Chronic hypoxia enhances the phrenic nerve response to arterial chemoreceptor stimulation in anesthetized rats

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Dwineill, M. R., and F. L. Powell. Chronic hypoxia enhances the phrenic nerve response to arterial chemoreceptor stimulation in anesthetized rats. J. Appl. Physiol. 87(2): 817–823, 1999.—Chronic exposure to hypoxia results in a time-dependent increase in ventilation called ventilatory acclimatization to hypoxia. Increased O2 sensitivity of arterial chemoreceptors contributes to ventilatory acclimatization to hypoxia, but other mechanisms have also been hypothesized. We designed this experiment to determine whether central nervous system processing of peripheral chemoreceptor input is affected by chronic hypoxic exposure. The carotid sinus nerve was stimulated supramaximally at different frequencies (0.5–20 Hz, 0.2-ms duration) during recording of phrenic nerve activity in two groups of anesthetized, ventilated, vagotomized rats. In the chronically hypoxic group (7 days at 80 Torr inspired PO2), phrenic burst frequency (fB, bursts/min) was significantly higher than in the normoxic control group with carotid sinus nerve stimulation frequencies >5 Hz. In the chronically hypoxic group, peak amplitude of integrated phrenic nerve activity (JPhr, percent baseline) or change in JPhr was significantly greater at stimulation frequencies between 5 and 17 Hz, and minute phrenic activity (JPhr × fB) was significantly greater at stimulation frequencies >5 Hz. These experiments show that chronic hypoxia facilitates the translation of arterial chemoreceptor afferent input to ventilatory efferent output through a mechanism in the central nervous system.

hypoxic ventilatory response; acclimatization; central nervous system

Stimulation of the peripheral arterial chemoreceptors with hypoxia results in a reflex increase in ventilation. If the hypoxic stimulus is sustained for hours to weeks, ventilation continues to increase in a time-dependent manner termed ventilatory acclimatization to hypoxia (VAH). The peripheral arterial chemoreceptors, primarily the carotid bodies, are required for the initial increase in ventilation during hypoxic exposure (4). An increase in the ventilatory response to isocapnic hypoxia has been demonstrated in awake rats (1), goats (8), and cats (32) after chronic hypoxia. However, the mechanisms involved in the continued increase in ventilation remain unresolved, and various sites in the reflex pathway may be involved.

It is known that the carotid body becomes more sensitive to hypoxia, resulting in a greater input to the respiratory centers in the central nervous system (CNS) via the carotid sinus nerve. Chronic hypoxia changes the anatomy and ultrastructure, neurotransmitters, and ion channels in the carotid body (15, 16, 29, 30), and all these changes could contribute to an increased sensitivity to hypoxia in the carotid body. Single-fiber recordings from the carotid sinus nerve in anesthetized goats demonstrate that carotid sinus nerve afferent discharge frequency increases during 4 h of continuous isocapnic hypoxia (21). Single-fiber recordings from anesthetized cats during 2–3 h of hypoxia were not different from control recordings; however, after 28 days of continuous hypoxia, the carotid sinus nerve afferent discharge was significantly greater than the control levels (3). Whole carotid body neural output in anesthetized cats was significantly greater after 48 h of hypoxia (32). Increases in carotid sinus nerve activity indicate that the carotid body is becoming more sensitive to hypoxia during chronic hypoxia, and this will increase ventilation for a given arterial PO2.

Another mechanism of VAH that has been hypothesized is an increase in the sensitivity of respiratory centers in the CNS with chronic hypoxia (7). This is suggested, for example, by experiments showing that the ventilatory response to intravenous doxapram, a peripheral chemoreceptor stimulant, was significantly increased after chronic exposure to hypoxia in humans. Mechanisms of VAH in the CNS could involve neurotransmitters in the nucleus tractus solitarius (NTS), which is the primary site of afferent input from arterial chemoreceptors (9–11, 17). For example, dopamine is released in the NTS in response to severe hypoxia in anesthetized rabbits (13), and glutamate is released in the NTS in response to hypoxia in awake rats (20). Systemic N-methyl-D-aspartate receptor blockade has been shown to attenuate the hypoxic ventilatory response (HVR) in awake rats (22), and dopamine receptor blockade in the CNS decreases the HVR in anesthetized cats (27). However, the role of these neurotransmitters in chronic hypoxia has not been investigated.

The objective of this study was to determine whether chronic hypoxic exposure alters the quantitative relationship between arterial chemoreceptor input and phrenic nerve output, which we will term the CNS gain of the HVR. The experiment is designed to be independent of any potential changes in other afferent inputs or lung mechanics. Lung pressure-volume curves can change in humans during short-term acclimatization to hypoxia (12), but this has not been studied in rats.

METHODS
Experimental preparation. We studied two groups of adult Harlan Sprague Dawley rats: normoxic controls (n = 9, 424 ± 7 g) and chronically hypoxic rats that were subjected to hypoxia for 7 days, 380 Torr barometric pressure, and 80 Torr inspired PO2 (n = 8, 391 ± 9 g). Seven additional normoxic control rats were studied to test for effects of different carotid...
sinus nerve stimulation currents: current controls (419 ± 20 g). All animals were anesthetized initially with isoflurane, a tracheal cannula was inserted, and the animals were artificially ventilated (model 680 rodent respirator, Harvard) with 50% O2-balance N2 while tracheal pressure was measured (model P23 ID pressure transducer, Statham). Femoral arterial and venous catheters were inserted for arterial blood pressure measurement (model P23 ID pressure transducer, Statham), arterial blood gas sampling, and intravenous fluid (50:50% bicarbonate-Ringer lactate) injection. After catheterization the animals were switched slowly from isoflurane to urethane (1.6/kg iv) over a 20-min period. Additional anesthetic (urethan) was given intravenously as needed, as judged by a change in arterial blood pressure after a toe pinch. End-tidal P CO2 (PETO2) was measured with a flow-through capnograph (model 1265 Capnoequar, Novametrix). Rectal temperature was measured, and body temperature was maintained close to 37°C with a heated circulating-water pad.

A ventral approach was used for insertion of the tracheal cannula and the femoral arterial and venous lines and for bilateral vagotomy. The left carotid sinus nerve and left phrenic nerve were isolated using a dorsal approach. The carotid sinus nerve was cut proximal to the carotid body, freed from surrounding connective tissue, and placed on a bipolar platinum hook electrode. To avoid current spread, care was taken to carefully free as long a piece of carotid sinus nerve as possible. The left phrenic nerve was isolated, cut distally, desheathed, and placed on a bipolar silver hook electrode. Both nerves were immersed in mineral oil to prevent desiccation.

Experimental protocol. Once the surgical preparation was complete, the animal was allowed to stabilize for 40–60 min and then paralyzed with pancuronium bromide (2.5 mg/kg iv). The ventilator was adjusted to maintain PETCO2 at 3 Torr above the CO2 threshold for phrenic nerve activity. Carotid sinus nerve stimulation level was determined by finding the minimum current needed to evoke a response in the phrenic nerve output (threshold current) at 20 Hz, 0.2-ms pulse duration (848 stimulator and PS16 photoelectric stimulus isolation unit, Grass). The stimulus current for the remainder of the protocol was set 2.5–3 times above this threshold. A maximum CO2 response was elicited by elevating PETCO2 to 70–80 Torr (inspiratory fraction of CO2 = 0.10) during 30% O2 breathing. Fifteen minutes after the end of the CO2 test, the stimulation protocol began. The carotid sinus nerve was stimulated at the predetermined current at 0.5, 1, 2, 5, 8, 11, 14, 17, and 20 Hz for 45 s with 4 min between each stimulation. Four minutes after the final stimulation, the maximum CO2 response (inspiratory fraction of CO2 = 0.10, PETCO2 = 70–80 Torr) was repeated. In some of the animals, arterial blood gas samples were taken at the beginning and end of the stimulation protocol during measurement of PETCO2 to confirm that constant PETCO2 throughout the stimulation protocol reflected constant arterial P CO2 (Paco2).

For the seven current control rats, the criteria used to establish the phrenic threshold and carotid sinus nerve stimulation level were the same as in the normoxic and chronically hypoxic groups. The carotid sinus nerve was stimulated using the three-times-threshold level at 17–20 Hz. The current was then increased another 1.2–6.6 times (i.e., 3.6–20 times threshold) to approximate the stimulation current for the chronically hypoxic group (see RESULTS). These rats were used to determine whether the stimulation current was supramaximal.

Data analysis. Phrenic burst frequency (fR), peak amplitude of integrated phrenic nerve activity (JPhr), and their product (JPhr × fR, neural minute activity) were averaged over 10 bursts recorded immediately before stimulation, during the first 10 bursts of the 45-s stimulation, and during the final 10 bursts of the 45-s stimulation. The fR was expressed as an absolute value. JPhr and JPhr × fR were normalized as a percentage of baseline phrenic nerve activity, measured immediately before the first stimulation, and as a percentage of the maximal phrenic nerve response (70–80 Torr PETCO2).

A two-factor multivariate repeated-measures analysis (StatView, version 4.53) was used to determine significant differences in the phrenic nerve variables between the control and chronically hypoxic rats. Unpaired t-tests were used to determine significant differences in PETCO2 at the phrenic apneic threshold and the minimum stimulation current for the carotid sinus nerve thresholds between the two groups of rats. P < 0.05 was considered significant. Values are means ± SE.

RESULTS

Apneic threshold and stimulus currents. The CO2 apneic threshold for phrenic nerve activity was significantly different between the control and chronically hypoxic rats (P < 0.004). The apneic threshold was 29.1 ± 0.8 and 33.9 ± 1.2 Torr PETCO2 for normoxic control and current control rats, respectively. The apneic threshold for the chronically hypoxic rats was 25.2 ± 0.8 Torr PETCO2.

The threshold current for a phrenic response to carotid sinus nerve stimulation (20 Hz, 0.2-ms pulse duration) was 1.9 ± 0.4 mA in normoxic control rats. Threshold current was significantly greater in chronically hypoxic rats (3.0 ± 0.4 mA, P < 0.05). In current control rats, threshold current was 1.0 ± 0.4 mA. The stimulation current used during the protocol was set at 2.5–3 times the threshold current or 5.3 ± 2.6 and 8.8 ± 1.3 mA in normoxic control and chronic hypoxic rats, respectively. In current control rats, measurements were made with stimulation currents 3 times the threshold (3.1 ± 1.2 mA, 17 Hz) and again with current increased further to 3.6–20 times the threshold value (0.7–9 mA). There were no significant differences in fR or phrenic burst amplitude when current was increased above three times threshold. Hence, stimulus currents three times threshold produced maximum effects independent of the threshold value or absolute current, and we used this criterion to adjust the stimulus level in all experiments.

To determine whether current spread during carotid sinus nerve stimulation could influence phrenic nerve output, we crushed the central end of the carotid sinus nerve at the end of the experiment in two rats. No change in fR or peak phrenic nerve activity was observed when the carotid sinus nerve was stimulated after being crushed.

Phrenic output during carotid sinus nerve stimulation. The fR, JPhr, and JPhr × fR increased with
increasing carotid sinus nerve stimulation frequency in the normoxic and chronically hypoxic groups (Fig. 1). The $f_{r}$ during the first 10 peaks of the stimulation was significantly greater in the chronically hypoxic group at carotid sinus nerve stimulation frequencies up to 5 Hz ($P < 0.05$; Fig. 2A). During the last 10 peaks of the stimulation, $f_{r}$ in chronically hypoxic rats was significantly greater at 14- and 17-Hz stimulation frequencies ($P < 0.05$; Fig. 2B).

$\int \Phi_{fr}$ (as a percentage of baseline) during the first 10 peaks was not significantly different at any stimulation level. During the last 10 peaks of the stimulation, $\int \Phi_{fr}$ was significantly greater in the chronically hypoxic group at 8- to 20-Hz carotid sinus nerve stimulation frequencies ($P < 0.05$; Fig. 2A). The change in $\int \Phi_{fr}$ from baseline ($\Delta \int \Phi_{fr}$, percent baseline) was significantly greater in the chronically hypoxic group during the last 10 peaks at stimulation levels between 5 and 17 Hz ($P < 0.05$). $\int \Phi_{fr}$ expressed as a percentage of the maximum value during the CO$_2$ test, was not significantly different between the control and chronically hypoxic groups. $\int \Phi_{fr} \times f_{r}$ (percent baseline) was significantly greater in the chronically hypoxic group during the last 10 peaks at carotid sinus nerve stimulation frequencies up to 20 Hz ($P < 0.05$; Fig. 4). $\Delta (\int \Phi_{fr} \times f_{r})$ was significantly greater in chronically hypoxic rats under the same conditions.

Arterial blood gases. Arterial blood gas samples were taken before and after the stimulation protocol in several rats. This was done to ensure that PaCO$_2$ was being held constant by maintaining PETCO$_2$ constant. PETCO$_2$ was within 1–2 Torr of PaCO$_2$ in most cases. In the control rats, PETCO$_2$ averaged 33.6 ± 0.6 Torr when PaCO$_2$ was 34.1 ± 1.1 Torr. In the chronically hypoxic rats, PETCO$_2$ was 27.4 ± 0.6 Torr when PaCO$_2$ was 28.7 ± 0.9 Torr.

Arterial blood pressure. Mean arterial blood pressure (MABP) was measured before the stimulation began, immediately on stimulation, and just before termination of the stimulation. MABP decreased during each stimulation period in proportion to the stimulus level in both groups of rats. However, MABP was significantly lower in the control rats than in the chronically hypoxic rats during the 8- to 20-Hz stimulations (Table 1). MABP tended to decrease throughout the course of the experiment in the control rats, but the chronically hypoxic rats maintained their MABP more constant throughout the entire protocol. The change in MABP between the control and the beginning of the stimulation was significantly different between the normoxic and chronically hypoxic rats at stimulation frequencies of 5–11 Hz. At higher (14–20 Hz) and lower (0.5–2 Hz) stimulation frequencies the difference was not significantly different between groups.

**DISCUSSION**

The results from this study demonstrate that chronic exposure to hypobaric hypoxia increases the CNS gain of the HVR in the anesthetized rat. This mechanism may contribute to the increased isocapnic HVR previously reported for awake rats after chronic hypobaric hypoxia (1). VAH after prolonged hypoxic exposure may also involve increased carotid body sensitivity. An increase in carotid body sensitivity to hypoxia has been reported in cats (32) and goats after chronic hypoxia (8). The results in the present study suggest that, in addition to changes occurring at the carotid bodies, chronic hypoxia increases ventilation and the HVR by a mechanism in the CNS.

**Critique of methods.** The time course for VAH in rats is similar to that in humans (23). During 1 wk of hypoxia, minute ventilation progressively increases.

![Fig. 1. Integrated phrenic nerve activity (∫Φfr) during control, 10% CO2, and carotid sinus nerve stimulations in a chronically hypoxic rat. Response to 10% CO2 (70–80 Torr end-tidal Pco2) is maximum response after a steady state had been achieved. Carotid sinus nerve was stimulated at frequencies between 0.5 and 20 Hz at a constant current for 45 s (horizontal bars).](http://jap.physiology.org/10.1152/japplphysiol.01395.2016)
and plateaus at an acclimatized level. For this reason, we chose to use 1 wk of hypobaric hypoxia to ensure that the rats were acclimatized. During the surgical preparation, before the start of the stimulation protocol, the rats were maintained under hyperoxic (50% O2) conditions to ensure stability of the preparation. Deacclimatization, or a reduction in the increased drive to breathe after return to normoxia from chronic hypoxia, may have begun during this hyperoxic period, possibly reducing the effect of acclimatization on the peripheral chemoreceptors and central integration of the peripheral afferent input. Consequently, deacclimatization would be expected to diminish, rather than enhance, the difference we observed. However, fR and ePhr responses were significantly enhanced by chronic hypoxia, suggesting that the present results could be even greater without any influences of deacclimatization.

Electrical stimulation of the carotid sinus nerve does not mimic a physiological stimulus such as hypoxia. However, the range of stimulation frequencies used in the present study are within the range of frequencies recorded from single fibers and whole nerve activity during normoxia and hypoxia (3, 21, 24, 32). It has been reported that electrical carotid sinus nerve stimulation and hypoxia elicit similar time-dependent responses in phrenic nerve activity in anesthetized rats and cats, i.e., long-term facilitation (2, 14, 19).

The criteria used to determine the phrenic nerve baseline were the same in both groups of animals; however, the CO2 apneic thresholds were significantly different. This was not surprising, since ventilation in the awake rat after prolonged hypoxic exposure is increased and PaCO2 is decreased (1). Hence, our protocol simulates the physiological condition with a change in the PaCO2 set point. The criteria used to determine the carotid sinus nerve stimulation current were also the same in both groups. Although the absolute cur-
rents used to stimulate the carotid sinus nerve were significantly different in the two groups, the stimulation was supramaximal in both. In the current control rats, there were no differences in the response to a given stimulation frequency when the current was increased above three times threshold, up to a maximum of 9 mA (the mean stimulation current used for the chronically hypoxic rats). This indicates that although the carotid sinus nerve stimulation threshold can vary from rat to rat, the differences between the normoxic and chronic hypoxic groups were not due to different stimulation currents. Furthermore, there is no evidence for current spread to other afferent nerves in this preparation (14). The phrenic nerve response to carotid sinus nerve stimulation was eliminated after the carotid sinus nerve was crushed in our laboratory as well.

A major concern with studies designed to compare differences in neurograms between animals is in the normalization of the neurogram. Baseline phrenic nerve activity is determined by increasing P_eTCO2, a few Torr above the apneic threshold. This is done by adjusting the settings on the ventilator. Small changes in P_aCO2 can have a significant influence on baseline phrenic nerve activity. To avoid this problem, the phrenic neurogram can be normalized to peak phrenic nerve activity recorded during a maximum CO2 response (70–80 Torr P_eTCO2). However, one potentially confounding problem with normalization of the phrenic neurogram is that the phrenic nerve amplitude during a maximum response to inspired CO2 might increase after chronic exposure to hypoxia. The ventilatory response to 9% inspired CO2 increases in awake rats with chronic hypoxia (n = 5, P = 0.005; unpublished observations). However, the relationship between maximum phrenic nerve amplitude and ventilation is not known in these conditions. Therefore, we decided it was more conservative to use baseline phrenic nerve activity for normalizing the phrenic nerve amplitude. Also, we know that absolute baseline phrenic nerve activity must increase with chronic hypoxia, because baseline minute ventilation increases (1) and resting ventilation is not near any mechanical limit before or after chronic hypoxia. If we corrected for this change in baseline, the significant effect of chronic hypoxia on phrenic amplitude would be even greater.

Species differences. The rat has been shown to be a reliable animal model to study VAH. Similar to humans (26), goats (8), and cats (32), the isocapnic HVR after VAH is increased in the rat (1). When CO2 is allowed to fall during the HVR, ventilation increased, but to a lesser degree than during the isocapnic HVR (1). The time course of VAH in the awake rat is similar to that in humans (23). Goats acclimatize very rapidly, within 4–6 h (8), making comparison of time courses with other species difficult. Comparison with results from experiments using cats is also difficult, because not all laboratories find the same time course for ventilatory chemoreflex acclimatization in this species. For example, one laboratory found an increase in O2 sensitivity of carotid body chemoreceptors in cats exposed to hypoxia (70 Torr inspired PO2) for 4 wk (3). However, another laboratory reports blunted O2 sensitivity of carotid body chemoreceptors and ventilation in cats exposed to the same level of hypoxia for 3–4 wk (31) but increased O2 sensitivity after 48 h of hypoxia (32). This group also reports no increase in CNS translation of carotid sinus nerve activity into ventilatory output after 48 h of hypoxia (32). On the basis of these results, the CNS response to hypoxia in cats may vary with duration and level of hypoxia, making it somewhat difficult to compare cats with rats and humans.

Mechanism of change in CNS gain of HVR. These results demonstrate that mechanisms in addition to the known increase in carotid body chemoreceptor O2 sensitivity (8, 32) contribute to the increased HVR during chronic hypoxia. In the present study the carotid sinus nerve is sectioned distally from the carotid body before being electrically stimulated, therefore eliminating any effects of carotid body chemoreceptors on phrenic nerve activity. The CNS is the most likely site for this effect of chronic hypoxia on the translation of chemoreceptor afferent input to respiratory motor output.

Potential CNS mechanisms that can be ruled out as explaining the effect of chronic hypoxia on the gain of

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**Table 1.** Mean arterial blood pressure measured under control conditions, during the first 10 peaks of stimulation, and during final 10 peaks of stimulation in normoxic control and chronically hypoxic rats

<table>
<thead>
<tr>
<th>Stimulation Frequency, Hz</th>
<th>Control</th>
<th>Normoxia</th>
<th>Chronic hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Arterial Blood Pressure, mmHg</td>
<td>Start</td>
<td>End</td>
</tr>
<tr>
<td>0.5</td>
<td>139.5 ± 4.8</td>
<td>138.8 ± 4.9</td>
<td>138.7 ± 4.8</td>
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<tr>
<td>1</td>
<td>134.8 ± 4.8</td>
<td>132.9 ± 6.9</td>
<td>132.9 ± 7.1</td>
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<tr>
<td>2</td>
<td>136.7 ± 5.0</td>
<td>134.9 ± 4.9</td>
<td>134.3 ± 4.9</td>
</tr>
<tr>
<td>5</td>
<td>131.8 ± 6.5</td>
<td>125.6 ± 6.9</td>
<td>124.7 ± 7.3</td>
</tr>
<tr>
<td>8</td>
<td>133.6 ± 5.2</td>
<td>121.2 ± 6.2</td>
<td>124.9 ± 6.2</td>
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<tr>
<td>11</td>
<td>129.5 ± 6.3</td>
<td>114.7 ± 7.6</td>
<td>119.0 ± 8.2</td>
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<tr>
<td>14</td>
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<td>112.0 ± 7.7</td>
<td>117.3 ± 7.4</td>
</tr>
<tr>
<td>17</td>
<td>123.6 ± 7.5</td>
<td>100.6 ± 8.6</td>
<td>107.2 ± 9.0</td>
</tr>
<tr>
<td>20</td>
<td>122.6 ± 7.6</td>
<td>96.1 ± 8.9</td>
<td>102.7 ± 9.5</td>
</tr>
</tbody>
</table>

Values are means ± SE. Start, 1st 10 peaks of stimulation; End, final 10 peaks of stimulation; chronic hypoxia, 7 days of hypobaric hypoxia. Normoxia was significantly less than chronic hypoxia during 1st 10 stimulation bursts (start) between 8 and 20 Hz, P < 0.05.
the HVR include a multiplicative interaction between central CO2 sensitivity and peripheral O2 sensitivity. Chronic hypoxia increases central CO2 sensitivity, as evidenced by the decrease in apneic CO2 threshold we observed and decreased Paco2 in awake animals (1). However, rats do not appear to show a multiplicative hypoxic-hypercapnic interaction, as do humans (5), and furthermore, the multiplicative O2-CO2 interaction in humans is thought to occur in the arterial chemoreceptors and not in the CNS (6).

Another potential mechanism that is probably not involved is “hyperventilation-induced hyperpnea.” This phenomenon is observed as a persistent increase in ventilation after artificial hyperventilation (28). However, in preliminary reports of 6 h of voluntary hyperventilation in humans, no change was found in the gain of the HVR (25).

Effects of chronic hypoxia on blood pressure. To consider whether the effect of chronic hypoxia on the HVR is unique to this reflex, we also examined our blood pressure data for changes in the baroreflex. When the carotid sinus nerve was stimulated, the chemoreceptors and the baroreceptors were activated, resulting in an increase in phrenic nerve activity due to chemoreceptor stimulation as well as a fall in arterial blood pressure due to baroreceptor stimulation. MABP fell in proportion to the stimulation frequency and chronically hypoxic rats. However, the magnitude of the baroreflex response (i.e., the decrease in arterial pressure) was significantly greater in the normoxic rats than in the chronically hypoxic rats at stimulation frequencies between 5 and 11 Hz. Stimulation of the carotid sinus nerve in our protocol was not adjusted to be a supramaximal stimulus for the baroreceptor reflex. Therefore, we cannot rule out different numbers of nerve fibers being recruited and different numbers of action potentials reaching the CNS between the two groups. However, hypoxia may change the relationship between baroreceptor stimulation and arterial blood pressure, suggesting that other sensory systems may also be altered by chronic hypoxia.

In conclusion, 1 wk of hypobaric hypoxia increases the CNS gain of the HVR in adult rats. The mechanism of this change is not known, but it may involve neurotransmitter systems in the NTS.

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