Chronic intermittent hypoxia alters NMDA and AMPA-evoked currents in NTS neurons receiving carotid body chemoreceptor inputs

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Abstract

SLEEP APNEA IS A SIGNIFICANT health risk with ~10% of the population affected by this disorder (44, 45). Whether central or peripheral in origin, the apnea results in arterial hypoxemia. Chronic exposure to intermittent hypoxia (CIH) during the nocturnal period is widely used in animals to mimic the arterial hypoxemia of sleep apnea. Humans with sleep apnea and animals exposed to CIH have elevated blood pressures and augmented sympathetic nervous system responses to acute exposures to hypoxia. To test the hypothesis that exposure to CIH alters neurons within the nucleus of the solitary tract (NTS) that integrate arterial chemoreceptor afferent inputs, we measured whole cell currents induced by activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and N-methyl-d-aspartate (NMDA) receptors in enzymatically dispersed NTS neurons from normoxic (NORM) and CIH-exposed rats (alternating cycles of 3 min at 10% O2 followed by 3 min at 21% O2 between 8 AM and 4 PM for 7 days). To identify NTS neurons receiving carotid body afferent inputs the anterograde tracer 4-(dihexadecylamino)styril-N-methylpyridinium iodide (DiA) was placed onto the carotid body 1 wk before exposure to CIH. AMPA dose-response curves had similar EC50 but maximal responses in increased neurons isolated from DiA-labeled CIH (20.1 ± 0.8 μM, n = 9) compared with NORM (6.0 ± 0.3 μM, n = 8) rats. NMDA dose-response curves also had similar EC50 but maximal responses decreased in CIH (8.4 ± 0.4 μM, n = 8) compared with NORM (19.4 ± 0.6 μM, n = 9) rats. These results suggest reciprocal changes in the number and/or conductance characteristics of AMPA and NMDA receptors. Enhanced responses to AMPA receptor activation could contribute to enhanced chemoreflex responses observed in animals exposed to CIH and humans with sleep apnea.

KEY WORDS: chemoreflex; electrophysiology; sleep apnea

METHODS

Experiments were performed on 47 adult, male Sprague-Dawley rats (275–350 g, Charles River Laboratories, Wilmington, MA) housed two per cage in a fully-accredited (Association for Assessment and Accreditation of Laboratory Animal Care and United States Department of Agriculture) laboratory animal room with free access to food and water. Before being used for any procedures, rats were given at least 1 wk to acclimate. All experimental protocols were approved by the Institutional Animal Care and Use Committee.

Rats were anesthetized with medetomidine (0.5 mg/kg ip; Pfizer) and ketamine (75 mg/kg ip; Fort Dodge Laboratory) 1–2 wk prior to placement in the intermittent hypoxia chambers and under asptic conditions, crystals of the anterograde fluorescent tracer DiA (Molecular Probes, Eugene OR) were applied to the carotid body region and sealed in place with Kwik-Sil (World Precision Instruments, Sarasota, FL) to visualize chemoreceptor synaptic terminals and neurons receiving these synaptic contacts, as we and others have previously described (29, 40). Anesthesia was terminated by atipamezole (1 mg/kg ip; Pfizer) at the conclusion of the surgical procedures. Postoperative analgesics (Nubaine, im) were available as needed.

CIH. Rats were divided into two groups: normoxic controls (NORM; n = 30) and rats exposed to CIH (n = 23). Hypoxic rats were housed in hypoxia chambers with ad libitum food and water access and were exposed to 8 h of CIH, using the paradigm described by Fletcher and colleagues (10, 11). Chambers were flushed with 100% N2 until a fractional O2 concentration of 8–10% was reached and maintained for 3 min. After that, the chambers were flushed with room air (21% O2) for 3 min. Exposures to CIH occurred during the light period (08:00–16:00) for 7 days. The animals were not exposed to CIH during the remainder of the light period (16:00–21:00) and during the dark period (21:00–07:00). NORM animals were kept in the same types of chambers as CIH-exposed rats, and gas switching occurred on the same time frame as in CIH-exposed rats. However, in NORM rats, gas switching was between sources of room air so that NORM rats always inspired room air (21% O2).

Dispersion of caudal NTS neurons. Experiments were performed 7 days after placement in the intermittent hypoxia chambers. Rats were

Address for reprint requests and other correspondence: S. Mifflin, Dept. of Pharmacology, Mail Code 7764, Univ. of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78229-3900 (e-mail: mifflin@uthscsa.edu).

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de Paula PM, Tolstykh G, Mifflin S. Chronic intermittent hypoxia alters NMDA and AMPA-evoked currents in NTS neurons receiving carotid body chemoreceptor inputs. Am J Physiol Regul Integr Comp Physiol 292: 2259–2265, 2007. First published March 1, 2007; doi:10.1152/ajpregu.00760.2006.—Chronic exposure to intermittent hypoxia (CIH) has been used in animals to mimic the arterial hypoxemia that accompanies sleep apnea. Humans with sleep apnea and animals exposed to CIH have elevated blood pressures and augmented sympathetic nervous system responses to acute exposures to hypoxia. To test the hypothesis that exposure to CIH alters neurons within the nucleus of the solitary tract (NTS) that integrate arterial chemoreceptor afferent inputs, we measured whole cell currents induced by activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and N-methyl-d-aspartate (NMDA) receptors in enzymatically dispersed NTS neurons from normoxic (NORM) and CIH-exposed rats (alternating cycles of 3 min at 10% O2 followed by 3 min at 21% O2 between 8 AM and 4 PM for 7 days). To identify NTS neurons receiving carotid body afferent inputs the anterograde tracer 4-(dihexadecylamino)styril-N-methylpyridinium iodide (DiA) was placed onto the carotid body 1 wk before exposure to CIH. AMPA dose-response curves had similar EC50 but maximal responses in increased neurons isolated from DiA-labeled CIH (20.1 ± 0.8 μM, n = 9) compared with NORM (6.0 ± 0.3 μM, n = 8) rats. NMDA dose-response curves also had similar EC50 but maximal responses decreased in CIH (8.4 ± 0.4 μM, n = 8) compared with NORM (19.4 ± 0.6 μM, n = 9) rats. These results suggest reciprocal changes in the number and/or conductance characteristics of AMPA and NMDA receptors. Enhanced responses to AMPA receptor activation could contribute to enhanced chemoreflex responses observed in animals exposed to CIH and humans with sleep apnea.

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anesthetized with isoflurane, and the brain stem was rapidly removed and placed in ice-cold Krebs buffer. The brain stem was cut into 440-µm-thick transverse sections using a vibratome, and sections were incubated in PIPES buffer with trypsin (Sigma type XI, 3–5 mg/10 ml) for 55 min at ~34°C. After enzyme treatment, slices were rinsed three times with incubation solution and maintained at room temperature (22–25°C) in a continuously oxygenated jar with PIPES buffer. Slices were placed on a glass slide, and the caudal NTS region was identified and excised by using a scalpel blade. The excised NTS region was gently triturated in a DMEM buffer using a series of fire-polished pipettes. Following titration, an aliquot of the DMEM/neuron suspension was placed in the perfusion bath and allowed to settle for 5 min, after which superfusion of the cells was begun with a normal physiological solution.

**Electrophysiology.** Whole cell patch-clamp recordings were performed on acutely dissociated NTS neurons in an external solution consisting of (in mM) 140 NaCl; 33 d-glucose; 10 HEPES; 3 KCl; 2 CaCl$_2$; and 2.4 MgCl$_2$ (pH 7.4 with NaOH; osmolality 315–320 mosM). For NMDA protocols, MgCl$_2$ was omitted. Electrodes were formed from thin-walled quartz glass with a laser-based puller (Sutter Instrument, Novato, CA) to resistances of 3–7 MΩ. Electrodes were filled with (in mM): 150 CsCl; 0.1 NaGTP; 4 NaATP; and 12 HEPES (pH 7.2 with CsOH; osmolality 280–300 mosM). Neurons were viewed under appropriate wavelength fluorescence, and cells exhibiting DiA-labeled somatic appositions were studied, as well as cells that did not exhibit DiA somatic appositions. Cells were voltage clamped at −60 mV by using an Axopatch 200B amplifier (Axon Instruments). Currents were expressed as current density (pA/pF) to normalize for cells of different sizes. Currents were low-pass-filtered at 1 kHz, sampled at 10 kHz, and analyzed using the pCLAMP version 8.1 software suite (Axon Instruments). If leak current was more than 50 pA, data were not included in the analysis. Prior to each drug application, a membrane test (Axon Instruments) was performed to ensure that access resistance did not change over time or during different experimental conditions.

We repeated the drug application protocol described in our previous publications (40, 41). AMPA-containing solutions consisted of (in mM) 132 NaCl; 33 d-glucose; 10 TEA-Cl; 10 HEPES; 3 KCl; 2 CaCl$_2$; and 0.5 mM TTX. NMDA-containing solutions consisted of (in mM): 140 NaCl; 33 d-glucose; 10 HEPES; 3 KCl; 2 CaCl$_2$; and 0.5 mM glycine; and 0.5 mM TTX. AMPA and NMDA were applied by gravity flow to isolated cells via a two-barrel square glass pipette using a fast-step perfusion apparatus (Warner Instruments) so that rise time for the drug responses were <100 ms. Each pipette was attached via tubing to separate valves connecting each pipette to up to six (for AMPA) or seven (for NMDA) solutions. The first pipette was attached via tubing to separate valves connecting each pipette to up to six (for AMPA) or seven (for NMDA) solutions. The first pipette was positioned adjacent and as close as possible to the target cell. A steady stream of AMPA-free or NMDA-free TTX containing 0.5 mM bath solution abolished action potential discharge and protected the neuron from leakage from the second downstream drug-containing pipette. The second pipette was connected to six or seven different concentrations of AMPA or NMDA, respectively. To apply AMPA or NMDA, the pipette assembly was rapidly moved, repositioning the TTX-containing pipette upstream to the cell and the AMPA or NMDA-ejecting pipette adjacent to the cell. The pipette assembly was returned to the starting position after a user-defined interval. Dose-response relationships were obtained during sequential, 5-s applications of 1, 3, 10, 30, 120, and 300 µM AMPA or 1, 3, 10, 30, 100, 300, and 1,000 µM NMDA with at least 2-min intervals between all drug applications. Preliminary experiments indicated that after exposure to drug, neurons returned to control values within 2 min. Time-control experiments indicated that neuron properties and drug responses were stable over the period of recording, and if neuronal properties and drug responses changed during the course of the recording, the neuron was excluded from analysis.

Intracellular polyamines, such as spermine, have been shown to modulate current flow through both NMDA and AMPA receptors (28). Therefore, current-voltage (I-V) relationships were obtained by using electrodes that contained spermine (100 µM) and electrodes that did not contain spermine.

**Data analysis.** Dose-response curves were fit using

\[
I = I_{\text{min}} + \frac{I_{\text{max}} - I_{\text{min}}}{[1 + (A/EC_{50})^n]} 
\]

where $I$ is the AMPA- or NMDA-induced current, $I_{\text{max}}$ is the maximal AMPA or NMDA current, $I_{\text{min}}$ is the minimal AMPA or NMDA current, $A$ is the concentration of AMPA or NMDA, and $n$ is the Hill slope. Curve fits were obtained by a nonlinear regression using a four-parameter logistic function. Statistical significance of differences between dose-response curves were determined using two-way ANOVA with the Holm-Sidak test used for post hoc comparisons. Percentile data were analyzed by Student’s t-test following logarithmic transformation. All values are expressed as means ± SE, and significance was accepted at $P < 0.05$.

**RESULTS**

The membrane capacitance of neurons provides an approximate index of cell size, and there was no difference in neurons obtained from normoxic (9.77 ± 0.97 pF, $n = 45$) compared with hypoxic (12.02 ± 1.07 pF, $n = 34$) ($P = 0.126$) rats.

**Responses to AMPA.** Sequential increases in the concentration of AMPA increased the steady-state level of inward current (Fig. 1A). Curve fitting of the dose-response curves for cells exhibiting DiA labeling revealed no difference in the $EC_{50}$ of cells isolated from NORM rats (13.4 ± 2.9 µM, $n = 9$) compared with cells isolated from CIH rats (19.6 ± 2.3 µM, $n = 8$) ($P = 0.07$) (Fig. 2). However, the maximum AMPA-evoked current was significantly greater in cells isolated from CIH rats (20.1 ± 0.8 pA/pF) compared with NORM (6.0 ± 0.3 pA/pF) ($P < 0.001$).

Similar findings were obtained in NTS neurons that did not exhibit DiA labeling. Curve fitting of the dose-response curves for such cells revealed no difference in the $EC_{50}$ of cells isolated from NORM rats (13.0 ± 2.4 µM, $n = 26$) compared with cells isolated from CIH rats (12.6 ± 0.6 µM, $n = 30$) ($P = 0.86$) (Fig. 2). However, the maximum AMPA-evoked current was significantly greater in cells isolated from CIH rats (15.8 ± 0.2 pA/pF) compared with NORM (5.7 ± 0.3 pA/pF) ($P < 0.001$).

**I-V relationships for AMPA-evoked currents** were linear in neurons from NORM and CIH rats (Fig. 3) and inclusion of spermine in the patch pipette (100 µM) did not change the shape of the I-V relationship ($n = 7$). The slope of the I-V curve was less in NTS neurons isolated from NORM [0.05 ± 0.01 (pA/pF)/mV, $n = 15$] compared with CIH rats [0.33 ± 0.01 (pA/pF)/mV, $n = 11$] ($P = 0.001$). AMPA-evoked currents (30 µM, $n = 6$) were blocked by the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (100 µM).

Application of cyclothiazide (100 µM) to block AMPA receptor desensitization markedly enhanced the amplitude of AMPA-evoked currents in NTS neurons isolated from NORM (318 ± 37%, $n = 5$) and CIH (268 ± 92%, $n = 8$) rats (Fig. 4). The percentage increase in AMPA-evoked current by cyclothiazide was not different compared with neurons from NORM and CIH rats ($P = 0.71$).

**Responses to NMDA.** Sequential increases in the concentration of NMDA increased the steady-state level of inward current (Fig. 5). Curve fitting of the dose-response curves for...
cells exhibiting DiA labeling revealed no difference in the EC50 of cells isolated from NORM rats (75.9 ± 7.3 µM, n = 8) compared with cells isolated from CIH rats (55.0 ± 8.0 µM, n = 9) (P = 0.08) (Fig. 6). However, the maximum NMDA-evoked current was significantly reduced in cells isolated from CIH rats (8.4 ± 0.4 pA/pF) compared with NORM (19.4 ± 0.6 pA/pF) (P < 0.001).

Similar findings were obtained in NTS neurons that did not exhibit DiA labeling. Curve fitting of the dose-response curves for such cells revealed no difference in the EC50 of cells isolated from NORM rats (67.6 ± 11.2 µM, n = 23) compared with cells isolated from CIH rats (70.3 ± 6.5 µM, n = 15) (P = 0.86) (Fig. 6). However, the maximum NMDA-evoked current was reduced in cells isolated from CIH rats (8.0 ± 0.2 pA/pF) compared with NORM (15.2 ± 0.7 pA/pF) (P < 0.001).

I-V relationships for NMDA-evoked currents studied in Mg2+-free solution were linear in neurons from NORM and CIH rats (Fig. 7). The slope of the I-V curve was less in neurons isolated from CIH [0.14 ± 0.01 (pA/pf)/mV, n = 11] compared with NORM rats [0.33 ± 0.02 (pA/pf)/mV, n = 13] (P = 0.001).

**DISCUSSION**

Exposure to CIH is used to mimic the arterial hypoxemia that accompanies sleep apnea. CIH in rats results in elevated basal levels of sympathetic outflow and blood pressure, as well as enhanced responses to acute exposures to hypoxia (3, 10, 11, 16). These same changes have been reported in humans with sleep apnea (6, 17, 18, 24–26).

It has been proposed that enhanced arterial chemoreflex activity can lead to persistent increases in sympathetic outflow, which could contribute to the hypertension associated with CIH and/or sleep apnea. The carotid body chemoreceptors could contribute to persistent activation of sympathetic outflow following CIH, as enhanced arterial chemoreceptor responses...

Fig. 2. Dose-response relationships of AMPA-evoked current in non-DiA-labeled neurons from rats exposed to normoxia (black circles) and rats exposed to IH (white circles) and in DiA-labeled neurons from rats exposed to normoxia (black squares) and rats exposed to IH (white squares).

Fig. 3. Current-voltage (I-V) relationships obtained during application of 100 µM AMPA in neurons isolated from rats exposed to normoxia (black line) and IH (grey line). I-V curves were generated by voltage ramps from −120 to +10 mV over 50 ms. Gray shading indicates means ± SE. Addition of spermine to the electrode solution did not change the I-V relationships.

Fig. 1. α-Amino-3-hydroxy-5-methyl-4-isoxazol-epropionate (AMPA)-activated currents in nucleus of the solitary tract (NTS) neurons isolated from a rat exposed to normoxia (A) and a rat exposed to intermittent hypoxia (IH) for 7 days (B). The capacitance of the neuron in A was 5.6 pF and for the neuron in B was 4.0 pF; therefore, the neurons were approximately the same size. Cells isolated from normoxic rats had smaller current responses to AMPA than cells isolated from intermittent hypoxic rats. Micrographs in (C) illustrate brightfield image of an NTS neuron (left; calibration bar = 10 µm) and fluorescent DiA somatic appositions (right).
to hypoxia have been observed following comparable exposures to CIH rats (16, 33) and in humans with sleep apnea (25, 26). Enhanced chemoreceptor responses to acute hypoxia have been reported after exposure to CIH (29–31). Since the transmitter released by chemoreceptor afferents within the NTS is assumed to be glutamate (42, 46, 47), our results suggest that central alterations in glutamatergic transmission within the NTS could also contribute to enhanced chemoreflex responses.

The results of the present study indicate that following 7 days of exposure to CIH, the responses of NTS neurons to activation of AMPA receptors are enhanced within the voltage range of $-30$ mV to $-120$ mV. These changes occur with no obvious alteration in the level of desensitization of AMPA receptors at rest. In addition, following exposure to CIH, NTS neuronal responses to activation of NMDA receptors are reduced in the voltage range of $-20$ to $-120$ mV. The responses to AMPA and NMDA in the subpopulation of NTS neurons with DiA-labeled presumptive boutons were not different from those observed in non-DiA-labeled neurons, suggesting that the observed changes in NTS neurons were independent of whether the neuron received a peripheral arterial chemoreceptor input.

The lack of rectification in the $I-V$ relationship during application of AMPA suggests that the receptors contain the GluR2 subunit (36) and that this does not change as a result of exposure to CIH. Intracellular polyamines, such as spermine, are responsible for inward rectification of calcium-permeable AMPA receptors (28). The lack of rectification observed in the present studies was not due to dialysis of endogenous polyamines, as addition of spermine to the electrode solution did not alter the $I-V$ relationship. GluR1 and GluR2/3 AMPA receptor subunits have been localized within the NTS (2, 13, 42).

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**Fig. 4.** Effect of cyclothiazide (CYZ) on AMPA currents. A: responses of an NTS neuron to application of AMPA alone and with CYZ. B: percentage increase in AMPA (100 μM) current during bath application of CYZ (100 μM) in rats exposed to normoxia ($n = 5$ cells) and IH ($n = 8$ cells). CYZ-enhanced responses to AMPA were not different between the 2 groups ($P = 0.71$).

**Fig. 5.** $N$-methyl-$d$-aspartate (NMDA)-activated currents in NTS neurons isolated from rats exposed to normoxia (A) and IH (B) rats. The capacitance of the neuron in A was 21.5 pF and for the neuron in B was 25.1 pF; therefore, the neurons were approximately the same size. Cells isolated from rats exposed to normoxia had greater current responses to NMDA than cells isolated from rats exposed to IH.
to CIH would provide insights into the functional conse-
quences of enhanced AMPA and reduced NMDA induced currents.

Besides oxidative stress, what other potential stimuli might initiate the changes in amino acid receptor responses we observed? Enhanced discharge of arterial chemoreceptors at rest and in response to hypoxia following exposures to CIH could increase levels of excitatory amino acids (23), GABA (39), angiotensin (34), or other neuromodulators/hormones stimulated by arterial chemoreflexes. Therefore, the changes in excitatory amino acid receptor characteristics could be hormonally mediated. In addition, NTS neurons have been shown to directly respond to hypoxia (27).

The observed changes could reflect a change in the number of binding sites for AMPA and NMDA. In addition, the phosphorylation status of the receptors could alter the amount of current conducted. Changes within the NTS in signal transduction systems that can mediate amino acid receptor phosphorylation have been reported in rats following acute exposures to hypoxia (8, 12, 14, 15), and these systems, and/or others, might enhance AMPA-mediated responses in NTS neurons following CIH. Phosphorylation can increase the affinity and conductance of AMPA receptors (7, 37). Phosphorylation can also alter responses by increasing receptor trafficking resulting in insertion of AMPA receptors in the membrane (4, 5). Our data are more consistent with changes in receptor number or receptor subunit composition that lead to increased conductance, as responses at each dose of AMPA were increased with no changes in EC50, or the affinity, of the receptor. Increases in NMDA receptor subunit expression within the NTS have been reported following similar exposures to CIH (32) and are not consistent with the reduced NMDA receptor responses observed in the present study. However, the study examined receptor expression in a fairly large area of the dorsal hindbrain, and the result may therefore not be representative of what occurs in a subpopulation of neurons within the area. Analysis of the role of these potential factors in the changes we report would be a useful future direction.

The fact that the changes in AMPA- and NMDA-evoked responses were observed in NTS neurons that did not exhibit DiA labeling suggests that the changes are not dependent upon...
whether or not the neuron receives an arterial chemoreceptor afferent input. The ubiquitous changes in AMPA and NMDA responses in both DiA- and non-DiA-labeled neurons suggest that the NTS neuronal response to all inputs should be enhanced, providing the inputs to activate AMPA receptors. For example, NTS neurons could exhibit enhanced responses to arterial baroreceptor, cardiopulmonary, and gastrointestinal afferent inputs or descending excitatory inputs from the hypothalamus. Analysis of other reflex responses in rats exposed to CIH could answer this question.

However, it is possible that the changes in excitatory amino acid responses we report are only functionally significant if the alterations in neuronal responses are coupled with enhanced afferent input. This would confer functional specificity to those neurons receiving arterial chemoreceptor afferent inputs. Additional changes might occur in other ligand-gated or voltage-gated channels that further influence the ultimate response of a neuron to a depolarizing input. As one example, we have reported that the sensitivity of NTS neurons to activation of GABA<sub>B</sub> receptors is reduced following chronic exposures to sustained hypoxia (40). Since the response of many NTS neurons to activation of arterial chemoreceptor inputs is a depolarization followed by GABA<sub>A</sub> receptor-mediated hyperpolarization (22), in rats chronically exposed to sustained hypoxia the reduced GABAergic inhibition could lead to an enhanced NTS neuronal response to chemoreceptor activation. The extent to which exposures to CIH alter GABAergic inhibition of NTS neurons, or any other determinant of neuronal function, could influence the ultimate response of the neuron. The present report should be considered a first step on the road to documenting adaptations of central neurons following exposures to CIH. Attempts to try and completely define neuronal responses on the basis of one observed alteration may be misleading.

In conclusion, exposure to CIH for 7 days alters the response of NTS neurons to exogenous application of the inotropic excitatory amino acid agonists AMPA and NMDA. The changes occur in, but were likely not restricted to, NTS neurons receiving arterial chemoreceptor afferent inputs. If these changes are reflected in synaptic inputs to NTS neurons then this observation could provide an explanation for the enhanced chemoreceptor reflexes observed after chronic exposures to intermittent hypoxia (16, 26, 38).

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