Expression of AMPA Glutamate Receptors on RVLM-projecting nTS Neurons in Response to Chronic Intermittent Hypoxia

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Abstract

Obstructive sleep apnea (OSA) is a serious condition affecting 10% of the adult population. Individuals with OSA experience periods of arterial hypoxemia during sleep. Chronic intermittent hypoxia (CIH) in rats is a model for OSA. Humans with OSA and rats with CIH both show an increase in sympathetic nerve activity and hypertension, which have been attributed to an augmented carotid body chemoreflex. Chemoreceptor afferents form a synapse on second-order neurons in the nucleus of the solitary tract (nTS) before projecting to other areas of the brain. Projections from the nTS to the rostral ventrolateral medulla (RVLM), which contain presympathetic neurons and mediate sympathetic tone, may be responsible for the increase in sympathetic nerve activity with CIH. The primary excitatory neurotransmitter released by chemoreceptor afferents is glutamate, which binds to alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) glutamate receptors within the nTS. Our lab has shown that synaptic glutamatergic plasticity within the nTS in response to CIH is associated with the physiological effects observed with CIH. Based on our previous results and the observed increase in sympathetic nerve activity in response to CIH, *we **hypothesize** that AMPA glutamate receptors in the nTS are up-regulated after CIH.* To test this hypothesis, rats will be exposed to CIH for 10 days, perfused with fixative, and tissue sections of the nTS will be generated for immunohistochemistry of AMPA receptor subunits in retrogradely labeled RVLM-projecting cells. Western blot analysis of nTS tissue will be performed to confirm expression levels after exposure to CIH.

Introduction

The carotid body chemoreflex serves as a physiological mechanism to increase respiration, blood pressure, and sympathetic nervous system activity when arterial hemoglobin saturation declines past 75%. Decreases in arterial O_2 (hypoxia) are sensed by arterial chemoreceptors, which increase afferent chemosensory impulses to the brainstem nucleus of the solitary tract (nTS). There, chemosensory afferent fibers form a synapse on second-order neurons in the nTS. Afferent information is processed and integrated in the nTS before being sent to other cardiorespiratory nuclei.

One area of the brain receiving direct projections from the nTS as part of the carotid body chemoreflex is the rostral ventrolateral medulla (RVLM). The RVLM contains presympathetic neurons that project directly to preganglionic sympathetic neurons. As such, the RVLM is critical in maintaining baseline sympathetic nerve activity by direct control of the sympathetic nerves (Fig 1). The RVLM is also vital to the chemoreflex-induced increase in blood pressure.

Patients with obstructive sleep apnea (OSA) exhibit alterations in respiration, elevated sympathetic nerve activity, and sustained hypertension. These alterations are due to an increased sensitivity of the chemoreflex. Animal models of OSA using chronic intermittent hypoxia (CIH) have shown similar physiologic effects. Patients with OSA and rats exposed to CIH experience cyclic periods of arterial hypoxemia during their nocturnal hours. The cyclic periods of hypoxia activate the chemoreflex and culminate in persistently increased chemoreceptor afferent signaling to the nTS. The primary excitatory neurotransmitter released by chemoreceptor afferents is glutamate, which binds to alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) glutamate receptors. AMPA glutamate receptors are ionotropic, heterotetrameric receptors consisting of at least one GluR1 subunit, with a combination of GluR2, GluR3, or GluR4 subunits. Continual stimulation of nTS neurons by glutamate from chemoreceptive afferents is thought to cause a change in the number or subunit composition of glutamate receptors within the nTS. In addition, previous research in the lab has shown alterations in glutamatergic signaling and receptor efficacy in response to CIH within the nTS. However, it is not known whether such alterations occur in neurons that project to the RVLM. Because of the importance of the RVLM under normal conditions, and the increase in sympathetic tone and hypertension associated with CIH, we hypothesize that CIH will induce an increase in glutamate receptors on nTS neurons that project to the RVLM.

Fig. 1: Chemoreflex Pathway

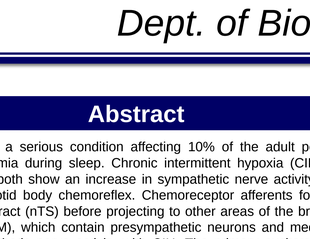

Fig. 1: Afferent fibers from carotid body project to nTS. From the nTS, output neurons send information to the RVLM to maintain sympathetic tone and reflex response. *Fig. adapted from Ackland GL, Kasymov V, Gourine AV.* Biochem Soc Trans. 2007 Nov;35(Pt 5):1264-8

Methods

Microinjection of retrograde tracers: Using aseptic technique, anesthetized Sprague-Dawley rats were placed in a stereotaxic device for injection of the retrograde tracer Fluorogold (2%, 30nl) bilaterally into the RVLM. Targeted RVLM sites were based on the rat atlas of Paxinos & Watson (2005). Microinjection sites were functionally verified by a pressor response (+10 mm Hg) to glutamate injection (10mM, 30nl). Animals were allowed to recover for 10 days before exposure to hypoxia.

Exposure to hypoxia: Six rats were randomly allocated to one of two groups, 3 normoxic and 3 chronic intermittent hypoxic (CIH). All rats were housed in standard rat cages that allowed free movement and access to food and water ad libitum. For CIH, the rat cage was placed inside a commercially available hypoxic chamber (Biospherix). Exposure to hypoxia consisted of alternating cycles of 21% O_2 (5 min) and 6% O_2 (45 sec) from 0900 to 1700 hours for 10 days. This protocol mimics a moderate case of OSA. Normoxic rats were kept within the same room as the hypoxic system so they experienced the same sounds and light stimuli.

Immunohistochemistry: Rats were deeply anesthesized and perfused transcardially with 4% paraformaldehyde. The brain was removed and stored in phosphate buffered saline (PBS). Sections of the brain stem (30 μm) were cut using a vibratome and stored in 48 well plates in PBS. Immunohistochemistry was performed in a 1 in 6 series on pairs of normoxic and CIH tissue sections. Tissue sections were blocked for 30 minutes in 10% normal donkey serum (NDS) and 0.3% Triton-PBS (0.1M, pH 7.4). Tissue was washed in PBS and incubated in blocking solutions of Avidin/Biotin. Tissue was incubated for 24 hours in primary antibody [either mouse α-GluR1 (1:100 Santa Cruz) or rabbit α-GluR2 (0.5g/mL Chemicon)] in 1% NDS and 0.3% Triton-PBS at 21 C. After incubation in primary antibody, tissue was washed in PBS and incubated for 2 hours in secondary antibody (1:200 donkey α-mouse or α-rabbit Biotinylated IgG with 1% NDS and 0.3% Triton-PBS) and washed in PBS. Tissue was incubated for 1 hour in Streptavidin Cy2 or Streptavidin Cy3. Tissue was washed in PBS before being mounted on gelatin coated slides and coverslipped with Prolong Gold (Invitrogen).

Data analysis: Fluorescence microscopy was utilized to observe staining, and images were taken and analyzed using ImageJ software. Blinded cell counts were done on tissue sections of the mid-Area Postrema region (Fig 2). Colocalization analysis was used to identify Fluorogold labeled cells expressing GluR2 subunits (Fig 3). Data are presented as average +/- SE.

Fig. 2: CIH induces an increase in GluR2

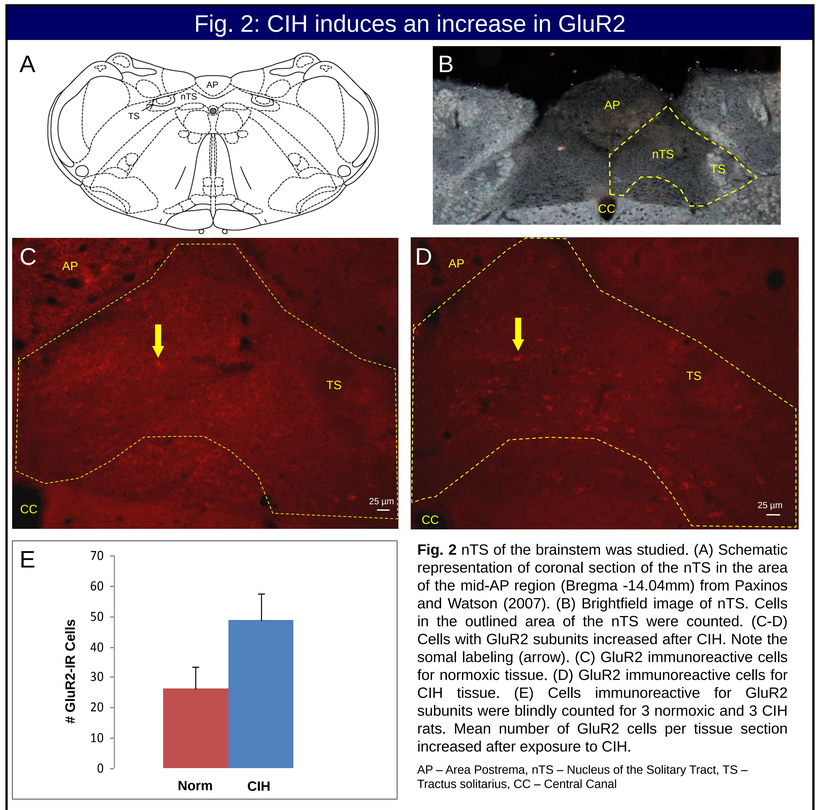

Fig. 2 nTS of the brainstem was studied. (A) Schematic representation of coronal section of the nTS in the area of the mid-AP region (Bregma -14.04mm) from Paxinos and Watson (2007). (B) Brightfield image of nTS. Cells in the outlined area of the nTS were counted. (C-D) Cells with GluR2 subunits increased after CIH. Note the somal labeling (arrow). (C) GluR2 immunoreactive cells for normoxic tissue. (D) GluR2 immunoreactive cells for CIH tissue. (E) Cells immunoreactive for GluR2 subunits were blindly counted for 3 normoxic and 3 CIH rats. Mean number of GluR2 cells per tissue section increased after exposure to CIH.

AP – Area Postrema, nTS – Nucleus of the Solitary Tract, TS – Tractus solitarius, CC – Central Canal

Fig. 3: CIH induces an increase in GluR2 in nTS cells projecting to the RVLM

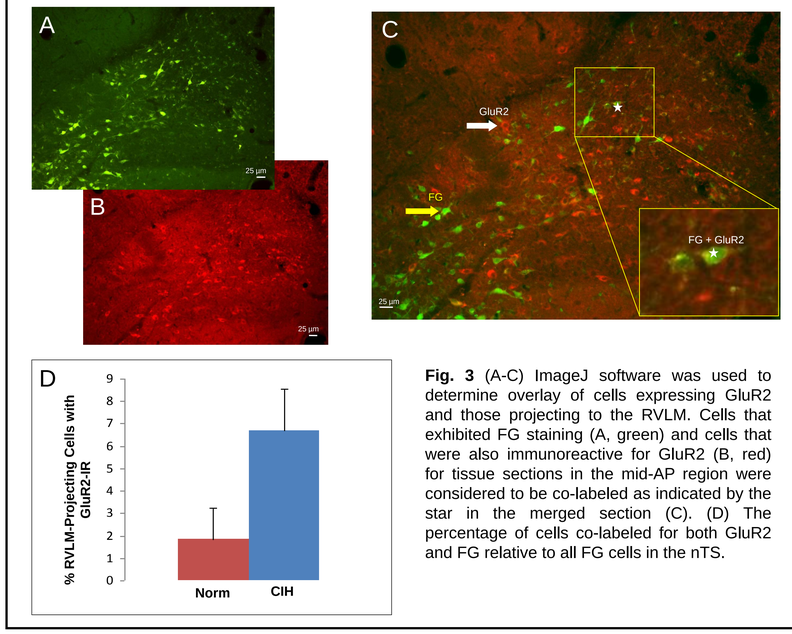

Fig. 3 (A-C) ImageJ software was used to determine overlay of cells expressing GluR2 and those projecting to the RVLM. Cells that exhibited FG staining (A, green) and cells that were also immunoreactive for GluR2 (B, red) for tissue sections in the mid-AP region were considered to be co-labeled as indicated by the star in the merged section (C). (D) The percentage of cells co-labeled for both GluR2 and FG relative to all FG cells in the nTS.

Fig 4: RVLM injection site was identified functionally and anatomically

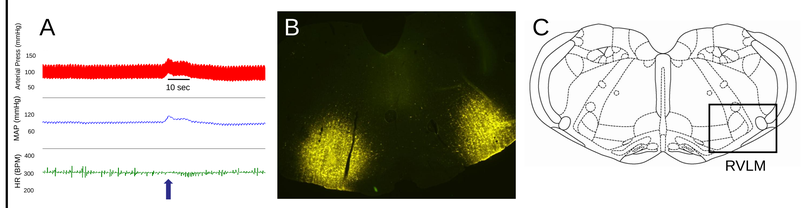

Fig. 4 (A) Bi-lateral injection of FG into the RVLM was functionally verified by an increased pressor response to glutamate injection, which occurred where indicated by the arrow. (B) Images of the injection sites were compared to Paxinos and Watson (2007) rat atlas schematic of the RVLM (C, Bregma -12.24mm) to anatomically verify injection site.

Fig 5: Control immunohistochemistry tissue sections exhibit no GluR2 immunoreactivity

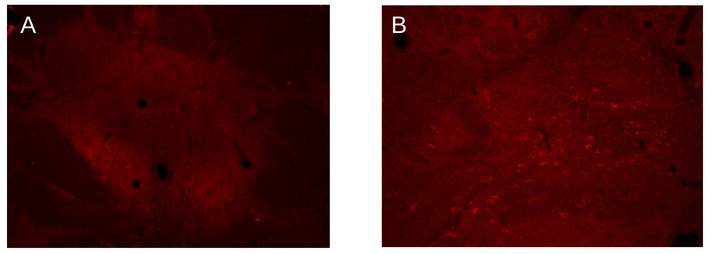

Fig. 5 Control tissue sections during immunohistochemistry were not exposed to primary antibody. Images of control (A) and non-control (B) were obtained at the same light exposure level.

Summary

- Chronic intermittent hypoxia (CIH) induces an increase in cells expressing the GluR2 subunit of the AMPA-type glutamate receptor in the nTS.
- There is an increase in cells expressing the GluR2 subunit that project to the RVLM in response to CIH.

Conclusions

Through previous research, our lab has shown that CIH induces an alteration in glutamatergic signaling and receptor efficacy within the nTS. Results from this study have indicated that the GluR2 subunit of AMPA glutamate receptors is increased in response to CIH. In addition, RVLM-projecting neurons seem to exhibit an increase in GluR2. The GluR2 subunit of AMPA glutamate receptors has been associated with decreasing the calcium permeability normally associated with AMPA glutamate receptors. We speculate that the elevation in GluR2 subunits following CIH is a form of homeostatic plasticity to maintain the neuron's resting membrane potential in a more hyperpolarized state by decreasing calcium influx. Future studies will address this notion.

Studies in Progress

- Immunohistochemistry (IHC) has been performed on GluR1 subunits and data is being analyzed. Preliminary data shows high GluR1 subunits within the region of the nTS and AP border.
- GluR2 subunit IHC is being analyzed to determine if rostral-caudal location within the nTS affects number of subunits and/or degree to which they are expressed on RVLM-projecting neurons.
- Immunohistochemistry will be performed on GluR3 and GluR4 subunits
- Western blot analysis of normoxic and CIH rats will quantify levels of glutamate receptor expression.

Acknowledgements

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