Hypoxia activates nucleus tractus solitarii neurons projecting to the paraventricular nucleus of the hypothalamus

T. Luise King¹, Cheryl M. Heesch¹,², Catharine G. Clark¹, David D. Kline¹,², Eileen M. Hasser¹,²,³.

TLK, CMH, DDK and EMH designed the study; TLK, CGC, EMH collected and analyzed data, supervised by EMH; TLK, CMH, DDK and EMH prepared and edited the manuscript and provided conceptual advice.

¹Department of Biomedical Sciences and ²Dalton Cardiovascular Research Center, ³Department of Medical Pharmacology and Physiology.
University of Missouri, Columbia, MO 65211

Running Head: Chemoreflex activation of PVN-projecting nTS neurons

Contact Information:
Eileen M. Hasser, Ph.D.
Dalton Cardiovascular Research Center
University of Missouri
134 Research Park Dr.
Columbia, MO 65211
Tel: 573-882-7789
Fax: 573-884-4232
E-mail: HasserE@missouri.edu
Peripheral chemoreceptor afferent information is sent to the nucleus tractus solitarii (nTS), integrated, and relayed to other brain regions to alter cardiorespiratory function. The nTS projects to the hypothalamic paraventricular nucleus (PVN), but activation and phenotype of these projections during chemoreflex stimulation is unknown. We hypothesized that activation of PVN-projecting nTS neurons occurs primarily at high intensities of hypoxia. We assessed ventilation and cardiovascular parameters in response to increasing severities of hypoxia. Retrograde tracers were used to label nTS PVN-projecting neurons and, in some rats, RVLM-projecting neurons. Immunohistochemistry was performed to identify nTS cells that were activated (Fos-immunoreactive, Fos-IR), catecholaminergic, and GABAergic following hypoxia. Conscious rats underwent 3hrs normoxia (n=4, 21% O₂) or acute hypoxia (12, 10, or 8% O₂; n=5 each). Hypoxia increased ventilation and the number of Fos-IR nTS cells (21%, 13±2; 12%, 58±4; 10%, 166±22; 8%, 186±6). Fos expression after 10% O₂ was similar whether arterial pressure was allowed to decrease (-13±1 mmHg) or was held constant. The percentage of PVN-projecting cells activated was intensity-dependent, but contrary to our hypothesis, PVN-projecting nTS cells exhibiting Fos-IR were found at all hypoxic intensities. Notably, at all intensities of hypoxia, approximately 75% of the activated PVN-projecting nTS neurons were catecholaminergic. Compared to RVLM-projecting cells, a greater percentage of PVN-projecting nTS cells was activated by 10% O₂. Data suggest that increasing hypoxic intensity activates nTS PVN-projecting cells, especially catecholaminergic, PVN-projecting neurons. The nTS to PVN catecholaminergic pathway may be critical even at lower levels of chemoreflex activation and more important to cardiorespiratory responses than previously considered.

**Key Words:** Chemoreflex, Ventilation, Blood Pressure, Fos, Catecholaminergic neurons, GABAergic neurons
INTRODUCTION

The arterial chemoreceptor reflex responds to decreases in arterial oxygen levels and regulates ventilation, sympathetic nerve activity, and blood pressure. This reflex is critical in physiological adaptations to acute and chronic hypoxia, and also in pathophysiological conditions such as obstructive sleep apnea (OSA), hypertension, and heart failure (37, 65, 71). The first synapse of chemoreceptor afferent nerves in the central nervous system occurs in the nucleus tractus solitarii (nTS). The nTS is a highly integrative nucleus, and chemoreceptor input is modulated within the nucleus before output neurons relay this information to brain regions involved in autonomic and respiratory regulation (3, 66). The phenotype and activity of these output neurons thus are critical in shaping cardiorespiratory reflex responses.

The nTS sends monosynaptic projections directly to the rostral ventrolateral medulla (RVLM) (41, 44, 45) and this is thought to be the primary pathway involved in chemoreflex regulation of sympathetic nerve activity and breathing (31, 41, 43, 45). However, the contributions of other areas in the brain to chemoreflex responses have not been fully characterized. A brain region that plays an important role in autonomic and neuroendocrine integration is the paraventricular nucleus of the hypothalamus (PVN) (38, 77). PVN neurons are activated by hypoxia and hypercapnia (12, 24, 49). In addition, the PVN modulates cardiorespiratory function, including blood pressure (47, 57), sympathetic nerve activity (17), and respiratory timing and activity (39, 50, 69), via direct projections to the RVLM (6, 7, 17, 32), spinal cord (7, 61, 73), phrenic motor neurons and neurons located in the pre-Botzinger complex (50). There is also evidence that the PVN is required for full expression of chemoreflex responses. Lesion (57) or interruption of neuronal activity in the PVN (62) blunts the magnitude and duration of the pressor and sympathoexcitatory responses evoked by chemoreflex activation. Taken together, these data support the concept that the PVN plays an important role in central processing of the arterial chemoreflex. However, the source of input carrying chemoreceptor afferent information to the PVN remains to be established.

The PVN receives projections from a variety of brain regions important in cardiorespiratory control (67), including direct anatomical connections from the nTS (64). Because the nTS receives afferent input from the peripheral chemoreceptors, we
hypothesized that nTS neurons that project to the PVN are activated in response to hypoxia. Previous studies have shown that most PVN-projecting nTS neurons receive polysynaptic connections from visceral afferents. These neurons exhibit variable synaptic responses to solitary tract activation that include small excitatory post synaptic currents and high rates of failed synaptic transmission (9). Thus, successful transmission of peripheral sensory afferent signals from the nTS to the PVN is less likely and may require a stronger stimulus compared to monosynaptically connected pathways. We therefore hypothesized that PVN-projecting nTS neurons would be activated primarily at higher intensities of hypoxia (lower levels of inspired oxygen).

This study used Fos immunohistochemistry combined with retrograde tracers to examine activation of PVN-projecting nTS neurons by increasing hypoxic intensity. Because catecholaminergic neurons are important in cardiorespiratory reflexes (72) and a substantial number project from the nTS to the PVN (67, 69), we determined if PVN-projecting nTS neurons activated by hypoxia were catecholaminergic. Furthermore, GABAergic neurons are found throughout the nTS (5, 15, 28) and inhibition at the level of the nTS is important in modulating cardiorespiratory responses (29). Therefore, we determined if GABAergic nTS neurons project to the PVN and whether they are activated by hypoxia.

Our findings indicate that increasing the intensity of acute (three hours) hypoxia activates progressively more nTS cells, including PVN-projecting neurons. In contrast to our hypothesis, nTS neurons projecting to the PVN were activated even at the lowest intensity of hypoxia studied. With more severe hypoxic stimuli, there was further recruitment of these cells, especially catecholaminergic, PVN-projecting neurons. These results suggest that PVN-projecting nTS neurons contribute to cardiorespiratory responses and play an increasingly important role as chemoreflex stimulation intensifies.
METHODS

Animals

Experiments were performed on adult male Sprague–Dawley rats (280-350 g, n=43, Harlan, Indianapolis, IN, USA) that were maintained on a twelve hour light–dark cycle with food and water provided ad libitum. All experiments were done in accordance with the “Guiding Principles for the Care and Use of Vertebrate Animals” established by the American Physiological Society and were approved by the University of Missouri Institutional Animal Care and Use Committee. Rats were allowed at least seven days to acclimate to their surroundings prior to experimental procedures.

Surgical procedures

For protocols requiring prior instrumentation or treatment, rats were anesthetized with Isoflurane (AErane, Baxter, Deerfield, IL, USA; 5% for induction and 2–2.5% for maintenance). All procedures were performed using aseptic technique. Rats were treated post operatively with fluids (3 ml, 0.9% saline s.c.), Baytril (2.5 mg/kg i.m., Bayer, Shawnee Mission, KS, USA), and Buprenex (0.03 mg/kg s.c., Reckitt Benckiser Pharmaceuticals, Richmond, VA, USA) to maintain hydration status, prevent infection, and for pain management, respectively. Upon recovery from anesthesia animals were returned to their cages.

Microinjection of retrograde tracers. Rats (n=22) were placed in a stereotaxic apparatus (Kopf, Tujunga, CA, USA), and a midline incision (~ 2 cm) was made along the dorsal surface of the skull. Muscle and fascia were bluntly dissected to visualize bregma and lambda which were then positioned in the horizontal plane. A small drill hole was made in the skull and the dura cut for introduction of a single barrel glass pipette (OD 10-20 μm) filled with the retrograde tracer Fluoro-Gold (FG, 2% in deionized water, Fluorochrome Inc., Denver, CO, USA). The target stereotaxic coordinates for PVN microinjections were: 1.8 - 2.0 mm caudal to bregma, ± 0.5 mm lateral from the midline and 7.8 mm ventral to the dura. Fluoro-Gold (60-90 nL) was microinjected bilaterally into the PVN using a custom built pressure injection system. The volume of retrograde tracer injected was quantified by monitoring the movement of the meniscus
within the pipette using a 150× microscope (Rolyn Optics, Corvina, CA, USA) with a calibrated eyepiece micrometer.

In three animals, PVN injections were performed as described above, and microinjections were also made bilaterally in the RVLM. In these animals, we used two retrograde tracers, FG and cholera toxin B (CtB, 1% in deionized water, List Biological Laboratories, Inc., Campbell, CA). The site of injection (PVN or RVLM) for the individual tracers was varied. In these animals, a catheter was inserted into the aorta via the femoral artery for monitoring arterial pressure and was removed after the surgery. For RVLM injections, the dorsal surface of the medulla was exposed via a limited occipital craniotomy and calamus scriptorius (CS) visualized. The head was then deflected downward until CS was 2.4 mm posterior to interaural 0, positioning the medulla in a horizontal position (41, 56). Target stereotaxic coordinates for the RVLM were: 0.7 - 0.8 mm rostral and 1.6 - 1.8 mm lateral to CS, and 3.6 - 4.2 mm ventral to the dorsal surface of the brain. The RVLM was identified functionally by pressor responses (≥ 15 mmHg) to microinjection of L-glutamate (10 mM, 30 nL) through one barrel of a double barreled pipette. Without moving the pipette, the retrograde tracer (30 nL) was microinjected through the second barrel.

Retrograde tracers were injected over one minute and the pipette remained in the tissue for at least five minutes to minimize movement of tracer up the injection tract. The pipette was then removed and the incision site closed. The animals were allowed 7 - 10 days for surgical recovery and for transport of the retrograde tracer. During this period, daily clinical examination and body weight measurements were performed.

**Blood pressure measurements.** To assess mean arterial blood pressure (MAP) and heart rate (HR) changes in response to acute hypoxia, a separate group of rats (n=11) without injection of retrograde tracer was instrumented with a telemetry device (TA11PA-C40, Data Sciences International, USA). Via a midline incision (3 - 4 cm), the abdominal aorta was visualized and the catheter probe of the telemetry device was inserted. The site was sealed with a cellulose patch and tissue adhesive. The transmitter was secured to the abdominal muscle using nonabsorbable suture, and the skin incision closed. Animals were allowed to recover for at least 10 days. Arterial pressure, HR, and the strength and quality of the telemetry signal were evaluated daily.
after surgery. Five days before immunohistochemistry experiments in which MAP was maintained constant during acute hypoxia (see below), four of these animals also were instrumented with a femoral venous catheter.

In a separate group of animals (n=5) catheters (PE10 fused to PE50) were placed in the femoral artery and vein for measurement of arterial pressure and drug administration, respectively. Catheters were secured, tunneled subcutaneously and exteriorized at the back of the neck then filled with heparinized saline (10 units/mL). Animals were allowed 5 days for recovery before plethysmography experiments in which MAP was held constant.

**Oxygen saturation and respiratory reflex responses to increasing severity of hypoxia**

Ventilation was assessed in conscious, freely moving rats (n=6) by whole body plethysmography. Animals were placed in a Plexiglas plethysmography chamber (Model: 600-2400-001, Data Sciences International, USA) that contained inlet and outlet ports to permit airflow through the chamber. The animal and reference chambers were connected to a differential pressure transducer (Validyne Engineering Corp., Northridge, CA, USA; model: DP 45-34) to measure respiratory parameters (PowerLab Data Acquisition System, ADInstruments, Colorado Springs, CO, USA). We used a leaky, flow-through plethysmography chamber and the pressure signal recorded was proportional to volume changes (19); body temperature and metabolic rate were not quantified. In order to obtain a measure of tidal volume (tidal volume index), we integrated the area under the inspiratory pressure curve and normalized to body weight. Ventilatory parameters measured included: respiratory rate (RR, breaths per min), tidal volume index and minute ventilation index (RR x tidal volume index). Oxygen saturation was measured using a collar pulse oximeter (MouseOx, Starr Life Sciences Corp., USA).

All animals were acclimatized to the chamber environment for two to three hours at least five times prior to the experiment. On the day of the experiment, rats were weighed and allowed to acclimate in the chamber for 60 minutes. Ventilation and oxygen saturation were monitored continuously at baseline and during exposure (ten minutes) to 21, 12, 10 and 8% O₂-balanced N₂. Ventilatory parameters were measured
from an average of about 20 consecutive breaths independent of sniffs, sighs or movement artifacts within the last minute of exposure to each level of oxygen (40, 42). This protocol was repeated after a 30 minute recovery period breathing room air. The order of gas exposure was varied to produce a balanced design.

**Maintenance of arterial pressure.** Preliminary experiments indicated that exposure to acute hypoxia decreased MAP. To evaluate if changes in MAP affected respiratory responses, we measured the hypoxic ventilatory response independent of changes in MAP in five animals. Respiratory variables were measured using plethysmography as described above during normoxia, acute hypoxia (10% inspired O\(_2\)) and acute hypoxia during infusion of the vasoconstrictor phenylephrine (PE; 0.6 mg/mL, 0.1-0.5 mL/hr, i.v.) through the venous catheter in order to maintain MAP constant.

**nTS neuronal activation to increasing severity of hypoxia**

Five to seven days after injection of retrograde tracer, conscious rats in their home cages were acclimated to the hypoxia chamber (Biospherix Inc., Redfield, NY, USA) for 1-3 hours per day for 2 days to allow them to become accustomed to the environmental stimuli associated with the chamber. On the day of the experiment, conscious rats (n=22) with previous PVN or PVN and RVLM microinjections were allowed to acclimate for 30 minutes in the chamber (21% O\(_2\)). The gas mixture then was adjusted to bring the air in the chamber to: 21% O\(_2\) (normoxic control, n=4), 12% O\(_2\) (n=5), 10% O\(_2\) (n=5) or 8% O\(_2\) (n=5) and maintained at that percentage for three hours via a negative feedback control system, similar to previous studies (12, 23, 41, 78). Double labeled animals (n=3) were only exposed to 10% O\(_2\). Gas levels and temperature were monitored throughout the protocol.

**Maintenance of arterial pressure.** To evaluate neuronal activation due to acute hypoxia, independent of changes in arterial pressure, in a separate group of rats, MAP and HR were monitored continuously using telemetry during two hours of normoxia (21% O\(_2\); n=7), 10% O\(_2\) (n=8), or 10% O\(_2\) with infusion of PE (0.6 mg/mL, 0.1-0.5 mL/hr, i.v.; n=4) to maintain MAP constant. In seven of these rats, MAP and HR were measured under more than one condition, with at least five days between experiments. Following experiments in which MAP was held constant, animals were perfused and
brains processed for Fos immunohistochemistry. Fos expression in these animals was compared to animals in which MAP was not held constant.

**nTS immunohistochemistry**

*Transcardial perfusion and tissue preparation.* Immediately following exposure to hypoxia, animals were deeply anesthetized with Isoflurane and transcardially perfused with heparinized, Dulbecco's Modified Eagle Medium (Sigma, 125 mL, pH 7.4) bubbled with oxygen, followed by 4% paraformaldehyde (PFA, Sigma, 500 mL, pH 7.4). The brains were removed and post-fixed in 4% PFA overnight. Thirty micron hindbrain sections were cut on a vibrating microtome (VT 1000S; Leica, Germany). Immunohistochemistry was performed on every sixth section (sections separated by 180 μm).

*Immunohistochemistry protocols.* Two sets of immunohistochemistry protocols were performed on tissue from animals with PVN microinjections of retrograde tracer. Fos-immunoreactivity (IR) was used as a marker for cells within the nTS that were activated after hypoxia (23). Neurons that projected to the PVN were identified by the presence of retrograde tracer. Tyrosine hydroxylase (TH), the rate limiting enzyme in catecholamine synthesis, was used as a marker for catecholaminergic neurons. GABAergic neurons were identified using immunoreactivity for glutamic acid decarboxylase isoform 67 (GAD67), a GABA synthesizing enzyme.

For Fos and GAD67, free floating coronal sections at room temperature were rinsed (3×10 min) in 0.01 M PBS (phosphate buffered saline, pH 7.4) and incubated in 0.3% hydrogen peroxide (30 minutes) to quench endogenous peroxidases before preblocking (30 minutes) with 10% normal donkey serum (NDS; Jackson ImmunoResearch Inc., West Grove, PA, USA) in 0.01 M PBS. Sections were rinsed and incubated (72 hours) in antibodies against GAD67 (mouse anti-GAD67, 1:5000; MAB5406, Millipore Inc., Billerica, MA) and Fos (rabbit anti-Fos, 1:3000; PC38, Calbiochem; Darmstadt, Germany) with 3% NDS in 0.01 M PBS. Sections were then rinsed and incubated for two hours in donkey anti-mouse biotinylated IgG (1:300; Jackson ImmunoResearch) and Cy3-conjugated donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch) with 3% NDS in 0.01 M PBS. The sections were again washed and
incubated in a series of steps for Tyramide Signal Amplification (one hour Streptavidin-HRP, 15 min TSA-biotin using TSA kit from Perkin Elmer SAT700001). Finally, sections were rinsed, then incubated with neutravidin/avidin Oregon Green (1:300, Invitrogen Oregon Green® 488, Carlsbad, CA) with 3% NDS in 0.01 M PBS for 2 hours. After a final rinse, the sections were mounted on gel-coated slides, air-dried, coverslipped with ProlongGold (Invitrogen, P36930), and sealed with nail polish. Immunohistochemistry involving GAD67 was always performed within 1 week of perfusion of the tissue.

For Fos/TH immunohistochemistry, free floating sections underwent a series of rinse/blocking steps as described above. Sections were incubated for 24 hours in 1% NDS and 0.3% Triton-0.01M PBS containing primary antibodies against Fos (rabbit anti-Fos, 1:3000, Calbiochem) and TH (mouse anti-TH, 1:1000, Millipore Inc.). The following day, sections were rinsed then incubated for two hours in Cy3-conjugated donkey anti-rabbit IgG and Cy2-conjugated donkey anti-mouse IgG (1:200 Jackson ImmunoResearch) with 1% NDS in 0.3% Triton-0.01M PBS. Sections then were rinsed and mounted on gel-coated slides as described above. In addition to animals with PVN microinjections of retrograde tracers, tissue from rats in which MAP was maintained constant during hypoxia also underwent this protocol.

In PVN and RVLM microinjected animals the retrograde tracers FG and CtB were visualized using guinea pig anti-FG (Protos Biotech, New York, NY, USA, 1:500) and goat anti-cholera toxin B subunit (List Biological Laboratories, Inc., 1:2000), respectively. Sections from these animals also underwent a Fos/TH immunohistochemistry protocol similar to that described above. Briefly, sections were incubated for 24 hours in a solution containing antibodies against FG, CtB, Fos and TH. They then underwent a two hour incubation in AMCA-conjugated donkey anti-guinea pig IgG, Cy2-conjugated donkey anti-goat IgG, Cy5-conjugated donkey anti-mouse IgG and Cy3-conjugated donkey anti-rabbit IgG (all from Jackson ImmunoResearch).

Antibody specificity. Individual immunohistochemistry protocols were conducted simultaneously on tissue from hypoxic and normoxic animals and in each protocol at least one section served as a control (was not incubated in a primary or secondary antibody). Previous experiments verified optimal primary and secondary antibody concentrations. Antibodies were verified for their specificity by the vendor indicated
using western blots (anti-Fos) or preincubation with immunogen (anti-FG), or by our laboratory (5) and others (28) (anti-TH or anti-GAD67, respectively) using western blot. In addition, as in previous studies (1), we confirmed the specificity of the FG and CtB antibodies in pilot studies in which we compared the intrinsic fluorescence of FG or fluorescent-conjugated CtB with that of the appropriate antibody. An exact match of the antibody with the intrinsic fluorescence was observed.

**Verification of microinjection sites.** In all animals with retrograde tracer injections, the forebrain or brainstem was sectioned and examined to verify the location of PVN or RVLM injection sites, respectively. A standard brain atlas (59) was used to anatomically verify the location of each injection site. Only the animals in which the microinjections were localized to the PVN (and RVLM in double labeled animals) were used for immunohistochemistry protocols.

**Microscopy and image analysis**

*Image acquisition.* Brainstem sections were examined using an Olympus epifluorescent microscope (BX51). Filter sets for AMCA, Oregon Green or Cy2, Cy3, Cy5, and Fluoro-Gold were used as appropriate to visualize positive labeling. The image was brought into focus using the filter set for Cy3 (Fos-IR). Images in this same focal plane were then captured under each filter set using a cooled monochrome digital camera (ORCA-AG, Hamamatsu, Bridgewater, NJ, USA). Images subsequently were combined and analyzed with Image J (ver. 1.41, NIH) using a custom made plugin (GAIA Group, Novato, CA, USA, http://gaiag.net/index.html). Image brightness and contrast only were adjusted for image clarity.

*nTS analysis.* For immunohistochemical analysis, we evaluated the caudal nTS, the primary site of cardiorespiratory afferent projections. The section containing the caudal pole of the area postrema was identified and designated as calamus scriptorius (defined as “0”). Seven sections of the nTS were examined extending from caudal to rostral (-540 to +540 µm relative to calamus scriptorius). Because in some animals the injections were not centered on the PVN bilaterally and since the projection from nTS to PVN is primarily ipsilateral (68), we evaluated the nTS unilaterally, ipsilateral to the appropriate injection site. On the digital image of each section, the region of the nTS
was outlined unilaterally and cells were counted manually using Image J. Two individuals blinded to the experimental protocol performed the counting and counts for each section were averaged. The following criteria were used to identify positively labeled cells: Fos-IR was identified as round or ovoid-shaped nuclear staining with a visible nucleolus. GAD67-IR somas exhibited cytoplasmic staining surrounding a blank nucleus. TH-IR cells exhibited cytosolic labeling with visible processes and a blank nuclear region. FG or CtB-positive cells displayed bright or punctate cytosolic labeling. When the above criteria were met under more than one filter set, the cells were considered double-labeled. Cells that contained positive signal under all three filter sets were considered triple-labeled. Immunohistochemical data were analyzed as the total (sum) of counts from all sections examined within the nTS, and also at each caudal-rostral level of the nTS evaluated. In experiments studying the role of changes in MAP on expression of Fos due to hypoxia, Fos-IR in specific subregions (commissural, medial, ventrolateral, ventral, dorsolateral, and central subnuclei) of the nTS also was evaluated by comparing sections to a standard brain atlas (59).

Statistical analysis.

Statistical analyses were performed using SigmaPlot (11.0, Systat Software, San Jose, CA, USA). All data are presented as mean ± SE. Significance was accepted at p < 0.05. A one–way repeated measures ANOVA was used to compare the number of Fos, TH, GAD67 and FG labeled cells, colabeling, and percentages of colabeling among the three intensities of hypoxia and normoxia. The percent of colabeling was calculated by dividing the number of colabeled cells by the total number of cells of each individual phenotype. For example, (Fos and FG colabeled cells)/(total number of FG labeled cells) X 100 = the percent of projecting cells activated. A one-way repeated measures ANOVA was also used to evaluate respiratory changes in response to increasing intensity of hypoxia. Caudal-rostral and subregional distributions of labeling were analyzed by two-way repeated measures ANOVA. Blood pressure and heart rate responses to hypoxia were analyzed with a two-way repeated measures ANOVA. When appropriate, ANOVAs were followed by post-hoc analysis using Fisher’s LSD test.
RESULTS

Chemoreflex respiratory responses during increasing intensities of hypoxia

In conscious rats, exposure to increasing hypoxia progressively decreased arterial oxygen saturation, accompanied by an intensity-dependent increase in respiratory rate, tidal volume index, and minute ventilation index. The response of an individual rat to activation of the chemoreflex with three intensities of acute hypoxia is shown in Figure 1A. Respiratory variables increased within the first minute of hypoxic challenge and were graded with the intensity of hypoxia. Group data are shown in Figure 1B-E. There was a progressive decrease in arterial blood oxygen saturation and graded increases in respiratory rate, tidal volume index and minute ventilation index to increasing hypoxia. All parameters returned to baseline within 5 minutes when rats were allowed to recover at 21% O2. The increase in minute ventilation index was due to both increased respiratory rate and tidal volume index.

Immunohistochemistry of the nTS after graded hypoxia

Hypoxia increases Fos immunoreactivity in the nTS. Fos-immunoreactivity (IR) was used to examine activation of nTS cells by graded hypoxia. Figure 2A includes examples of Fos-IR in brainstem sections containing the nTS from animals that were exposed to three hours of normoxia (21% O2) or increasing intensities of hypoxia (12, 10, 8% inspired O2). On the right, a diagrammatic representation and bright field image depict the regions of the nTS shown in the photomicrographs. In control animals exposed only to normoxia (21% O2) the nTS contained very few Fos-IR cells, indicating a low level of basal Fos expression. The number of Fos-IR cells increased in relation to the intensity of hypoxia. Mean data showing the increase in total number (sum) of all Fos-IR cells counted in seven sections of the nTS are depicted in Figure 2B. Significant increases in Fos-IR in the nTS were apparent at 12% O2. Further intensifying the hypoxic stimulus produced a progressive increase in the number of cells activated.

A distinct caudal-rostral pattern of activation in response to hypoxia was seen in the nTS. Two-way repeated measures ANOVA revealed main effects of hypoxic intensity and caudal-rostral level but the interaction (p=0.07) did not reach statistical
significance. A greater proportion of Fos-IR was localized in the caudal nTS and decreased in more rostral sections. The greatest expression of Fos was observed at -540 µm caudal to CS while the least amount of Fos-IR was seen at 540 µm rostral to CS (Figure 2C).

**Maintenance arterial blood pressure during acute hypoxia does not affect the hypoxic ventilatory response or nTS Fos-IR**

Because preliminary studies indicated that acute hypoxia produced moderate depressor responses which may alter respiration and Fos-IR via interactions with the baroreflex, the hypoxic ventilatory response and nTS neuronal activation were compared in rats after acute hypoxia (10% O₂) or hypoxia with MAP held constant by PE infusion (10% O₂ + PE). Normoxia did not alter MAP or HR (Figure 3A and B). Acute hypoxia in conscious rats induced an early increase in HR, and a sustained depressor response which was prevented by simultaneous infusion of PE. Maintaining MAP constant did not alter oxygen saturation (10% O₂: 70.1±0.9%; 10% O₂ + PE: 70.5±1.0%) or ventilatory responses to hypoxia, including respiratory rate (10% O₂: 148±9; 10% O₂ + PE: 144±8 breaths per minute), tidal volume index (10% O₂: 0.447±0.02; 10% O₂ + PE: 0.454±0.01 arbitrary units), and minute ventilation index (10% O₂: 67±7; 10% O₂ + PE: 66±4 arbitrary units).

The total number of Fos-IR nTS neurons following hypoxia was similar whether MAP was allowed to fall (10% O₂: 184±9) or was held constant (10% O₂ + PE: 176±6). In addition, the caudal-rostral distribution of Fos-IR in the nTS was similar between groups (Figure 3C). To further evaluate potential differences in Fos expression, we also compared the distribution of Fos-IR cells within nTS subnuclei of the two hypoxic groups (Figure 3D) and the number of activated catecholaminergic nTS cells. In both groups, Fos-IR was significantly greater in the medial, dorsolateral and ventrolateral nTS compared to other subnuclei, with the greatest number of Fos-IR cells in the medial subnucleus. Importantly, Fos-IR in all subregions was similar whether arterial pressure was allowed to fall during hypoxia or was maintained constant. The number of activated catecholaminergic cells also was similar between the hypoxic groups (10% O₂: 57±3;
10% O₂ + PE: 54±7). Thus, the modest decrease in MAP did not alter the hypoxic ventilatory response nor nTS neuronal activation due to 10% hypoxia.

Neuronal phenotypes activated by acute hypoxia

Because the PVN plays an important role in modulation of cardiorespiratory responses to chemoreflex activation, and the nTS projects to the PVN, we examined whether hypoxia resulted in Fos-IR in nTS cells projecting to the PVN (FG labeled). We also evaluated whether activated and/or PVN-projecting neurons were catecholaminergic (TH-IR) or GABAergic (GAD67-IR). Labeling for all of these phenotypes was observed in the nTS. As expected, the number of TH-IR and GAD67-IR cells was independent of severity of hypoxia (Table 1).

Verification of injection sites. Before retrograde labeling in the nTS was evaluated, histological verification of microinjection sites was performed in all animals. Figure 4A contains representative photomicrographs from two individual animals that received FG injections in the PVN (left) or RVLM (right). Schematics (59) of the PVN (B) or RVLM (D) illustrate the center of injection sites in all the animals. For RVLM microinjections, injection sites were also identified functionally by pressor responses (17± 2 mmHg) to microinjection of glutamate (10 mM, 30 nL) before injection of the retrograde tracer. Only the animals in which the microinjections were located within the PVN (and RVLM in double labeled animals) were used in immunohistochemistry studies. Location of injections (Figure 4B and C) and the number of retrogradely labeled (FG) cells (Table 1) were similar in animals from the normoxic and all hypoxic groups.

PVN-projecting nTS neurons are activated by hypoxia. Microinjection of the retrograde tracer FG into the PVN resulted in labeling at all levels of the nTS evaluated. The caudal-rostral distribution of PVN-projecting cells within the nTS was similar in all groups, with more PVN-projecting neurons at caudal levels of the nTS and fewer more rostrally (data not shown). Further characterization of PVN-projecting nTS neurons revealed that almost none of the PVN-projecting neurons was GABAergic (FG + GAD67-IR, Table 1). However, combined data indicate that almost half (47±2%) of PVN-projecting neurons (FG) were colabeled with TH-IR (FG+TH).
Increased expression of Fos-IR in PVN-projecting nTS neurons was observed as hypoxic intensity was increased. Figure 5A includes examples of Fos-IR (pseudocolored red) and FG labeling (pseudocolored blue) in the nTS of animals that were exposed to normoxia (21% O₂) and each intensity of hypoxia. There were very few PVN-projecting cells that were activated after breathing 21% O₂. Note the increase in colabeling of Fos-IR and FG at increased intensities of hypoxia.

The number of PVN-projecting nTS cells that co-expressed Fos was significantly increased at all levels of hypoxia, with greater Fos-IR at higher intensities. Exposure to hypoxia significantly increased the number of nTS cells colabeled with FG and Fos-IR to 11±1, 30±2 and 34±3 cells, for 12, 10, and 8% O₂, respectively, compared to normoxic animals (3±1). The number of FG and Fos-IR colabeled cells was significantly greater at 10% and 8% compared to 12% O₂; however there was no difference between 10 and 8% O₂. We also examined the percentage of PVN-projecting nTS cells that co-expressed Fos (Figure 6A). For all intensities of hypoxia, the percent of PVN-projecting cells that was activated was significantly increased compared to normoxia. Furthermore, breathing either 10% or 8% O₂ increased Fos-IR compared to 12% O₂ but there was no difference between the two highest intensities of hypoxia.

**Catecholaminergic nTS neurons are activated by hypoxia.** Catecholaminergic (TH-IR) cells were found throughout the caudal-rostral extent of the nTS. Combined data indicate that nearly a third (31±2%) of catecholaminergic nTS cells were PVN-projecting (TH-IR and FG colabeled; Table 1). There were no differences among groups in the number of cells exhibiting colabeling for TH-IR and FG labeling.

Co-localization of Fos-IR in catecholaminergic neurons was observed in the nTS after exposure to every intensity of hypoxia. Figure 5B contains photomicrographs of the nTS showing labeling for catecholaminergic (TH-IR, pseudocolored green) and activated cells (Fos-IR, pseudocolored red) from normoxic and hypoxic animals at three intensities of hypoxia. There were very few TH-IR cells that were activated following normoxia. Note that the number of colabeled cells increased with exposure to higher intensities of hypoxia.

The number of catecholaminergic nTS cells that was activated (TH- and Fos-IR) increased significantly at all intensities of hypoxia. Compared to normoxia (4±2 cells)
the number of co-labeled cells increased significantly to 21±3, 57±3 and 69±2 cells for 12, 10, and 8% O₂, respectively. There was a graded effect of hypoxia, with each level of hypoxia activating significantly more catecholaminergic neurons compared to lower intensities. We also examined TH- and Fos-IR colabeling as a percentage of the number of catecholaminergic nTS cells (Figure 6B). At all intensities of hypoxia, the percent of catecholaminergic cells exhibiting Fos-IR was significantly greater than after normoxia. Furthermore, at each increasing intensity of hypoxia there was a significantly greater percent of TH-IR cells that was activated.

PVN-projecting catecholaminergic neurons are activated by hypoxia. Fos-IR due to hypoxia in catecholaminergic vs. non-catecholaminergic PVN-projecting nTS neurons also was examined. Figure 5C includes photomicrographs showing examples of PVN-projecting catecholaminergic nTS cells that also exhibited Fos-IR in animals exposed to normoxia or increasing hypoxia. There were few triple labeled cells (Fos-IR, TH-IR, FG) in the normoxic animals, while the number of cells that were triple-labeled increased with hypoxia. The number of PVN-projecting catecholaminergic nTS cells that was activated increased significantly at each level of hypoxia (21%: 3±1, 12%: 11±1, 10%: 25±4, 8%: 34±4). Figure 6C includes mean data indicating the percent of PVN-projecting catecholaminergic or non-catecholaminergic nTS cells that was activated by hypoxia. At all intensities of hypoxia, the percent of FG and TH-IR colabeled cells that also exhibited Fos-IR was significantly greater than normoxia, and activation was graded with intensity of hypoxia. After breathing 8% O₂, approximately two-thirds of PVN-projecting catecholaminergic cells were activated. Compared to catecholaminergic cells, there were significantly fewer non-catecholaminergic PVN-projecting cells activated at each intensity of hypoxia. Of all activated PVN-projecting nTS neurons, at each intensity of hypoxia, the majority (approximately 75%) were catecholaminergic.

Activation of GABAergic neurons by hypoxia. GABAergic neurons were observed throughout the nTS and there was no difference in the caudal-rostral distribution of GAD67-IR cells between normoxic and hypoxic animals (data not shown). Almost no GABAergic cells also projected to the PVN (Table 1). Photomicrographs showing examples of cells in the nTS with GAD67-IR (pseudocolored green) and Fos-IR (pseudocolored red) from normoxic and hypoxic animals at three intensities of hypoxia
are included in Figure 5D. Activated GABAergic neurons were observed in the nTS after exposure to all intensities of hypoxia. There were very few GAD67-IR cells that were also Fos-IR after breathing 21% O₂ (1±0) and the number of colabeled cells increased with hypoxia (12%: 8±1, 10%: 16±2, 8%: 14±3). The percent of GABAergic nTS cells that was also Fos-IR after hypoxia is shown in Figure 6D. At all intensities of hypoxia, the percent of GAD67-IR cells that was colabeled with Fos-IR was significantly greater than normoxia. Unlike the other activated phenotypes, however, maximum percent activation of GABAergic cells occurred after breathing 10% O₂ and, although changes were small, the percent of activated GABAergic cells decreased significantly at 8% (14±2%) compared to 10% O₂ (19±3%).

PVN-projecting vs. RVLM-projecting nTS neurons

Previous studies from our laboratories (41) determined that hypoxia (10% O₂) results in Fos expression in RVLM-projecting nTS neurons. In the current study, animals received microinjections of retrograde tracers in both the PVN and RVLM and we compared directly the activation by hypoxia (10% inspired O₂) of nTS neurons that projected to these two regions. Immunohistochemical analysis (Figure 7A and B) revealed separate populations of PVN- and RVLM-projecting nTS neurons that were activated (Fos-IR) by hypoxia. Fewer nTS cells projected to the PVN (63±3) than to the RVLM (158±7) and no cells were identified that projected to both the PVN and RVLM. Following hypoxia, the number of RVLM-projecting cells that was Fos-IR (40±3) was significantly greater than the number of colabeled PVN-projecting cells (29±2). However, relative to the specific population of projecting cells, the percent of PVN-projecting nTS cells activated by hypoxia (10% O₂) was significantly greater than the percent of RVLM-projecting cells activated (Figure 7C).
DISCUSSION

This study determined whether a direct pathway between the nTS and PVN is activated by increasing severity of hypoxia. We hypothesized that PVN-projecting nTS neurons are activated by hypoxia, but because the majority of these neurons receive weak, polysynaptic inputs from visceral afferents, (9), a strong hypoxic stimulus would be required to activate them. Data indicate that even the lowest intensity of hypoxia studied (12% inspired O₂) activated nTS neurons that project to the PVN. Furthermore, increasing the hypoxic intensity progressively augmented activation of nTS neurons, including PVN-projecting neurons. Catecholaminergic PVN-projecting neurons were particularly sensitive to hypoxia. These neurons may therefore play a more important role in cardiorespiratory responses to chemoreflex activation than previously understood.

Increasing intensity of hypoxia augments ventilatory responses and activation of nTS neurons

Because increasing the severity of hypoxia produced progressive increases in ventilation and neuronal activation in the nTS, it is likely that the activated cells are involved in chemoreflex function. The pattern of activation, with more Fos-IR in the caudal nTS, is consistent with previous studies showing that this area of the nTS is innervated by carotid body afferent fibers (27). These results also support previous work evaluating Fos expression in the nTS after stimulation of the chemoreflex in rats exposed to two to three hours of moderate to severe hypoxia (12, 34, 41, 78).

The progressive increase in Fos-IR in the nTS due to increasing hypoxia is most likely related primarily to chemoreflex activation rather than the influence of other afferents such as the arterial baroreceptors. In this study, hypoxia (10% O₂) resulted in a brief increase in heart rate and a small, sustained decrease in arterial pressure that occurred after five minutes. While some studies report an increase in MAP due to chemoreflex activation (11, 18, 34), our results are consistent with work in which systemic hypoxia resulted in relatively small decreases (10-20 mmHg) in arterial pressure (26, 51, 52, 60, 72). The decrease in MAP may be due to hypoxia-mediated vasodilation, either directly or indirectly via the production of vasodilator metabolites.
such as adenosine, carbon dioxide, or nitric oxide (51, 52). Importantly, both the hypoxic ventilatory response and the number and distribution of Fos-IR nTS cells following hypoxia was similar whether MAP was allowed to decrease or was held constant. Thus it is unlikely that baroreflex responses to the modest decrease in MAP contributed substantially to hypoxia-induced Fos expression in the nTS. Of course, it is possible that the same nTS neurons are activated by both chemoreflex and blood pressure changes; however separate populations of neurons appear to be activated by these distinctly different stimuli (18, 54, 55). Although we cannot eliminate the possibility that other factors could contribute, taken together, our data suggest that neuronal activation in the nTS after acute hypoxia was predominantly due to chemoreflex activation.

Involvement of PVN-projecting nTS neurons in the peripheral chemoreflex pathway

The PVN plays an important role in the modulation of breathing and sympathetic nerve activity induced by chemoreflex activation. Microinjection of excitatory amino acid or adrenergic antagonists into the PVN of anesthetized rats (47) or prior bilateral electrolytic lesion of the PVN in awake rats (57) significantly blunts the immediate pressor response following peripheral chemoreceptor activation with injection of potassium cyanide or inorganic phosphate. Furthermore, blockade of the PVN using lidocaine attenuates the arterial pressure, renal sympathetic nerve activity, and phrenic nerve response evoked by potassium cyanide-induced stimulation of arterial chemoreceptors (62). An important question not addressed by previous studies is the source of input to the PVN during chemoreceptor stimulation.

Retrogradely labeled PVN-projecting cells were observed in all caudal-rostral levels of the nTS evaluated. The greatest number of PVN-projecting neurons was found in the caudal nTS and gradually decreased more rostrally. A consideration in the current experiments is that, although some studies indicate that FG is not taken up by fibers of passage (13, 70), there are reports suggesting otherwise (22, 79). Therefore, it is possible that some FG-labeled nTS neurons may project to brain regions other than the PVN via fibers that course through the PVN. Nevertheless, the current findings are in consensus with previous studies demonstrating anatomical connections between the nTS and PVN (20, 21, 33, 67). Also consistent with previous studies indicating that a
large proportion of PVN-projecting cells in the nTS are catecholaminergic (20, 33, 67), we found that almost half of PVN-projecting nTS neurons were TH-IR. In contrast, almost none of the PVN-projecting neurons was GABAergic. As expected, the number and phenotype of PVN-projecting nTS neurons in the present study were similar among groups, independent of exposure to hypoxia.

The observation that acute hypoxia produced an intensity-dependent activation of PVN-projecting nTS neurons suggests that a direct pathway from the nTS to the PVN is important in mediating cardiorespiratory responses to hypoxia. This does not eliminate a potential role for other pathways however. The PVN not only receives direct projections from the nTS, but also from the ventrolateral medulla (21), circumventricular organs (2, 10), parabrachial complex and the periaqueductal gray (53, 58). All of these projections could be important in conveying information contributing to autonomic and neuroendocrine responses elicited by hypoxia. Nevertheless, the graded and substantial activation of PVN-projecting nTS neurons in the current experiments suggests an important role for the nTS-PVN pathway in chemoreflex responses.

Because in vitro studies indicate that the fidelity of transmission of visceral afferent information to PVN projecting nTS neurons is relatively low (9), we expected that severe hypoxia might be required to activate these neurons. However, PVN-projecting nTS neurons were activated by even the lowest intensity of hypoxia examined. It is possible that the mildest hypoxic stimulus (12% inspired O₂) used in this study was of sufficient intensity, particularly when applied for a period of three hours, to activate these polysynaptically connected nTS neurons. Alternatively, there is a subpopulation of PVN-projecting nTS neurons that are monosynaptically connected to visceral afferents (9), and these neurons may be activated at the lower intensities of chemoreflex activation. With increasing severity of hypoxia, nTS neurons receiving polysynaptic inputs may be recruited. Interestingly, the overall activation of PVN-projecting nTS neurons appeared to be maximal when breathing 10% O₂, with no further increase due to more intense hypoxia. It is possible that these polysynaptic connections may serve as a low-pass filter, limiting activation of PVN-projecting neurons. Further studies evaluating activation of nTS neurons following exposure to a greater range of hypoxic intensities are required to address these questions.
Phenotypes of nTS neurons in the peripheral chemoreflex pathway

**Catecholaminergic neurons.** Brainstem catecholaminergic neurons have been implicated in mediating adaptive autonomic and neuroendocrine responses to cardiorespiratory challenges (4, 14, 25, 63), including hypoxia (26, 34, 41, 78). We confirmed and extended this previous work, showing that increasing severity of hypoxia produced a progressive increase in activation of nTS catecholaminergic neurons and that a substantial proportion of these neurons project to the PVN. Remarkably, of PVN-projecting catecholaminergic cells, about thirty percent were activated by moderate hypoxia (12% O₂) and activation of these cells more than doubled with more severe hypoxic challenges. In contrast to PVN-projecting neurons as a whole, the subpopulation of catecholaminergic PVN-projecting neurons exhibited progressive activation at all intensities of hypoxia. Significantly fewer non-catecholaminergic PVN-projecting neurons were activated by hypoxia. Thus, hypoxia preferentially activates catecholaminergic PVN-projecting nTS cells and these neurons may specifically contribute to chemoreflex responses throughout the range of hypoxic challenges.

The catecholaminergic inputs to the PVN presumably relay sensory information and influence respiratory, neurosecretory and/or autonomic responses during chemoreflex activation, likely by activation of PVN adrenergic receptors (47). For example, stimulation of alpha adrenergic receptors increases the excitability of spinally-projecting PVN neurons by augmenting glutamatergic and attenuating GABAergic neurotransmission (16, 48). Furthermore, blockade of PVN adrenergic receptors blunts the cardiovascular response to carotid chemoreceptor stimulation with inorganic phosphate (47). Thus, adrenergic inputs may augment sympathetic responses during hypoxia, although the source of adrenergic inputs to the PVN was unknown. Together, data from the current study indicate that catecholaminergic projections from the nTS to the PVN are activated by hypoxia, and thus these nTS projection neurons may influence cardiorespiratory responses to chemoreflex activation.

Interestingly, the transcription factor Phox2b is expressed in a population of catecholaminergic neurons of the nTS, but rarely in neurons that are GAD67-IR (36). Furthermore, Phox2b is expressed in a group of brainstem neurons involved in chemoreception, including hypoxia-sensitive nTS neurons that project to the
retrotrapezoid nucleus, and it has been suggested that Phox2b defines a series of neurons critical to both central and peripheral chemoreception (30, 75). Whether Phox2b is expressed in activated catecholaminergic neurons that project to the PVN remains to be clarified.

**GABAergic neurons.** GABAergic neurons are interspersed throughout the nTS and contribute to cardiorespiratory regulation (28, 29, 35). They serve as local interneurons to modulate visceral afferent input (8, 46), project to other brainstem regions and the spinal cord (5, 74, 79), and are activated following visceral afferent stimulation (15, 79). Consistent with previous studies, (5, 8, 28, 74), we found GAD67-IR neurons distributed throughout the nTS. However, none of these GABAergic neurons projected to the PVN, suggesting that the nTS is not a major source of direct inhibitory input to the PVN. This is in contrast to findings that a proportion of nTS projections to other brain regions important in cardiorespiratory regulation, including the RVLM and CVLM, are GABAergic (5, 15, 76, 79).

Although GABAergic nTS neurons were activated by all intensities of hypoxia examined, activation was appreciably less than the other phenotypes studied. In contrast to other phenotypes, recruitment of GABAergic neurons peaked at lower levels of stimulation and decreased with more severe hypoxia. It is possible that this apparent decrease in activation of inhibitory neurons at higher intensities of hypoxia may result in disinhibition, contributing to further activation of other cellular phenotypes and augmentation of ventilation. Overall, evidence from the current study supports a role for GABAergic neurons in the modulation of chemoreflex function within the nTS.

**Activation of PVN- vs. RVLM-projecting nTS neurons during hypoxia**

Previous work from our laboratory (41) and others (34) demonstrates that RVLM-projecting cells in the nTS are activated by hypoxia (10% O2). However, the relative importance of the PVN- vs. RVLM-projecting pathways from the nTS is not known. We found that virtually no nTS cells projected to both the RVLM and PVN, consistent with the concept that few nTS neurons send collateral projections to different brain regions (33). However, both types of projection neurons were activated by a near maximal hypoxic stimulus (10% O2). At this intensity of hypoxia, the proportionate activation of
the PVN-projecting pathway was greater than the classical nTS to RVLM chemoreflex pathway. Thus, the nTS to PVN pathway also may be vital in mediating the full expression of chemoreflex responses. Future experiments will compare these pathways at less severe hypoxic intensities to determine if differential activation of RVLM-projecting versus PVN-projecting nTS neurons occurs at various levels of hypoxia.

**Perspectives and Significance**

These studies evaluated the neural pathways involved in cardiorespiratory responses to arterial chemoreceptor activation, particularly neurons projecting from the nTS to PVN. PVN-projecting nTS neurons were activated by all levels of hypoxia evaluated. Strikingly, catecholaminergic PVN-projecting neurons were preferentially activated by hypoxia compared to non-catecholaminergic neurons. Despite the substantial number of GABAergic neurons in the nTS, essentially none of them projected to the PVN. Finally, we considered the possibility of differential activation of brain region specific nTS projection neurons and found that the proportion of PVN-projecting nTS neurons activated during hypoxia was greater than the proportion projecting to the RVLM. The projection from the nTS to the PVN may be critical in mediating cardiorespiratory responses to hypoxia even during mild to moderate hypoxia. Thus, the PVN, activated by an nTS to PVN catecholaminergic pathway, may be more important to integrated chemoreflex responses than previously thought.
ACKNOWLEDGEMENTS

We would like to thank Sarah A. Friskey for her outstanding technical expertise.

GRANTS

Supported by RO1 HL98602 (EMH, CMH, DDK)
REFERENCE LIST


68. **Sawchenko PE, Swanson LW.** The organization and biochemical specificity of afferent projections to the paraventricular and supraoptic nuclei. *Prog Brain Res* 60: 19-29, 1983.


FIGURE CAPTIONS

**Figure 1 - Ventilatory responses to increasing intensities of hypoxia.** (A) 10 second raw tracings from a representative rat, showing the changes in oxygen saturation (top) and breathing (bottom) in response to chemoreflex activation using three intensities of acute hypoxia (12, 10, and 8% inspired O₂). (B-E) Group data (n=6); Effects of graded intensities of hypoxia upon respiratory variables: (B) Arterial oxygen saturation, (C) Respiratory rate, (D) Tidal Volume Index and (E) Minute Ventilation Index. Measurements reflect the average from 20 breaths during the last minute of exposure to each hypoxia intensity. p<0.05; * vs. 21% O₂; ** vs. 21 and 12% O₂; *** vs. 21, 12, and 10% O₂. Ventilatory parameters during periods of recovery are denoted by open circles.

**Figure 2 - Increasing intensity of hypoxia augments Fos-IR in the nTS.** (A) Photomicrographs taken from coronal sections at the postremal level of the nTS. Images depict Fos-IR nuclei (pseudocolored red) following exposure to normoxia (upper left) and increasing intensities of acute hypoxia (12% O₂, 10% O₂, 8% O₂). White outlines identify area of the nTS where cells IR for Fos were counted. On the right, diagrammatic representation and bright field image illustrating the region of the nTS shown in the photomicrographs. (B) Total number of Fos-IR cells counted in all seven sections of the nTS following exposure to normoxia (n=4) and each intensity of acute hypoxia (n=5 each). Note the graded increase in number of Fos-IR cells in response to increasing hypoxia. (C) Caudal-rostral distribution of Fos-IR nTS cells for each intensity of acute hypoxia. Two-way repeated measures ANOVA indicated significant main effects of hypoxic intensity and caudal-rostral level. "0" represents calamus scriptorius (the caudal-most portion of the area postrema). p<0.05; * vs. 21% O₂; ** vs. 21 and 12% O₂; *** vs. 21, 12, and 10% O₂. In (C), significance represents post hoc analysis of main effect of hypoxic intensity. nTS: nucleus tractus solitarii, AP: Area Postrema, TS: Solitary tract, DMX: Dorsal motor nucleus of the vagus. Scale bars=100 µm (photomicrographs) and 1mm (bright field).
**Figure 3 - Maintenance of arterial blood pressure during acute hypoxia does not affect nTS Fos-IR.** Mean arterial pressure (MAP, A) and heart rate (HR, B) during a period of 120 minutes of normoxia (21% O₂, n=7), systemic hypoxia without (10% O₂, n=8) or with phenylephrine infusion (10% O₂ + PE, n=4). The period of hypoxia began at time “0”. PE infusion prevented the hypoxia-induced depressor response. (C) Caudal-rostral distribution and (D) subregional distribution of Fos-IR cells in the nTS in response to hypoxia without (10% O₂) or with (10% O₂ + PE) MAP maintained at normoxic levels. There were no significant differences in caudal-rostral or subregional distribution of Fos-IR cells between groups. In A, *, p<0.05; 10% O₂ vs. 21% and 10% O₂ + PE. In B, *, p<0.05; 10% O₂ and 10% O₂ + PE vs. 21% O₂. In D, for both 10% O₂ and 10% O₂ + PE, p<0.05; SolM (a) > SolDL (b) > SolVL (c) > SolC, SolV, SolCe. SolC: commissural nTS, SolM: medial nTS, SolVL: ventrolateral nTS, SolV: ventral nTS, SolDL: dorsolateral nTS, SolCe: central nTS.

**Figure 4 - Microinjection sites were identified and verified anatomically.** (A) Epifluorescent photomicrographs of representative injection site of 60 nL of 2% FG injected bilaterally in the PVN (left) or 30 nL of FG in the RVLM (right). Arrows indicate the center of the injection sites. (B) Location of PVN injection sites unilaterally, ipsilateral to the side on which labeled cells in the nTS were counted. Filled circles represent hypoxic animals and open circles normoxic animals. Grey circles represent animals that had a retrograde tracer injected in both the PVN and RVLM. (C) Location of all RVLM injection sites, plotted unilaterally, ipsilateral to the side counted. Numbers indicate the location in millimeters caudal to Bregma. f: fornix, 3V: third ventricle, opt: optic tract, nA: nucleus ambiguus, 4V: fourth ventricle. Scale bars=100 µm

**Figure 5 - Phenotypes of nTS neurons activated following increasing intensities of acute hypoxia.** Representative merged photomicrographs showing labeling for Fos (pseudocolored red) and (A) FG (pseudocolored blue), (B) TH (pseudocolored green), (C) FG and TH, and (D) GAD67 (pseudocolored green) in the nTS from animals exposed to normoxia (21% O₂), 12% O₂, 10% O₂, or 8% O₂. In a given section, images for each fluorophore were taken in the same focal plane. Landmarks in the region of the
nTS are labeled in photomicrographs from animals breathing 21% O2. Abbreviations are the same as in Figure 2. Examples of Fos-IR cells colabeled with FG, TH, FG and TH (triple labeled cell), or GAD67 are denoted by arrows. Higher magnification of boxed areas in some panels also shows colabeling of Fos with FG and/or TH. Scale bars=100 µm (A,B,C), 50 µm (D).

**Figure 6 - Relative activation of nTS neuronal phenotypes in response to increasing intensities of acute hypoxia.** The percent of nTS cellular phenotypes that also were activated (Fos-IR) following exposure to normoxia (n=4) or increasing intensities of acute hypoxia (12% O2, 10% O2, 8% O2; n=5 each): (A) PVN-projecting [(Fos+FG)/(FG) X100], (B) catecholaminergic [(Fos+TH)/(TH) X100], (C) PVN-projecting catecholaminergic [(Fos+TH+FG)/(TH+FG) X100] or PVN-projecting noncatecholaminergic [(Fos+(FG and TH-negative))/(FG and TH-negative) X100] and (D) GABAergic [(Fos+GAD67)/(GAD67) X100] nTS neurons. (C) Solid bars, PVN-projecting catecholaminergic cells; striped bars, PVN-projecting non-catecholaminergic cells. Within the catecholaminergic group of PVN projecting neurons there was a graded effect of hypoxia. Within the noncatecholaminergic group (C) there was no difference between 10% and 8% O2. p<0.05; for a specific phenotype, * vs. 21% O2; ** vs. 21 and 12% O2; *** vs. 21, 12, and 10% O2. †, p<0.05 vs. catecholaminergic cells within each hypoxic group.

**Figure 7 - Acute hypoxia activates RVLM- and PVN-projecting nTS neurons.** (A) Representative merged photomicrograph showing immunoreactivity and labeling for Fos (pseudocolored red), TH (pseudocolored green), PVN-projecting (pseudocolored blue), and RVLM-projecting (pseudocolored gray) cells in the nTS from an animal exposed to acute hypoxia (10% inspired O2). (B) Higher magnification of boxed region in photomicrograph (A) above showing colabeled cells. Arrow: activated catecholaminergic cell, Arrow head: activated RVLM-projecting cell, Double arrow head: activated PVN-projecting catecholaminergic cell. (C) The percent of RVLM- or PVN-projecting cells colabeled with Fos-IR (n=3). *, p<0.05, PVN-projecting versus RVLM-
projecting. PVN: paraventricular nucleus of hypothalamus, RVLM: rostral ventrolateral medulla. Scale bar =100 µm
FIGURE 2

A

Normoxia 12% O2

10% O2 8% O2

AP nTS DMX nTS DMX

Caudal - Rostral nTS (μm)

B

# Fos-IR cells in the nTS

% Oxygen in inspired air

21% O2 12% O2 10% O2 8% O2

C

# Fos-IR cells

Caudal - Rostral nTS (μm)

-540 -360 -180 0 180 360 540

- 21% O2 - 12% O2 - 10% O2 - 8% O2

* ** ***
FIGURE 3

A

B

C

D

21% O₂
10% O₂
10% O₂ + PE

21% O₂
10% O₂
10% O₂ + PE

10% O₂
10% O₂ + PE

Caudal - Rostral nTS (μm)

# Fos-IR cells

# Fos-IR cells

SolIC SolIM SolVL SolIV SolIDL SolCe
FIGURE 4

A

B

C

PVN

3V

opt

-1.6 mm

opt

4V

-1.8 mm

nA

-12.24 mm

4V

-12.36 mm

RVLM

3V

nA

opt

PVN

-2.0 mm

PVN

RVLM

nA
FIGURE 5

21% O₂  12% O₂  10% O₂  8% O₂

nTS  AP  DMX  nTS  AP  DMX  nTS  AP  DMX

FOS & GAD67  FOS, FG, & TH  FOS & TH  FOS & FG
FIGURE 6

A

% PVN projecting cells activated

% Oxygen in inspired air

B

% Catecholaminergic cells activated

% Oxygen in inspired air

C

% PVN projecting catecholaminergic or non-catecholaminergic cells activated

% Oxygen in inspired air

D

% GABAergic cells activated

% Oxygen in inspired air
FIGURE 7

A

B

C

% Projecting cells activated

0 10 20 30 40 50

nTS to RVLM projecting nTS to PVN projecting

*
Table 1. Total number of positively labeled cells counted in seven nTS sections.

<table>
<thead>
<tr>
<th></th>
<th>21% O₂</th>
<th>12% O₂</th>
<th>10% O₂</th>
<th>8% O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td># FG cells</td>
<td>84±7</td>
<td>76±7</td>
<td>85±8</td>
<td>93±10</td>
</tr>
<tr>
<td># TH cells</td>
<td>145±15</td>
<td>148±4</td>
<td>147±6</td>
<td>149±4</td>
</tr>
<tr>
<td># GAD67 cells</td>
<td>106±16</td>
<td>68±11</td>
<td>83±10</td>
<td>98±16</td>
</tr>
<tr>
<td># FG + TH cells</td>
<td>51±4</td>
<td>39±3</td>
<td>44±6</td>
<td>51±6</td>
</tr>
<tr>
<td># FG + GAD67 cells</td>
<td>0±0</td>
<td>0±0</td>
<td>1±1</td>
<td>1±1</td>
</tr>
</tbody>
</table>

PVN-projecting (FG; FG labeled), catecholaminergic (TH; TH-IR), GABAergic (GAD67; GAD67-IR) and colabeled cells in normoxic animals (n=4) and in animals exposed to one of three levels of hypoxia (n=5 each). Values are mean ±S.E.