Serotonin neurons of the caudal raphe nuclei contribute to sympathetic recovery following hypotensive hemorrhage

Ling-Hsuan Kung,1,2 Jaimee Glasgow,2,3 Anna Ruszaj,2 Thackery Gray,1,3 and Karie E. Scrogin1,2,3

1Neuroscience Graduate Program, Department of 2Pharmacology and Experimental Therapeutics and 3Cell Biology, Neurobiology, and Anatomy Graduate Program, Stritch School of Medicine, Loyola University Chicago, Maywood, Illinois

Submitted 9 November 2009; accepted in final form 2 February 2010

doi:10.1152/ajpregu.00738.2009.—Serotonin is thought to contribute to the syncopal-like response that develops during severe blood loss by inhibiting presypathetic neurons of the rostroventrolateral medulla (RVLM). Here, we tested whether serotonin cells activated during hypotensive hemorrhage, i.e., express the protein product of the immediate early gene c-Fos, are critical for the normal sympathetic response to blood loss in unanesthetized rats. Serotonin-immunoreactive cells of the raphe obscurus and raphe magnus, parapyramidal cells of the B3 region, subependymal cells of the ventral periaqueductal gray region were activated by hypotensive hemorrhage, but not by hypotension alone. In contrast to findings in anesthetized animals, lesion of hindbrain serotonergic cells sufficient to produce >80% loss of serotonin nerve terminal immunoreactivity in the RVLM accelerated the sympatholytic response to blood loss, attenuated recovery of sympathetic activity after termination of hemorrhage, and exaggerated metabolic acidosis. Hindbrain serotonin lesion also attenuated ventilatory and sympathetic responses to stimulation of central chemoreceptors but increased spontaneous arterial baroreflex sensitivity and decreased blood pressure variability. A more global neurotoxic lesion that also eliminated tryptophan hydroxylase-immunoreactive cells of the ventral periaqueductal gray region had no further effect on the sympatholytic response to blood loss. Together, the data indicate that serotonin cells of the caudal hindbrain contribute to compensatory responses following blood loss that help maintain oxygenation of peripheral tissue in the unanesthetized rat. This effect may be related to facilitation of chemoreflex responses to acidosis.

PROGRESSIVE HEMORRHAGE leads to a complex, multiphasic autonomic response, the origins of which are not well understood. The initial sympathetic activation that accompanies mild blood loss is mediated primarily by arterial baroreceptor unloading and is quite likely augmented by cardiopulmonary reflex activation (27, 31, 34). After significant blood loss, these compensatory responses suddenly abate, resulting in a sympatholytic-like event, characterized by rapid sympathetic withdrawal and cardiac vagal activation (1, 27, 33). If blood loss continues, autonomic compensation is slowly reestablished, eventually culminating in tachycardia and sympathoexcitation (45). However, if severe hypovolemia persists much beyond the point of maximal secondary sympathetic activation, the vasculature becomes unresponsive to catecholamines, leading to a decapsulation that is difficult to treat (20).

Several lines of evidence suggest that serotonin release in the central nervous system contributes to the early syncopal-like sympathetic withdrawal that accompanies significant blood loss. For instance, the hypotensive response to hemorrhage is delayed and attenuated in anesthetized animals subjected to global serotonin depletion with systemic administration of the tryptophan hydroxylase inhibitor p-chlorophenylalanine (p-CPA) (2, 6, 23). Systemic administration of the broad-spectrum serotonin receptor antagonist methysergide rapidly reverses the hypotensive response to blood loss and improves survival of anesthetized cats subjected to severe hemorrhage (6). More recently, it was found that unilateral injection of the serotonin (5-hydroxytryptamine) type 1A (5-HT1A) receptor antagonist WAY-106635 into the pressor region of the rostral ventrolateral medulla (RVLM) attenuates and delays the sympathoexcitatory response to blood loss in anesthetized rats (4). Together, these findings suggest that a sympatholytic effect of 5-HT1A receptor activation in the RVLM mediates the syncopal-like hemodynamic response to severe hemorrhage.

However, this view remains controversial, given that significant serotonin depletion fails to produce an observable delay or attenuation of the depressor response to hemorrhage in unanesthetized rabbits (8). Moreover, systemic and intracerebroventricular administration of 5-HT1A receptor agonists, including the potent full 5-HT1A receptor agonist 8-hydroxy-2-(di-n-propylamino)tetratin (8-OH-DPAT), mimics the ability of methysergide to delay the onset of the sympatholytic response to blood loss in conscious rats (36). The ability of methysergide or 8-OH-DPAT to delay hypotension is prevented by prior treatment with WAY-100635. As such, the effect of methysergide is most likely due to an agonist action on 5-HT1A receptors, rather than a blockade of endogenous serotonin. More recent evidence indicates that systemic administration of partial and full 5-HT1A receptor agonists can rapidly reverse the sympathoexcitation response to hypotensive shock (28, 45). Thus studies in unanesthetized animals call into question the role of endogenous serotonin in the sympatholytic response to hemorrhage. In fact, they suggest that serotonin receptor activation may also contribute to sympathetic recovery from hypotensive hemorrhage.

However, it is possible that a redundant, nonserotonergic pathway that is normally suppressed during anesthesia contributes to the depressor response in unanesthetized rats. Alternatively, it is possible that, in prior studies that utilized unanesthetized models, serotonin depletion was not sufficient to affect the sympathoexcitation response. To further address these possibilities, we determined whether targeted destruction of serotonin cell groups normally activated by hypotensive hem-
orrhage is required for normal sympathetic responses to hemorrhage in unanesthetized rats. Indexes of baroreflex sensitivity were also assessed to determine the effect of lesion on baroreflex integrity. Central chemoreflex sensitivity was also measured to provide functional evidence of serotonin depletion on the basis of recent evidence demonstrating the importance of ventromedial medullary serotonin cells in PCO₂ detection in conscious animals (5).

METHODS

Animals

Male Sprague-Dawley rats (300–350 g body wt; Harlan, Indianapolis, IN) were acclimated to the housing facility while given ad libitum access to food and water ≥1 wk before surgery. The facility was maintained at a constant temperature of 22 ± 2°C with a 12:12-h light-dark cycle. All experiments were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Surgery

Protocol 1. For studies assessing serotonin cell activation during hemorrhage, all rats were anesthetized with pentobarbital sodium (65 mg/kg ip) and implanted with unilateral arterial and venous femoral catheters (PE-50 heat-welded to a length of PE-10) 3–5 days before the experiment. The catheters were externalized at the neck and sutured in place.

Protocol 2. For neurotoxin studies, caudal hindbrain serotonin nuclei were initially targeted for selective serotonin lesion on the basis of outcomes from protocol 1. All rats were pretreated with the dopamine and noradrenaline reuptake blocker nomifensine (15 mg/kg in 20% β-cyclodextran) ≥30 min before neurotoxin injection to prevent toxin-induced destruction of noradrenergic and dopaminergic cells. Rats were anesthetized with pentobarbital sodium (65 mg/kg ip) and placed in a stereotaxic apparatus. The incisor bar was placed 11 mm below the level of the interaural cavity. The back of the skull was exposed, and a portion of the occipital bone was removed to expose the brain stem. The occipital membrane was incised, and a micropipette injector (~20 μm OD) was centered at the caudal tip of calamus scriptorius. 5,7-Dihydroxytryptamine (5,7-DHT; 5 mM in 0.1% ascorbic acid) or vehicle was injected into three rostrocaudal planes: 1.9, 2.9, and 3.9 mm rostral to the caudal end of calamus scriptorius. Three 200-nl injections were made 3 mm below the dorsal surface of the brain stem (relative to calamus scriptorius) at each rostrocaudal plane, 1.1 mm to the left and right of the midline and at the midline, for a total of nine injections targeted to the ventromedial serotonergic cell groups of the caudal brain stem in each rat. The neck muscle and skin were closed in separate layers.

After 2 wk, all rats were reanesthetized with pentobarbital sodium (65 mg/kg ip) and implanted with vascular catheters 1 day before the experiment, as described in protocol 2. During the same surgery, rats were implanted with a single renal sympathetic recording electrode during vascular catheter implantation surgery, as described above (35).

Data Acquisition

Arterial pressure, heart rate (HR), RSNA, and dEMG (where applicable) were recorded on a Macintosh G4 Powerbook computer using PowerLab data acquisition software (Chart version 5.2.1, ADInstruments, Colorado Springs, CO). Arterial pressure was measured with a disposable pressure transducer (Transpac IV, Abbott Labs, North Chicago, IL) and a PowerLab bridge amplifier (ADInstruments). HR was calculated using peak-to-peak detection of the pulse pressure wave. Sympathetic activity and dEMG were sampled (4,000 Hz) and amplified (×10–20,000) with PowerLab Bioamplifiers (ADInstruments). The recorded neurograms were filtered (1–10,000 Hz), rectified, and integrated over a 20-ms time constant. Background noise in the RSNA recordings was determined at the end of each experiment by measurement of the remaining signal following ganglionic blockade (hexamethonium chloride, 30 mg/kg iv). Background noise was subtracted from nerve activity values to provide a measurement of RSNA. Background noise in the dEMG electrode recording was determined during expiration, when phrenic motor nerve activity was absent. Measurements of RSNA and dEMG obtained during the experiment were normalized to baseline determined over a 10-min period directly before the start of interventions. Only RSNA and dEMG data from animals with a >2:1 signal-to-noise ratio in the respective neurograms were included in the data analysis. Respiratory rate was determined by peak-to-peak detection of the integrated dEMG signal.
In hindbrain- and sham-lesioned rats, spontaneous baroreflex sensitivity (BRS) was determined from 5-min segments of blood pressure and HR collected before hemorrhage. Data were analyzed with Nevрокard SA-BRS software version 3.2.4 to determine BRS by the sequence method (29). Gain was determined as the average slope of linear regressions obtained from a minimum of three sequences that satisfied the following constraints: three or more consecutive interbeat intervals (IBI) with variation in the same direction, >0.5 ms that correlated (r² > 0.85) with systolic, diastolic, or mean arterial blood pressure variations of >0.5 mmHg, with a three-beat delay. These parameters were chosen on the basis of prior analyses demonstrating that they retrieved the most sequences with the highest gain in intact rats (12). Cross-spectral analyses were performed on IBI and blood pressure data using a 128-point fast Fourier transformation with a smoothed Hamming window. Coherence between IBI and blood pressure variability was determined as the square root of the ratio of IBI to blood pressure power spectra with 50% overlap and zero padding of 8. Values are reported as the alpha index in the low-frequency (0.06–0.6 Hz) and high-frequency (0.6–3.0) domains. In addition, blood pressure variability was determined in the low-frequency domain and in the time domain, the latter of which was determined as the standard deviation of blood pressure between normal beats (SDNN).

Alveolar-arterial PO2 differences were calculated to estimate the degree of ventilation-perfusion mismatch in blood samples taken during hemorrhage in hindbrain- and sham-lesioned animals. Alveolar PO2 (PaO2) was estimated using the following equation: \( \text{PaO}_2 = \text{FiO}_2 \times (P_a - P_{aw}) - P_{aCO}_2/R \), where the fraction of inspired O2 (FiO2) was estimated as 0.21, barometric pressure (Pb) as 760 mmHg, water vapor pressure (Pwv) as 47 mmHg, and respiratory quotient (R) as 0.8. Arterial PO2 and PCO2 (PaO2 and PaCO2) were determined from arterial blood gas measurements obtained before and during hemorrhage (39). Arterial blood gases were determined using a blood gas analyzer (I-STAT 1, I-STAT, East Windsor, NJ).

Experimental Protocols

Protocol 1: serotonin neural activation. Rats were handled daily after surgery to flush catheters and reduce handling stress. On the day of the experiment, rats were randomized to one of the three groups and connected to the recording instrument through an overhead swivel system to allow blood withdrawal or blood pressure recording while the animals rested unrestrained in their home cage. All groups were habituated to the set-up for ≥3 h before the experiment. For the hypotensive hemorrhage group, arterial blood was withdrawn using an automated withdrawal pump set at a rate of 3.2 ml-min⁻¹kg⁻¹ for 6 min and adjusted to a slower withdrawal rate of 0.52 ml/min for an additional 4 min. This procedure produces a consistent hypotensive response after ~3.5 min and maintains pressure at 40–50 mmHg for the remainder of active blood withdrawal. A second group was given hydralazine (10 mg/kg iv) while blood pressure was recorded from the arterial catheter. In this case, the rats were exposed to the withdrawal pump noise but were not connected to the pump. In a third group, arterial pressure was recorded during exposure to the withdrawal pump noise without further intervention. At 90 min after the start of the withdrawal pump, the rats were rapidly anesthetized with pentobarbital sodium (65 mg/kg iv) and perfused transcardially as described in protocol 1 for hemorrhage.

Protocol 2: global serotonin lesion and sympathetic response to hemorrhage. On the basis of outcomes in protocol 2, additional experiments were performed on animals subjected to more global serotonin lesion. Animals prepared with the more global lesion (see Protocol 1) were connected to the recording instruments as described in protocol 1 for hemorrhage, except dEMG was not recorded and blood samples were not assayed for blood gases. Rats were subjected to hemorrhage identical to that described in protocol 1. At 40 min after the start of hemorrhage, the rats were anesthetized with pentobarbital sodium (65 mg/kg iv) and perfused transcardially as described in protocol 1 to enable collection of brain tissue for immunohistochemical verification of serotonin lesion.

Immunohistochemistry

For double-labeling of Fos and serotonin immunoreactivity, one of every six 40-μm sections was incubated in 0.2% Triton X-100 (4 min), 3% H2O2 (10 min), 6% goat serum, and rabbit anti-c-Fos primary antibody (1:1,000 dilution; Santa Cruz Biotechnology) for 48 h at 4°C. Sections were incubated in biotinylated goat anti-rabbit IgG (1:1,000 dilution; Vector Laboratories) for 1 h at room temperature and then with avidin-biotin complex (Vector Laboratories) for 50 min. Immunoreactivity was exposed with nickel sulfate-intensified 3,3’-diaminobenzadine tetrahydrochloride (DAB) chromagen to produce a black nuclear label. Sections were rinsed overnight and subjected to the same procedures with rabbit anti-serotonin antibody (1:250,000 dilution; Immunostar). Immunoreactivity was exposed with DAB in the absence of nickel to produce a brown cytoplasmic label.

Brain sections from rats in protocols 1 and 2 were processed to expose tryptophan hydroxylase immunoreactivity. One of every six sections was incubated as described above for c-Fos, except sheep anti-tryptophan hydroxylase primary antibody (1:1,000 dilution; Chemicon) was followed by biotinylated goat anti-sheep IgG (1:1,000 dilution; Vector). To verify that the neurotoxin did not affect noradrenergic neurons, one of every six sections was incubated in mouse anti-tyrosine hydroxylase (1:1,000 dilution; Chemicon). Sections were washed and incubated in the appropriate biotinylated IgG made in goat for 1 h and incubated with avidin-biotin complex, as described above. The specificity of the primary antibodies was verified by characteristic labeling of known serotonergic and adrenergic cell bodies.
Serotonin nerve terminal density was determined in rats subjected to hindbrain lesion. One of every six sections from lesioned- and sham-lesioned rats was incubated in 6% donkey serum, 0.2% Triton X-100, and rabbit anti-serotonin antibody (1:200,000 dilution; Immunostar) for 48 h at 4°C. Sections were then washed and incubated in 6% donkey serum, 0.2% Triton X-100, and donkey anti-rabbit Dylight 649 (1:200 dilution; Jackson ImmunoResearch) for 2 h. Sections were rinsed in PBS and mounted on gel-coated slides, and coverslips were immediately applied with Fluoromount (Sigma). Sections were stored at 4°C for 14 days before microscopy. Some sections containing the RVLM were double-labeled for serotonin and phenylethanolamine-N-methyltransferase (PNMT) to enable examination of serotonin fiber density in the C1 region. In this case, the above-described protocol was followed, except mouse anti-PNMT antibody (1:4,000 dilution; Abnova) was included in the primary incubation and donkey anti-mouse Cy3 (1:200 dilution; Jackson ImmunoResearch) was included in the second incubation.

Microscopy

Fos and serotonin double-labeling. Cells showing serotonin and Fos immunoreactivity were counted in one of every six sections throughout the anatomic extent of each serotonin-rich nucleus or region. These regions included the dorsal and medial raphe nuclei, the ventrolateral periaqueductal gray (vPAG) region, the raphe magnus and its lateral extension around the pyramids (hereafter referred to as the parapyramidal region), the raphe obscurus, cells in the subependymal layer at the ventrolateral border of the pyramids (hereafter referred to as the subependymal region), and the raphe pallidus (38).

Tryptophan hydroxylase and serotonin immunoreactivity in lesion studies. Because, in our experience, serotonin cell bodies are better visualized with a tryptophan hydroxylase label, cell bodies showing tryptophan hydroxylase immunoreactivity were counted throughout the regions described above. Inasmuch as serotonin nerve terminals are better visualized with a serotonin label, serotonin immunoreactivity was also assessed in terminal projections to brain stem areas known to regulate autonomic function, including the RVLM and the nucleus tractus solitarius (NTS). In rats subjected to hindbrain neurotoxin or vehicle administration, serotonin-immunoreactive fiber density in the NTS was determined unilaterally at the level of the medial and commissural NTS subnuclei, ~13.4 and 14.1 mm caudal to bregma, respectively. Serotonin fiber density was also determined in the RVLM at three levels: 11.8, 12.2, and 12.8 mm caudal to bregma. Fiber density was determined by thresholding a composite image made from thirty 1-μm deconvolved optical sections taken from each tissue section. Images were obtained with an Olympus IX80 inverted epifluorescence microscope with a motor-driven stage and a ×10 objective. Background levels were determined from unlabeled neuropil measured at the same rostrocaudal level in three to five tissue sections from different animals and averaged. All sections at the same level were subjected to the same degree of background subtraction before thresholding. All thresholding within a region was performed at the same level. Tissue from equal numbers of lesioned and control animals was processed in parallel in two separate cohorts. All images were acquired at the same exposure length: 14 days after processing. Deconvolution was performed with Vaytek Microtome software using the rapid nearest-neighbor method within each optical section. Image Pro analysis software was used to merge deconvolved optical sections into a single composite image. Serotonin nerve terminal density was measured from images in which the sections were carefully oriented to keep the midline of the section aligned in a vertical plane with the camera. An area of interest (AOI) was placed over the 8-bit image, and the percent area of the thresholded image that was occupied by the thresholded signal was determined using National Institutes of Health ImageJ software. For measurements of the commissural NTS, an AOI box 825 pixels wide and 518 pixels high was placed such that the lower left corner of the AOI touched the most dorsomedial point of the central canal. For measurement of the medial NTS, an AOI oval, with a maximal pixel width of 900 and maximal pixel height of 600, was centered such that the leading left edge of the AOI was centered over the right lateral border of the fourth ventricle, where it meets the right NTS. For measurements in the RVLM, an AOI 900 pixels wide and 800 pixels high was placed 450 pixels from the midline, such that the right lower corner touched the right ventrolateral subependymal surface of the section. With use of this method, AOIs were consistently centered over the pressor region of the RVLM, as determined by the density and orientation of PNMT-positive cells, which make up the C1 bulboospinal neurons that project to preganglionic sympathetic cells of the spinal cord.

Data Analysis

The percentage of serotonin neurons that express Fos was determined in each serotonin-rich nucleus and compared between groups by one-way ANOVA. The number of serotonin-immunoreactive neurons that express Fos was assessed along the rostral-caudal extent of each serotonin-rich nucleus. Within subjects, cell counts of three sections (240 μm apart) were summed to provide a representative cell count over 720 μm. This method was used to minimize effects of small differences in the anatomic start point of serial sections. Group differences were compared by two-way ANOVA with repeated measures. Group differences were determined by Newman-Keuls post hoc tests. Effect of lesion on blood pressure, HR, RSNA, integrated demG, respiratory rate, and serotonin nerve terminal fiber density and tryptophan hydroxylase-immunoreactive cell number was determined by two-way ANOVA with repeated measures. When significant interactions and main effects were found, Newman-Keuls post hoc tests were performed.

RESULTS

Protocol 1

Blood pressure and HR responses of animals subjected to no intervention, hydralazine injection, or hypotensive hemorrhage are shown in Fig. 1. To minimize postsurgical stress and ensure that all groups were subjected to the same surgical procedures, all animals included in the Fos assessment were implanted with single arterial catheters to enable blood withdrawal or to control for the effects of arterial catheter implantation on Fos expression. Summary data and statistical analyses of cardiovascular responses for protocol 1 are not available, since measurements of blood pressure in rats subjected to hemorrhage would have required placement of a second arterial
catheter to enable simultaneous measurement of blood pressure and blood withdrawal. To avoid the confounding effects of different surgical interventions between groups, immunohistochemical data from the rat subjected to hypotensive hemorrhage in Fig. 1 were not included in the analysis.

As shown in Fig. 1, the hemorrhage protocol produced transient bradycardic and hypotensive responses that recovered after termination of blood withdrawal 10 min after the start of hemorrhage. The hemodynamic profile of this animal is highly consistent with summary data from sham-lesioned animals in protocol 2 and our previous studies (28, 37). Hydralazine injection produced a progressive hypotensive response that reached a nadir similar to that observed during hypotensive hemorrhage. However, the hypotensive effect was maintained for the duration of the experiment. Hypotension following hydralazine was accompanied by a progressive tachycardic response indicative of persistent arterial baroreflex activation. This single example is highly consistent with results from prior studies that used the same dosing regimen (30). Blood pressure and HR responses in the control rat declined slightly over the course of the 90-min recording period.

The raphe obscurus, the parapyramidal region, and the vlPAG region showed a greater percentage of serotonin-immunoreactive cells that expressed Fos after hypotensive hemorrhage than after hydralazine or control treatment (Table 1). Repeated-measures analysis through the rostral-caudal extent of each serotonin-rich nucleus showed significantly higher numbers of serotonin neurons expressing Fos in the raphe obscurus, the subependymal region, the parapyramidal region, the raphe magnus (Fig. 2), and the vlPAG region. Representative labeling of Fos and serotonin immunoreactivity in these nuclei are shown for individual rats subjected to control con-

Table 1. Percentage of serotonin-immunoreactive neurons that also show c-Fos immunoreactivity

<table>
<thead>
<tr>
<th>Region</th>
<th>Control (n = 7)</th>
<th>Hydralazine (n = 8)</th>
<th>Hemorrhage (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raphe pallidus</td>
<td>11.9 ± 2.1</td>
<td>14.4 ± 1.9</td>
<td>24.7 ± 4.8</td>
</tr>
<tr>
<td>Raphe obscurus</td>
<td>7.2 ± 1.2</td>
<td>6.2 ± 0.4</td>
<td>12.1 ± 1.8*‡</td>
</tr>
<tr>
<td>Subependymal region</td>
<td>12.6 ± 2.9</td>
<td>13.1 ± 2.2</td>
<td>24.9 ± 4.0</td>
</tr>
<tr>
<td>Parapyramidal region</td>
<td>15.9 ± 3.0</td>
<td>17.9 ± 2.0</td>
<td>28.0 ± 4.3*‡</td>
</tr>
<tr>
<td>Raphe magnus</td>
<td>13.3 ± 2.0</td>
<td>13.2 ± 1.6</td>
<td>19.3 ± 2.5</td>
</tr>
<tr>
<td>Median raphe</td>
<td>12.3 ± 1.5</td>
<td>13.4 ± 2.3</td>
<td>19.3 ± 4.4</td>
</tr>
<tr>
<td>Dorsal raphe</td>
<td>9.3 ± 1.7</td>
<td>7.7 ± 0.9</td>
<td>14.9 ± 4.0</td>
</tr>
<tr>
<td>vlPAG region</td>
<td>14.2 ± 3.1</td>
<td>10.1 ± 1.2</td>
<td>23.7 ± 3.6*‡</td>
</tr>
</tbody>
</table>

Values are group means ± SE of serotonin-immunoreactive cells per 40-μm section obtained every 240 μm throughout the extent of the indicated nucleus. *P < 0.05 vs. control. †P < 0.05 vs. hydralazine. ‡P < 0.01 vs. hydralazine.

Fig. 2. Numbers of serotonin-immunoreactive cells averaged across groups and numbers of serotonin-immunoreactive cells that also showed Fos immunoreactivity in brains from rats subjected to surgery but no intervention, hydralazine treatment, or hypotensive hemorrhage. Profiles are shown for those nuclei that had significantly greater Fos label after hemorrhage than after no intervention or hydralazine treatment (Table 1). Repeated-measures analysis through the rostral-caudal extent of each serotonin-rich nucleus showed significantly higher numbers of serotonin neurons expressing Fos in the raphe obscurus, the subependymal region, the parapyramidal region, the raphe magnus (Fig. 2), and the vlPAG region. Representative labeling of Fos and serotonin immunoreactivity in these nuclei are shown for individual rats subjected to control con-
ditions, hydralazine treatment, and hypotensive hemorrhage in Fig. 3. Serotonin cells of hydralazine-treated rats did not show increased Fos expression beyond that observed in control animals in any of the serotonin-rich regions.

On the basis of these results, injections of 5,7-DHT were targeted to the caudal hindbrain serotonergic nuclei, primarily at the midline raphe magnus and obscurus region and laterally at the parapyramidal and subependymal regions in the first set of lesion studies. Lesion did not significantly affect body weight or hemodilution during the course of hemorrhage (Table 2). Blood pressure, HR, and RSNA of rats injected with vehicle showed typical biphasic responses during hemorrhage, characterized by a short normotensive phase, during which sympathetic activity increased, followed by a rapid fall in all three variables (Fig. 4). After reaching their nadir between 6 and 8 min after the start of blood withdrawal, the three variables began to recover and reached a steady state within 20 min of hemorrhage termination. The respiratory response was characterized by tachypnea during the sympatholytic phase, which began to normalize before the end of hemorrhage and continued to decline slightly below baseline, where it remained for the rest of the recording period. The integrated dEMG response was somewhat variable. In general, it rose initially during the sympathoexcitatory phase, declined to baseline, and fluctuated thereafter. In general, dEMG was elevated above baseline, but an overall ANOVA failed to show a significant effect of time in control animals.

Hindbrain neurotoxic lesion did not significantly affect the blood pressure or HR response to hemorrhage or recovery. Qualitatively, baseline sympathetic burst frequency or amplitude did not appear different between groups (data not shown). The maximal rise in sympathetic activity during blood withdrawal did not differ between groups (106.6 ± 19.0 and 69.6 ± 23% of baseline for control and lesioned groups). The sympatholytic response developed more quickly in the lesioned animals, but the nadirs were similar. Sympathetic activity in lesioned rats recovered to a lower steady state overall (P <

Table 2. Body weight at start of the experiment and hematocrit and plasma protein concentration before and after the experiment

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body Wt, g</th>
<th>Hematocrit, %</th>
<th>Plasma Protein, g/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Baseline</td>
<td>60 min</td>
<td>Baseline 60 min</td>
</tr>
<tr>
<td>Control</td>
<td>15</td>
<td>377.2 ± 4.7</td>
<td>39.7 ± 0.6</td>
<td>30.6 ± 0.7*</td>
</tr>
<tr>
<td>Lesion</td>
<td>10</td>
<td>389.7 ± 6.9</td>
<td>39.8 ± 1.7</td>
<td>29.5 ± 1.0*</td>
</tr>
</tbody>
</table>

Values are group means ± SE before (baseline) and 60 min after start of hemorrhage. *P < 0.01 vs. baseline within group.

Fig. 3. Representative photomicrographs of selected serotonin-rich regions that express significantly greater levels of Fos following hemorrhage in control, hydralazine-treated, and hemorrhaged rats. Serotonin immunoreactivity is shown as a gray cytoplasmic label and Fos as a black nuclear label. Arrows in lower-magnification (×10) images indicate area of inset obtained at higher magnification (×40). Scale bar represents scale in all images.
includes 7 rats. Values are group means
number of rats in lesion group is 8 for all parameters, except RSNA, which
control group is 13 for MAP, HR, and RR, 10 for RSNA, and 11 for dEMG;
(5,7-DHT, lesion,
lower level (animals during active blood loss but then declined to an overall
0.01). Respiratory rate of lesioned rats paralleled that in control
animals during active blood loss but then declined to an overall
lower level (P < 0.05). Integrated dEMG declined from the
start of hemorrhage and remained below baseline and below
that in control rats throughout recovery (P < 0.01).

Blood gases and acid-base balance did not differ between
groups before hemorrhage. In sham-lesioned rats, a significant
decrease in pH was only observed in the blood sample taken 60
min after start of blood withdrawal (Table 3). Lactate accumu-
lation and loss of base excess developed within the first 10 min
of blood loss (P < 0.01). In control animals, lactate and base
excess began to return to baseline by the end of the recording
period. Loss of base excess and lactate accumulation were
exaggerated in lesioned animals and remained exaggerated
through the end of the experiment (P < 0.01). Consistent with
these findings, arterial pH was significantly reduced compared
with control rats at 10 and 60 min after the start of hemorrhage
(P < 0.05 and P < 0.01, respectively).

PaO2 increased during the first 10 min of hemorrhage and
remained elevated throughout recovery in sham-lesioned rats
(P < 0.01). Lesioned rats showed a similar increase in PaO2
initially that further increased and exceeded that in control
animals during recovery (P < 0.01). PaCO2 declined initially
during hemorrhage (P < 0.01) and recovered in the posthem-
orrhage period similarly in both groups.

Exposure to hyperoxic hypercapnia increased sympathetic
activity and ventilation but reduced HR in sham-lesioned rats
(Figs. 5 and 6). Increased ventilation was characterized by
increased rate and amplitude of phrenic nerve bursts with
increasing CO2. Lesion attenuated the ventilatory (P < 0.05)
and sympathetic (P < 0.01) responses to 8% CO2. However,
lesion did not affect bradycardia or tachypnic responses to
increasing CO2. Arterial pH and PaCO2 did not differ between
groups over the course of the chemoreflex experiment (Table 4).

Average spontaneous baroreflex gain of the HR reflex was
increased in lesioned rats when assessed with the sequence
method (P < 0.05) when the IBI was related to systolic, mean,
or diastolic pressure (Table 5). There was a tendency for an
increased baroreflex gain in lesioned groups when assessed by
the spectral method (P < 0.06). Similarly, the power of blood
pressure variability in the low-frequency domain tended to be
lower in lesioned animals, but the difference was not signifi-
cant (P < 0.07). However, blood pressure variability in the
time domain was significantly reduced in lesioned rats when
systolic, mean, or diastolic pressure was assessed (P < 0.05).
The low-frequency-to-high-frequency ratio of HR variability
also did not differ between groups.

Inasmuch as that hindbrain serotonin lesion did not delay the
sympatholytic response to blood loss, the experiment was
repeated in rats subjected to a more global serotonin lesion to
determine whether pontine serotonin neurons contributed to the
hypotensive response to hemorrhage. As shown in Fig. 7,
baseline blood pressure was slightly, but not significantly,
lower in rats subjected to the more global lesion. Neither the
latency to onset of the hypotensive response nor the nadir in
pressure during hemorrhage was affected by the global lesion.
Blood pressure recovered to a slightly lower level in lesioned
animals, but the difference was not significant. Similarly,
the lesion did not affect the latency of the bradycardic response
following the start of hemorrhage. However, a spurious re-
response in one lesioned rat led to a slight, but nonsignificant,
attenuation in the extent of the bradycardic response. The
latency to onset of the sympatholytic response was also una-
affected by lesion. However, the nadir reached by the end of
hemorrhage was significantly greater and recovery of sympa-
thetic activity was significantly lower in lesioned rats.

Hindbrain and global neurotoxin injection resulted in large
reductions in the numbers of tryptophan hydroxylase-immuno-
reactive cells throughout the caudal serotonin-rich region of the
brain stem (Fig. 8). The degree of depletion following neuro-
toxin injection was most extensive between 13.5 and 11 mm
caudal from bregma. In general, the extent of hindbrain sero-
tonin cell depletion was greater when the neurotoxin was
targeted to the hindbrain than when it was injected into the
ventricles. The major histological difference produced by the

Fig. 4. Mean arterial pressure (MAP), HR (beats/min), renal sympathetic nerve
activity (RSNA), integrated diaphragmatic EMG (dEMG), and respiratory rate
(RR, breaths/min) during hemorrhage (gray shaded area) and subsequent
recovery in rats treated with vehicle (control, ) and 5,7-dihydroxytryptamine
(5,7-DHT, lesion, ) targeted to the caudal raphe nuclei. Number of rats in
control group is 13 for MAP, HR, and RR, 10 for RSNA, and 11 for dEMG;
number of rats in lesion group is 8 for all parameters, except RSNA, which
includes 7 rats. Values are group means ± SE. *P < 0.05, **P < 0.01 between
groups.
two methods was a greater depletion of serotonin cells in the more rostral portions of the raphe obscurus with the hindbrain-targeted injection, whereas ventricular administration produced a more diffuse depletion throughout the nucleus. The hindbrain lesion did not affect tryptophan hydroxylase-positive cell numbers in the pontine serotonin nuclei, whereas ventricular administration of the neurotoxin produced extensive depletion in the dorsal raphe and vlPAG regions (Fig. 10).

Tyrosine hydroxylase-immunoreactive cells in the locus ceruleus and A1 region showed no apparent difference in morphology after treatment with the neurotoxin (Fig. 10, A–D). Tyrosine hydroxylase-immunoreactive cell number in the A2 and A5 regions was not affected by neurotoxin injection. Cell counts in the locus ceruleus were not determined because of the intense staining and inability to differentiate single cells. The number of tyrosine hydroxylase-immunoreactive cells in the A1 region was not determined, since it was not possible to differentiate between noradrenergic cells of the A1 region and adrenergic cells of the C1 region within the same section using DAB labeling techniques. However, the apparent density of staining did not differ in either region (Fig. 10, A–D).

Serotonin fiber density was reduced by >80% at three rostral-caudal levels within the C1 region of the RVLM (Fig. 11, E and F, Table 6). Serotonin nerve terminal immunoreactivity in the NTS at the level of the commissural and medial NTS subnuclei was diminished by 50–60% after targeted caudal hindbrain neurotoxin administration.

**DISCUSSION**

Serotonin cells of five serotonin-rich regions were selectively activated during hypotensive hemorrhage in unanesthetized rats. These regions include four caudal medullary regions: the raphe obscurus, cells of the subependymal layer at the ventrolateral border of the pyramidal tracts, and the raphe magnus and its lateral extension around the pyramidal tracts. In addition, more rostral serotonin cells in the vlPAG region were also selectively activated by acute hypotensive hemorrhage. Selective destruction of serotonin cells in the caudal raphe nuclei sufficient to produce substantial loss of serotonin innervation of the RVLM slightly accelerated the sympatholytic response to blood loss. More global lesion, which included serotonin cells of the vlPAG region, had no effect on the depressor and sympatholytic responses to blood loss. Instead, rats with selective lesion of caudal hindbrain serotonin cells or more global serotonin cell destruction showed an attenuated recovery of sympathetic activity after hemorrhage compared with control rats.

These findings contradict several lines of evidence that suggest that serotonin release contributes to the syncopal-like response to severe blood loss. In prior studies, systemic administration of the tryptophan hydroxylase inhibitor p-CPA was found to attenuate the hypotensive and bradycardic responses to severe hemorrhage in the pentobarbital-anesthetized cat (6). Systemic p-CPA treatment also prevented the sympa-
activate 5-HT1A receptors expressed by bulbospinal presympathetic neurons. This led to the view that blood loss stimulates serotonin neurons to delay the depressor response to blood loss in rats hemorrhaged under pentobarbital sodium anesthesia (4). Together, these studies showed that serotonin depletion on the depressor response to simulated blood loss in chloralose-anesthetized rats (23). A more recent study found that unilateral injection of a selective 5-HT1A receptor antagonist into the pressor region of the RVLM prevented the sympatholytic response and delayed the depressor response to blood loss in unanesthetized animals. These findings confirm evidence from a similar study in which examination of cells limited to the obex delays the initial hypotensive response in urethane-anesthetized rats. In the present study, hindbrain neurotoxin injections were effective in depleting serotonin cells rostral to obex, but they had little effect on more caudal cells. Taken together, the evidence is consistent with the view that neurons in the ventromedial medulla that mediate the depressor response to hemorrhage are either not serotonergic or contribute to a redundant serotonin-dependent pathway that mediates sympatholysis under anesthesia, whereas an alternative nonserotonergic pathway is activated in the unanesthetized rat. Alternatively, it is plausible that a nonserotonergic pathway that remains inactive under anesthesia compensates for serotonin-mediated sympathetic effects after lesion in unanesthetized animals.

The data further suggest that serotonin cells are activated (express Fos) to counteract the hemodynamic disturbances associated with blood loss, rather than as the trigger that induces sympathetic withdrawal. The stimulus (or stimuli) that induced serotonin cell activation is not clear. It was not likely hypothesizing serotonin cell activation that either not serotonergic or contribute to a redundant serotonin-dependent pathway that mediates sympatholysis under anesthesia, whereas an alternative nonserotonergic pathway is activated in the unanesthetized rat. Alternatively, it is plausible that a nonserotonergic pathway that remains inactive under anesthesia compensates for serotonin-mediated sympathetic effects after lesion in unanesthetized animals.

Prior work showed that inactivation of cells in the ventromedial medullary raphe region interferes with normal recovery of blood pressure and HR after blood loss (10, 13). Chemical inactivation of the ventromedial raphe region that lies caudal to the obex exaggerates hypotension and slows recovery of blood pressure and HR, whereas inactivation of regions rostral to obex delays the initial hypotensive response in urethane-anesthetized rats. In the present study, hindbrain neurotoxin injections were effective in depleting serotonin cells rostral to obex, but they had little effect on more caudal cells. Taken together, the evidence is consistent with the view that neurons in the ventromedial medulla that mediate the depressor response to hemorrhage are either not serotonergic or contribute to a redundant serotonin-dependent pathway that mediates sympatholysis under anesthesia, whereas an alternative nonserotonergic pathway is activated in the unanesthetized rat. Alternatively, it is plausible that a nonserotonergic pathway that remains inactive under anesthesia compensates for serotonin-mediated sympathetic effects after lesion in unanesthetized animals.

The data further suggest that serotonin cells are activated (express Fos) to counteract the hemodynamic disturbances associated with blood loss, rather than as the trigger that induces sympathetic withdrawal. The stimulus (or stimuli) that induced serotonin cell activation is not clear. It was not likely hypothesizing serotonin cell activation that either not serotonergic or contribute to a redundant serotonin-dependent pathway that mediates sympatholysis under anesthesia, whereas an alternative nonserotonergic pathway is activated in the unanesthetized rat. Alternatively, it is plausible that a nonserotonergic pathway that remains inactive under anesthesia compensates for serotonin-mediated sympathetic effects after lesion in unanesthetized animals.

Prior work showed that inactivation of cells in the ventromedial medullary raphe region interferes with normal recovery of blood pressure and HR after blood loss (10, 13). Chemical inactivation of the ventromedial raphe region that lies caudal to the obex exaggerates hypotension and slows recovery of blood pressure and HR, whereas inactivation of regions rostral to obex delays the initial hypotensive response in urethane-anesthetized rats. In the present study, hindbrain neurotoxin injections were effective in depleting serotonin cells rostral to obex, but they had little effect on more caudal cells. Taken together, the evidence is consistent with the view that neurons in the ventromedial medulla that mediate the depressor response to hemorrhage are either not serotonergic or contribute to a redundant serotonin-dependent pathway that mediates sympatholysis under anesthesia, whereas an alternative nonserotonergic pathway is activated in the unanesthetized rat. Alternatively, it is plausible that a nonserotonergic pathway that remains inactive under anesthesia compensates for serotonin-mediated sympathetic effects after lesion in unanesthetized animals.

The data further suggest that serotonin cells are activated (express Fos) to counteract the hemodynamic disturbances associated with blood loss, rather than as the trigger that induces sympathetic withdrawal. The stimulus (or stimuli) that induced serotonin cell activation is not clear. It was not likely hypothesizing serotonin cell activation that either not serotonergic or contribute to a redundant serotonin-dependent pathway that mediates sympatholysis under anesthesia, whereas an alternative nonserotonergic pathway is activated in the unanesthetized rat. Alternatively, it is plausible that a nonserotonergic pathway that remains inactive under anesthesia compensates for serotonin-mediated sympathetic effects after lesion in unanesthetized animals.

Prior work showed that inactivation of cells in the ventromedial medullary raphe region interferes with normal recovery of blood pressure and HR after blood loss (10, 13). Chemical inactivation of the ventromedial raphe region that lies caudal to the obex exaggerates hypotension and slows recovery of blood pressure and HR, whereas inactivation of regions rostral to obex delays the initial hypotensive response in urethane-anesthetized rats. In the present study, hindbrain neurotoxin injections were effective in depleting serotonin cells rostral to obex, but they had little effect on more caudal cells. Taken together, the evidence is consistent with the view that neurons in the ventromedial medulla that mediate the depressor response to hemorrhage are either not serotonergic or contribute to a redundant serotonin-dependent pathway that mediates sympatholysis under anesthesia, whereas an alternative nonserotonergic pathway is activated in the unanesthetized rat. Alternatively, it is plausible that a nonserotonergic pathway that remains inactive under anesthesia compensates for serotonin-mediated sympathetic effects after lesion in unanesthetized animals.

The data further suggest that serotonin cells are activated (express Fos) to counteract the hemodynamic disturbances associated with blood loss, rather than as the trigger that induces sympathetic withdrawal. The stimulus (or stimuli) that induced serotonin cell activation is not clear. It was not likely hypothesizing serotonin cell activation that either not serotonergic or contribute to a redundant serotonin-dependent pathway that mediates sympatholysis under anesthesia, whereas an alternative nonserotonergic pathway is activated in the unanesthetized rat. Alternatively, it is plausible that a nonserotonergic pathway that remains inactive under anesthesia compensates for serotonin-mediated sympathetic effects after lesion in unanesthetized animals.
delivered to the cerebroventricles. Values are group means subsequent recovery in rats treated with vehicle (control) and 5,7-DHT (lesion) group did not increase Fos expression in serotonin cells (30). It induction period was equal to that experienced by the hemorrhage comparable to that in present study. Hydralazine-induced hypotension in which the average pressure drop over the 2-h Fos induction period was equal to that experienced by the hemorrhage group did not increase Fos expression in serotonin cells (30). It could be argued that the more rapid hypotensive response to hemorrhage provided a stronger stimulus for Fos induction in serotonin cells than was provided by the slower hypotension that developed with hydrazine. However, to our knowledge, there are no available data that demonstrate activation of serotonin cells in response to selective baroreceptor unloading.

Similarly, it is not likely that loss of serotonin neurons inhibited sympathetic recovery following hemorrhage by disrupting the baroreflex. Instead, our data indicate that medullary serotonin cells may actually inhibit baroreflex sensitivity. Lesion of serotonin cells enhanced spontaneous baroreflex control of HR assessed by the sequence method. Spontaneous baroreflex assessment by this method tends to be biased toward the vagal component of the reflex, because of its more rapid response to changes in blood pressure. Thus the sequence method may not adequately reflect alterations in sympathetic baroreflex function. Although we did not directly test baroreflex control of sympathetic function, we did note a reduction in the SDNN of blood pressure in lesioned animals. Baroreflex-mediated oscillations in sympathetic vasomotor control provide an important buffer that limits blood pressure variability (17). Reduced fluctuations in blood pressure suggest that sympathetic baroreflex control of vasomotor tone was enhanced in lesioned animals. Indexes of baroreflex gain and sensitivity using frequency analysis methods failed to demonstrate a significant effect of lesion. However, this appeared to be due to the higher variability associated with spectral methods. Interestingly, the gain of HR and renal sympathetic baroreflex responses to nitroprusside is increased after more discrete lesions of serotonin nerves (16). We also observed significant loss of serotonin nerve terminals in the NTS with 5,7-DHT (16). We also observed significant loss of serotonin nerve terminals with the medial subnucleus of the NTS, where the majority of baroreceptor afferents terminate. Thus the attenuated recovery of sympathetic activity following hemorrhage could have been due to enhanced baroreflex suppression of sympathetic activity during recovery of blood pressure. This seems unlikely, however, inasmuch as HR responses were similar in lesioned and sham-lesioned animals. Moreover, ventilation normally increases with decreasing pressure because of baroreceptor unloading, yet ventilation slowed with the onset of blood withdrawal in lesioned animals (3). Deficits in ventilation and sympathoexcitation seemed to parallel one another, suggesting that altered sympathetic function may have been a consequence of suppressed central respiratory drive.

Serotonin lesion may have disrupted peripheral chemoreflex function and, thus, ventilatory and sympathetic responses to hemorrhage. Hypoxia is a potent stimulus for Fos expression in

![Fig. 7. MAP, HR, and RSNA during hemorrhage (gray shaded area) and subsequent recovery in rats treated with vehicle (control) and 5,7-DHT (lesion) delivered to the cerebroventricles. Values are group means ± SE of number of rats shown in parentheses; number of rats in control group is 9 for MAP and HR and 7 for RSNA; number of rats in lesion group is 8 for MAP and HR and 6 for RSNA. Significant difference between groups: *P < 0.05, **P < 0.01.](http://ajpregu.physiology.org/ Downloaded from http://ajpregu.physiology.org/)

R948 SEROTONIN AND SYMPATHETIC RECOVERY FROM HEMORRHAGE

Table 5. Spontaneous baroreflex gain, HR, and blood pressure variability in lesioned and control rats

<table>
<thead>
<tr>
<th></th>
<th>Sequences, ms/mmHg</th>
<th>Alpha</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Up</td>
<td>Down</td>
<td>All</td>
<td>LF</td>
<td>HF</td>
</tr>
<tr>
<td>Control</td>
<td>15</td>
<td>0.98 ± 0.11</td>
<td>0.84 ± 0.06</td>
<td>0.94 ± 0.09</td>
<td>0.37 ± 0.04</td>
<td>0.75 ± 0.13</td>
</tr>
<tr>
<td>SBP</td>
<td>9</td>
<td>1.18 ± 0.13</td>
<td>1.23 ± 0.09</td>
<td>1.21 ± 0.10</td>
<td>0.39 ± 0.05</td>
<td>0.98 ± 0.20</td>
</tr>
<tr>
<td>MBP</td>
<td>1.27 ± 0.15</td>
<td>1.18 ± 0.09</td>
<td>1.24 ± 0.11</td>
<td></td>
<td>0.42 ± 0.05</td>
<td>1.01 ± 0.20</td>
</tr>
<tr>
<td>DBP</td>
<td>1.40 ± 0.18*</td>
<td>1.13 ± 0.14*</td>
<td>1.30 ± 0.16*</td>
<td></td>
<td>0.48 ± 0.10</td>
<td>1.09 ± 0.23</td>
</tr>
<tr>
<td>Lesion</td>
<td></td>
<td>1.87 ± 0.27*</td>
<td>1.65 ± 0.18*</td>
<td>1.77 ± 0.22*</td>
<td>0.53 ± 0.11</td>
<td>1.51 ± 0.39</td>
</tr>
<tr>
<td>SBP</td>
<td>1.85 ± 0.26*</td>
<td>1.60 ± 0.15*</td>
<td>1.74 ± 0.20*</td>
<td></td>
<td>0.55 ± 0.12</td>
<td>1.53 ± 0.34</td>
</tr>
</tbody>
</table>

Values are group means ± SE. Spontaneous baroreflex gain was determined using the sequence method, including up, down, and all sequences that met our criteria. Spontaneous baroreflex gain was determined using the spectral method (correlation coefficient between heart rate interval and blood pressure) in the low-frequency (alpha LF) and high-frequency (alpha HF) domains. Power of blood pressure variability in the low-frequency domain (LF power), variability of beat-to-beat pressure (BP SDNN) and the ratio of low-frequency to high-frequency heart rate variability were also determined. SBP, MBP, and DBP, systolic, mean, and diastolic blood pressure.

![Sequence Diagram](http://ajpregu.physiology.org/ Downloaded from http://ajpregu.physiology.org/)
Fig. 8. Typical tryptophan hydroxylase immunoreactivity in raphe pallidus (A and B), raphe obscurus (C and D), subependymal cells of the parapyramidal region (E and F), raphe magnus (G and H), and parapyramidal cells (I and J) from 40-μm sections obtained from rats subjected to hindbrain injection of vehicle (A, C, E, G, and I) or 5,7-DHT (B, D, F, H, and J). Cell profiles of tryptophan hydroxylase-positive cells are shown for each serotonin-rich nucleus throughout the extent of the nucleus. Profiles for hindbrain and cerebroventricular vehicle-injected rats were combined (control). Separate profiles for rats subjected to targeted hindbrain lesion or global lesion are also shown. Values are group means ± SE. Significant difference between hindbrain lesion and control: *P < 0.05, **P < 0.01. Significant difference between global lesion and control: +P < 0.05, ++P < 0.01. Significant difference between hindbrain lesion and global lesion: #P < 0.05.

Serotonin neurons and produces patterns of activation similar to those observed with our hemorrhage protocol (7). Although we did not observe direct evidence of arterial hypoxia during the course of the study, arterial blood pH was reduced, suggesting that peripheral chemoreceptors were activated, particularly during recovery. However, the role of serotonin neurons in modulating the sensitivity of the peripheral chemoreflex has not been well studied and remains controversial. In humans,
dietary tryptophan depletion does not affect chemoreflex activation or sensitivity (40). In contrast, the raphe obscurus, which sends serotonergic projections to the NTS, when stimulated suppresses ventilatory responses to potassium cyanide, possibly by activating 5-HT3 receptors (47).

More compelling data suggest that caudal serotonin cells are important in central chemoreception, particularly in the absence of anesthesia. Recently, it was found that the integrity of raphe magnus serotonin cells is necessary for normal ventilatory responses to hypercapnia in conscious rats (5). We extended these findings by demonstrating that serotonin cells of the caudal raphe are necessary for normal ventilatory and sympathetic responses to selective activation of the central chemoreceptors with hyperoxic hypercapnia. Moreover, hypercapnia produces a pattern of serotonin cell Fos expression similar to that observed after our hemorrhage protocol (22). In our hemorrhage study, we did not observe overt signs of respiratory acidosis. In fact, \( P_{CO_2} \) fell during active blood loss, likely in response to the increased ventilation that developed with hypotension. However, \( P_{CO_2} \) subsequently rose \( \sim 30\% \) during the posthemorrhage recording period, despite slightly higher ventilation and increased \( P_{aO_2} \). Thus the rise in \( P_{CO_2} \)

Fig. 9. Representative photomicrographs of pontine raphe demonstrating tryptophan hydroxylase immunoreactivity in dorsal raphe (A–C), median raphe (D–F), and vIPAG region (G–I) in rats treated with vehicle [A, D, and G (control)] or 5,7-DHT [B, E, and H (hindbrain lesion)] in hindbrain raphe regions or cerebroventricles [C, F, and I (global lesion)]. J: total tryptophan-immunoreactive cell numbers in dorsal raphe (DR) and median raphe (MR), as well as vIPAG region, averaged across groups.
likely resulted from the buffering of metabolic acidosis. Lesioned rats showed attenuated ventilatory and sympathetic responses during a similar rise in PaCO₂, consistent with the possibility that central chemoreceptor activation contributes to normal ventilatory and sympathetic responses to blood loss.

Lesioned animals developed higher PaO₂ by the end of hemorrhage, despite evidence of reduced ventilation throughout recovery. This may have resulted from reduced ventilation-perfusion mismatch, inasmuch as lesioned animals also demonstrated a lower estimated PAO₂-PaO₂ difference by the end of the recording period. Lesioned animals also experienced more severe tissue hypoxia, as evidenced by an exaggerated accumulation of lactate and lower pH during recovery from hemorrhage. Lung perfusion was higher in lesioned animals than in controls, and/or ventilatory mismatch was reduced in lesioned animals compared with controls. Lower extracellular pH in pulmonary tissue of lesioned animals appeared to experience more severe tissue hypoxia, inasmuch as they showed an exaggerated accumulation of lactate and lower pH during recovery from hemorrhage. Inasmuch as blood pressure was not different between sham and lesioned animals and PaO₂ was elevated in lesioned animals, the evidence suggests that lesioned animals experienced reduced O₂ delivery to peripheral tissue due, in part, to greater peripheral vascular resistance. Although this idea remains to be confirmed with direct measures of cardiac output, the data suggest that caudal hindbrain serotonin neurons may help preserve cardiac output following hemorrhage, possibly by facilitating sympathetic-dependent increases in venous return.

It remains to be seen how arterial vascular resistance was elevated when sympathetic activity was attenuated. We used renal sympathetic activity as an index of sympathetic vasomotor tone, despite its more well-characterized role in volume regulation. It is clear that changes in RSNA often do not track with changes in sympathetic drive to other vascular beds that are important in maintaining peripheral resistance (26). Nevertheless, we have found that changes in RSNA parallel those of splanchnic nerve activity during progressive blood loss and subsequent hemorrhagic shock (43). Thus the uncoupling of blood pressure recovery from RSNA recovery was surprising. This would suggest that vasoactive hormones may have maintained blood pressure when sympathetic-mediated vasoconstriction was attenuated in lesioned rats.
Rostral and caudal raphe neurons of the ventromedial medulla provide major projections to preganglionic sympathetic cells, including those that stimulate brown adipose tissue and thermogenesis (24). Although body temperature was not measured in our studies, a report in awake rabbits indicates that blood loss similar in magnitude to that used in our studies results in a rapid decrease in core body temperature, metabolism, and O2 consumption (11). Ambient cooling sufficient to reduce core temperature also reduces RSNA in anesthetized rats (18, 32). Thus a loss of raphe-mediated thermogenesis could have contributed to an exaggerated fall in core temperature and sustained inhibition of renal sympathetic activity following hemorrhage. However, reductions in metabolism and O2 consumption in hemorrhaged rabbits precede, and thus could have contributed to, loss of core temperature, suggesting that thermogenesis may normally be suppressed during blood loss (11). Transient hypotensive hemorrhage was found to reduce the threshold for shivering in barbiturate-anesthetized cats, as does hypoxia in conscious cats (9, 19). Furthermore, anatomic data indicate that the majority of ventromedial raphe cells that express Fos after exposure to thermogenic stimuli are glutamatergic, rather than serotonergic (24). Thus it seems unlikely that serotonin cell lesion attenuated recovery of renal sympathetic activity by preventing thermogenic responses to blood loss.

It should be noted that 5,7-DHT destroys the entire cell and, in so doing, depletes not only serotonin, but also important cotransmitters that have been implicated in control of ventilation, including thyrotropin-releasing hormone and substance P (14). It remains to be determined whether loss of serotonin per se contributes to the observed effects. Moreover, 5,7-DHT is not entirely selective for serotonin neurons. The neurotoxin has a high affinity for the serotonin reuptake transporter, through which the toxin is thought to gain access to the cell to promote oxidative stress and cellular degeneration (41). The neurotoxin 5,7-DHT also has affinity for norepinephrine and dopamine transporter proteins (15). Noradrenergic neurons of the A1, A2, and A5 cell groups express norepinephrine reuptake transporter protein and also regulate sympathetic function (21). However, in our study, prior administration of nomifensine appeared to be effective in preventing cell death or morphological changes of tyrosine hydroxylase-immunoreactive cells. Thus it is unlikely that diminished sympathetic recovery was due to toxic effects on noradrenergic cells.

One possibility not directly examined in these studies is that the lesion interfered with preganglionic sympathetic function. Although neurotoxin could have diffused down to the spinal cord to affect preganglionic cells with ventricular administration of the drug, it is highly unlikely that drug from parenchymal injections diffused as far as the thoracic spinal cord to affect spinal interneurons or preganglionic sympathetic cells that regulate postganglionic RSNA. The initial sympathoexcitatory response to blood loss was virtually identical in rats that received 5,7-DHT or vehicle into the ventricular system. Thus lesion sufficient to attenuate sympathetic recovery following hemorrhage did not impair the ability to produce at least moderate degrees of sympathoexcitation. Together, these findings suggest that preganglionic sympathetic cell function was not compromised by lesions sufficient to attenuate sympathetic recovery after hemorrhage.

In summary, the present study provides further evidence that serotonin may not contribute to the depressor response to blood loss in unanesthetized animals. Although this conclusion is not unequivocal, the data provide compelling evidence to support the view that caudal medullary serotonergic neurons contribute, instead, to ventilatory and sympathetic reflex responses to acidosis during hypotensive hemorrhage, which may in turn facilitate beneficial hemodynamic responses to support microcirculation during hemorrhagic shock.

**Perspectives**

In prior studies, we demonstrated that the serotonin 5-HT1A receptor agonist 8-OH-DPAT produced a robust sympathetic-mediated increase in venous tone and cardiac output and improved blood gas parameters in rats subjected to hypovolemic shock (42). In the present study, endogenous serotonin was also shown to facilitate recovery of sympathetic drive and attenuate the development of metabolic acidosis following hemorrhage. These findings support the intriguing possibility that endogenous serotonin released during hemorrhage acts on 5-HT1A receptors to mediate sympathetic compensation and facilitate increased venous return in unanesthetized animals. These results may have significant implications for design of therapeutic approaches to hemorrhagic shock.

**ACKNOWLEDGMENTS**

The authors thank Dr. Charles Webber for helpful discussions and insightful comments during the writing of the manuscript.

Present affiliations: T. Gray, Department of Anatomy, American University of the Caribbean, St. Martin; A. Ruszaj, School of Natural Resources and Environment, University of Michigan, Ann Arbor, MI.

**GRANTS**

This work was supported by National Heart, Lung, and Blood Institute Grants HL-072354 and HL-076162 (K. Scroggin) and American Heart Association Grant 0815529G (L.-H. Kung).

**DISCLOSURES**

No conflicts of interest are declared by the author(s).
REFERENCES