further play a role in understanding the physiological mechanisms underlying the ventilatory response to long term hypoxia.

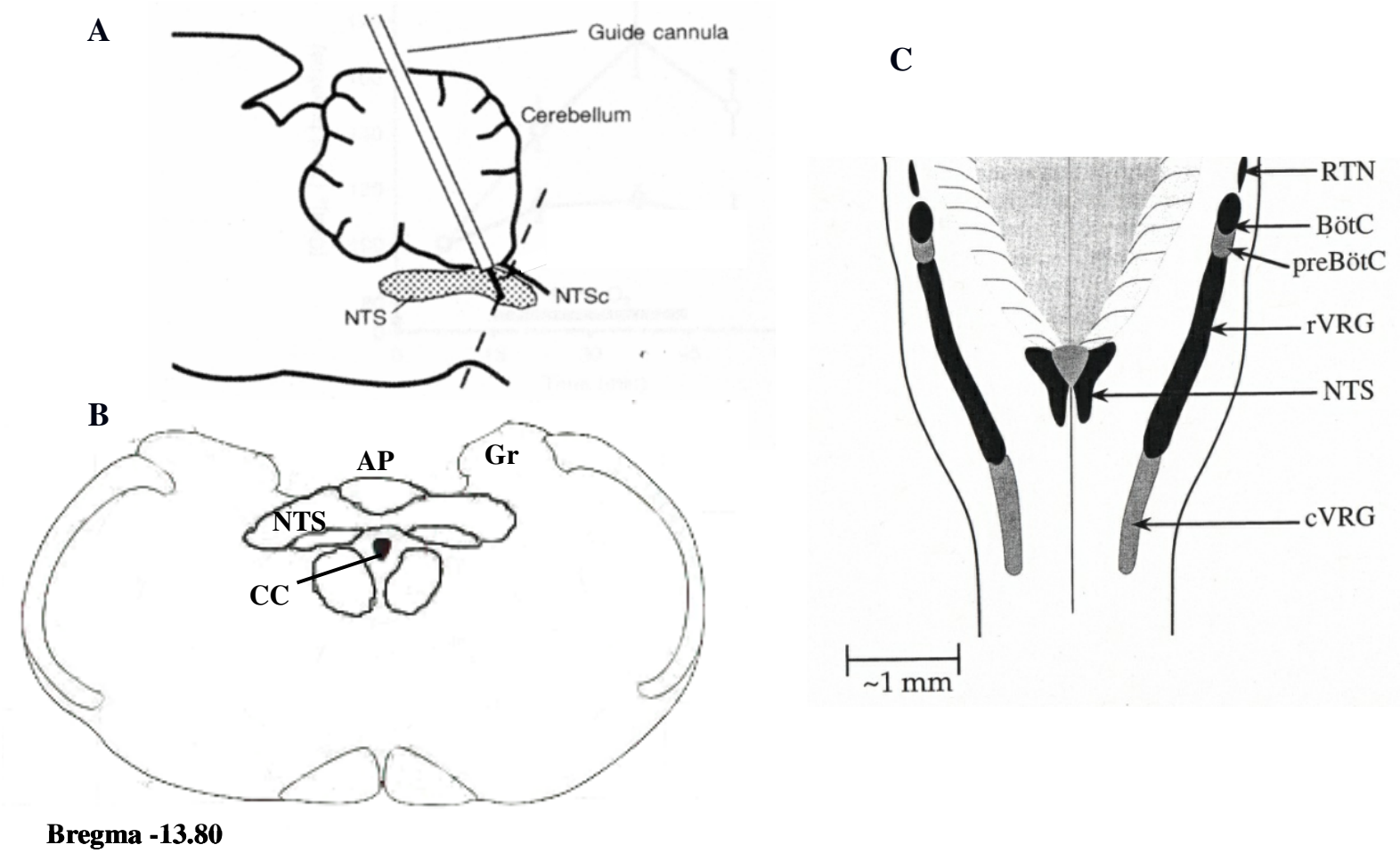

Figure 1. Stereotaxic techniques and locations. **(A)** Lateral View of the brainstem showing the location of the guide cannula. **(B)** Coronal section of the brainstem at 13.80 mm caudal to bregma. NTS = Nucleus of the Solitary Tract; AP = Area Postrema; Gr = Granule Nucleus; CC = Central Canal. **(C)** Dorsal view of the rat brainstem illustrating some respiratory nuclei.

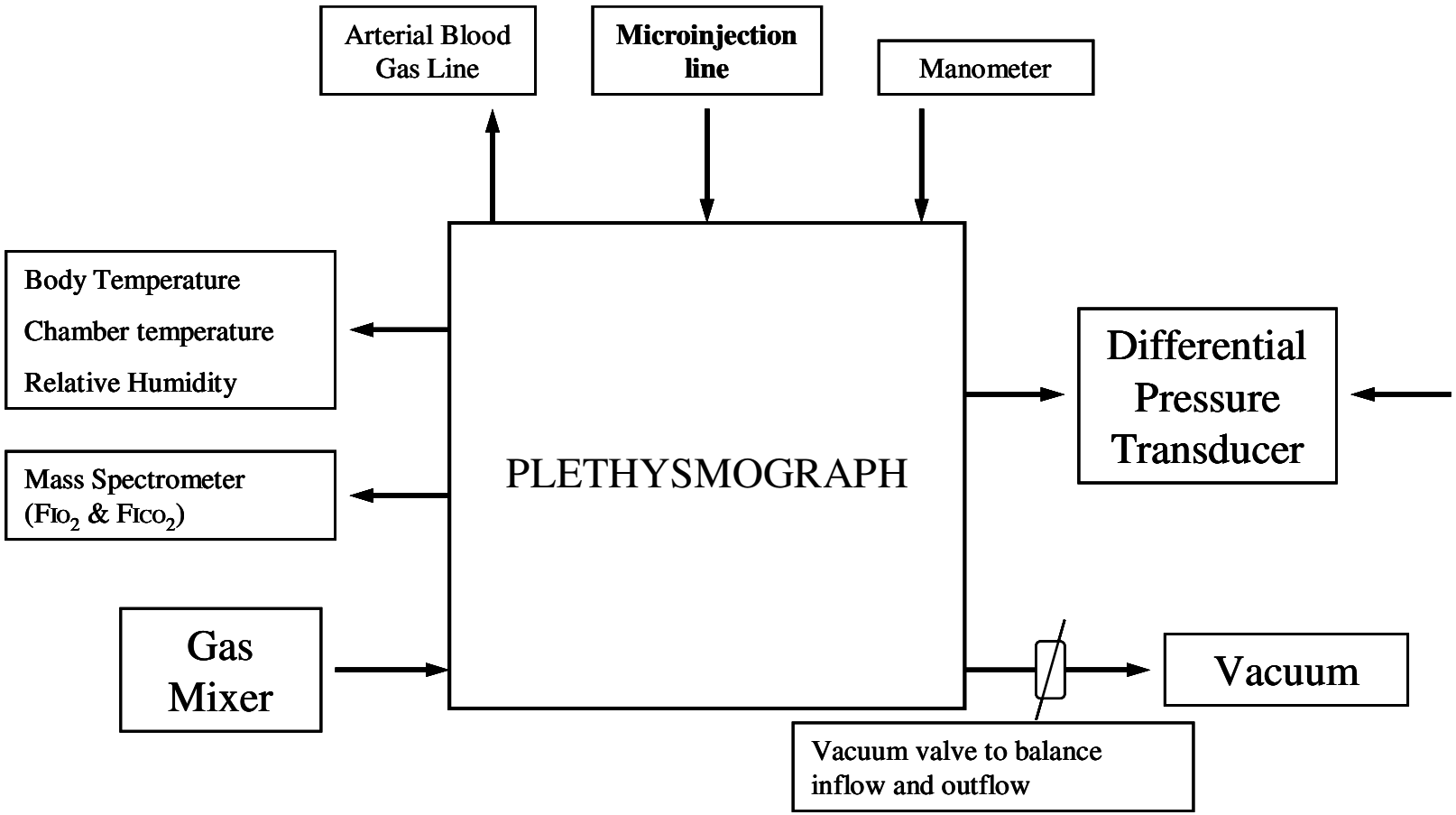

Figure 2. The Plethysmograph and its components.

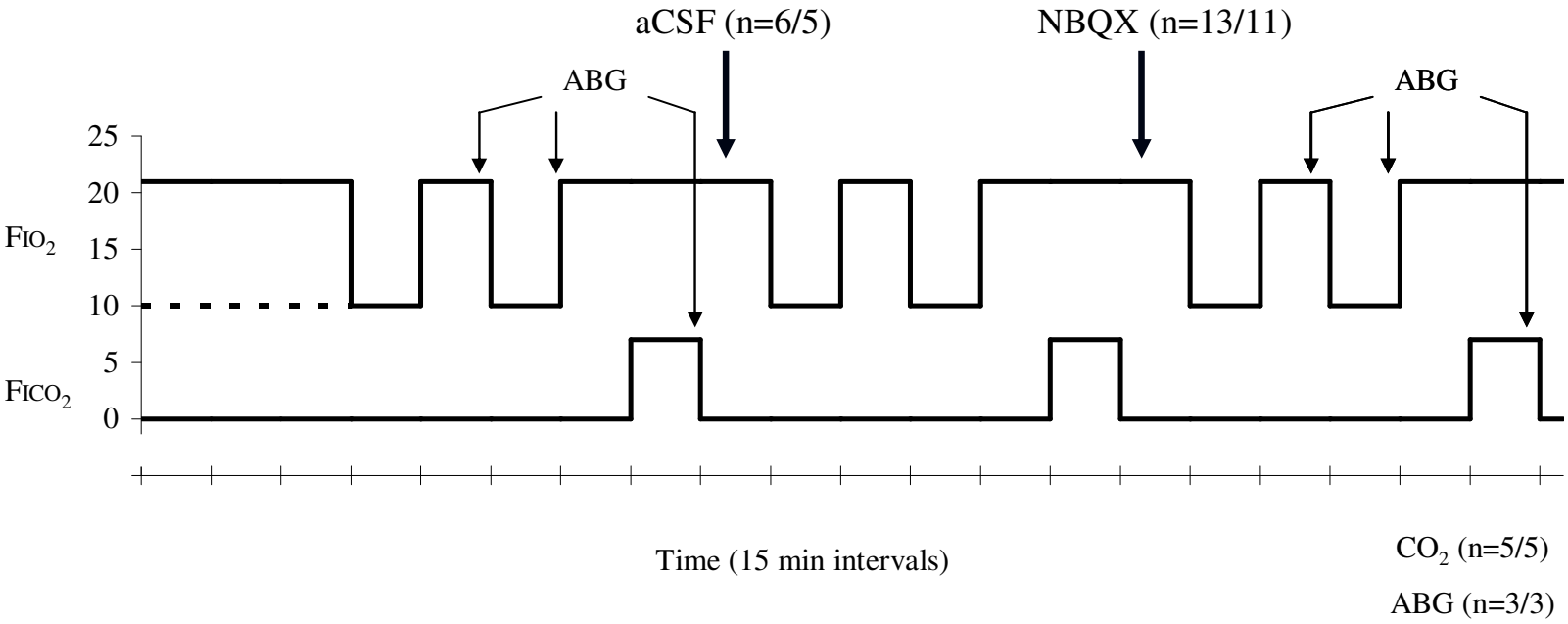

Figure 3. Experimental Protocol. Represents changes in fractions of inspired O_2 and CO_2 during the experiment. Each animal was allowed 40 minutes to habituate to the plethysmograph at their chronic inspired O_2 level (N, solid line; CH, dotted line). Arterial blood gas samples (ABG) were taken at different inspired O_2 and CO_2 levels. Artificial cerebrospinal fluid was microinjected in a subset (n=N/CH) of animals.

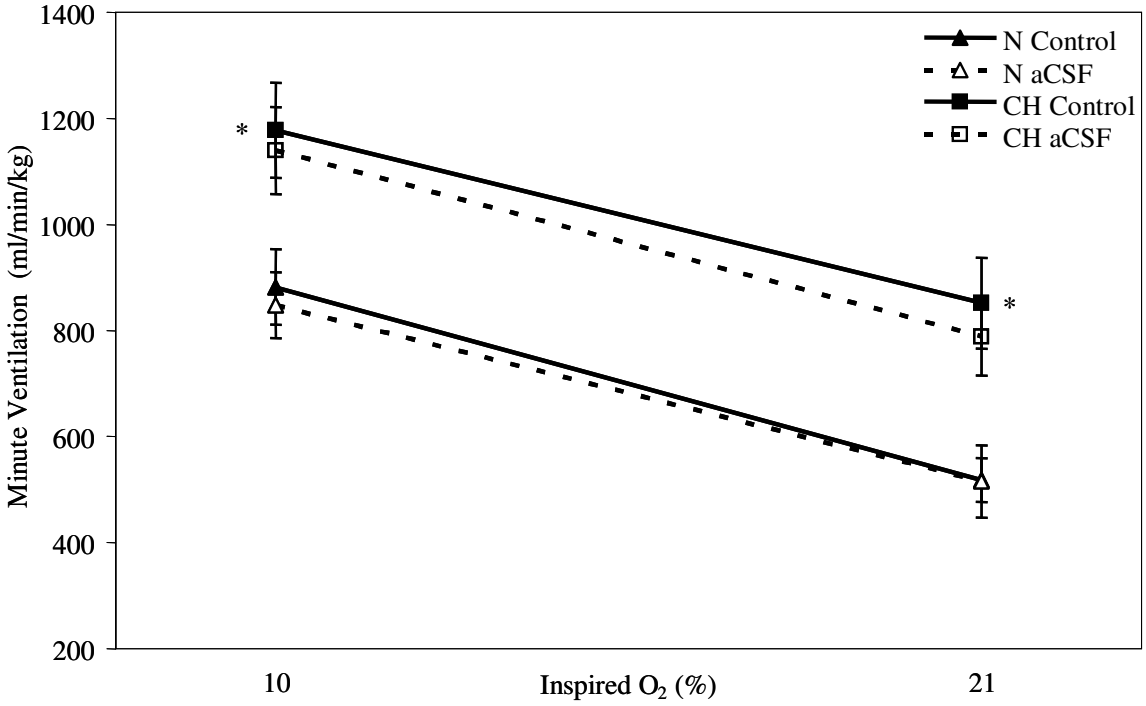

Figure 4. Effect of artificial cerebrospinal fluid (aCSF) on total minute ventilation. Changes in total minute ventilation (V_I) for normoxic control (N, n=6) and chronically hypoxic (CH, n=5) rats before and after microinjection of aCSF. Mean ± SEM. Significance is $P < 0.05$. * = significantly different from normoxic value.

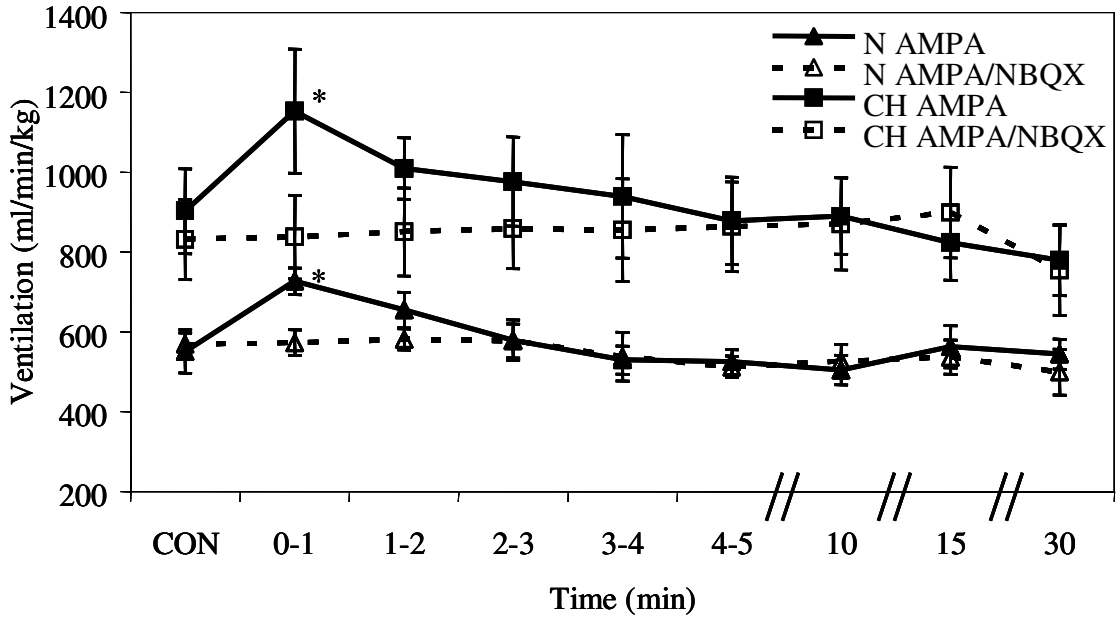

Figure 5. Effects of chronic hypoxia and AMPA in the presence and absence of NBQX on total minute ventilation. Shows the time response to bilateral microinjections of AMPA in the presence (dotted lines) and absence (solid lines) of NBQX for normoxic (N, n=4) and chronically hypoxic (CH, n=6) rats. The time axis is not linear. All measurements were taken in 21% O2. Mean ± SEM. Significance is $P < 0.05$. * = significantly different from "CON" value.

Figure 6A

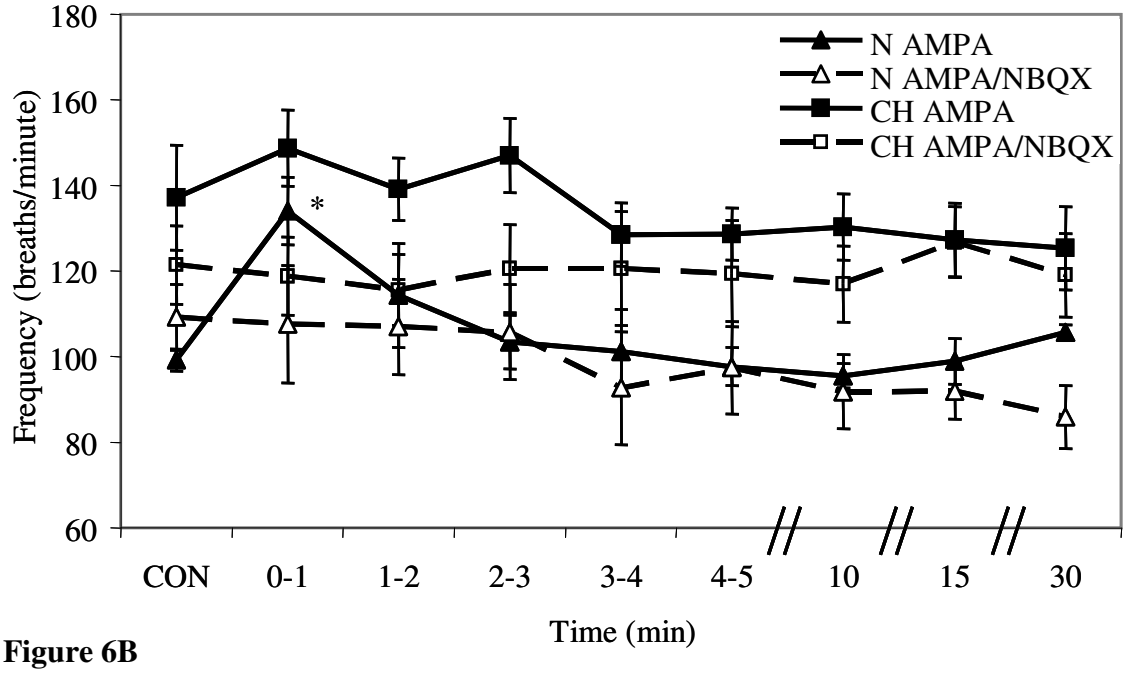

Figure 6B

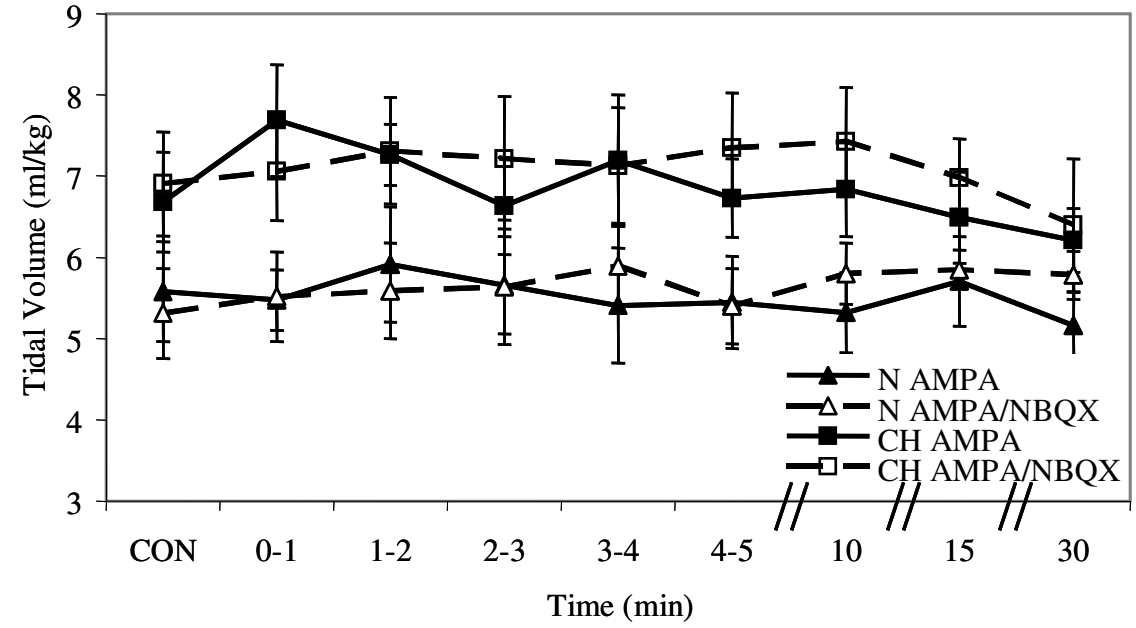

Figure 6. Effects of chronic hypoxia and AMPA in the presence and absence of NBQX on respiratory frequency and tidal volume. **(A)** Changes in the frequency (f_R) in response to AMPA microinjection in the presence (dotted lines) and absence (solid lines) of NBQX for normoxic (N, n=4) and chronically hypoxic (CH, n=6) rats. **(B)** Changes in the tidal volume (V_T) in response to AMPA microinjection in the presence and absence of NBQX. The time axis is not linear. Mean ± SEM. Significance is $P < 0.05$. * = significantly different from "CON" value.

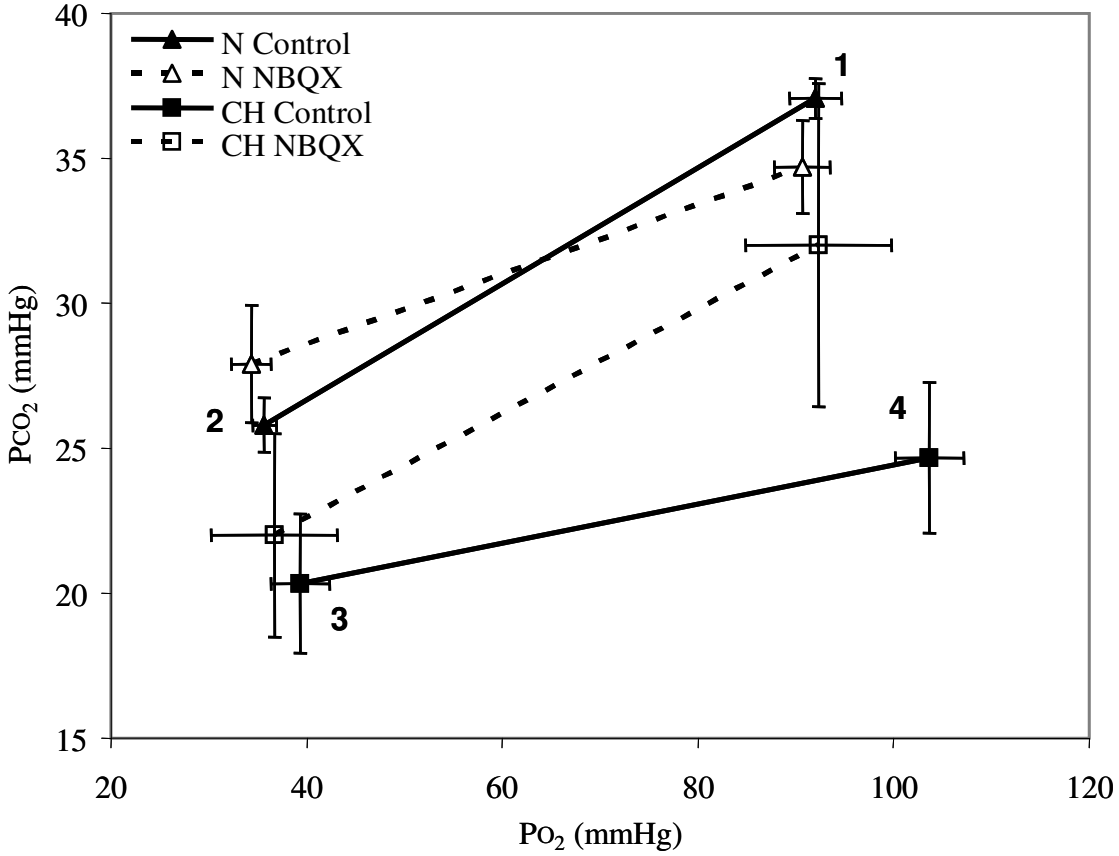

Figure 7. Arterial Blood Gases. Average changes in P_{O_2} and P_{CO_2} in response to microinjection of NBQX into the NTS of normoxic control and chronically hypoxic rats breathing acute levels of 10% and 21% O_2. All blood gases are corrected for body temperature. The numbers in the diagram refer to the following: (1) normoxic rats breathing 21% O_2; (2) normoxic rats breathing 10% O_2; (3) chronically hypoxic rats breathing 10% O_2; (4) chronically hypoxic rats breathing 21% O_2. Mean ± SEM. Significance is $P < 0.05$. * = significantly different from normoxic value.

Figure 8A

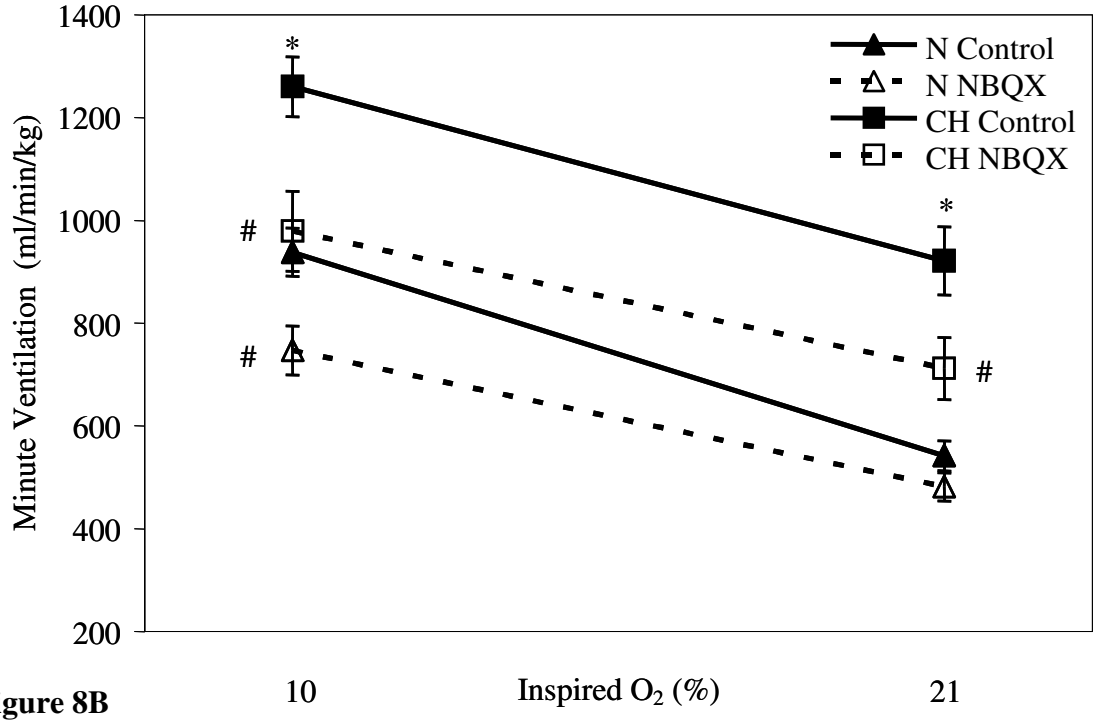

Figure 8B

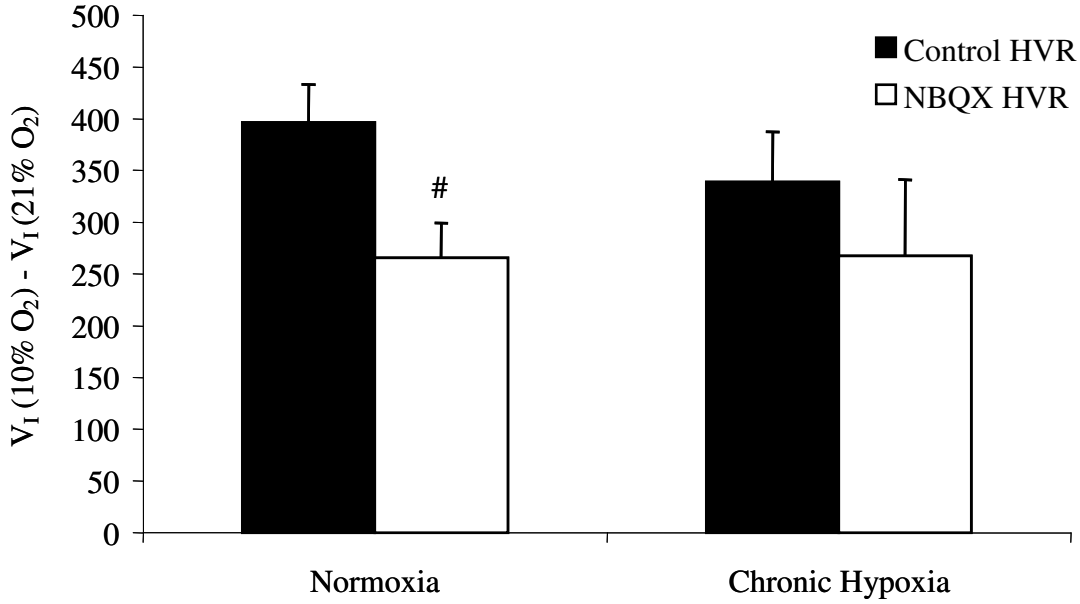

Figure 8. Effects of chronic hypoxia and NBQX on total minute ventilation. **(A)** Changes in total minute ventilation (V_I) for normoxic control (N, n=13) and chronically hypoxic (CH, n=11) rats before and after microinjection of NBQX. **(B)** Effect of chronic hypoxia and NBQX on the magnitude of the hypoxic ventilatory response (HVR). Mean ± SEM. Significance is $P < 0.05$. * = significantly different from normoxic value. $^{\#}$ = significantly different from control value.

Figure 9A

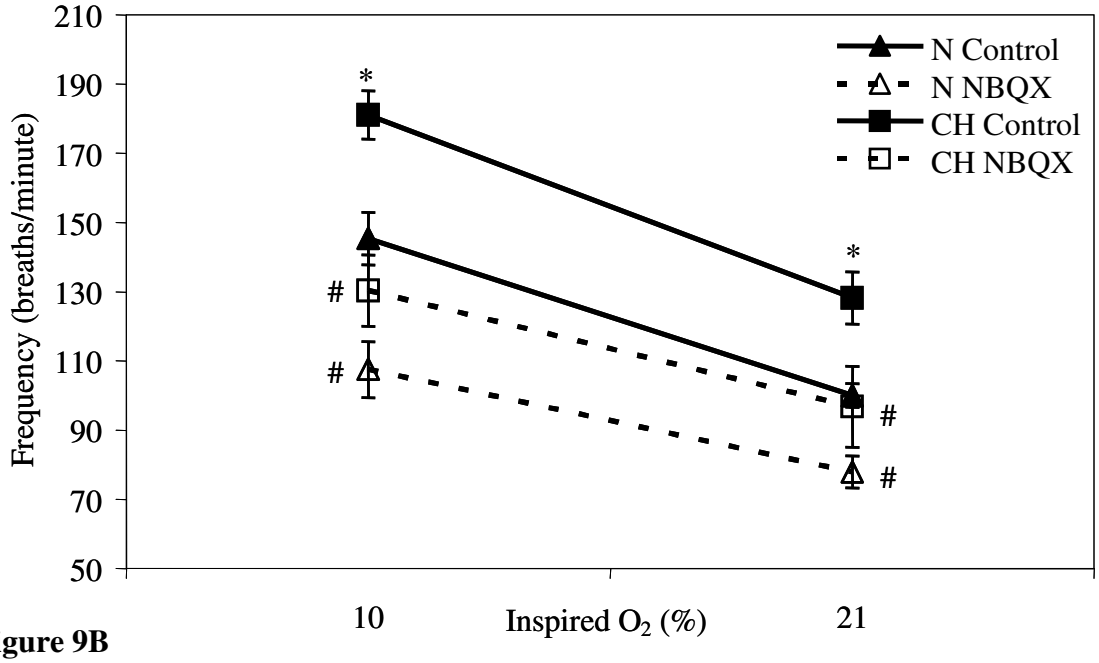

Figure 9B

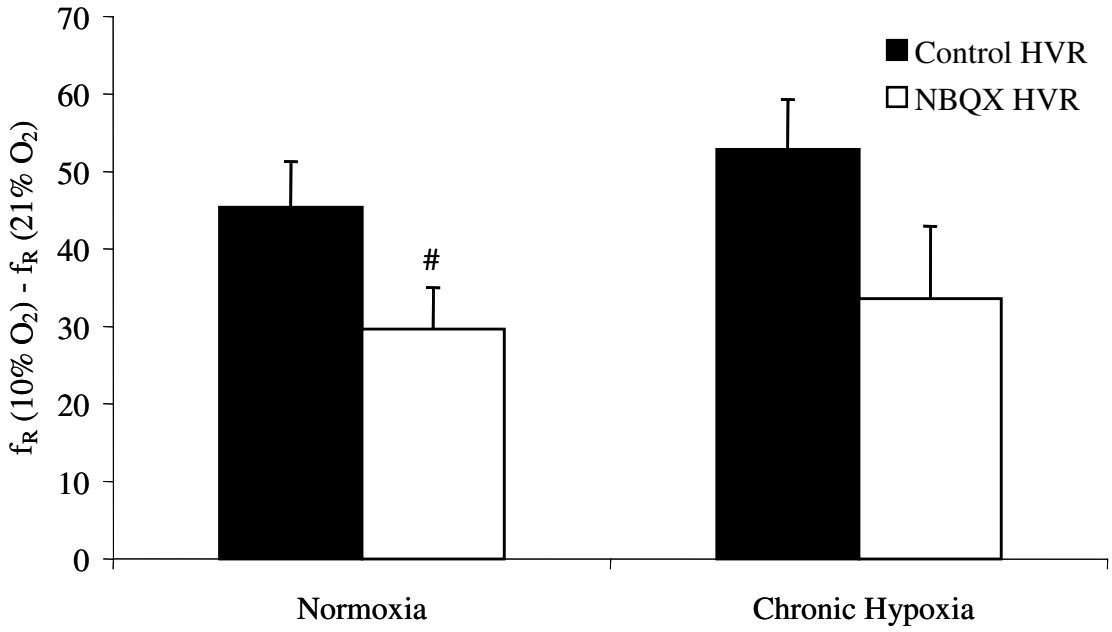

Figure 9. Effects of chronic hypoxia and NBQX on respiratory frequency. **(A)** Changes in the frequency response (f_R) for normoxic control (N, n=13) and chronically hypoxic (CH, n=11) rats before and after microinjection of NBQX. **(B)** Effect of NBQX on the magnitude of the frequency component of the hypoxic ventilatory response (HVR). Mean ± SEM. Significance is $P < 0.05$. * = significantly different from normoxic value. # = significantly different from control value.

Figure 10A

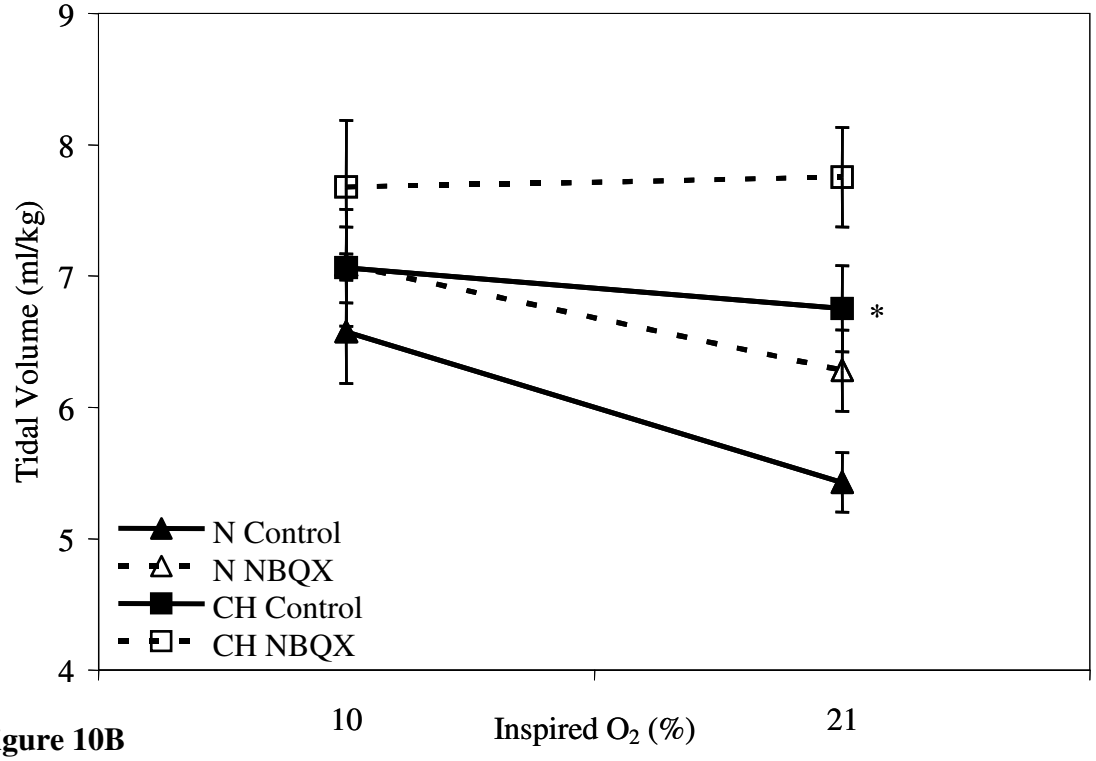

Figure 10B

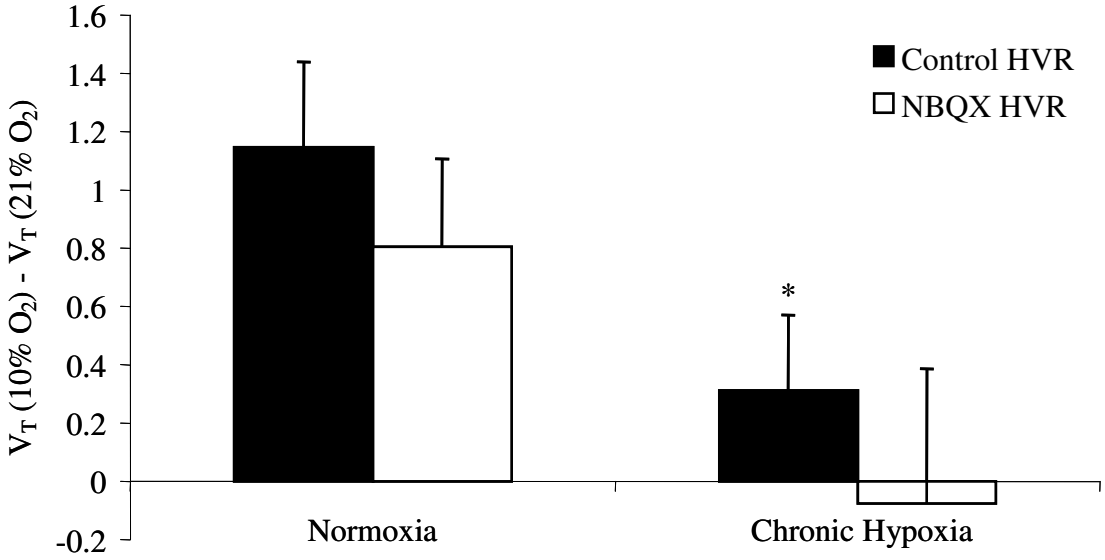

Figure 10. Effects of chronic hypoxia and NBQX on tidal volume. **(A)** Changes in the tidal volume response (V_T) for normoxic control (N, n=13) and chronically hypoxic (CH, n=11) rats before and after microinjection of NBQX. **(B)** Effect of NBQX on the magnitude of the tidal volume component of the hypoxic ventilatory response (HVR). Mean ± SEM. Significance is $P < 0.05$. * = significantly different from normoxic value. # = significantly different from control value.

Figure 11A

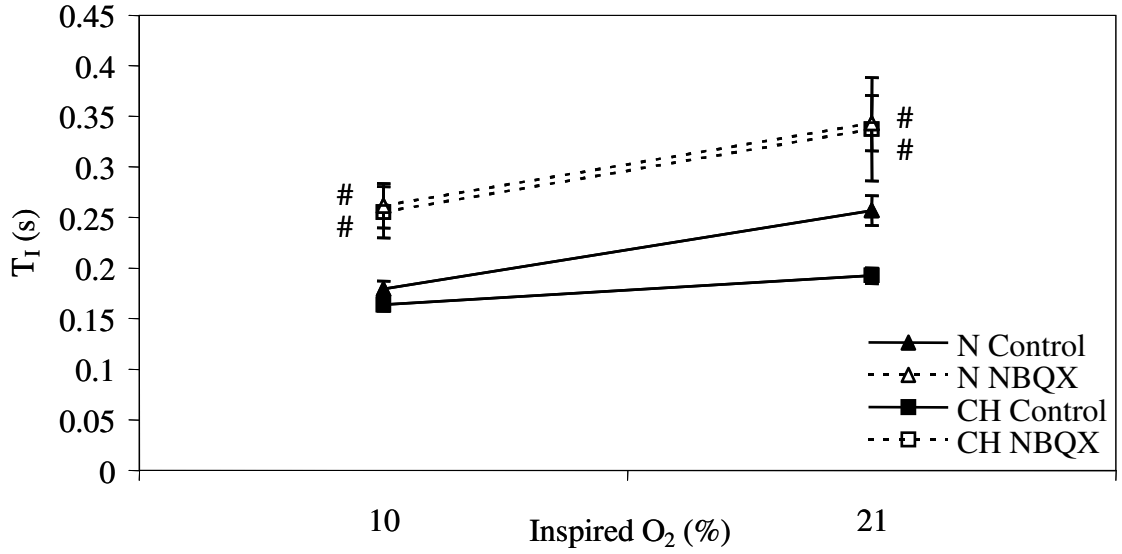

Figure 11B

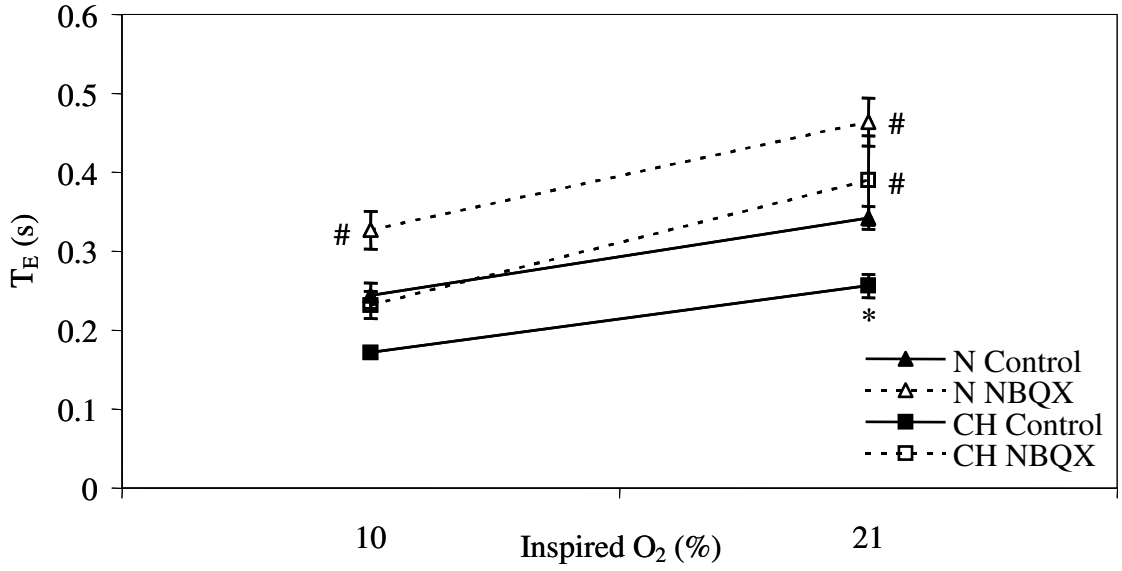

Figure 11. Effects of chronic hypoxia and NBQX on inspiratory time and expiratory time. Changes in the **(A)** inspiratory time response (T_I) and **(B)** expiratory time response (T_E) for normoxic control (N, n=13) and chronically hypoxic (CH, n=11) rats before and after microinjection of NBQX. Mean ± SEM. Significance is $P < 0.05$. * = significantly different from normoxic value. $^{\#}$ = significantly different from control value.

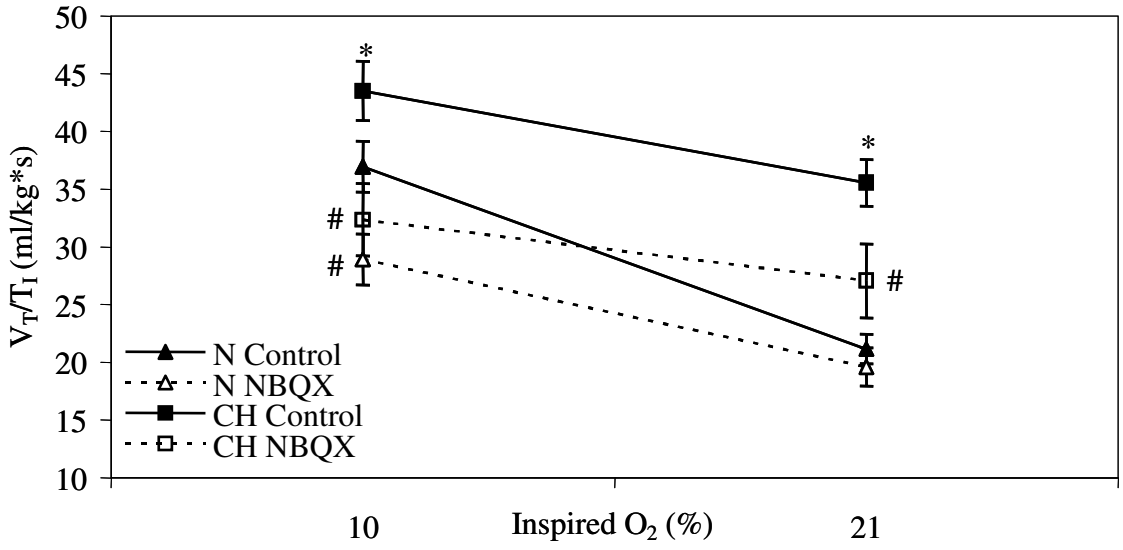

Figure 12. Effects of chronic hypoxia and NBQX on the ventilatory drive to breathe. Changes in the index for the drive to breath (V_T/T_I) for normoxic control (N, n=13) and chronically hypoxic (CH, n=11) rats before and after microinjection of NBQX. Mean ± SEM. Significance is $P < 0.05$. * = significantly different from normoxic value. $^{\#}$ = significantly different from control value.

Figure 13A

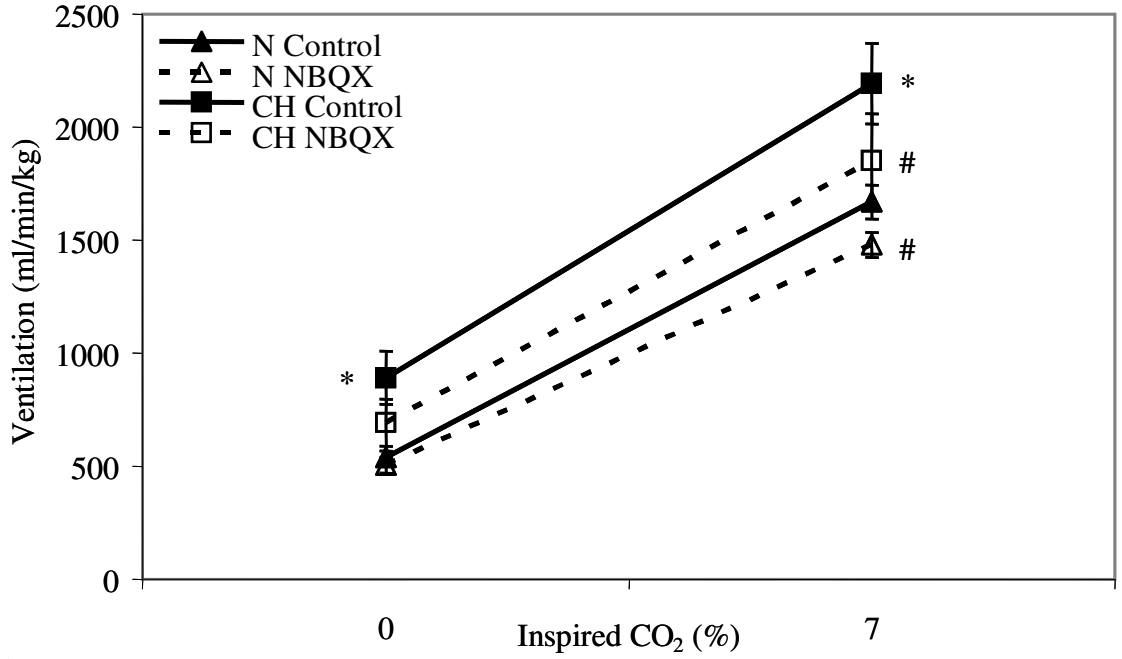

Figure 13B

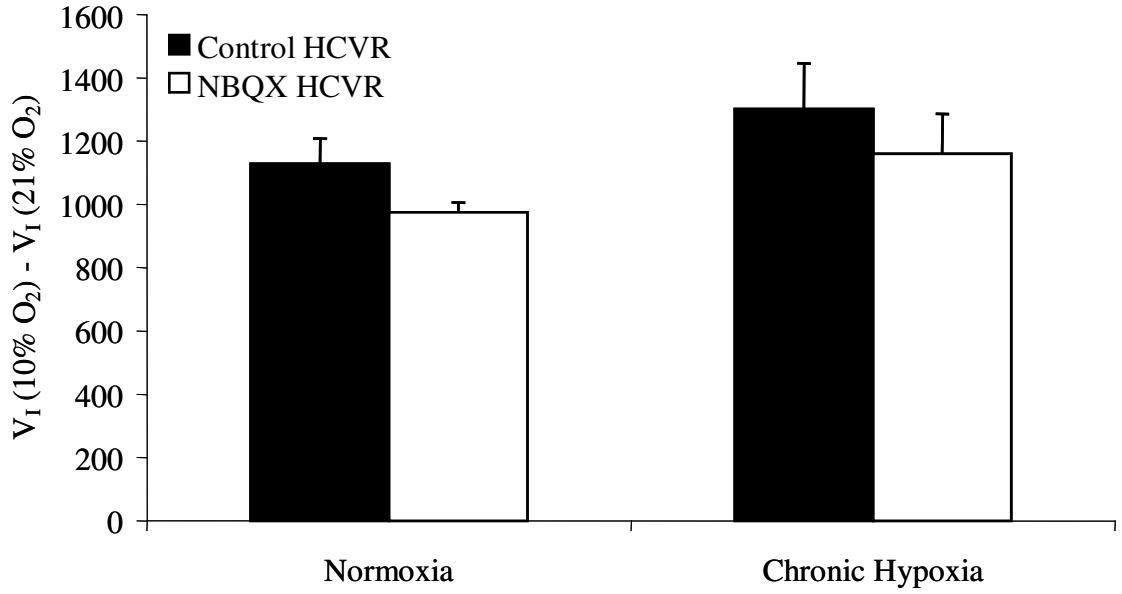

Figure 13. Effects of chronic hypoxia and NBQX on total minute ventilation in hypercapnia. **(A)** Changes in total minute ventilation (V_I) in hypercapnia for normoxic control (N, n=5) and chronically hypoxic (CH, n=5) rats before and after microinjection of NBQX. **(B)** Effect of NBQX on the magnitude of the ventilatory response to hypercapnia (HCVR). Mean ± SEM. Significance is $P < 0.05$. * = significantly different from normoxic value. $^{\#}$ = significantly different from control value.

Figure 14A

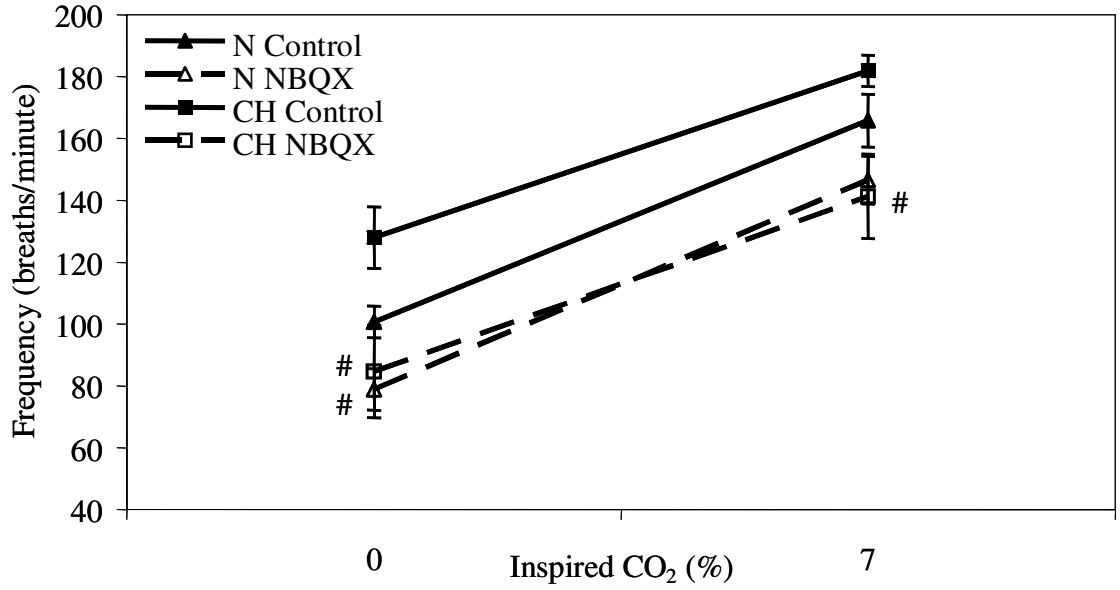

Figure 14B

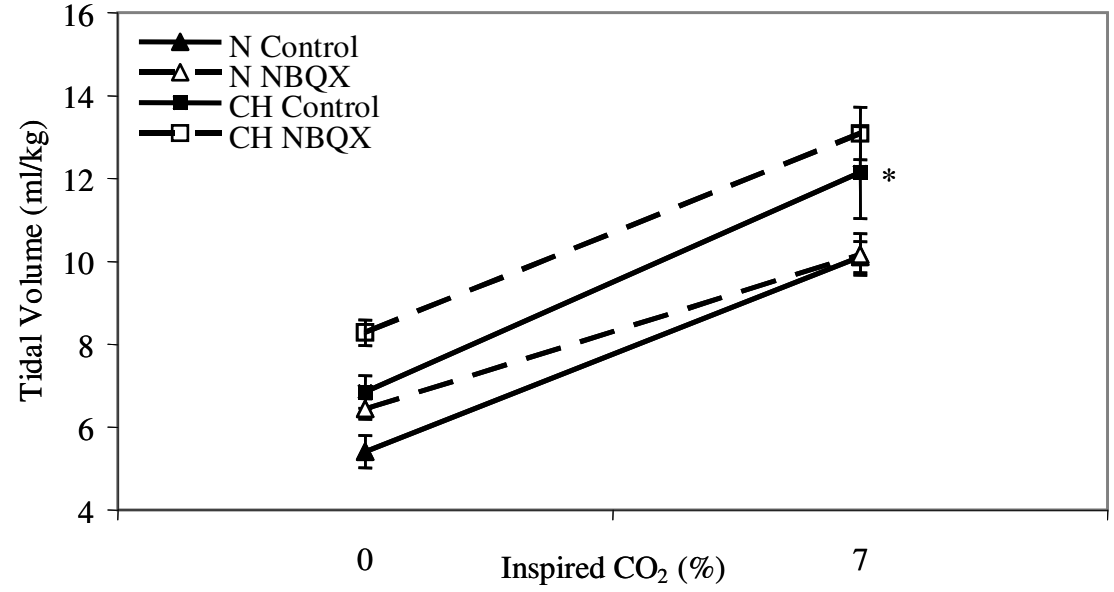

Figure 14. Effects of chronic hypoxia and NBQX on respiratory frequency and tidal volume in hypercapnia. Changes in **(A)** frequency response (f_R) and **(B)** tidal volume (V_T) in hypercapnia for normoxic control (N, n=5) and chronically hypoxic (CH, n=5) rats before and after microinjection of NBQX. Mean ± SEM. Significance is $P < 0.05$. * = significantly different from normoxic value. # = significantly different from control value.

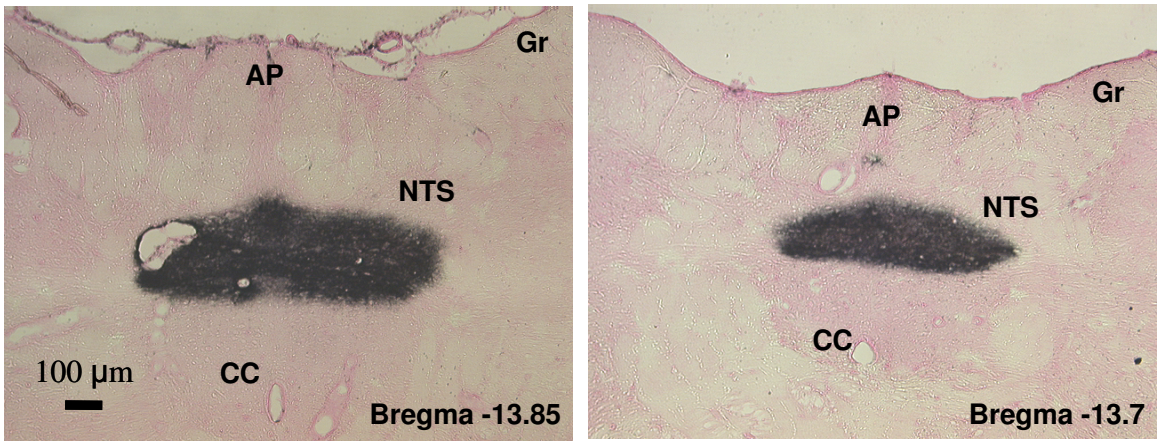

Figure 15. Localization of the microinjection site with colloidal gold. Shows a coronal section of the brainstem of two different rats, representative of the group. The micrograph shows the sites of successful microinjection with colloidal gold in the caudal region of the NTS. Aqueous Eosin Y is used as a cytoplasmic counterstain. Magnification to 6.3x.

V. REFERENCES

Aaron, E., Powell, F., 1993. Effect of chronic hypoxia on hypoxic ventilatory response in awake rats. J Appl Physiol 74, 1635-1640.

Almado, C., Machado, B., 2005. Respiratory and autonomic responses to microinjection of NMDA and AMPA into the commissural subnucleus of the NTS of awake rats. Brain Res 1063, 59-68.

Ang, R., Hoop, B., Kazemi, H., 1992. Role of glutamate as the central neurotransmitter in the hypoxic ventilatory response. J Appl Physiol 72, 1480-1487.

Barnard, P., Andronikou, S., Pokorski, M., Smatresk, N., Mokashi, A., Lahiri, S., 1987. Time-dependent effect of hypoxia on carotid body chemosensory function. J Appl Physiol 63, 685-691.

Berger, A., Cooney, K., 1982. Ventilatory effects of kainic acid injection of the ventrolateral solitary nucleus. J Appl Physiol 52, 131-140.

Bisgard, G., Neubauer, J., 1995. Peripheral and central effects of hypoxia in the control of breathing. In: Dempsey, J.A., Pack, A.I. (Ed.) Regulation of Breathing, vol. 79. Marcel Dekker, New York, pp. 617-668

Bliss, T., Collingridge, G., 1993. A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361, 31-39.

Bonham, A., 1995. Neurotransmitters in the CNS control of breathing. Respir Physiol 101, 219-230.

Borday, V., Foutz, A., Nordholm, L., Denavit-Saubié, M., 1998. Respiratory effects of glutamate receptor antagonists in neonate and adult mammals. Eur J Pharmacol 348, 235-246.

Bouverot, P., 1985. Adaptation to altitude-hypoxia in vertebrates. Springer-Verlag, New York.

Cheng, Z., Guo, S., Lipton, A., Gozal, D., 2002. Domoic acid lesions in nucleus of the solitary tract: time-dependent recovery of hypoxic ventilatory response and peripheral afferent axonal plasticity. J Neurosci 22, 3215-3226.

Chung, S., Ivy, G., Reid, S., 2006. GABA-mediated neurotransmission in the nucleus of the solitary tract alters resting ventilation following exposure to chronic hypoxia in conscious rats. Am J Physiol Regul Integr Comp Physiol 291, R1449-1456.

Coates, E., Li, A., Nattie, E., 1993. Widespread sites of brain stem ventilatory chemoreceptors. J Appl Physiol 75, 5-14.

Dean, J., Lawing, W., Millhorn, D., 1989. CO2 decreases membrane conductance and depolarizes neurons in the nucleus tractus solitarii. Exp Brain Res 76, 656-661.

Dempsey, J., Forster, H., 1982. Mediation of Ventilatory Adaptations. Physiol Rev 62, 262-346.

Donoghue, S., Felder, R., Jordan, D., Spyer, K., 1984. The central projections of carotid baroreceptors and chemoreceptors in the cat: a neurophysiological study. J Physiol 347, 397-409.

Drorbaugh, J., Fenn, W., 1955. A barometric method for measuring ventilation in newborn infants. Pediatrics 16, 81-87.

Dwinell, M., Huey, K., Powell, F., 2000. Chronic hypoxia induces changes in the central nervous system processing of arterial chemoreceptor input. Adv Exp Med Biol 475, 477-484.

Dwinell, M., Powell, F., 1999. Chronic hypoxia enhances the phrenic nerve response to arterial chemoreceptor stimulation in anesthetized rats. J Appl Physiol 87, 817-823.

Eden, G., Hanson, M., 1987. Maturation of the respiratory response to acute hypoxia in the newborn rat. J Physiol 392, 1-9.

Finley, J., Katz, D., 1992. The central organization of carotid body afferent projections to the brainstem of the rat. Brain Res 572, 108-116.

Gasic, G., Hollmann, M., 1992. Molecular neurobiology of glutamate receptors. Annu Rev Physiol 54, 507-536.

Glaum, S., Miller, R., 1992. Metabotropic glutamate receptors mediate excitatory transmission in the nucleus of the solitary tract. J Neurosci 12, 2251-2258.

Gozal, D., Gozal, E., Simakajornboon, N., 2000. Signaling pathways of the acute hypoxic ventilatory response in the nucleus tractus solitarius. Respir Physiol 121, 209-221.

Housley, G., Martin-Body, R., Dawson, N., Sinclair, J., 1987. Brain stem projections of the glossopharyngeal nerve and its carotid sinus branch in the rat. Neuroscience 22, 237-250.

Housley, G., Sinclair, J., 1988. Localization by kainic acid lesions of neurones transmitting the carotid chemoreceptor stimulus for respiration in rat. J Physiol 406, 99-114.

Isenovic, E., Radulovacki, M., Carley, D., 2007. Impact of intertrigeminal region AMPA receptor blockade on respiratory responses in rats. Respir Physiol Neurobiol 158, 39-44.

Jacky, J., 1978. A plethysmograph for long-term measurements of ventilation in unrestrained animals. J Appl Physiol 45, 644-647.

Kessler, J., Baude, A., 1999. Distribution of AMPA receptor subunits GluR1-4 in the dorsal vagal complex of the rat: a light and electron microscope immunocytochemical study. Synapse 34, 55-67.

Kessler, M., Baudry, M., Lynch, G., 1989. Quinoxaline derivatives are high-affinity antagonists of the NMDA receptor-associated glycine sites. Brain Res 489, 377-382.

Li, A., Nattie, E., 1995. Prolonged stimulation of respiration by brain stem metabotropic glutamate receptors. J Appl Physiol 79, 1650-1656.

Lin, J., Suguihara, C., Huang, J., Hehre, D., Devia, C., Bancalari, E., 1996. Effect of N-methyl-D-aspartate-receptor blockade on hypoxic ventilatory response in unanesthetized piglets. J Appl Physiol 80, 1759-1763.

Machado, B., Bonagamba, L., 1992. Microinjection of L-glutamate into the nucleus tractus solitarii increases arterial pressure in conscious rats. Brain Res 576, 131-138.

McCrimmon, D., Mitchell, G., Dekin, M., 1995. Glutamate, GABA, and Serotonin in Ventilatory Control. In: Dempsey, J., Pack, A. (Eds.), Regulation of Breathing, vol. 79. Marcel Dekker, New York, pp. 151-218

McGuinness, N., Anwyl, R., Rowan, M., 1991. Trans-ACPD enhances long-term potentiation in the hippocampus. Eur J Pharmacol 197, 231-232.

Miller, B., Felder, R., 1988. Excitatory amino acid receptors intrinsic to synaptic transmission in nucleus tractus solitarii. Brain Res 456, 333-343.

Miller, R., 1991. Metabotropic excitatory amino acid receptors reveal their true colors. Trends Pharmacol Sci 12, 365-367.

Mitchell, G., Johnson, S., 2003. Neuroplasticity in respiratory motor control. J Appl Physiol 94, 358-374.

Mizusawa, A., Ogawa, H., Kikuchi, Y., Hida, W., Kurosawa, H., Okabe, S., Takishima, T., Shirato, K., 1994. In vivo release of glutamate in nucleus tractus solitarii of the rat during hypoxia. J Physiol 478 (Pt 1), 55-66.

Monaghan, D., Cotman, C., 1985. Distribution of N-methyl-D-aspartate-sensitive L-[3H]glutamate-binding sites in rat brain. J Neurosci 5, 2909-2919.

Mulle, C., Sailer, A., Swanson, G., Brana, C., O'Gorman, S., Bettler, B., Heinemann, S., 2000. Subunit composition of kainate receptors in hippocampal interneurons. Neuron 28, 475-484.

Nakanishi, S., 1994. Metabotropic glutamate receptors: synaptic transmission, modulation, and plasticity. Neuron 13, 1031-1037.

Nattie, E., Li, A., 2008. Central chemoreception is a complex system function that involves multiple brainstem sites. J Appl Physiol.

Nielsen, A., Bisgard, G., Vidruk, E., 1988. Carotid chemoreceptor activity during acute and sustained hypoxia in goats. J Appl Physiol 65, 1796-1802.

Ohtake, P., Torres, J., Gozal, Y., Graff, G., Gozal, D., 1998. NMDA receptors mediate peripheral chemoreceptor afferent input in the conscious rat. J Appl Physiol 84, 853-861.

Olson, E.J., Dempsey, J., 1978. Rat as a model for humanlike ventilatory adaptation to chronic hypoxia. J Appl Physiol 44, 763-769.

Olson, E.J., Vidruk, E., Dempsey, J., 1988. Carotid body excision significantly changes ventilatory control in awake rats. J Appl Physiol 64, 666-671.

Paxinos, G., Watson, C., 1986. The Rat Brain in Stereotaxic Coordinates. Academic Press, Inc., San Diego.

Powell, F., 2007. The influence of chronic hypoxia upon chemoreception. Respir Physiol Neurobiol 157, 154-161.

Powell, F., Huey, K., Dwinell, M., 2000. Central nervous system mechanisms of ventilatory acclimatization to hypoxia. Respir Physiol 121, 223-236.

Powell, F., Milsom, W., Mitchell, G., 1998. Time domains of the hypoxic ventilatory response. Respir Physiol 112, 123-134.

Prabhakar, N., Jacono, F., 2005. Cellular and molecular mechanisms associated with carotid body adaptations to chronic hypoxia. High Alt Med Biol 6, 112-120.

Rahn, H., Otis, A., 1949. Man's respiratory response during and after acclimatization to high altitude. Am J Physiol 157, 445-462.

Reid, S., Powell, F., 2005. Effects of chronic hypoxia on MK-801-induced changes in the acute hypoxic ventilatory response. J Appl Physiol 99, 2108-2114.

Reis, D., Granata, A., Perrone, M., Talman, W., 1981. Evidence that glutamic acid is the neurotransmitter of baroreceptor afferent terminating in the nucleus tractus solitarius (NTS). J Auton Nerv Syst 3, 321-334.

Schoepp, D., Bockaert, J., Sladeczek, F., 1990. Pharmacological and functional characteristics of metabotropic excitatory amino acid receptors. Trends Pharmacol Sci 11, 508-515.

Smith, A.S., Dempsey, J.A., Hornbein, T.F., 2001. Control of Breathing at High Altitude. In: Hornbein, T.F., Schoene, R.B. (Eds.), High Altitude, vol. 161. Marcel Dekker, New York, pp. 139-173

Talman, W., Perrone, M., Reis, D., 1981. Acute hypertension after the local injection of kainic acid into the nucleus tractus solitarii of rats. Circ Res 48, 292-298.

Tansley, J., Fatemian, M., Howard, L., Poulin, M., Robbins, P., 1998. Changes in respiratory control during and after 48 h of isocapnic and poikilocapnic hypoxia in humans. J Appl Physiol 85, 2125-2134.

Vardhan, A., Kachroo, A., Sapru, H., 1993. Excitatory amino acid receptors in commissural nucleus of the NTS mediate carotid chemoreceptor responses. Am J Physiol 264, R41-50.

Wang, Z., Dinger, B., Stensaas, L., Fidone, S., 1995. The role of nitric oxide in carotid chemoreception. Biol Signals 4, 109-116.

Watkins, J., Evans, R., 1981. Excitatory amino acid transmitters. Annu Rev Pharmacol Toxicol 21, 165-204.

Weil, J., 1986. Ventilatory control at high altitude. In: Cherniack, N., Widdicome, J. (Eds.), Handbook of Physiology, Sec. 3, vol. II. American Physiological Society, Bethesda, MD, pp. 703-727

Weil, J., Zwillich, C., 1976. Assessment of ventilatory response to hypoxia: methods and interpretation. Chest 70, 124-128.

Whitney, G., Ohtake, P., Simakajornboon, N., Xue, Y., Gozal, D., 2000. AMPA glutamate receptors and respiratory control in the developing rat: anatomic and pharmacological aspects. Am J Physiol Regul Integr Comp Physiol 278, R520-528.

Youssef, F., Addae, J., McRae, A., Stone, T., 2001. Long-term potentiation protects rat hippocampal slices from the effects of acute hypoxia. Brain Res 907, 144-150.