5-HT$_{1A}$ receptors of the nucleus tractus solitarii facilitate sympathetic recovery following hypotensive hemorrhage in rats

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Vantrease JE, Dudek N, DonCarlos LL, Scrogin KE. 5-HT$_{1A}$ receptors of the nucleus tractus solitarii facilitate sympathetic recovery following hypotensive hemorrhage in rats. Am J Physiol Heart Circ Physiol 309: H335–H344, 2015. First published May 15, 2015; doi:10.1152/ajpheart.00117.2015.—The role of serotonin in the hemodynamic response to blood loss remains controversial. Caudal raphe serotonin neurons are activated during hypotensive hemorrhage, and their destruction attenuates sympathetic increases following blood loss in unanesthetized rats. Caudal raphe neurons provide serotoninnegative projections to the nucleus tractus solitarii (NTS), and disruption of serotonin-positive nerve terminals in the NTS attenuates sympathetic recovery following hemorrhage. Administration of 5-HT$_{1A}$-receptor agonists following hemorrhage augments sympathetic-mediated increases in venous tone and tissue hypoxia. These findings led us to hypothesize that severe blood loss promotes activation of 5-HT$_{1A}$ receptors in the NTS, which facilitates sympathetic recovery and peripheral tissue perfusion. Here, we developed an adeno-associated viral vector encoding an efficacious small hairpin RNA sequence targeting the rat 5-HT$_{1A}$ receptor. Unanesthetized rats subjected to NTS injection of the anti-rat 5-HT$_{1A}$ small hairpin RNA-encoding vector 4 wk prior showed normal blood pressure recovery, but an attenuated recovery of renal sympathetic nerve activity (~6.4 ± 12.9 vs. 42.6 ± 15.6% baseline, $P < 0.05$) 50 min after 21% estimated blood volume withdrawal. The same rats developed increased tissue hypoxia after hemorrhage, as indicated by prolonged elevations in lactate (2.77 ± 0.5 vs. 1.34 ± 0.2 mmol/L, 60 min after start of hemorrhage, $P < 0.05$). 5-HT$_{1A}$ mRNA levels in the commissural NTS were directly correlated with renal sympathetic nerve activity ($P < 0.01$) and inversely correlated with lactate ($P < 0.05$) 60 min after start of hemorrhage. The data suggest that 5-HT$_{1A}$ receptors in the commissural NTS facilitate tissue perfusion after blood loss likely by increasing sympathetic-mediated venous return.

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thetic effects in hemorrhaged animals, we posit that endogenous serotonin acts in the NTS during hemorrhage to augment compensation of sympathetic drive following blood loss.

It is not known what receptor population mediates these beneficial effects of serotonin during compensation from hemorrhage. A number of serotonin receptor subtypes are expressed in the NTS (3, 8, 18, 21, 28). Previous experiments have shown that the 5-HT_{1A}-receptor agonist, 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT), elicits a rapid recovery of sympathetic activity when administered centrally or systemically to unanesthetized, hypovolemic rats (32). 8-OH-DPAT-mediated increases in sympathetic activity increase venous tone and culminate in increased cardiac output (35). Since 8-OH-DPAT appears to mimic the hemodynamically beneficial effects of endogenous serotonin release during blood loss, we predicted that 5-HT_{1A}-receptor activation in the NTS would be necessary for normal sympathetic compensation following hypotensive hemorrhage. To test this hypothesis, we used an adeno-associated viral vector (AAV) encoding a small hairpin RNA (shRNA) to selectively reduce 5-HT_{1A}-receptor message in neurons of the NTS and determined the effects of knockdown on cardiovascular variables and blood gases during hemorrhage.

**METHODS**

**Screening of Candidate Sequences for 5-HT_{1A}-Receptor Silencing**

Five shRNA sequences targeting rat 5-HT_{1A}-receptor mRNA were screened (S' → 3'): 1) GTGAAAGGAAAGACGGTGAA; 2) CCGAGTGGGCAACTCCAAA; 3) GCTCAATCCGTATTTAT; 4) GAGTGCAGCAGAGGGCACCACAAGA; and 5) GCC-TATCACCAGGACCTAT. Sequences were cloned into a mammalian expression vector containing a mouse RNA polymerase III U6 promoter upstream of the shRNA-encoding insert. The vector also coded for humanized Renilla reniformis green fluorescent protein (hrGFP) driven by a cytomegalovirus promoter (pAAV-U6-CMV-GFP, University of Iowa, Viral Vector Core). The full-length rat 5-HT_{1A}-receptor gene (provided by Dr. Paul Albert, University of Ottawa) was cloned into a mammalian expression vector containing a mCherry reporter gene (mCherry-Ch1-17T43, gift from Dr. R. Y. Tsien, University of California, San Diego, CA) to screen shRNA-mediated knockdown of the resulting mCherry-5-HT_{1A}-receptor fusion protein. Human embryonic kidney (HEK)-293 cells were cotransfected with the mCherry-5-HT_{1A}-receptor fusion protein and each of the shRNA-hrGFP-encoding plasmids. Controls were transfected with a sequence encoding a shRNA targeting the rat tryptophan hydroxylase 2 gene (TPH2), which is not expressed in HEK-293 cells (CCACCATTGG-GAGGCTGAA). Integrated fluorescence density was determined 72 h after transfection in three separate visual fields and averaged for each plate using Image J software [National Institutes of Health (NIH), USA]. Each sequence was screened in three separate experiments conducted on different days. Cells were subsequently lysed and harvested for determination of 5-HT_{1A}-receptor mRNA levels using semiquantitative PCR.

Total RNA was isolated with Trizol Reagent (Invitrogen). CDNA was synthesized by reverse transcription (first-strand cDNA synthesis, Fermentas) and amplified using the following primers: GAPDH forward 5' = CCACGTCCACAATGGCCATG; 5-HT_{1A}-receptor forward 5' = CCTCAGGAGGCTGACACACATGG; and reverse 5' = CCCTCTTTCTTTCCCTTTCTCTCT-3'. PCR cycling parameters for amplification of rat 5-HT_{1A}-receptor cDNA were as follows: 94°C for 1 min, 30 cycles of 94°C for 15 s, 45°C for 30 s, 72°C for 45 s, and 72°C for 10 min. For GAPDH, cycling parameters were as follows: 94°C for 1 min, 32 cycles of 94°C for 15 s, 58°C for 30 s, and 72°C for 10 min. Bands were imaged with a Kodak Image Station 4000-mm Pro. Band density was calculated using Image J (NIH, USA). Samples were normalized to GAPDH cDNA.

5-HT_{1A}-receptor protein was determined by Western blot using a lysate from cells that were cotransfected with the untagged receptor and each of the shRNA-encoding sequences. Labeling was conducted with a custom 5-HT_{1A}-receptor primary antibody (0.7 mg/ml, Pacific Immunology, Ramona, CA). Proteins were separated by electrophoresis (10% sodium dodecyl sulfate polyacrylamide gel) and transferred to a nitrocellulose membrane. Membranes were blocked (nonfat dry milk and 0.1% Tween 20 in Tris-buffered saline) for 1 h and incubated overnight with rabbit anti-5-HT_{1A}-receptor polyclonal antibody (1:10,000), or mouse anti-actin antiserum (1:40,000, MP Biomedicals, Santa Ana, CA) at 4°C. Membranes were incubated with horseradish peroxidase-conjugated anti-mouse (0.8 mg/ml) and antirabbit (1.3 mg/ml) secondary antibodies (1:2,000 and 1:5,000, respectively, Pierce) for 1 h at room temperature. Membranes were developed with Super-Signal West Pico substrate (Thermo) and exposed on autoradiographic film. The band density was quantified with Image J (NIH, USA) and normalized to actin.

The most efficient shRNA sequence and a scrambled version of the same sequence (ScranchRNA) without homology to any known sequence in the rat genome were incorporated into serotype 2/8 AAV vectors for use in in vivo studies (Gene Transfer Vector Core, University of Iowa). The baculovirus system was used for virus production, as described by Urabe et al. (38).

**Animals**

All protocols conformed to the American Physiological Society’s Guiding Principles for Research Involving Animals and Human Beings and the Principles of Laboratory Animal Care, as adopted and promulgated by the US NIH, and were approved by the Institutional Animal Care and Use Committee of Loyola University Chicago, Stritch School of Medicine. Experiments were performed on 37 male Sprague-Dawley rats (275–300 g, Harlan, Indianapolis, IN). Animals were acclimated to the animal care facility under standard conditions (22°C ambient temperature, 12:12-h light-dark cycle) with food and water available ad libitum at least 1 wk before viral injection surgery.

**AAV Vector Injections**

Before surgery, AAV vectors were diluted in buffer (400 nM NaCl, 20 mM Tris base) to 1 × 10^15 vg/ml and dialyzed in 0.9% saline. Heparin (1,000 U/ml) was added to virus in a 1:7 ratio (vol/vol) to facilitate viral infection (33). Rats were anesthetized with ketamine-xylazine (85:13 mg/kg im), and supplemented with ketamine (30 mg/kg) as needed. Rats were secured in a stereotaxic apparatus with the nose bar positioned 11.0 mm below a flat skull plane. Injections were localized 0.5 mm ventral to obex at two rostro-caudal planes along the NTS: +0.5 rostral and 0 mm relative to obex, 0.7 and 0.25 mm lateral from the midline for the rostral and more caudal injections, respectively (26). Virus was infused bilaterally (750 nl/site, 200 nl/min) through pulled glass pipettes. Rats were given buprenorphine (50 µg/kg sc) every 8 h for 2 days. Ampicillin (150 mg/kg sc) was given twice daily for 3 days. Rats recovered for 4 wk before the experiment.

**Vascular Catheter and Renal Sympathetic Recording Electrode Implantation**

On the day before the experiment, ~4 wk after viral injection, rats were anesthetized with isoflurane (1.5–2.5%) and instrumented with bilateral femoral arterial and unilateral venous femoral catheters (PE-50 fused with PE-10 tubing) to enable blood withdrawal, measurement of arterial pressure, and blood sampling. A fiber bundle of the left renal sympathetic nerve was isolated through a flank incision.
and placed on a bipolar, stainless steel electrode (bare diameter = 0.005 in.; A-M Systems, Everett, WA) and isolated in Kwiksi! silicon (World Precision Instruments). All incisions were closed (3-0 silk), and all catheters and lead wires were externalized at the nape of the neck. Rats were given ampicillin (150 mg/kg sc) and ketoprofen (5 mg/kg sc) and allowed to recover overnight before the experiment.

Experimental Protocols

Hypotensive hemorrhage. Vascular lines and electrode leads were connected to a withdrawal pump, blood sampling syringe, and amplifier through an overhead swivel system, while the rats rested unrestrained in their home cage. Rats were allowed to rest after instrumentation for at least 1 h, after which blood withdrawal commenced at a rate of 3.2 ml·min⁻¹·kg⁻¹ for 6 min, and then 0.52 ml·min⁻¹·kg⁻¹ for an additional 4 min, for a total withdrawal of 21% of estimated blood volume over 10 min (36). Femoral venous blood samples (150 μl) were taken 20 min before hemorrhage, as well as 10 and 60 min after the start of blood withdrawal to measure hematocrit, venous blood gases, and acid-base status with an i-STAT 1 analyzer (i-STAT, East Windsor, NJ). Plasma protein concentrations were determined by a handheld clinical refractometer (ATAGO U.S.A.). At the end of the experiment, rats were given hexamethonium chloride (30 mg/kg iv) for determination of background noise in the nerve the end of the experiment, rats were given hexamethonium chloride (30 mg/kg iv) for determination of background noise in the nerve recording.

Rats were anesthetized with an overdose of pentobarbital sodium (100 mg/kg iv). In a subset of animals, brains were extracted and flash frozen in cold isopentane and stored at −80°C for PCR. The remaining rats were perfused transcardially with 90 ml of sodium nitrate (6.7 mM), followed by 90 ml of 4% paraformaldehyde in 0.01 M phosphate-buffered saline. Brains were removed and postfixed for 1 h in the same solution. The brain stem was blocked and postfixed overnight. The following day, brains were transferred to 30% sucrase and stored at 4°C. Brains were cut (40 μm) and collected serially into six wells of cryoprotectant (30% sucrase and 30% ethylene glycol in 0.1 M phosphate buffer) and stored at −20°C for histology.

Spontaneous baroreflex sensitivity. Five-minute segments of baseline blood pressure and heart rate were recorded before hemorrhage and analyzed with SA-BRS software (Nevrokard, version 3.2.4) to determine spontaneous baroreflex gain using the sequence method (25). Baroreflex gain was determined as the average slope of linear regression obtained from at least three sequences. Accepted sequences had three or more consecutive interbeat intervals (IBI) with variation in the same direction, and >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 (12). A 128-point fast-Fourier transformation with a smoothed Hamming window was used to conduct cross-spectral analysis on IBI and blood pressure. The relationship was determined as the square root of the ratio of IBI and blood pressure with 50% overlap and zero padding of eight values. Values were reported as the α-index in the low-frequency (0.06–0.6 Hz) and high-frequency (0.6–3.0 Hz) domains (27). Blood pressure and heart rate variability were also determined in the time domain as the standard deviation of systolic, mean, or diastolic blood pressure or heart rate during normal IBIs (SD of normal R-R intervals). Blood pressure variability in the low-frequency domain was measured as an index of the sympathetic contribution to baseline blood pressure (17).

Data acquisition. Arterial pressure, heart rate, and renal sympathetic nerve activity (RSNA) were recorded on a Macintosh G4 Powerbook with 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 Laboratories, North Chicago, IL) and a PowerLab bridge Amplifier (ADInstruments). Heart rate was calculated using peak-to-peak detection of the pulse pressure wave. Sympathetic activity was sampled (4,000 Hz) and amplified (10,000×) with a PowerLab Bioamplifier (ADInstruments). The raw signal was filtered (1-1,000 Hz), rectified, and integrated over a 20-ms time constant. Only nerve recordings with a signal-to-noise ratio greater than 2:1 were included in the data analysis.

Quantitative real-time PCR from brain tissue. Bilateral tissue punches (0.75 mm inner diameter) were obtained from 500-μm-thick sections containing the commissural and medial NTS, as well as the hypoglossal nucleus using landmarks described in Paxinos and Watson (26). Total RNA was extracted from tissue homogenized in Trizol Reagent (Life Technologies, NY). RNA was treated with DNAase I enzyme (Fermentas) and reverse transcribed using recombinant M-MuLV reverse transcriptase (Fermentas). Quantitative PCR was performed on 2-μg cDNA using SsoAdvanced SYBR Green (Bio-Rad) in conjunction with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Primers (5’ → 3’) are as follows: for GAPDH, forward, GACATGGCCGCTGGAGAAC, and reverse, AGCCAGGATGC CCTTATGT; for 5-HT1A, forward, AAGAAGAGCTGACAGGGA, and reverse, CAG AGGAGGTGCTCTTGG. Samples were run in triplicate and in parallel with a no template control. The following general PCR protocol was used: 95°C for 30 s, 40 cycles of 95°C for 5 s, and 56°C for 30 s, depending on primer set. Relative mRNA levels were determined using the 2⁻ΔΔCt method (19). 5-HT1A-receptor mRNA levels from each sample were normalized to GAPDH mRNA levels and then compared with the group mean of ScramshRNA-injected rats. Levels of 5-HT1A-receptor mRNA were determined in the target regions (commissural and medial NTS), as well as in off-target regions enriched in 5-HT1A receptor (raphe obscurus and dorsal raphe). Since the α-adrenergic 2A receptors (αAR2A) in the NTS are recognized to have important effects on blood pressure regulation and ventilation during chemoreflex activation, their expression was also determined in commissural NTS to identify potential off-target or compensatory effects of 5-HT1A shRNA injection. Primers for αAR2A were as follows: forward, CCGGATAAGCAGGTC GGGAAC, and reverse, TTGCACACCGTGAGACG, with an annealing temperature of 63°C.

Localization of hrGFP expression and immunohistochemistry for serotonin immunoreactivity. In a subset of rats (n = 4), hrGFP expression was examined in serotonin-immunoreactive cells of the caudal raphe to determine whether potential retrograde transport of virus led to transduction of serotonin cell bodies enriched in 5-HT1A receptor. Forty-micrometer sections were incubated in rabbit anti-serotonin antiserum with 0.4% Triton X-100 (1:80,000; Immunostar) for 48 h at 4°C, then washed and incubated in goat anti-rabbit Dylight-649 (1.5 mg/ml, 1:500, Jackson ImmunoResearch) for 3 h at 37°C. Mounted sections were imaged with an Olympus IX81 inverted microscope fitted with epifluorescence. GFP expression was mapped to identify the location of transduced cells using a Leica DMR microscope equipped with epifluorescence, a Retiga video camera, and NeuroLucida software (MBF Biosciences, Willston, VT).

Statistical Analysis

Values are presented as group means ± SE. Group differences were determined by Student’s t-test. Two-way ANOVAs with repeated measures were used to assess cardiovascular and blood-gas measures over time. Holm-Šídák post hoc tests were used to determine group differences. When group data failed the Shapiro-Wilk normality test, the Mann-Whitney U rank sum test was used instead. P values of <0.05 were considered significant.

RESULTS

shRNA Screening

Of five shRNA sequences screened, only one [group injected with vector encoding anti-5-HT1A receptor shRNA (1ARshRNA) #2] produced a visible decrease in fluorescence in HEK-293 cells transfected with the mCherry-5-HT1A-receptor fusion 5-HT1A receptors of the NTS in hemorrhage. H3373

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protein (Fig. 1A). Total fluorescence intensity was decreased in cells transduced with 1ARshRNA compared with TPH2 shRNA (TPHshRNA) (8.4 ± 0.8 vs. 27.0 ± 2.3 arbitrary units, P < 0.01). Transfection with 1ARshRNA#2 also decreased 5-HT\textsubscript{1A} mRNA levels (Fig. 1B; P < 0.05). Our custom polyclonal rabbit 5-HT\textsubscript{1A} rabbit antibody labeled a ~46-kDa protein in HEK-293 cells transduced with the full-length, untagged rat 5-HT\textsubscript{1A} receptor (Fig. 1C). The band size is consistent with the expected size of the rat 5-HT\textsubscript{1A} receptor (1). The band was absent in lysates from cells cotransfected with 1ARshRNA#2 and from untransfected cells (Fig. 1C). Cells cotransfected with the receptor and the anti-TPHshRNA continued to express the protein (Fig. 1D). Transfection with 1ARshRNA#2 produced a 97% knockdown of 5-HT\textsubscript{1A}-receptor protein compared with the TPHshRNA (P < 0.01). AAV encoding 1ARshRNA sequence also produced extensive knockdown of mCherry fluorescence in HEK cells transduced with the mCherry-5-HT\textsubscript{1A}-receptor fusion protein compared with AAV encoding a scrambled version of the 1ARshRNA sequence (data not shown).

Two sets of bilateral injections of virus were made within the medial and commissural NTS for a total of four injections in each animal (sites shown in Fig. 2, A and B). Injection of virus encoding either the 1ARshRNA#2 sequence (1ARshRNA) or a scrambled version of the same sequence resulted in extensive neuronal transduction, as shown by robust hrGFP expression within neural structures of the NTS 4 wk after administration (Fig. 2C). A similar distribution of transduced cells was observed following injection of control or 1ARshRNA encoding virus, with the highest density of transduction in and around the caudal NTS (Fig. 2D). There was limited evidence of transgene expression in the underlying hypoglossal nucleus (example in Fig. 2C). Evidence of hrGFP expression in the caudal raphe regions was observed, particularly around serotonin-positive cell bodies of the raphe magnus (Fig. 3, A and B). Very little hrGFP appeared around the raphe obscurus (Fig. 3, C and D). However, there was no colocalization of hrGFP and serotonin immunolabel in any caudal raphe cell bodies. No hrGFP expression was observed in or around the dorsal raphe (data not shown).

Injection of virus encoding the 1ARshRNA reduced 5-HT\textsubscript{1A}-receptor mRNA in micropunches obtained from the commissural and medial NTS in a subset of rats of each group (Fig. 4A), but had no effect on tissue punches obtained from the raphe obscurus (Fig. 4B). Injection of the 1ARshRNA-encoding virus reduced 5HT\textsubscript{1A}-receptor mRNA levels in the commissural NTS and medial NTS by >50 and ~30%, respectively (Fig. 4C). There was no difference in 5-HT\textsubscript{1A}-receptor mRNA levels in tissue obtained from raphe obscurus, the dorsal raphe, or the hypoglossal nucleus (Fig. 4C). The level of αAR\textsubscript{2A}-receptor mRNA was not different between groups in either the commissural NTS or medial NTS (Fig. 4D).

Hypotensive Hemorrhage

Treatment had no effect on body weight (358 ± 5 vs. 368 ± 5 g, respectively, for rats treated with virus encoding the scrambled or 1ARshRNA sequences, respectively) before the start of hemorrhage. Of 31 rats injected with virus and successfully implanted with vascular catheters, 24 showed good artifact-free baseline blood pressure recordings, sufficient for testing of spontaneous baroreflex activity. Neither spontaneous baroreflex gain, nor heart rate, nor blood pressure variability analysis revealed any group difference before blood loss (Table 1).
All rats subjected to acute hypovolemic hemorrhage showed a characteristic multiphasic response to blood withdrawal (Fig. 5, A and B), i.e., a short normotensive phase characterized by augmented sympathetic activity and reflex tachycardia. After withdrawal of \(\sim 9–12 \text{ ml/kg}\) of estimated blood volume, a rapid decline in blood pressure, heart rate, and RSNA developed in both groups. After termination of blood withdrawal, blood pressure and heart rate recovered over the next 10–15 min before reaching a plateau. Treatment with the 1ARshRNA-encoding virus did not affect baseline blood pressure or heart rate, or the response to, and subsequent recovery from, blood withdrawal (Fig. 5B). Among animals treated with virus encoding the scrambled sequence, RSNA recovered in parallel with blood pressure and heart rate. RSNA of rats treated with virus encoding the scrambled sequences exceeded baseline levels and stabilized at a plateau \(\sim 40–60\%\) above baseline. In contrast, nerve activity of rats treated with 1ARshRNA-encoding virus stabilized at levels \(\sim 20\%\) below baseline for the remainder of the experiment. Rats treated with the 1ARshRNA-encoding virus showed reduced RSNA compared with rats treated with virus coding for the scrambled sequence for the duration of the recovery period \((P < 0.01)\). Significant positive correlations were found between RSNA (determined as %change from baseline) 60 min after the start of hemorrhage and 5-HT1A-receptor mRNA levels in both the commissural and medial NTS samples (Fig. 5C).

Venous blood sampled at baseline and 10 and 60 min after the start of hemorrhage showed similar changes in hematocrit and protein plasma levels between the two groups (Table 2). Lactate levels were elevated 10 min after the start of blood withdrawal, and the rise was similar between groups (Fig. 6A). By 60 min after start of hemorrhage, lactate levels had declined nearly back to baseline in rats treated with the ScramshRNA-encoding virus. However, lactate remained elevated in rats treated with the 1ARshRNA-encoding virus (Fig. 6A). Lactate levels determined 60 min after the start of blood withdrawal were negatively correlated with 5-HT1A-receptor mRNA in the commissural NTS, but not in the medial NTS (Fig. 6B). Sympathetic activity and lactate levels 60 min after the start of hemorrhage were inversely related \((R^2 = -0.61, P < 0.05)\). There were no further differences in acid-base balance or venous blood-gas variables between groups (Table 3).
DISCUSSION

Rats with a 30–50% reduction in 5-HT<sub>1A</sub>-receptor mRNA in the commissural NTS and medial NTS showed an attenuated recovery of sympathetic activity following a moderate level and rate of blood loss (~21% of estimated blood volume over 10 min), 5-HT<sub>1A</sub>-receptor mRNA levels were inversely related to the level of lactic acidosis and directly related to RSNA activity 60 min after the start of hemorrhage ($P < 0.05-0.01$). The relationships between receptor mRNA levels and lactate or sympathoexcitation were most robust when tissue from the commissural and medial NTS showed an attenuated sympathetic activity in the LF and HF domains and the LF-to-HF ratio. RRI, R-R interval; nu, normalized units.

Table 1. Spontaneous baroreflex gain and blood pressure variability

<table>
<thead>
<tr>
<th>BP Measure</th>
<th>Up Sequences, ms/mmHg</th>
<th>Down Sequences, ms/mmHg</th>
<th>All Sequences, ms/mmHg</th>
<th>Alpha (LF)</th>
<th>Alpha (HF)</th>
<th>LF Power, mmHg&lt;sup&gt;2&lt;/sup&gt;</th>
<th>BP SDNN</th>
<th>LF RRI, nu</th>
<th>HF RRI, nu</th>
<th>LF-to-HF Ratio</th>
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<tr>
<td>ScramshRNA (13)</td>
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<tr>
<td>SBP</td>
<td>1.68 ± 0.27</td>
<td>1.33 ± 0.19</td>
<td>1.57 ± 0.22</td>
<td>0.78 ± 0.10</td>
<td>1.78 ± 0.33</td>
<td>11.47 ± 1.75</td>
<td>4.64 ± 0.47</td>
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<td>MBP</td>
<td>1.94 ± 0.28</td>
<td>1.84 ± 0.23</td>
<td>1.90 ± 0.24</td>
<td>0.78 ± 0.09</td>
<td>2.04 ± 0.32</td>
<td>9.91 ± 1.32</td>
<td>4.20 ± 0.37</td>
<td>37.34 ± 5.28</td>
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<td>DBP</td>
<td>2.02 ± 0.26</td>
<td>1.76 ± 0.22</td>
<td>1.90 ± 0.23</td>
<td>0.81 ± 0.11</td>
<td>1.92 ± 0.27</td>
<td>9.57 ± 1.12</td>
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<td>SBP</td>
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<td>1.76 ± 0.24</td>
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<td>MBP</td>
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<td>DBP</td>
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Values are group means ± SE determined for systolic (SBP), mean (MBP), and diastolic (DBP) blood pressure where applicable; no. of rats per group are in parentheses. Scram group injected with vector encoding scrambled small hairpin RNA (shRNA) sequence; 1AR group injected with vector encoding anti-5-HT<sub>1A</sub> receptor shRNA. Spontaneous heart rate baroreflex gain was determined using the sequence method, including up and down sequences, as well as the average of all sequences that met baroreflex criterial outlined in text. Spontaneous heart rate baroreflex gains were also determined from the spectral method (correlation coefficient between heart rate interval and blood pressure) in the low (alpha LF) and high-frequency (alpha HF) domains. Power of blood pressure (BP) variability in the LF domain (LF power) and in the time domain [BP standard deviation of normal-to-normal R waves (SDNN)] are shown, as well as the heart rate variability in the LF and HF domains and the LF-to-HF ratio. RRI, R-R interval; nu, normalized units.
findings, coupled with the results of the present study, provide compelling evidence that serotonin released into the NTS during blood loss activates 5-HT \textsubscript{1A} receptors to stimulate beneficial hemodynamic responses that increase perfusion of peripheral tissue, as assessed by lactate levels.

Rats injected with virus encoding the 1ARshRNA sequence displayed the typical initial multiphasic response to blood loss, suggesting that 5-HT \textsubscript{1A} receptors in the NTS are likely not involved in the regulation of the first sympathoexcitatory response or the sympatholytic phase of hypotensive hemorrhage. The data appear to be involved in the second sympathoexcitatory response that develops after the sympatholytic phase. The data are consistent with the view that 5-HT \textsubscript{1A} receptors in the NTS facilitate oxygen delivery to peripheral tissue. Specifically, exaggerated lactic acidosis was found at the end of the second sympathoexcitatory phase of blood loss in rats treated with the 1ARshRNA-encoding vector, suggesting that deficits in oxygen delivery to peripheral tissue developed following termination of blood withdrawal in these animals. Furthermore, the inverse relationship between lactic acid accumulation and sympathetic recovery suggests that sympathetic drive may aid in peripheral tissue oxygenation. Reduced oxygen delivery results from reduced oxygen in arterial blood and/or deficits in perfusion of peripheral tissue. In this study, we did not measure arterial blood gases. However, in our prior study, we found that caudal serotonin cell lesion actually increased arterial PO\textsubscript{2} during and after hemorrhage recovery. As such, peripheral tissue hypoxia observed in this prior study was most likely due to reduced perfusion rather than to a deficit in blood oxygenation. Here, we measured venous blood gases since they better reflect peripheral metabolic acidosis. No group differences were found in either venous PO\textsubscript{2} or venous PCO\textsubscript{2} at any point, suggesting that arterial blood oxygen was similar between groups during and after hemorrhage. We cannot rule out the possibility that tissue O\textsubscript{2} extraction, and thus arterial PO\textsubscript{2}, differed between groups. However, given our evidence that caudal serotonin cell lesion did not impair arterial blood oxygenation, it seems more likely that the greater tissue hypoxia observed in rats treated with the 1ARshRNA-encoding vector was due to reduced perfusion of peripheral tissue. We found that deficits in NTS 5-HT\textsubscript{1A} receptor mRNA increased peripheral tissue hypoxia in the absence of any

Table 2. Hematocrit and plasma protein concentrations before and after hypotensive hemorrhage in rats injected with adeno-associated viral vector encoding a scrambled sequence or shRNA targeting the rat 5-HT\textsubscript{1A} receptor

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Hematocrit, %</th>
<th>Plasma Protein, g/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>ScrambRNA</td>
<td>11</td>
<td>43 ± 1</td>
<td>6.3 ± 0.2</td>
</tr>
<tr>
<td>0 min</td>
<td></td>
<td>33 ± 1*</td>
<td>4.7 ± 0.1*</td>
</tr>
<tr>
<td>1ARshRNA</td>
<td>11</td>
<td>33 ± 1*</td>
<td>4.9 ± 0.1*</td>
</tr>
<tr>
<td>0 min</td>
<td></td>
<td>30 ± 2*</td>
<td>4.9 ± 0.1*</td>
</tr>
<tr>
<td>10 min</td>
<td></td>
<td>41 ± 2</td>
<td>6.2 ± 0.1</td>
</tr>
<tr>
<td>60 min</td>
<td></td>
<td>31 ± 2*</td>
<td>4.6 ± 0.1*</td>
</tr>
</tbody>
</table>

Values are group means ± SE; n, no. of rats per group. ScrambRNA, scrambled shRNA; 1ARshRNA, shRNA targeting the rat 5-HT\textsubscript{1A} receptor. *P < 0.01 vs. 0 min within group.
change in blood pressure compensation. After treatment with the 1ARshRNA-encoding vector, blood pressure compensation from hemorrhage appears normal, but, in fact, is likely mediated more by arterial peripheral resistance, rather than by a more hemodynamically beneficial increase in cardiac output. Young et al. (37) noted that, during hemorrhage, sympathetic activity contributes substantially to constriction of the venous vasculature, while angiotensin II and vasopressin contribute to blood pressure increases primarily through increased arterial vasoconstriction. Our observation of an inverse relationship between sympathetic activity and lactate accumulation further supports the view that sympathetic activity promotes rather than hinders peripheral tissue perfusion. Together, these data suggest that endogenous serotonin released during hemorrhage acts on 5-HT1A receptors in the NTS to maintain tissue perfusion and blood pressure compensation. After treatment with the 1ARshRNA-encoding vector, blood pressure compensation from hemorrhage appears normal, but, in fact, is likely mediated more by arterial peripheral resistance, rather than by a more hemodynamically beneficial increase in cardiac output.

Table 3. Effects of hemorrhage on venous blood-gas variables and acid-base status in ScramshRNA- and 1ARshRNA-injected rats

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>pH</th>
<th>Pco2, mmHg</th>
<th>Po2, mmHg</th>
<th>BE, mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>ScramshRNA</td>
<td>13</td>
<td>7.49±0.01</td>
<td>43.31±0.44</td>
<td>34.85±1.58</td>
<td>10.00±0.66</td>
</tr>
<tr>
<td>0 min</td>
<td></td>
<td>7.44±0.01*</td>
<td>42.35±0.82</td>
<td>26.62±1.38*</td>
<td>4.46±0.55*</td>
</tr>
<tr>
<td>10 min</td>
<td></td>
<td>7.46±0.01§</td>
<td>46.19±0.54§</td>
<td>31.69±1.14*</td>
<td>9.00±0.78§</td>
</tr>
<tr>
<td>60 min</td>
<td></td>
<td>7.46±0.01§</td>
<td>46.19±0.54§</td>
<td>31.69±1.14*</td>
<td>9.00±0.78§</td>
</tr>
<tr>
<td>1ARshRNA</td>
<td>13</td>
<td>7.50±0.01</td>
<td>42.52±0.66</td>
<td>34.85±1.49</td>
<td>10.08±0.70</td>
</tr>
<tr>
<td>0 min</td>
<td></td>
<td>7.44±0.01*</td>
<td>41.89±0.96</td>
<td>27.92±1.14*</td>
<td>4.08±1.06*</td>
</tr>
<tr>
<td>10 min</td>
<td></td>
<td>7.45±0.01§</td>
<td>46.40±0.76§</td>
<td>31.85±1.64*</td>
<td>8.00±1.12§</td>
</tr>
<tr>
<td>60 min</td>
<td></td>
<td>7.45±0.01§</td>
<td>46.40±0.76§</td>
<td>31.85±1.64*</td>
<td>8.00±1.12§</td>
</tr>
</tbody>
</table>

Values are group means ± SE; n, no. of rats per group. BE, base excess. *P < 0.05, §P < 0.01 vs. 0 min within group. †P < 0.05, ‡P < 0.01 vs. 10 min within group. In a small number of cases, blood gases could not be determined in animals that underwent hemorrhage due to either a nonpatent venous catheter, or an insufficient volume of venous blood at sampling.
The effects of 5-HT1A-receptor activation remains to be determined. The phenotype of commissural NTS cells that mediate those from our previous work (15, 16, 35, 36) provide complementary evidence that serotonin neurons release serotonin in the raphe obscurus or raphe magnus, coupled with the normal 5-HT1A mRNA levels in the caudal NTS. As such, physiological effects of vector injection were likely not due to downregulation of the structurally similar αAR2A receptor that is recognized to facilitate peripheral chemoreflex responses (11).

5-HT1A receptors primarily couple to Gαi proteins to foster membrane hyperpolarization (2). Thus it seems most likely that 5-HT1A receptors act to attenuate the activity of inhibitory cells that, in turn, suppress sympathetic compensation during hemorrhage. The particularity of commissural NTS cells that mediate the effects of 5-HT1A-receptor activation remains to be determined. However, recent work has confirmed that GABAergic rather than excitatory cells of the NTS express 5-HT1A receptors, and that application of the 5-HT1A-receptor ligand, 8-OH-DPAT, acts on 5-HT1A receptors in a brain stem slice preparation to cause mild activation of excitatory cells in the commissural NTS (24). It remains to be determined whether the response might be enhanced during input from peripheral chemoreceptors, particularly in light of evidence that prolonged hypoxia stimulates GABA release in the commissural NTS (34). Thus 5-HT1A-mediated inhibition of GABA release could potentially facilitate chemoreflex responses during prolonged hypoxia, such as that experienced during hemorrhage.

In summary, findings from the present study, together with those from our previous work (15, 16, 35, 36) provide compelling evidence that serotonin neurons release serotonin in the commissural NTS and act on 5-HT1A receptors to promote a beneficial hemodynamic response during compensation from blood loss. A renewed appreciation for the role of the venous vasculature in hypovolemic compensation has emerged in recent years (5, 6). Our findings suggest that agonists of the 5-HT1A receptor may have potential therapeutic benefit in early hemorrhagic shock through actions in the NTS.

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

AUTHOR CONTRIBUTIONS
Author contributions: J.E.V., N.D., L.L.D., and K.E.S. conception and design of research; J.E.V. and N.D. performed experiments; J.E.V., N.D., L.L.D., and K.E.S. analyzed data; J.E.V., N.D., L.L.D., and K.E.S. interpreted results of experiments; J.E.V., N.D., L.L.D., and K.E.S. prepared figures; J.E.V., N.D., L.L.D., and K.E.S. drafted manuscript; J.E.V., N.D., L.L.D., and K.E.S. edited and revised manuscript; J.E.V., N.D., L.L.D., and K.E.S. approved final version of manuscript.

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