The Effects of Chronic Intermittent Hypoxia on NMDA Specific Glutamate Receptor Subunits in the Nucleus Tractus Solitarius

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Abstract

Patients with obstructive sleep apnea (OSA) have increased basal sympathetic nervous system activity, blood pressure, and cardiorespiratory responses to acute hypoxia. These effects are thought to be due to chemoreceptor activation. Afferent neurons projecting from the carotid body chemoreceptors synapse in the nucleus tractus solitarius (nTS) and release glutamate as their primary excitatory neurotransmitter. Chemoreceptor information is processed within the nTS and sent to other brain regions. These include the rostral ventrolateral medulla (RVLM), which is critical to basal and reflex control of sympathetic nerve activity. Previous work shows increased glutamatergic transmission in the nTS after chronic intermittent hypoxia (CIH) in rats, a model of OSA. N-methyl-D-aspartate glutamate receptors (NMDARs) are modulatory receptors and may contribute to nTS plasticity in response to CIH. *We **hypothesize** that CIH increases NMDAR expression in the nTS, including on neurons projecting to the RVLM.* For identification of neurons projecting from the nTS to the RVLM, a retrograde tracer will be injected into the RVLM prior to CIH. Rats will be subjected to ten days of CIH by placing them into a chamber that cyclically changes the oxygen levels to mimic OSA. After CIH, rats will be sacrificed and perfused with paraformaldehyde. Control rats are exposed to normal oxygen levels. Coronal sections of the nTS region will be cut and immunohistochemistry used to evaluate NMDAR subunits NMDAR1-3. We will examine distribution of NMDARs in the nTS, including on RVLM-projecting neurons. NMDAR proteins will be quantified from fresh nTS tissue using western blot techniques.

Introduction

Obstructive Sleep Apnea (OSA), affecting approximately 9% of women and 24% of men, is a sleep disorder in which cyclic cessation in breathing is observed. This pattern is induced by repetitive upper airway blockages or temporary collapses of the trachea while sleeping. Humans with OSA have symptoms such as increased basal sympathetic nerve activity and hypertension. These symptoms are thought to be due to activation of arterial chemoreceptors initiated primarily by a decrease in blood oxygen levels. Chemoreceptor information is projected in afferent fibers to the nucleus tractus solitarius (nTS) region of the brainstem where glutamate is released as the primary excitatory neurotransmitter. Chemo-afferent information is then processed within the nTS and sent to other regions of the brain to influence respiration, sympathetic nerve activity, and blood pressure. The rostral ventrolateral medulla (RVLM), which is considered the primary controller of sympathetic nervous system activity, receives direct projections from the nTS and is thought to be critical to chemoreflex function. Prior studies using chronic intermittent hypoxia (CIH) in rats, an animal model of OSA, have shown an increase in glutamatergic transmission within the nTS region of the brainstem. We hypothesized that CIH causes an increased number or altered composition of glutamate receptors on cells in the nTS, including neurons projecting to the RVLM. N-methyl-D-aspartate (NMDA) receptors are known to be one of the modulatory receptors within the ionotropic subgroup of glutamate receptors. There are 6 known subunit types of NMDA receptors with the NR1 subunit obligatory to receptor function. We focused on NMDA glutamate receptors since it has been shown that the early response to hypoxia induces c-Fos protein expression, an index of neuronal activation, primarily on cells with NMDA glutamate receptors within the nTS of the rat.

Methods

Retrograde Tracer

The retrograde tracer FluoroGold (FG, 2%, 30 nl) was microinjected into the RVLM region of the brainstem of anesthetized rats using aseptic technique. The RVLM was identified by published coordinates. To functionally identify the appropriate brain region, glutamate (10 mM, 30 nl) was microinjected into the region and an increase in blood pressure (see Figure 3, arrow) was recorded.

Exposure to CIH

Rats (n=3) were subjected to 10 days of CIH by placing standard rat cages into a commercially available chamber (Biospherix) which cycled between 21% and 6% inspired oxygen for 8 hours/day. This model mimics moderate OSA. Control (normoxic) rats (n=3) were exposed to normal oxygen levels in an identical chamber placed next to the test chamber.

Tissue Preparation

Following exposure to CIH, rats were deeply anesthetized and transcardially perfused with 4% paraformaldehyde. The brains were removed and the brainstem was cut into 30 µm coronal sections on a vibratome.

Immunohistochemistry

Immunohistochemistry was performed in a 1 in 6 series. Tissue was first blocked with 10% Normal Donkey Serum followed by a sequence of Avidin and Biotin blocks. Following washes, tissue was exposed to primary antibody consisting of either Rabbit α NR1 (0.5 µg/ml, Millipore) or Rabbit α NMDAR2A (1:1000, Milllipore) overnight. Following washing with phosphate buffered saline, the NMDAR2A antibodies were amplified with a Biotin-Streptavidin complex. Both NR1 and NMDAR2A were visualized using Cy3 fluorescence (see Figure 2). Negative controls without primary antibodies were used for both hypoxic and normoxic animals.

Slide Preparation

Tissue sections were mounted on slides and coverslipped with Prolong Gold medium.

Data Analysis

Using fluorescence microscopy, unilateral images were obtained of the nTS region at calamus scriptorius (CS) and up to 600 µm both caudal and rostral to CS, (see Figure 4). Both FluoroGold and Cy3 fluorescent imaging was captured using the appropriate filter set for each tissue section. Images were overlaid and analyzed using ImageJ software. Data are presented as mean +/- SEM for 3 normoxic and 3 hypoxic rats.

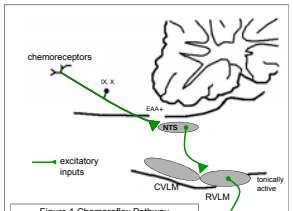

Figure 1 Chemoreflex Pathway
Chemoreceptors project to the nTS where they synapse with neurons projecting to the RVLM (Adapted from Dampney)

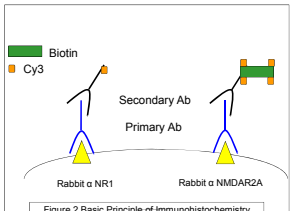

Figure 2 Basic Principle of Immunohistochemistry
Left: Primary Ab plus a secondary Ab with Cy3
Right: Primary Ab plus a secondary Ab with Biotin-Streptavidin Cy3 complex

Functional and Anatomical Identification of the RVLM

Figure 3 **Left:** Graph showing BP and HR response to glutamate injection (arrow, 10 mM) into the RVLM. Note the pressor response with little change in HR, **Middle:** Brightfield coronal image depicting area of the RVLM (white box) in the brain, **Right:** FG fluorescence of injection site in the RVLM.

RVLM-Projecting Neurons are located in the nTS

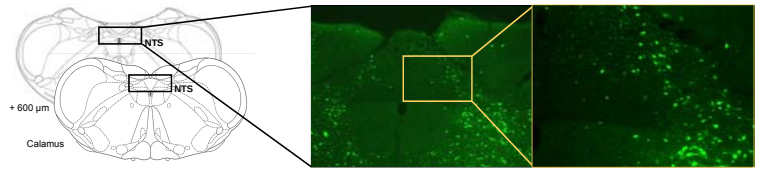

Figure 4 **Left:** Regions of the nTS at calamus scriptorius and 600 µm rostral to calamus were used in the current study, **Middle:** 4x image of nTS region showing fluorescent cells projecting from the RVLM, **Right:** 10x unilateral image of nTS for area of analysis.

NR1 receptors are located in the nTS and decrease with CIH

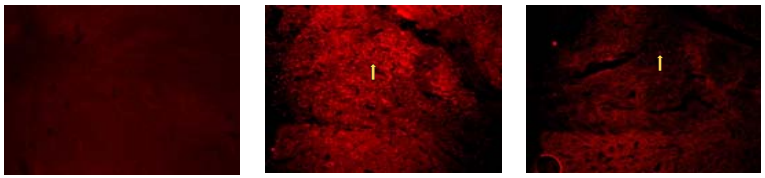

Figure 5 **Left:** Control tissue with no NR1 antibody, **Middle:** Normoxic tissue with punctate staining of NR1 glutamate receptors (arrow), **Right:** CIH tissue with punctate staining of NR1 glutamate receptors (arrow)

NR1 receptors colabel with RVLM-Projecting cells

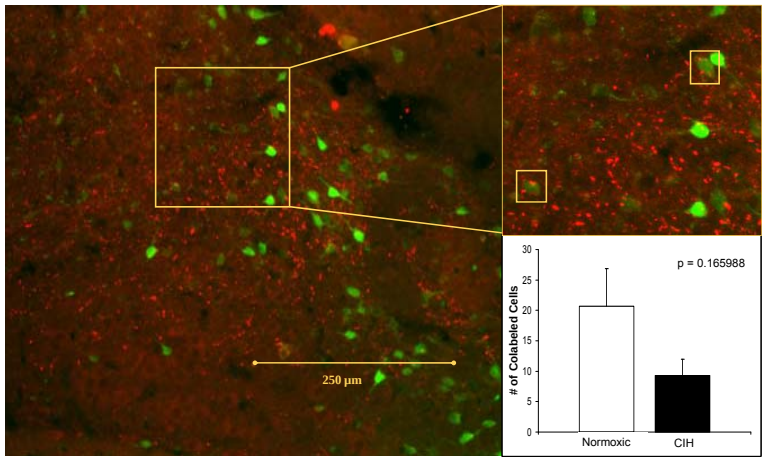

Figure 6 **Left:** Area of nTS showing FG labeled cells (green) and NR1 glutamate receptors (red), **Top Right:** Magnification depicting FG cells colabeled with NR1 receptors on the membrane, **Bottom Right:** CIH reduced the number of colabeled cells compared to normoxic rats

NR1 receptors decrease with CIH

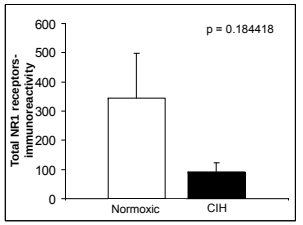

Figure 7 Graph depicting total number of NR1 receptors in the nTS of a normoxic versus a hypoxic rat. Note the decrease in NR1 receptors with CIH tissue.

Colabeling decreases with CIH

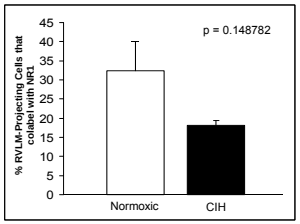

Figure 8 Graph depicting % of RVLM-Projecting cells (those labeled with FG) that colabel with NR1 receptors for both normoxic and hypoxic rats. Note the decrease in the % RVLM-Projecting cells with CIH tissue.

Summary of Data

- NR1 subunits of NMDA glutamate receptor are localized throughout the nTS region of the brain in normoxia and following CIH.
- CIH rats show a trend for a decreased number of NR1 glutamate receptors in the nTS region of the brain.
- Rats exposed to CIH appear to have less NR1 glutamate receptors on cells projecting to the RVLM region of the brain than rats exposed to normoxic conditions.

Conclusions

The trend for NR1 glutamate receptors (NMDAR1s) to decrease with CIH refutes our hypothesis. When active, NMDAR1s allow entry of calcium into the cell. From prior studies, we know that glutamate is increased in the chemoafferent-nTS synapse during hypoxia. The decrease in NMDA receptors may function to prevent excitotoxic cell death from elevated levels of calcium in the cell in response to CIH and increased levels of glutamate.

Future Directions

- Increase n in order to achieve significant difference in the study.
- Run Western Blots to quantify total NR1 glutamate receptor protein in the nTS region of the brain.
- Immunohistochemistry was completed on NR2A and NR2B glutamate subunit receptors. Image analysis is in progress.
- Study other NMDA glutamate receptor subunits.

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