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

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King TL, Heesch CM, Clark CG, Kline DD, Hasser EM. Hypoxia activates nucleus tractus solitarii neurons projecting to the paraventricular nucleus of the hypothalamus. Am J Physiol Regul Integr Comp Physiol 302: R1219–R1232, 2012. First published March 7, 2012; doi:10.1152/ajpregu.00028.2012.—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, rostral ventrolateral medulla (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 3 h 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, ∼75% of the activated PVN-projecting nTS neurons were catecholaminergic. Compared with 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.

chemoreflex; ventilation; blood pressure; Fos; catecholaminergic neurons; GABAergic neurons

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, 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 also 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 postsynaptic 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 with 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 whether 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 whether GABAergic nTS neurons project to the PVN and whether they are activated by hypoxia.

Our findings indicate that increasing the intensity of acute (3 h) 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) that were maintained on a 12-h light-dark cycle with food and water provided ad libitum. All experiments were done in accordance with the “Guiding Principles for Research Involving Animals and Human Beings” 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 7 days to acclimate to their surroundings before experimental procedures.

Surgical Procedures

For protocols requiring prior instrumentation or treatment, rats were anesthetized with isoflurane (5% for induction and 2–2.5% for maintenance; AErane, Baxter, Deerfield, IL). All procedures were performed using aseptic technique. Rats were treated postoperatively with fluids (3 ml, 0.9% saline sc), Baytril (2.5 mg/kg), and Buprenex (0.03 mg/kg sc, Reckitt Benckiser Pharmaceuticals, Richmond, VA) 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), 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, Bayer, Shawnee Mission, KS), and Buprenex (0.03 mg/kg sc), and Baytril (2.5 mg/kg) were injected subcutaneously into the neck. 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, and then filled with heparinized saline (10 U/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) that contained inlet and outlet ports to permit airflow through the chamber. The animal and reference chambers were connected to a differential pressure transducer (model: DP 45-34, Validyne Engineering, Northridge, CA) to measure respiratory parameters (PowerLab Data Acquisition System, ADInstruments, Colorado Springs, CO). 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. 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 the following: respiratory rate (RR, breaths/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).

All animals were acclimatized to the chamber environment for 2 to 3 h at least five times before the experiment. On the day of the experiment, rats were weighed and allowed to acclimate in the chamber for 60 min. Ventilation and oxygen saturation were moni-
tored continuously at baseline and during exposure (10 min) 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-min 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 whether 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 O2), and acute hypoxia during infusion of the vasoconstrictor phenylephrine (PE; 0.6 mg/ml, 0.1–0.5 ml/h iv) through the venous catheter 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 hypoxic chamber (Biospherix, Redfield, NY) for 1–3 h 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 RVLVM microinjections were allowed to acclimate for 30 min in the chamber (21% O2). The gas mixture was then adjusted to bring the air in the chamber to 21% O2 (normoxic control, n = 4), 12% O2 (n = 5), 10% O2 (n = 5), or 8% O2 (n = 5) and maintained at that percentage for 3 h via a negative feedback control system, similar to previous studies (12, 23, 41, 78). Double-labeled animals (n = 3) were only exposed to 10% O2. 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 2 h of normoxia (21% O2: n = 7), 10% O2 (n = 8), or 10% O2 with infusion of PE (0.6 mg/ml, 0.1–0.5 ml/h iv; n = 4) to maintain MAP constant. In seven of these rats, MAP and HR were measured under more than one condition, with at least 5 days between experiments. After experiments in which MAP was held constant, animals were perfused and brains processed for Fos immunohistochemistry. Fos expression in these animals was compared with 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 postfixed 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-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 phosphate-buffered saline (PBS, pH 7.4) and incubated in 0.3% hydrogen peroxide (30 min) to quench endogenous peroxidases before preblocking (30 min) with 10% normal donkey serum (NDS; Jackson ImmunoResearch, West Grove, PA) in 0.01 M PBS. Sections were rinsed and incubated (72 h) in antibodies against GAD67 (mouse anti-GAD67, 1:5,000; MAB5406, Millipore, Billerica, MA) and Fos (rabbit anti-Fos, 1:3,000; PC38, Calbiochem; Darmstadt, Germany) with 3% NDS in 0.01 M PBS. Sections were then rinsed and incubated for 2 h 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 then washed and incubated in a series of steps for Tyramide Signal Amplification (1 h streptavidin-horseradish peroxidase, 15 min TSA-biotin using TSA kit from Perkin Elmer SAT700001). Finally, sections were rinsed and then incubated with neutavidin/avidin Oregon Green (1:300, Invitrogen Oregon Green 488, Carlsbad, CA) with 3% NDS in 0.01 M PBS for 2 h. After a final rinse, the sections were mounted on gel-coated slides, air-dried, coverslipped with Prolong-Gold (Invitrogen, P36930), and sealed with nail polish. Immunohistochemistry involving GAD67 was always performed within 1 wk 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 h in 1% NDS and 0.3% Triton-0.01 M PBS containing primary antibodies against Fos (rabbit anti-Fos, 1:3,000, Calbiochem) and TH (mouse anti-TH, 1:1,000, Millipore). The following day, sections were rinsed and then incubated for 2 h 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-0.01 M 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 RVLVM-microinjected animals the retrograde tracers FG and CIB were visualized using guinea pig anti-FG (1:500; Protos Biotech, New York, NY) and goat anti-cholera toxin B subunit (1:2,000, List Biological Laboratories), respectively. Sections from these animals also underwent a Fos/TH immunohistochemistry protocol similar to that described above. Briefly, sections were incubated for 24 h in a solution containing antibodies against FG, CIB, Fos, and TH. They then underwent a 2-h incubation in amino-4-methyl coumarin 3-acetic acid (AMCA)-conjugated donkey anti-guinea pig IgG, Cy2-conjugated donkey anti-goat IgG, Cy3-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 CIB antibodies in pilot studies in which we compared the intrinsic fluorescence of FG or fluorescent-conjugated CIB 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 brain stem was sectioned and examined to verify the location of PVN or RVLVM injection sites, respectively. A standard brain atlas (59) was used to anatomically verify the location of each injection site. Only the animals in which
Microscopy and Image Analysis

Image acquisition. Brain stem sections were examined using an Olympus epifluorescent microscope (BX51). Filter sets for AMCA, Oregon Green, or Cy2, Cy3, Cy5, and FG 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). Images subsequently were combined and analyzed with Image J (ver. 1.41, NIH) using a custom-made plugin (GAIA Group, Novato, CA, http://gaia.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 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 cytoplasmatic 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 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). All data are presented as means ± 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 percentage 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) × 100 = the percentage 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 Fig. 1A. Respiratory variables increased within the first minute of hypoxic challenge and were graded with the intensity of hypoxia. Group data are shown in Fig. 1, B–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 min 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-IR in the nTS. Fos-IR was used to examine activation of nTS cells by graded hypoxia. Figure 2A includes examples of Fos-IR in brain stem sections containing the nTS from animals that were exposed to 3 h 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 Fig. 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 (Fig. 2C).

Maintaining 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 that 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 affect MAP or HR (Fig. 3, A and B). Acute hypoxia in conscious rats induced an early increase in HR and a sustained depressor response that was prevented by simultaneous infu-
sion of PE. Maintaining MAP constant did not alter oxygen saturation ($10\%$ $O_2$: $70.1 \pm 0.9\%$; $10\%$ $O_2 +$ PE: $70.5 \pm 1.0\%$) or ventilatory responses to hypoxia, including respiratory rate ($10\%$ $O_2$: $148 \pm 9$; $10\%$ $O_2 +$ PE: $144 \pm 8$ breaths per minute), tidal volume index ($10\%$ $O_2$: $0.447 \pm 0.02$; $10\%$ $O_2 +$ PE: $0.454 \pm 0.01$ arbitrary units), and minute ventilation index ($10\%$ $O_2$: $67 \pm 7$; $10\%$ $O_2 +$ PE: $66 \pm 4$ arbitrary units).

The total number of Fos-IR nTS neurons following hypoxia was similar whether MAP was allowed to fall ($10\%$ $O_2$: $184 \pm 9$) or was held constant ($10\%$ $O_2 +$ PE: $176 \pm 6$). In addition, the caudal-rostral distribution of Fos-IR in the nTS was similar between groups (Fig. 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 (Fig. 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 with 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 ($10\%$ $O_2$: $57 \pm 3$; $10\%$ $O_2 +$ PE: $54 \pm 7$). 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 (Fig. 4A, left) or RVLM (right). Schematics (59) of the PVN (Fig. 4B) or RVLM (Fig. 4D) illustrate the center of injection sites in all the animals. For RVLM microinjections, injection sites were also identified.

Fig. 1. Ventilatory responses to increasing intensities of hypoxia. A: 10-s 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 $O_2$). B–E: group data ($n = 6$). Effects of graded intensities of hypoxia upon respiratory variables are the following. 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. $21\%$ $O_2$; ** vs. $21$, and $12\%$ $O_2$; *** vs. $21$, $12$, and $10\%$ $O_2$. Ventilatory parameters during periods of recovery are denoted by open circles.
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 (Fig. 4, B 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 coexpressed 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 with normoxic animals (3±1). The number of FG- and Fos-IR-colabeled cells was significantly greater at 10% and 8% compared with 12% O2; however, there was no difference between 10% and 8% O2. We also examined the percentage of PVN-projecting nTS cells that coexpressed Fos (Fig. 6A). For all intensities of hypoxia, the percentage of PVN-projecting cells that was activated was significantly increased compared with normoxia. Furthermore, breathing either 10% or 8% O2 increased Fos-IR compared with 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 nTS projecting catecholaminergic 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.

Colocalization 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 with normoxia (4 ± 2 cells) the number of colabeled 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 with lower intensities. We also examined TH- and Fos-IR colabeling as a percentage of the number of catecholaminergic nTS cells (Fig. 6B). At all intensities of hypoxia, the percentage of catecholaminergic cells exhibiting Fos-IR was significantly greater than after normoxia. Furthermore, at each increasing intensity of hypoxia there was a significantly greater percentage of TH-IR cells that was activated.

**PVN-projecting catecholaminergic neurons are activated by hypoxia.** Fos-IR due to hypoxia in catecholaminergic versus noncatecholaminergic 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, whereas 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 percentage of PVN-projecting catecholaminergic or noncatecholaminergic nTS cells that was activated by hypoxia. At all intensities of hypoxia, the percentage 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 with catecholaminergic cells, there were significantly fewer noncatecholaminergic PVN-projecting cells activated at each inten-

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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>

Values are means ± SE. Paraventricular nucleus (PVN)-projecting Fluoro-Gold (FG; FG labeled), catecholaminergic (tyrosine hydroxylase, TH; TH-immunoreactivity), 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). nTS, nucleus tractus solitarii.
sity of hypoxia. Of all activated PVN-projecting nTS neurons, at each intensity of hypoxia, the majority (~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 Fig. 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 (10%), and the number of colabeled cells increased with hypoxia (12%: 8 ± 1, 10%: 16 ± 2, 8%: 14 ± 3). The percentage of GABAergic nTS cells that was also Fos-IR after hypoxia is shown in Fig. 6D. At all intensities of hypoxia, the percentage 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 percentage of activated GABAergic cells decreased significantly at 8% (14 ± 2%) compared with 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 (Fig. 7, A 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. After 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 percentage of PVN-projecting nTS cells activated by hypoxia (10% O2) was significantly greater than the percentage of RVLM-projecting cells activated (Fig. 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 2 to 3 h 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 HR and a small, sustained decrease in arterial pressure that occurred after 5 min. 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 vaso-dilator 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 barore-
Fig. 6. Relative activation of nTS neuronal phenotypes in response to increasing intensities of acute hypoxia. The percentage 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: nTS-projecting [(Fos+FG)/(FG) × 100]; B: nTS-projecting catecholaminergic [(Fos+TH)/(TH) × 100]; C: nTS-projecting catecholaminergic [(Fos+TH+FG)/(TH+FG) × 100]; D: GABAergic [(Fos+GAD67)/(GAD67) × 100] nTS neurons. In C, solid bars represent nTS-projecting catecholaminergic cells; striped bars represent nTS-projecting noncatecholaminergic cells. Within the catecholaminergic group of nTS 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. 8% O2.

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 PN 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-
hypoxia, nTS neurons appeared to be maximal when breathing 10% 
recruited. Interestingly, the overall activation of PVN-project- 
ents (9), and these neurons may be activated at the lower 
neurons that are monosynaptically connected to visceral affer- 
Alternatively, there is a subpopulation of PVN-projecting nTS 
activation of these cells more than doubled with more severe 
neurons exhibiting progressive activation at all intensities of 
Significantly fewer noncatecholaminergic PVN-project- 
neurons were activated by hypoxia. Thus hypoxia 
preferentially activates catecholaminergic PVN-projecting nTS 
cells, and these neurons may specifically contribute to 
smore severe hypoxic challenges. In contrast to PVN-projecting neurons as a 
whole, the subpopulation of catecholaminergic PVN-project- 
neurons exhibited progressive activation at all intensities of 
Phox2b is expressed in activated catecholaminergic neurons 
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 
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 
30% were activated by moderate hypoxia (12% O2) and acti- 
activation of these cells more than doubled with more severe 
neurons that are GAD67-IR (36). Furthermore, 
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.

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 α-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 brain stem 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 regula-
tion (28, 29, 35). They serve as local interneurons to modulate 
visceral afferent input (8, 46), project to other brain stem 
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 unknown. 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 may also be vital in mediating the full expression of chemoreflex responses. Future experiments will compare these pathways at less severe hypoxic intensities to determine whether 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 with noncatecholaminergic 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.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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