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

Author
Carr, John Austin

Publication Date
2008

Peer reviewed|Thesis/dissertation
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

2008
The Thesis of John Austin Carr is approved and it is acceptable in quality and form for publication on microfilm and electronically:

___________________________________________________

___________________________________________________

Co-Chair

___________________________________________________

Co-Chair

University of California, San Diego

2008
For my parents,
I love you.
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>aCSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AMPA</td>
<td>alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>CH</td>
<td>chronic hypoxia</td>
</tr>
<tr>
<td>CSN</td>
<td>carotid sinus nerve</td>
</tr>
<tr>
<td>HCVR</td>
<td>hypercapnic ventilatory response</td>
</tr>
<tr>
<td>HVR</td>
<td>hypoxic ventilatory response</td>
</tr>
<tr>
<td>iGluR</td>
<td>ionotropic glutamate receptor</td>
</tr>
<tr>
<td>KA</td>
<td>kainic acid</td>
</tr>
<tr>
<td>mGluR</td>
<td>metabotropic glutamate receptor</td>
</tr>
<tr>
<td>N</td>
<td>normoxia (sea level controls)</td>
</tr>
<tr>
<td>NBQX</td>
<td>2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[f]quinoxaline-2,3-dione</td>
</tr>
<tr>
<td>NMDA receptor</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>Non-NMDA receptor</td>
<td>AMPA and kainate receptors</td>
</tr>
<tr>
<td>NTS</td>
<td>nucleus of the solitary tract</td>
</tr>
<tr>
<td>VAH</td>
<td>ventilatory acclimatization to hypoxia</td>
</tr>
</tbody>
</table>
## LIST OF SYMBOLS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>FICO₂</td>
<td>fraction of inspired CO₂</td>
</tr>
<tr>
<td>FIO₂</td>
<td>fraction of inspired O₂</td>
</tr>
<tr>
<td>f₉</td>
<td>respiratory frequency</td>
</tr>
<tr>
<td>N₂</td>
<td>nitrogen</td>
</tr>
<tr>
<td>O₂</td>
<td>oxygen</td>
</tr>
<tr>
<td>Pao₂</td>
<td>arterial partial pressure of O₂</td>
</tr>
<tr>
<td>Paco₂</td>
<td>arterial partial pressure of CO₂</td>
</tr>
<tr>
<td>P₉</td>
<td>barometric pressure</td>
</tr>
<tr>
<td>Pco₂</td>
<td>partial pressure of CO₂</td>
</tr>
<tr>
<td>PO₂</td>
<td>partial pressure of O₂</td>
</tr>
<tr>
<td>PIIO₂</td>
<td>partial pressure of inspired O₂</td>
</tr>
<tr>
<td>Tₐ</td>
<td>expiratory time</td>
</tr>
<tr>
<td>T₁</td>
<td>inspiratory time</td>
</tr>
<tr>
<td>VI</td>
<td>total inspired minute ventilation</td>
</tr>
<tr>
<td>VT</td>
<td>tidal volume</td>
</tr>
<tr>
<td>VT/T₁</td>
<td>index of the ventilatory drive to breath</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1. Stereotaxic techniques and locations ........................................................ 28
Figure 2. The Plethysmograph and its components ................................................. 29
Figure 3. Experimental Protocol .............................................................................. 30
Figure 4. Effect of artificial cerebrospinal fluid (aCSF) on total minute ventilation ................................................................................................. 31
Figure 5. Effects of chronic hypoxia and AMPA in the presence and absence of NBQX on total minute ventilation........................................................ 32
Figure 6. Effects of chronic hypoxia and AMPA in the presence and absence of NBQX on respiratory frequency and tidal volume......................... 33
Figure 7. Arterial Blood Gasses ............................................................................... 34
Figure 8. Effects of chronic hypoxia and NBQX on total minute ventilation............ 35
Figure 9. Effects of chronic hypoxia and NBQX on respiratory frequency.......... 36
Figure 10. Effects of chronic hypoxia and NBQX on tidal volume......................... 37
Figure 11. Effects of chronic hypoxia and NBQX on inspiratory time and expiratory time.......................................................................................... 38
Figure 12. Effects of chronic hypoxia and NBQX on the ventilatory drive to breathe........................................................................................................ 39
Figure 13. Effects of chronic hypoxia and NBQX on total minute ventilation in hypercapnia........................................................ 40
Figure 14. Effects of chronic hypoxia and NBQX on respiratory frequency and tidal volume in hypercapnia........................................................ 41
Figure 15. Localization of the microinjection site with colloidal gold ................. 42
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my parents for their unconditional support in my endeavors, and for providing me with every opportunity to succeed at UCSD. The values that you have instilled in me have opened many different doors to success. You believe in me sometime more than I believe in myself, and for that I am truly grateful. I could not have accomplished this without your love and support. I love you.

Kevin, Tyson and Kellen. You have been more a part of my life away from home than you may think. Thank you for your always being supportive and for dedicating your time to being at special events for me.

I want to express many thanks to Frank Powell for giving me the opportunity to not only complete a Master’s thesis in his lab, but also for the opportunity to even join his lab as an undergraduate student. You have taught me not only about science, but more importantly how science works in today’s world and at the next level. I am also grateful for the trust that you had in me to take off with this project.

I am indebted to Zhenxing Fu for all of her help over the last few years that really made this project possible. She taught me everything that I have needed to know from surgical preparations to special “tricks” to make data collection easier. Zhenxing has the special surgical touch that I tried so hard to find. Her help with the experiments, and guidance through all of the techniques necessary for doing the experiments was truly appreciated.
I also owe many thanks to Katie Wilkinson. I first started in the lab helping her with some experiments, and she has taught me so much science and the way in which it is practiced and presented today. I have truly enjoyed working with Katie and the many long days that we have spent doing back to back experiments.

I would like to thank every member of the Powell lab (and honorary friends of the Powell lab) for making for a truly enjoyable experience. The lab was a fun environment to work in and I learned more from the experience than I could have ever imagined. Many thanks to Trevor Cooper and Jeff Struthers for always making sure that the equipment was running properly and that there were no major problems.

This experience began many miles away from San Diego and the UCSD campus. It began for me in Mammoth Lakes in discussion with Dan and Leslie Dawson, who recommended that I introduce myself to Frank Powell. I would like to thank both Dan and Leslie for their recommendation which has opened the doors to so much more.

Special thanks to Dr. Nick Spitzer and Dr. Kathy French for being exceptionally willing to invest an interest in my project. I am truly thankful for your input and your ability to make this project the best that it could be. I have enjoyed working with Kathy in a classroom setting as well and am grateful to her for that opportunity.

Finally, I want to thank all of my friends that I have been so lucky to meet at UCSD. My experiences and memories at this university were made special because of the people that I have been able to experience them with. It would be impossible to meet a better group of people have spent that last few years with. I am especially thankful to Evie Rodriguez for dedicating so much of her time to help me through the writing phase. The last three months would have been so much longer without her company.
ABSTRACT OF THE THESIS

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

by

John Austin Carr

Master of Science in Biology

University of California, San Diego, 2008

Professor Frank L. Powell, Co-Chair
Professor Nicholas Spitzer, Co-Chair

Ventilatory acclimatization to hypoxia (VAH) increases both (1) the hypoxic ventilatory response (HVR) to acute decreases in arterial Po2, and (2) normoxic ventilatory drive when inspired O2 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\text{\textsubscript{O}}_2 = 70 \text{ 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% \text{O}_2), normoxia (21% \text{O}_2) and acute hypercapnia (7% \text{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\textsubscript{O\textsubscript{2}} (Bouverot 1985). This hypoxia-induced hyperventilation helps to maintain proper O\textsubscript{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\textsubscript{O\textsubscript{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 O₂-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 P₀₂ (Housley et al. 1988; Powell 2007). These receptors then transduce changes in arterial P₀₂ 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 O₂-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.

CO₂-sensitivity adds complexity to the acute HVR after chronic hypoxia. A reduced arterial P\textsubscript{CO₂} 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 CO₂ 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 Pa\textsubscript{CO₂} 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\textsubscript{2} responsiveness of peripheral chemoreceptor reflexes (Weil et al. 1976).

Unlike O\textsubscript{2}-sensing which is restricted to the peripheral chemoreceptors, there are central and peripheral chemoreceptors that respond to CO₂. CO₂ 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 CO₂ and pH changes, including the NTS itself with intrinsic CO₂-sensitivity (Dean et al. 1989; Coates et al. 1993). Changes in central CO₂-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),
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) $\alpha$-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\textsubscript{2}-sensitivity 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% O₂ 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 (P₁O₂= 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\textsubscript{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\textsubscript{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
O₂ and CO₂ concentrations with the balance being composed of N₂. 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 cmH₂O) 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 O₂ and CO₂ 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):
\[ V_T = \frac{V_{cal} \cdot P_m}{P_{cal}} \cdot \left[ \frac{P_B - P_{CH,O}}{T_A (P_B - P_{CH,O}) - T_C (P_B - P_{AH,O})} \right] \]

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); \( P_{CH,O} \) = chamber vapor pressure (mmHg); \( P_{AH,O} \) = 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 O2 levels before study. Two inspired O2 levels (\( F_{IO_2} = 10\% \) and 21%) were used in the study. We used these two stimuli to obtain a two-point poikilocapnic HVR (\( V_1 \) at 10% O2 minus \( V_1 \) at 21% O2). The 10% O2 level for the HVR measurements produce the same \( P_{IO_2} \) as that in the hypobaric chamber used for acclimatization to chronic hypoxia. \( V_1 \) was measured at 10-15 minutes after the rats’ were exposed to an acute inspired gas concentration. We used a 7% CO2 concentration in 21% O2 to measure the hypercapnic ventilatory response (HCVR, \( V_1 \) at 7% CO2 minus \( V_1 \) at 0% CO2).

3. Arterial Blood Gas Measurements

Single arterial batch samples (0.2 mL) were taken while the rats were breathing 10% or 21% O2 (n=3 for N group and n=3 for CH group), as well as 7% CO2 in 21% O2.
(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 (Pao$_2$), arterial CO$_2$ pressure (Paco$_2$), 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$_2$PO$_4$, 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 (FiO₂ of 10% O₂ vs. 21% O₂ or 0% CO₂ vs. 7% CO₂) 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 (V̇_{T}), 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% O₂ or 10% O₂ 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% O₂. AMPA significantly increased V₁ within the first minute of microinjection in both the CH and N groups. In normoxic rats, V₁ 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 V₁ in N rats breathing 21% O₂, but it significantly decreased V₁ by 20.3% in 10% O₂ (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 $V_T/T_1$ (Fig. 12) shadowed $V_I$. NBQX significantly reduced $V_T/T_1$ 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 ($V_T/T_1$) 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% O₂. The drive to breathe as indexed by \( V_T/T_1 \) (Fig. 12) again shadowed \( V_1 \). NBQX significantly reduced \( V_T/T_1 \) in CH animals acutely breathing both 10% and 21% O₂.

**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 \( P_{aO_2} \) with chronic hypoxia were insignificant, but \( P_{aO_2} \) was generally higher in each CH animal compared to N. Chronic hypoxia generally decreased \( P_{aCO_2} \), which was significant in 21% O₂. Decreased \( P_{aCO_2} \) 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 \( P_{aCO_2} \) in CH rats breathing 21% O₂.

**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% CO₂), \( V_1 \) 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 \( V_1 \) 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 $V_1$ 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. $P_{aCO_2}$ 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, $P_{aCO_2}$ tended to increase after microinjection of NBQX. Elevated $P_{aCO_2}$ 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 $P_{aCO_2}$ is expected for a given decrease in $V_1$ 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 $V_T/T_1$ mirror those expected when analyzing $V_1$ in all conditions. $V_T/T_1$ is an index for the ventilatory drive to breathe. For example, in normoxic rats, $V_T/T_1$ 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 $T_1$. 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 $V_1$ when breathing 7% $CO_2$ compared to normoxic animals. NBQX significantly decreased $V_1$ 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\textsubscript{2} (which is the same as 21% O\textsubscript{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\textsubscript{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\textsubscript{2} reflex mechanisms. In addition, the NTS also has intrinsic CO\textsubscript{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 O₂-sensitivity 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 O₂ sensitivity and increase in ventilation while breathing 10% O₂ 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 V₁ whenever V₁ 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\textsubscript{2} and CO\textsubscript{2} during the experiment. Each animal was allowed 40 minutes to habituate to the plethysmograph at their chronic inspired O\textsubscript{2} level (N, solid line; CH, dotted line). Arterial blood gas samples (ABG) were taken at different inspired O\textsubscript{2} and CO\textsubscript{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 ($V_T$) 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 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 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 9. Effects of chronic hypoxia and NBQX on respiratory frequency. (A) Changes in the frequency response (fR) 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 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 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_L) 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 13. Effects of chronic hypoxia and NBQX on total minute ventilation in hypercapnia. (A) Changes in total minute ventilation ($V_t$) 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 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


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


