Adrenergic C1 neurons monitor arterial blood pressure and determine the sympathetic response to hemorrhage

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SUMMARY

Hemorrhage initially triggers a rise in sympathetic nerve activity (SNA) that maintains blood pressure (BP); however, SNA is suppressed following severe blood loss causing hypotension. We hypothesized that adrenergic C1 neurons in the rostral ventrolateral medulla (C1⁰RVLM) drive the increase in SNA during compensated hemorrhage, and a reduction in C1⁰RVLM contributes to hypotension during decompensated hemorrhage. Using fiber photometry, we demonstrate that C1⁰RVLM activity increases during compensated hemorrhage and falls at the onset of decompensated hemorrhage. Using optogenetics combined with direct recordings of SNA, we show that C1⁰RVLM activation mediates the rise in SNA and contributes to BP stability during compensated hemorrhage, whereas a suppression of C1⁰RVLM activity is associated with cardiovascular collapse during decompensated hemorrhage. Notably, re-activating C1⁰RVLM during decompensated hemorrhage restores BP to normal levels. In conclusion, C1 neurons are a nodal point for the sympathetic response to blood loss.

In brief

Souza et al. show that adrenergic C1 neurons in the caudal brainstem are required for the compensatory response to hemorrhage. A reduction in C1 activity leads to hypotension preceding circulatory shock. This work shows that C1 neurons are a nodal point for the sympathetic response to blood loss.

Graphical Abstract

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AUTHOR CONTRIBUTIONS

G.M.P.R.S., P.G.G., and S.B.G.A. designed the research; G.M.P.R.S. conducted all the physiological experiments and data analysis; D.S.S. and R.L.S. conducted all histological preparation and analysis. G.M.P.R.S., P.G.G., D.S.S., R.L.S., and S.B.G.A. wrote the paper and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2022.110480.
INTRODUCTION

Hemorrhage is a leading cause of death following traumatic injuries, accounting for 1.5 million deaths per year worldwide (Lord et al., 2014; Cannon, 2018). The physiological response to blood loss involves interactions between peripheral and central mechanisms that maintain perfusion and adequate oxygenation of critical organs during hypovolemia (Schadt and Ludbrook, 1991; Convertino et al., 2021). The initial response to hemorrhage is marked by sympathetic activation, leading to an increase in heart rate (HR) and total peripheral resistance (TPR) (Evans et al., 2001; Convertino et al., 2021). Elevated HR and TPR during hemorrhage maintain blood pressure (BP) near normal levels despite reduced blood volume, a condition termed compensated hemorrhage (Schadt and Ludbrook, 1991; Evans et al., 2001; Schiller et al., 2017). However, if hemorrhage is not controlled, further blood loss triggers an abrupt reduction in HR and TPR mediated by a reflex sympathetic withdrawal, causing severe hypotension and often syncope (Evans et al., 2001; Convertino et al., 2021). This condition, termed decompensated hemorrhage, is the prelude to circulatory shock and death and is considered a “last-ditch” effort to increase venous return and reduce cardiac metabolism (Schiller et al., 2017; Cannon, 2018; Convertino et al., 2021). The neural mechanisms responsible for this drastic shift in physiological state (compensated to decompensated) are not well understood.

C1 neurons are adrenergic cells that are identified by the expression of phenylethanolamine N-methyltransferase (PNMT) and/or tyrosine hydroxylase (TH) located in the ventrolateral
medulla and that play an important role in the autonomic responses to physical and psychological stress (Abe et al., 2017; Wenker et al., 2017; Zhao et al., 2017; Stornetta and Guyenet, 2018). A subset of C1 neurons located in the rostral portion of the ventrolateral medulla (RVLM) regulates sympathetic nerve activity (SNA) through excitatory inputs to sympathetic preganglionic neurons (Guyenet, 2006; Guyenet et al., 2013). Most of these neurons (henceforth called C1\textsuperscript{RVLM}) are exquisitely sensitive to changes in BP, owing to negative feedback from the arterial baroreceptors, and regulate SNA controlling the HR and TPR (Guyenet et al., 2013, 2018). Indeed, stimulating C1\textsuperscript{RVLM} neurons in freely behaving rats increases BP through an increase in SNA (Kanbar et al., 2010; Abbott et al., 2013), and, at least under anesthesia, acute chemogenetic inhibition of these neurons reduces SNA and BP substantially (Marina et al., 2011). The exact contribution of C1\textsuperscript{RVLM} neurons to SNA and BP in unanesthetized resting or variously stressed mammals is not yet known. The ablation or acute inhibition of C1\textsuperscript{RVLM} neurons in intact, freely behaving rats has surprisingly little effect on resting BP, yet these manipulations impair the adequacy of the cardiovascular responses to hypotension (Madden et al., 2006), anesthesia, and hypoxia (Wenker et al., 2017), presumably owing to a greater reliance on SNA for BP maintenance under these circumstances. In sum, these studies indicate that C1\textsuperscript{RVLM} neurons are perhaps dispensable for maintaining BP at rest but are required for BP stability in the face of homeostatic challenges. Such questions that can only be answered via direct monitoring of the activity of these neurons in unanesthetized subjects.

C1 neurons are activated by hemorrhage or isovolemic hypotension based on the expression of c-Fos, a marker of increases in cell activity (Chan and Sawchenko, 1994, 1998; Li and Dampney, 1994; Dayas et al., 2001). However, it is not clear if elevated SNA and BP stability during hemorrhage involves C1\textsuperscript{RVLM}, and it is unknown if the onset of decompensated hemorrhage is related to a change in their activity. Given the proposed role of C1\textsuperscript{RVLM} in the sympathetic regulation of BP and evidence that these neurons are activated by hemorrhage, we hypothesize that C1\textsuperscript{RVLM} are required for the sympathetic response to blood loss. Further, we sought to test whether the shift from compensated to decompensated hemorrhage is associated with a reduction in C1 cell activity. To test these hypotheses, we first measured the real-time activity of C1\textsuperscript{RVLM} in freely behaving rats during hemorrhage using a genetically encoded calcium indicator, GCaMP7s, by fiber photometry. Next, we used optogenetics combined with SNA recordings in conscious rats to test the contribution of C1\textsuperscript{RVLM} to BP and SNA during hemorrhage.

**RESULTS**

**Real-time activity of C1 neurons during hypotension and hemorrhage**

Unilateral microinjections of a Cre-dependent vector encoding GCaMP7s in the RVLM of Th-Cre rats resulted in GCaMP7s expression in 76% ± 6% (range: 69%–87%) of C1\textsuperscript{RVLM} within the ipsilateral RVLM with a selectivity of 76% ± 12% (range: 50%–90%, n = 9; Figures 1A–1C). We first established that our recordings included C1\textsuperscript{RVLM} that monitor BP by evaluating changes in fluorescence during intravenous injection of phenylephrine (PHE), a vasoconstrictor, and sodium nitroprusside (SNP), a vasodilator. Transient hypotension elicited a robust graded increase in fluorescence (Figures 1D, 1E, and...
1G), whereas increasing BP caused a small but significant reduction in fluorescence (Figures 1F and 1H). We performed non-linear regression using a Boltzmann sigmoid function to determine the relationship between changes in emitted fluorescence and mean arterial pressure (MAP) (Figure 1I). This analysis revealed that fluorescence emitted by GCaMP7s expressed in C1RVLM is exquisitely sensitive to hypotension, with the greatest sensitivity of this response (i.e., the V_{50}) occurring over a range of BPs that fall below the resting MAP (Figure 1I). In short, the monitored GCaMP7 fluorescence largely originated from C1 neurons that receive strong negative feedback from arterial baroreceptors, the hallmark of the sympathetic premotor neurons that regulate BP (Guyenet, 2006; Guyenet et al., 2013).

After testing the baroreflex response, animals were subjected to controlled hemorrhage. C1RVLM activity, as monitored by GCaMP7 fluorescence, increased progressively during blood withdrawal and reached its maximum at the start of decompensated hemorrhage (Figure 2A). During decompensated hemorrhage, C1RVLM activity decreased along with HR and BP, ultimately reaching a steady state that was lower than the peak observed at the end of the compensated hemorrhage but greater than the values observed before blood loss (Figures 2A and 2B). In a separate group of rats (n = 12), we recorded lumbar sympathetic nerve activity (LSNA) during the same protocol of hemorrhage. LSNA followed a pattern of activation reminiscent of C1 neuron activity (Figures 2C, 2D, and 2E); it increased progressively during the compensated phase and exhibited a steep decline during the decompensated phase, as previously reported (Vantrease et al., 2015). Interestingly, C1RVLM activity remained higher during decompensated hemorrhage than during euvoletic conditions, whereas LSNA fell to levels similar to euvoletic conditions during this phase (Figures 2C, 2D, and 2E).

**Sympathetic and BP responses to C1 neuron inhibition during hemorrhage**

To determine the extent to which C1 neurons contribute to SNA and the maintenance of BP and HR during hemorrhage, we used loss-of-function optogenetics (Figures 3A and 3B). Bilateral microinjections of a Cre-dependent vector encoding ArchT3.0-EYFP in the RVLM of a Th-Cre rat (Figure 3A) resulted in EYFP expression in 58% ± 12% (range: 44%–72%) of C1RVLM with a selectivity of 84% ± 7% (range: 72%–90%, n = 9; Figure 3C). Acute bilateral inhibition of C1 neurons in euvoletic conditions produced a small but significant BP reduction without a change in HR, as previously reported (Wenker et al., 2017), and a correspondingly small reduction in LSNA (Table S1). During acute blood loss, C1RVLM inhibition produced progressively larger drops in BP and LSNA that were greatest immediately prior to the onset of decompensated hemorrhage (Figures 3 and 4; Table S1). Interestingly, C1 inhibition during compensated hemorrhage also caused bradycardia (Table S1; Figure 4D). Following the onset of decompensated hemorrhage, C1 neuron inhibition had no effect on BP and HR, but a significant reduction in LSNA persisted (Figures 3 and 4; Table S1). Based on this result and the fiber photometry data, C1 neurons are vigorously activated during compensated hemorrhage, and this effect contributes to the rise in SNA and HR that stabilizes BP. The results suggest that a reduction in the activity of C1 neurons also contributes to the fall in LSNA, HR, and BP that occurs during the decompensated phase. However, C1RVLM still drive LSNA during this phase because inhibition of these neurons produces a substantial fall in LSNA (Figures 4A and 4C). Thus, C1RVLM activity
is important to drive LSNA during compensated and decompensated hemorrhages, but a reduction in their activity likely contributes to the transition between these states. Finally, green laser illumination of the C1RVLM neurons expressing EYFP alone (no ArchT) had no effect on BP or HR at rest or during either phase of hemorrhage (Figure 7C, 7D, and 7F).

**Sympathetic and BP responses to C1 neuron activation during hemorrhage**

We next asked whether re-activating C1RVLM during decompensated hemorrhage would rescue SNA and restore BP. To test this hypothesis, we used excitatory optogenetics (ChR2) to stimulate C1RVLM while recording LSNA, BP, and HR during hemorrhage (Figures 5 and 6). Bilateral microinjections of a Cre-dependent vector encoding CHR2-EYFP in the RVLM of a Tt-Cre rat resulted in EYFP expression in 71% ± 4% (range: 66%–74%) of C1RVLM with a selectivity of 75% ± 13% (range: 51%–84%, n = 5; Figures 5A–5C). In euvoletic rats, the increase in BP during C1RVLM stimulation was frequency-dependent between 5 and 20 Hz, and the effects produced by left- or right-side C1RVLM stimulation were similar in amplitude and additive (Figures 5D and 5E). Bilateral C1 neuron stimulation at rest increased LSNA by 29% ± 11%. During compensated hemorrhage, LSNA was not significantly increased by C1RVLM stimulation (Table S1), and the change in BP elicited by the stimulation was attenuated (Figure 6B). This is presumably because C1 neurons and SNA are already strongly activated in this state (i.e., a ceiling effect). C1RVLM stimulation during decompensated hemorrhage increased LSNA, HR, and BP to a significantly larger degree than at rest (pre-hemorrhage) and during the compensated phase of hemorrhage (Figures 6B, 6C, and 6D). In fact, C1 activation during decompensated hemorrhage restored BP to near-normal values despite a severe deficit in blood volume. Blue laser illumination of C1 neurons transduced with control virus had no effect on BP at rest or during hemorrhage (Figures 7E, 7H, and 7I).

**DISCUSSION**

Using fiber photometry and loss-of-function optogenetics, we show that C1RVLM neurons have low activity in resting euvoletic unanesthetized rats and are vigorously activated by hypotension or blood loss. During compensated hemorrhage, C1RVLM activation stimulates SNA and prevents BP from falling, whereas a reduction of their activity and of SNA results in the decompensated phase of hemorrhage. Finally, re-activation of C1 neurons during decompensated hemorrhage rescues BP by increasing SNA and HR.

**C1 neurons defend primarily against hypotension**

The present calcium fluorescence measurements revealed that C1RVLM neurons are vigorously activated by isovolemic hypotension in conscious rats. Such activation has also been observed in anesthetized and other preparations using single-cell electrophysiological recordings. In those preparations C1RVLM neurons are also vigorously inhibited by increases in BP. The activity of C1RVLM neurons at rest, as determined by GCaMP7s, appears to be low in conscious, quietly resting euvoletic rats based on the modest inhibition of C1RVLM in response to increases in BP produced by PHE. Consistent with this interpretation, in conscious, resting euvoletic rats, BP drops very little during bilateral optogenetic inhibition of the C1 neurons (Wenker et al., 2017; present data) and is virtually
unchanged after massive lesions of $C1^{RVLM}$ (Madden et al., 2006). Thus, the contribution of $C1^{RVLM}$ neurons to maintaining BP appears to be low unless the animal is subjected to a physiological insult such as hypoxia (Wenker et al., 2017) or, as shown here, a sudden drop in BP or hemorrhage.

**C1 neurons underlie the biphasic response to blood loss through regulation of sympathetic activity**

Autonomic adjustments during compensated hemorrhage preserve BP and blood flow through essential organs despite the blood-volume reduction (Evans et al., 2001; Schiller et al., 2017). This is largely achieved through elevating HR and TPR. Inhibiting C1 neurons at rest modestly reduces LSNA and BP but has little effect on HR; by contrast, C1 inhibition during compensated hemorrhage markedly reduced LSNA, BP, and HR. This indicates that C1 neurons mediate the increase in SNA targeting the heart and resistance vessels during compensated hemorrhage.

The onset of decompensation is marked by sympathetic withdrawal causing a decline in HR and TPR (Evans et al., 2001; Convertino et al., 2021), which presumably helps maintain cardiac filling and stroke volume by reducing afterload and consequently increasing preload (Evans et al., 2001; Convertino et al., 2021). Our study shows that the onset of decompensated hemorrhage is caused in part by a reduction in $C1^{RVLM}$ activity. First, the reduction in $C1^{RVLM}$ neuron activity occurs concurrently with the fall in LSNA, HR, and BP. Second, optogenetic re-activation of $C1^{RVLM}$ during decompensated hemorrhage increases LSNA and HR and restores BP, suggesting that a fall in $C1^{RVLM}$ neurons activity underlies reductions in SNA, HR, and BP. Interestingly, during decompensated hemorrhage, $C1^{RVLM}$ activity remained considerably higher relative to pre-hemorrhage values than SNA. The comparatively greater fall in SNA relative to $C1^{RVLM}$ activity may indicate that the withdrawal of SNA during decompensated hemorrhage could be related to changes in activity occurring in other brain regions that directly regulate sympathetic tone or other non-C1 presympathetic neurons in the RVLM. Another possibility is that only a subset of $C1^{RVLM}$ cells, perhaps those that directly control cardiac, gut, and muscle SNA, reduce their activity selectively, whereas other types of $C1^{RVLM}$ neurons remain activated. Indeed, the varied electrophysiological properties and projections of $C1^{RVLM}$ neurons suggest that subsets differentially regulate the sympathetic efferent versus the neuroendocrine system (Verberne et al., 1999; Guyenet et al., 2013). Unlike SNA, activation of the hypothalamic-pituitary-adrenal axis and the release of both vasopressin and adrenal catecholamines are not suppressed during the decompensated phase of hemorrhage (Rocha and Rosenberg, 1969; Plotsky and Vale, 1984; Darlington et al., 1986; Badoer et al., 1992; Stornetta et al., 1999) (Bereiter et al., 1986). The differential regulation of the adrenal and renal SNA during decompensated hemorrhage (Victor et al., 1989) likely indicates a differential activation of the bulbospinal presympathetic neurons, most of which are $C1^{RVLM}$ cells. Finally, we observed a marked reduction in LSNA during C1 inhibition in decompensated hemorrhage, indicating some residual control of skeletal SNA by C1 neurons during decompensation.
Regulation of C1 neuron activity during hemorrhage: Potential mechanisms

The increase in SNA during controlled hemorrhage or in experimental lower body negative pressure occurs in proportion to the reduction of central venous pressure (Rea et al., 1991) and is largely attributed to unloading of the arterial baroreceptors (Ludbrook and Graham, 1984; Schadt and Ludbrook, 1991; Convertino et al., 2021). Similarly, C1RVLM activity during compensated hemorrhage may also reflect baroreceptor unloading. The discharge rate of arterial baroreceptors is reduced and their discharge pattern altered during hemorrhage even when the mean BP is unchanged (Hakumaki et al., 1985). This phenomenon could perhaps explain the increase in C1RVLM activity in the absence of a change in BP during compensated hemorrhage. C1RVLM activity may also be stimulated by inputs from atrial low-pressure receptors, which are activated by small amounts of blood loss that may precede the activation of the aortic baroreceptors (Gupta et al., 1966). Cerebral hypoperfusion could also contribute to the increase in C1RVLM neuron activity and SNA during hemorrhage as a result of brain tissue hypoxia (Guyenet et al., 2013; Marina et al., 2015).

Several theories have been advanced to explain the seemingly paradoxical reduction of SNA during the hypotensive phase of hemorrhage. The SNA reduction is often attributed to a reflex initiated by the activation of cardiopulmonary afferents other than the baroreceptors (Oberg and Thoren, 1972; Victor et al., 1989; Schadt and Ludbrook, 1991). Alternatively, the decline of C1RVLM activity may reflect changes in the balance of input arising from the baro-, cardiopulmonary-, and other afferents. A second theory posits that serotonin release in the RVLM contributes to the reduction in SNA during decompensated hemorrhage (Morgan et al., 1988; Dean and Bago, 2002) because the inhibition of serotonin synthesis (Morgan et al., 1988) or the blockade of 5-HT1A receptors in the RVLM (Dean and Bago, 2002) delays and attenuates the reduction in SNA. Thus, serotoninergic neurons activated by hemorrhage (Pelaez et al., 2002; Dean and Woyach, 2004) may inhibit C1RVLM neurons and, consequently, SNA during the decompensated stage. Finally, opiate signaling may also contribute to reduced SNA during decompensated hemorrhage (Schadt and Ludbrook, 1991). For example, a high dose of the opiate antagonist naloxone restores BP during hemorrhage-induced hypotension in rats (Faden and Holaday, 1979), suggesting that opioids may actively inhibit SNA in this state. Further studies will be required to identify the inputs that regulate C1 activity and precipitate decompensated hemorrhage during blood loss.

Limitations of the study

This study utilizes a genetically encoded calcium indicator, GCaMP7s, to measure the activity of C1RVLM neurons in intact, unanesthetized rats. This method has many advantages: (1) GCaMP7s is genetically targeted to C1 neurons, (2) stable recordings can be obtained in intact, unanesthetized animals, and (3) recordings provide a continuous measure of activity with reasonable temporal resolution.

This approach also has certain limitations. The decay of fluorescence following activation of GCaMP7s is slow relative to other comparable genetically encoded calcium indicators (Dana et al., 2019) and direct neuronal recordings. Hence, the time course for the decrease in C1 activity during the transition to decompensated hemorrhage may be more rapid than our recordings suggest. GCaMP7s is also not ideally suited to detect inhibition when cell activity...
is low at baseline, as we expect is the case for C1 neurons at rest, and this could contribute to an underestimation of the effect of baroreflex loading with PHE on C1 activity.

Fiber photometry measures the *en masse* fluorescence emitted by GCaMP7s-expressing neurons and therefore overlooks the potential response heterogeneity of individual C1<sub>RVLM</sub> neurons. C1<sub>RVLM</sub> activity monitored with GCaMP7s was robustly activated by isovolemic hypotension, indicative of a withdrawal of negative feedback from the arterial baroreflex that is generalized to most C1<sub>RVLM</sub> neurons. However, baroreceptor-regulated C1<sub>RVLM</sub> neurons probably include several functional subtypes (Morrison, 2001). These neurons could have distinct responses to isovolemic hypotension and hemorrhage that were not captured by fiber photometry.

A third technical consideration is the effect of blood flow on fluorescence emitted from the recording site. Hemoglobin absorbs light across the excitation and emission wavelengths used by GCaMP7s, so changes in RVLM blood flow during hemorrhage introduce a potential source of error. Blood-flow artifacts were minimized by applying an isosbestic correction for GCaMP (Lerner et al., 2015; Allen et al., 2017), which also corrects for photobleaching and some movement artifacts that occur during long recordings, as was necessary for these experiments (Lerner et al., 2015). Importantly, the results of our fiber-photometry recordings are congruent with the effect of optogenetic C1<sub>RVLM</sub> neuron inhibition on LSNA in this study. Hence, the changes in fluorescence during hemorrhage appear to track the relative activity of C1<sub>RVLM</sub> neurons with reasonable precision.

**Conclusion**

C1<sub>RVLM</sub> neurons have a low level of activity at rest in conscious rats and defend BP against hypotension and hypovolemia, presumably to maintain cerebral perfusion. Their robust activation during compensated hemorrhage is essential for BP stabilization, but their activity declines during the decompensated phase; this decline presumably contributes to the paradoxical SNA inhibition present during decompensation. Finally, optogenetic re-activation of C1<sub>RVLM</sub> neurons restores BP during the decompensated phase, even without fluid administration. This work emphasizes the importance of C1<sub>RVLM</sub> neurons for the sympathetic response during hemorrhage and provides evidence that these neurons monitor and regulate BP *in vivo*.

**STAR★METHODS**

**RESOURCE AVAILABILITY**

**Lead contact**—Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Stephen Abbott (sba6t@virginia.edu).

**Materials availability**—This study did not generate new unique reagents.

**Data and code availability**

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

We used male (n=33) and female (n=15) adult (90–180 days old) Th-Cre rats with a targeted insertion of IRES-Cre immediately after the translational stop in the open reading frame of Th (Sprague Dawley background; SD-Th<sup>tm1(IRES-Cre) Sage</sup>, TGRA8400; RRID:RGD_12905029), procured from Envigo RMS and maintained as Cre homozygous as recommended by the vendor. Rats were chosen because this species has been used extensively to model hemorrhage and permits both reliable SNA recordings and controlled blood withdrawal in freely behaving non-anesthetized subjects. The animals’ sex was similarly distributed in each experimental group except for the SNA recordings in freely behaving rats which were performed exclusively in male rats (n=7 for inhibition and n=5 for stimulation experiments) because their larger size enabled a more successful procedure. Rats weighed 250–300 g at the time of viral microinjections and 250–550 g at the time of the recordings. Animals were housed at 23°C-24°C under a standard artificial 12 h light-dark cycle with water and food provided ad libitum. All experiments were conducted in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals and approved by the University of Virginia Animal Care and Use Committee (protocol #4312).

**METHOD DETAILS**

**Microinjections and optic fiber placement in the RVLM**—Surgical procedures were conducted under aseptic conditions with body temperature maintained at 37°C with a servo-controlled heating pad. The depth of anesthesia was assessed by the absence of a withdrawal reflex to a firm tail or hind paw pinch and by monitoring breathing rate. For brain microinjections and fiber optic placement, rats were anesthetized with a mixture of ketamine (75 mg·kg<sup>−1</sup>), xylazine (5 mg·kg<sup>−1</sup>), and acepromazine (1 mg·kg<sup>−1</sup>, i.m.). Additional anesthetic was administered if required (25% of the original dose, i.m.). Brain injections were performed on a stereotaxic frame, with the bite bar set at 3.5 mm below the interaural line for a flat skull. In all rats, incisions were closed in two layers using absorbable sutures for internal closures, and steel clips and VetClose adhesive for skin. Antibiotic (ampicillin, 125 mg·kg<sup>−1</sup>, i.p.) and analgesic (ketoprofen, 3–5 mg·kg<sup>−1</sup>, s.c.) were administered every 24 h for 3 d following surgery.

Microinjections into the RVLM were placed behind the caudal pole of the facial motor nucleus as previously described (Souza et al., 2020). For fiber photometry experiments, a single unilateral microinjection of vector (60 nL) was placed 1800 μm lateral from the midline, 400 μm caudal from the caudal pole of the facial motor nucleus and between 8,200 to 8,500 μm ventral to the surface of the brain according to the ventral extent of the facial motor nucleus. The optic fiber used for photometry (400 μm core, 0.57 NA, Doric Lenses) was implanted with the tip placed 300–400 μm dorsal to the vector injection site and secured to the skull with dental cement. For optogenetics experiments, the viral vector was injected at three sites bilaterally (60 nL at each site) separated by 200 μm along the rostro-caudal axis, the most rostral being placed 200 μm caudal to the facial motor nucleus at a depth.
corresponding to the ventral extent of the facial motor nucleus. Bilateral optical fibers (200 μm core, 0.39 NA, Thorlabs) were placed 500 μm above each injection site.

**Viral vectors**—For the fiber photometry experiments, we used a Cre-dependent vector encoding GCaMP7s (pGP-AAV1-syn-FLEX-GCaMP7s-WPRE, Addgene plasmid 104491, titer injected: $4.7 \times 10^{11}$ GC·ml$^{-1}$). For the optogenetic inhibition we used a Cre-dependent vector encoding ArchT3.0 (rAAV2-eF1α-DIO-eArch3.0-eYFP, UNC Vector Core, titer injected: $4.0 \times 10^{12}$ vg·ml$^{-1}$). For optogenetics stimulation, we used a Cre-dependent vector encoding ChR2 (rAAV2-eF1α-DIO-hChR2(H134R)-eYFP, UNC Vector Core, titer injected: $4.0 \times 10^{12}$ vg·ml$^{-1}$). As a control we microinjected a vector that expressed eYFP only (rAAV2-eF1α-DIO-eYFP, UNC Vector Core, titer injected: $4.0 \times 10^{12}$ vg·ml$^{-1}$).

**Blood pressure and sympathetic nerve recordings**—Four to five weeks after microinjections and fiber implantation, rats were re-anesthetized with 2.5% isoflurane in pure oxygen. A small incision was made on the left leg for the implantation of two polyethylene catheters (PE-10 connected to a PE-50, Clay Adams), one in the femoral artery for blood withdrawal and one in the femoral vein for drug infusion. The catheters were tunneled under the skin and the tip was positioned in the dorsal aspect of the neck. On the right leg, the same approach was used to expose the femoral artery and a radiotelemetry probe (PA-C10; Data Sciences International) was implanted to monitor arterial pressure.

During the same surgery in some cases, a midline incision in the abdominal wall was performed to access the peritoneal cavity. The intestines and the vena cava were gently retracted, and the lumbar sympathetic chain was isolated between the L2 and L3 ganglia. Custom-made Teflon-coated platinum-iridium bipolar electrodes (uncoated diameter: 0.005 inches, A-M systems, catalog # 777000) were then positioned in contact with the lumbar sympathetic nerve and covered with biocompatible silicone Kwik-Cast (World Precision Instruments), as described elsewhere (Miki et al., 2002). The electrode wires were tunneled under the skin, exteriorized in the area between the shoulder blades, and secured using sutures. The connector was constructed with miniature pin connectors (A-M systems, catalog #520100), Kwik-Sil (World Precision Instruments), and heat-shrink tubing.

**Hemorrhage**—Hemorrhage was performed by withdrawing blood through the femoral catheter connected to a 3 mL syringe at a constant rate for up to 20 minutes (rate range 0.2–0.5 mL·min$^{-1}$). Blood withdrawal after the onset of decompensated hemorrhage continued until either diastolic BP fell to ~35 mmHg or a total blood loss of 18 mL·kg$^{-1}$ occurred. After cessation of blood withdrawal, most rats were deeply anesthetized with a mixture of ketamine, xylazine, and acepromazine (described above) and perfused for histology. In the protocol where SNA was recorded, 3–5 mL of warm Ringer-lactate solution was slowly infused until BP recovered to a mean BP >80 mmHg. At this point, rats were administered a ganglionic blocker (hexamethonium, 30 mg·kg$^{-1}$) to determine background electrical noise. The hemorrhage protocol was not designed to assess the recovery period. None of the animals subject to hemorrhage experienced syncope or died as a result of bleeding.

**Fiber photometry**—Fiber photometry was performed as described previously (Lerner et al., 2015). We used a 470 nm LED modulated at 211 Hz for GCaMP7s excitation and
a 405 nm LED modulated at 531 Hz for the isosbestic channel. Both light sources were coupled into a single core multiple-mode fiber (Doric Lenses, 400 μm, 0.57 NA) through a fluorescent minicube (Doric Lenses). Modulation and demodulation of LEDs and the emitted fluorescence was performed using two lock-in amplifiers (SR810 DSP, Stanford Research Systems). The power of each LED was measured with a power meter (Thorlabs) and set at a mean of ~5 μW when measured at the tip of the fiber that is connected to the rat. Fluorescence signals were collected by a photoreceiver (Newport).

Baroreflex tests were performed to screen rats prior to hemorrhage. Sodium nitroprusside (SNP, 10 μg·kg<sup>−1</sup>) and phenylephrine (PHE, 1 μg·kg<sup>−1</sup>) were administered intravenously to lower and raise BP. Only animals that presented an increase in fluorescence (>2%) in response to hypotension produced by SNP were proceeded to the hemorrhage protocol. Three of 13 cases were excluded as a stable fluorescence signal could not be established. These cases did not exhibit spontaneous fluctuations in signal that were characteristic of recordings and did not exhibit a response to BP manipulations.

Optogenetics—On the day of the experiment, optical fibers implanted in the rat were connected to a matching connecting fiber optic cable (multimode fiber, 200 μm core, 0.39NA) that in turn was connected to a computer-controlled laser diode. A green laser diode (532 nm, power output measured at the tip of the connecting fiber: 10 mW) was used to activate ArchT3.0 using a 10 sec continuous pulse every 2 minutes over the time course of blood withdrawal. A blue laser diode (470 nm, power output measured at the tip of the connecting fiber: 10 mW) was used to activate ChR2(H134R) with 5 ms pulses delivered at 10 Hz for 10 secs once every 2 minutes over the time course of blood withdrawal. Laser pulses were controlled using Spike 2 (version 8, Cambridge Electronic Design Limited) software.

Acquisition of physiological variables—All the signals were acquired and digitized with a CED 1401 A/D acquisition system using Spike 2 (Version 8, Cambridge Electronic Design). BP was digitized and acquired at 1,000 Hz. SNA was amplified and band pass filtered (50,000x, 100 – 3,000 Hz, CWE inc.) and digitized at 5,000 kHz. Excitation and isosbestic signals for fiber photometry were acquired at 1,000 Hz and smoothed with a time constant of 0.5 s.

Histology—At the end of the experiments, animals were deeply anesthetized with a mixture of ketamine, xylazine, and acepromazine and then perfused transcardially with 4% paraformaldehyde. Brains were removed and postfixed in the same fixative for 12–16 h at 4°C. Brains were sectioned (30 μm; transverse plane) the next day on a vibratome (VT-1000S, Leica Biosystems), and brain slices were stored in cryoprotectant at −20°C (Souza et al., 2020). Immunohistochemistry, performed on free-floating sections, was used to identify C1 neurons transduced with GCaMP7s, ArchT3.0-eYFP and ChR2-eYFP. All immunohistochemistry procedures were conducted at room temperature, unless otherwise noted. A 1-in-6 series of sections were rinsed, then blocked in a solution containing 100 mM tris, 150 mM saline, 10% horse serum (v/v) 0.1% Triton-X (v/v), then incubated with primary antibodies for 60 min at room temperature then 4°C overnight. The next day, sections were rinsed and then incubated with secondary antibodies for 60 min and
rinsed again before mounting on slides. Slides were covered with ProLong Gold anti-fade mounting medium (P36931, Thermo Fisher Scientific). Primary antibodies used to identify neurons transduced by opsins or GCaMP7 were chicken anti-GFP (1:1000, Aves Labs, catalog #GFP-1020, RRID: AB_0000240), to identify C1 neurons mouse anti-TH (1:10k, Millipore Sigma, catalog T1299, RRID:AB_477560) and rabbit anti-PMNT (1:5k, gift from M.C. Bohn, Northwestern University, Illinois, RRID:AB_2315181). Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, and used at 1:500 as follows: AlexaFluor-488 AffiniPure Donkey Anti-Chicken IgY (IgG) (H+L)(catalog #703–545-155, RRID:AB_2340375) to reveal chicken anti-GFP; AlexaFluor-488 AffiniPure F(ab')² Fragment Donkey Anti-Mouse IgG (H+L)(catalog #715–546-150, RRID:AB_2340849), or Cy3 AffiniPure F(ab')² Fragment Donkey Anti-Mouse IgG (H+L)(catalog #715–166-150, RRID:AB_2340816) to reveal mouse anti-TH and Cy3 AffiniPure Donkey Anti-Rabbit IgG (H+L) (catalog #711–165-152) to reveal rabbit anti-PMNT.

QUANTIFICATION AND STATISTICAL ANALYSIS

Fiber photometry—The relative fluorescence (ΔF/F₀) was obtained from demodulated 470 nm and 405 nm fluorescence using the following formula: ΔF/F₀ = (ΔF₄₀⁵/F₀)/(ΔF₄₇₀/F₀). Results are presented as normalized values of ΔF/F₀. For normalization, corrected ΔF/F₀ values were subtracted from the mean and divided by the standard deviation of values collected prior to hemorrhage or drug infusion as described below. Normalized ΔF/F₀ response to baroreflex activation presented in Figures 1F and 1G were calculated using the mean and standard deviation of fluorescence during a 10-second period immediately prior to infusion of SNP or PHE. The changes in fluorescence presented in Figure 1G were calculated using the peak response for SNP; and in Figure 1H, for PHE we used a 5–10 second average period that was associated with the increase in BP. X-Y plots of mean arterial blood pressure (MAP) and the change in normalized ΔF/F₀ from baseline during SNP and PHE infusions were fitted with a Boltzmann sigmoid curve for Figure 1I. To analyze the changes in fluorescence in response to hemorrhage we considered a 5-minute period before hemorrhage as a base period to calculate the normalized ΔF/F₀. The pre-hemorrhage values consisted of a subsequent 5-minute period when the animal was quietly resting. The values of the compensated stage were taken from a 2-minute period prior to the decompensation and the values of the decomposition period were taken from a period where BP and HR were stabilized.

Optogenetics—Average changes in BP, HR and in SNA produced by C1RVLM inhibition or stimulation were compared to the average value of 10 seconds before the stimulation. The trial in the compensated phase of hemorrhage considered for analysis was taken 2–4 minutes before the decompensation phase which corresponds to the maximum SNA activation and the greatest decrease in BP in response to C1RVLM inhibition and the trial during the decompensated hemorrhage was taken during a sustained period of hypotension. The same rationale was applied for the C1RVLM stimulation experiments.

Lumbar sympathetic nerve activity—We recorded the lumbar sympathetic nerve activity (LSNA) which regulates primarily the vascular tone of skeletal muscles. In quiet rest conditions LSNA is silenced by increasing BP indicating that this nerve is highly...
responsive to baroreceptor activation hence carries activity that regulates vascular tone and blood pressure. LSNA was chosen as opposed to other sympathetic nerves involved in cardiovascular regulation because the surgical approach to conduct direct nerve recordings is the least invasive and most reliable in our hands. LSNA recordings included in this study had to meet the following quality controls: a signal-to-noise ratio with a minimum 2-fold difference between the RMS multi-unit activity (smoothing: 0.03 s) recorded from the nerve at rest before blood loss and the level present after ganglionic blockade, presence of cardiac-related activity but absence of detectable electrocardiogram contamination. Periods of movement-related activity were considered as artifacts and were excluded from the analysis. For Figures 2C and 2E, LSNA was corrected for noise using ganglionic blockade and normalized to the value of LSNA prior to blood loss. For optogenetics experiments, LSNA was corrected for noise and normalized to a 10s period immediately prior to the light stimulus, which was considered as 100%.

**Neuronal mapping**—Neuronal mapping was conducted using a motor driven stage with Neurolucida software (version 2021, MBF Bioscience) with an AxioImager M2 microscope (Carl Zeiss) after methods previously described (Stornetta et al., 2006). Digital photomicrographs were acquired with the same microscope in grayscale using a C11440 Orca-Flash 4.0LT digital camera (Hamamatsu). Filter settings for Cy3 or Atto 550, Alexa-488, and Atto 647 fluorophores were as follows: Alexa-488, excitation of 500 nm, emission of 535 nm; Atto 550 or Cy3, excitation of 545, emission of 605nm; Atto 647, excitation of 640 nm, emission of 690 nm. Only cell profiles that included a nucleus in the plane of section were counted and/or mapped.

The RVLM was defined as the region extending from the rostral tip of the lateral reticular nucleus to the mid facial motor nucleus, corresponding to bregma levels between −12.96 mm and −11.52 mm in Paxinos and Watson (2014), 7th ed. This region captures a majority of bulbospinal C1 neurons (Guyenet et al., 2013). C1 neurons in the RVLM (C1RVLM) were identified by the presence of tyrosine hydroxylase (TH) and/or phenylethanolamine N-methyl transferase (PNMT). There was a high correspondence between TH and PNMT in the RVLM; based on counts in 10 rats, 76.6 ± 0.8% of cells co-stained for both PNMT and TH, 17.6 ± 0.6 % were PNMT-positive only, and 5.8 ± 0.3% % were TH-positive only. Viral transduction efficiency in fiber photometry experiments was defined as the proportion of the C1 neurons expressing GCaMP7s within 400 μm ventral to the tip of the fiber optic as a proportion of total ipsilateral C1RVLM neurons. The selectivity of GCaMP7s expression was defined as the proportion of cells expressing the GCaMP7s that also expressed TH and/or PNMT. Viral transduction efficiency in optogenetic experiments was defined as the proportion of C1RVLM neurons expressing the opsin reporter as a proportion of total bilateral C1RVLM neurons. The selectivity of transgene expression for opsins was defined as the proportion of cells expressing the opsin reporter that also expressed TH and/or PNMT. No minimum threshold for the expression of transgene was used to exclude animals from the study. For presentation, images were pseudo-colored, brightness and contrast were optimized for visibility, all pixels in the image were adjusted equally; these modifications were performed in Fiji software (Schindelin et al., 2012).
**Statistics**—Statistical comparisons were made using Prism software (version 9.0, GraphPad). Following tests for normality (D’Agostino-Pearson or Shapiro-Wilk), significant differences were determined using repeated measures one-way ANOVA with Tukey’s or Dunnet’s post-test as indicated in figure legends. Data in Table S1 was analyzed using repeated measures two-way ANOVA followed by Bonferroni’s post-test. All F- and p-values for the interaction effects and individual treatment effects are reported either in the text or in the corresponding figure legend. Data are reported as mean ± SD unless otherwise noted in the figure legends and differences were considered significant when p < 0.05.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**ACKNOWLEDGMENTS**

This work was supported by an American Heart Association grant (19POST34430205) to G.M.P.R.S. and National Institutes of Health grants HL148004 to S.B.G.A. and HL28785 and HL074011 to P.G.G. We would like to thank Dr. Sean D. Stocker from the University of Pittsburgh for his help and advice with the sympathetic nerve recordings in freely behaving rats.

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Highlights

- C1 neurons monitor blood pressure in freely behaving rats
- The response of C1 neurons to hemorrhage is biphasic
- Inhibition of C1 during compensated hemorrhage causes hypotension
- Activation of C1 restores blood pressure during decompensated hemorrhage
Figure 1. C1 neurons monitor blood pressure (BP) in freely behaving rats
(A) Approach for genetically targeted fiber photometry of C1 neurons located within the rostral ventrolateral medulla (C1RVLM).
(B) Experimental setup to record from C1 neurons while monitoring BP in a freely behaving rat.
(C) Image of transduced C1 neurons at the RVLM. Dotted square beneath the optical fiber is shown at a higher magnification in the 4 panels to the right. Stars indicate triple-labeled neurons. Scale bars: 400 μm (left panel) and 100 μm (4 right panels). OF, optical fiber tract; py, pyramidal tract; Amb, nucleus ambiguus.
(D) Characterization of barosensitive C1 neurons in fiber-photometry recordings. Hypotension induced by infusion of sodium nitroprusside (SNP) reliably increases ΔF/F₀ emitted by C1 neurons.
(E) Mean ± SEM of BP and ΔF/F₀ during transient hypotension induced by SNP (n = 10).
(F) Mean ± SEM of BP and ΔF/F₀ during increase in BP induced by phenylephrine (PHE; n = 6).
(G) Changes in BP and ΔF/F₀ induced by SNP (paired t test, p < 0.0001, t = 6.661, df = 9).
(H) Changes in BP and ΔF/F₀ induced by PHE (paired t test, p = 0.0478, t = 2.608, df = 5).
(I) Relationship between MAP and $\Delta F/F_0$ from a representative case (data fitted with a Boltzmann-sigmoid curve, $R^2 = 0.93$ $V_{50} = 100.2$, slope = $-3.96$; inset: first derivative of curve fitted in I).
Figure 2. C1 neuron activity during hemorrhage in freely behaving rats is dynamic and correlates with sympathetic vasomotor tone

(A) Average ± SEM of BP, HR, and ΔF/F₀ during hemorrhage (n = 10). ΔF/F₀ increases progressively with blood loss until the onset of decompensated hemorrhage, denoted by the marked change in MAP and HR, after which ΔF/F₀ decreases during the transition to decompensation hemorrhage.

(B) ΔF/F₀ during pre-hemorrhage and compensated and decompensated hemorrhage (repeated measures [RM] one-way ANOVA, p < 0.0001, F \(_{(1.3, 11.7)} = 27.74\); Tukey’s post-test for pre- versus compensated, p < 0.0001; pre- versus decompensated, p = 0.0203; compensated versus decompensated, p = 0.0061). Gray bars in (A) indicate periods where data for (B) was taken. *, different from the pre-hemorrhage; #, different from compensated hemorrhage.

(C) Changes in lumbar sympathetic nerve activity (LSNA) in freely behaving rats during hemorrhage (RM one-way ANOVA, p < 0.0001, F \(_{(1.8, 18.1)} = 32.6\); Tukey’s post-test for pre- versus compensated, p = 0.0004; pre- versus decompensated, p = 0.1468; compensated versus decompensated, p < 0.0001). *, different from the pre-hemorrhage; #, different from compensated hemorrhage.

(D) Recordings of BP and LSNA of a case representative of the group pre-hemorrhage and during the compensated and decompensated stages of hemorrhage. Dotted line represents electrical noise obtained after ganglionic blockade. Average ± SEM of BP and SNA during hemorrhage (n = 12). During the compensated period, LSNA increases with the progression of hemorrhage.
of blood loss, and BP is maintained. With further blood loss, SNA declines along with BP, characterizing the decompensated hemorrhage (BP: RM one-way ANOVA, \( p < 0.0001 \), \( F_{(11, 154)} = 6.28 \); SNA: RM one-way ANOVA, \( p < 0.0001 \), \( F_{(11, 154)} = 8.53 \); Dunnet’s post-test for BP and SNA: \(*p < 0.05; **p < 0.01, \text{ and } ***p < 0.0001\) for comparisons with a baseline value at 18 min before the decompensation point (zero).
Figure 3. Contribution of C1 neurons to the maintenance of BP and HR over the time course of hemorrhage

(A) Approach for genetically targeted C1 neuron for optogenetic inhibition experiments.
(B) Experimental setup to inhibit C1 neurons while monitoring BP and lumbar SNA in a freely behaving rat.
(C) Presence of ArchT3.0-EYFP in C1 neurons and location of the optical fiber tracts (OF) in the RVLM. Dotted square beneath OF is shown at a higher magnification in the three bottom panels. Stars indicate triple-labeled neurons (ArchT3.0, TH, and PNMT). Scale bars: 200 μm (top panel) and 100 μm (bottom panels). OF, optical fiber tract; py, pyramidal tract; Sp5, spinal trigeminal tract; ION, inferior olive nucleus.
(D) Effect of optogenetic inhibition of C1 neurons (532 nm, 10 mW, 10 s every 2 min) on BP and HR over the time course of hemorrhage (n = 9). Note that the effect of C1 neuron inhibition on BP increases with the progression of blood loss and its maximum (arrowheads) close to the decompensation point (zero).
(E) Excerpt from (D) showing the bigger drops in the BP responses to C1 neuron inhibition with the progression of blood loss. Note that HR starts to decrease in response to C1 neuron inhibition prior to the onset of decompensated hemorrhage.

(F) BP over the time course of hemorrhage (n = 9, RM one-way ANOVA, p < 0.0001, F(16, 117) = 31.2).

(G) Changes in MAP in response to C1 neuron inhibition over the time course of hemorrhage (RM one-way ANOVA, p < 0.0001, F(16, 117) = 8.7).

(H) Changes in HR (RM one-way ANOVA, p = 0.0298, F(16, 117) = 1.9; Dunnet’s post-test for MAP, ΔMAP, and ΔHR: *p < 0.05; **p < 0.01, and ***p < 0.0001) for comparisons with a baseline value at 18 min before the decompensation point (zero).
Figure 4. C1 neurons drive SNA during hemorrhage to maintain blood pressure

(A) Effect of bilateral C1RVLM neuron optogenetic inhibition (532 nm, 10 mW, 10 s every 2 min) on BP and SNA prior to blood loss and during each stage of hemorrhage. C1 neuron inhibition produces little effect at rest but reduces BP and SNA markedly during compensated hemorrhage. During the decompensated phase, C1 inhibition also reduces SNA substantially but the effect on BP is minimal. Dotted line represents electrical noise obtained after ganglionic blockade.

(B) Changes in BP (n = 9) produced by C1 neuron inhibition prior to and during hemorrhage (RM one-way ANOVA, p < 0.0001, F (1.8, 14.7) = 118.5; Tukey post-test: pre- versus compensated hemorrhage, p < 0.0001; pre- versus decompensated hemorrhage, p = 0.1524; compensated versus decompensated hemorrhage, p < 0.0001).

(C) Changes in SNA (n = 7) produced by C1 neuron inhibition prior to and during hemorrhage (RM one-way ANOVA, p = 0.0019, F (1.6, 9.9) = 13.60; Tukey post-test: pre- versus compensated hemorrhage, p = 0.0087; pre- versus decompensated hemorrhage, p = 0.0057; compensated versus decompensated hemorrhage, p = 0.5204).

(D) Changes in HR (n = 9) produced by C1 neuron inhibition prior to and during hemorrhage (RM one-way ANOVA, p = 0.0156, F (1.2, 10.2) = 7.583; Tukey post-test: pre- versus compensated hemorrhage, p = 0.0245; pre- versus decompensated hemorrhage, p = 0.1323; compensated versus decompensated hemorrhage, p = 0.1285). *, different from the pre-hemorrhage; #, different from compensated hemorrhage. One symbol denotes p < 0.05, two symbols p < 0.01, and three symbols p < 0.0001.
Figure 5. Effect of bilateral stimulation of C1 neurons on BP

(A) Approach for genetically targeted C1<sub>RVLM</sub> optogenetic stimulation.
(B) Experimental setup to stimulate C1<sub>RVLM</sub> while monitoring BP and LSNA in a freely behaving rat.
(C) Image of the expression of ChR2-EYFP in C1 neurons and the OFs in the RVLM. Dotted square beneath OF is enlarged for 3 panels at the bottom. Stars indicate triple-labeled neurons. Scale bars: 200 μm (top panel) 100 μm (bottom panels). OF, optical fiber tract; py, pyramidal tract; Sp5, spinal trigeminal tract; ION, inferior olive nucleus.

(D) C1 neuron stimulation (n = 6) produced a bigger increase in BP when stimulated bilaterally (RM one-way ANOVA, p < 0.0001, F<sub>(1.3, 6.9)</sub> = 71.65; Tukey post-test for right versus left side, p = 0.6653; right versus bilateral, p < 0.0001; left versus bilateral, p = 0.0005). *, different from right side, #, different from left side.

(E) Effect of bilateral C1 neuron stimulation on BP (n = 7) is frequency dependent (RM one-way ANOVA, p = 0.0012, F<sub>(1.1, 6.7)</sub> = 31.75; Tukey post-test for different frequencies of stimulation: 5 versus 10 Hz, p = 0.0001; 5 versus 20 Hz, p = 0.0017; 10 versus 20 Hz, p = 0.0212). *, different from 5 Hz; #, different from 10 Hz.
Figure 6. Activation of C1 neurons rescues LSNA and restores BP during decompensated hemorrhage

(A) Changes in BP and LSNA elicited by bilateral stimulation of C1 neurons pre- and during hemorrhage. Note that C1 neuron stimulation (470 nm, 10 mW, 10 Hz) during decompensated hemorrhage restores BP to pre-hemorrhage levels. Dotted line in LSNA represents electrical noise obtained after ganglionic blockade.

(B) Changes in BP (n = 6) elicited by C1 neuron stimulation prior to and during hemorrhage (RM one-way ANOVA, p < 0.0001, F(1.9, 9.6) = 31.06).

(C) Changes in LSNA (n = 5) elicited by C1 neuron stimulation prior to and during hemorrhage (RM one-way ANOVA, p = 0.0003, F(1.6, 6.4) = 40.35).

(D) Changes in HR elicited by C1 neuron stimulation prior to and during hemorrhage (RM one-way ANOVA, p = 0.0065, F(1.1, 5.6) = 17.23). *, different from pre-hemorrhage; #, different from compensated hemorrhage. One symbol denotes p < 0.05, two symbols p < 0.01, three symbols p < 0.0001.
Figure 7. Effect of light stimulation of C1 neurons transduced with control virus on BP and HR during hemorrhage

(A) Experimental setup to test the effect of light on BP and HR during hemorrhage. Control virus was injected bilaterally into the RVLM followed by bilateral optical fiber implant.

(B) One month later, BP was recorded while laser pulses (532 nm, 10 mW, 10 s pulse every 2 min or 470 nm, 10 mW, 10 Hz every 2 min) were delivered into RVLM during hemorrhage.

(C and D) Recording of a representative animal showing no effect of green laser (532 nm, 10 mW, 10 s continuous pulse every 2 min) applied to the RVLM on BP during the different stages of hemorrhage.

(E) Recording of a representative animal showing no effect of blue laser (470 nm, 10 mW, 10 Hz, 5 ms pulse every 2 min) applied to the RVLM on BP during the different stages of hemorrhage.

(F) Effect of green light on BP (n = 6) during the different stages of hemorrhage (RM one-way ANOVA, p = 0.6407, F(1.5, 7.7) = 0.3861; Tukey post-test for pre- versus compensated hemorrhage, p = 0.7061; pre- versus decompensated hemorrhage, p = 0.9840; compensated versus decompensated hemorrhage, p = 0.6667).

(G) Effect of green light on HR (RM one-way ANOVA, p = 0.6506, F(1.5, 7.4) = 0.3583; Tukey post-test for pre- versus compensated hemorrhage, p = 0.5523; pre- versus decompensated hemorrhage, p = 0.9964; compensated versus decompensated hemorrhage, p = 0.8452).
(H) Effect of blue light on BP (n = 5) during the different stages of hemorrhage (RM one-way ANOVA, p = 0.9232, F_{(1.7, 7.1)} = 0.063; Tukey post-test for pre- versus compensated hemorrhage, p = 0.9873; pre- versus decompensated hemorrhage, p = 0.9773; compensated versus decompensated hemorrhage, p = 0.9421).

(I) Effect of blue light on HR (RM one-way ANOVA, p = 0.1634, F_{(1.4, 5.8)} = 2.5; Tukey post-test for pre- versus compensated hemorrhage, p = 0.2292; pre- versus decompensated hemorrhage, p = 0.8871; compensated versus decompensated hemorrhage, p = 0.3202).
# KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Chicken anti-GFP    | Aves Labs | Cat#GFP-1020; RRID: AB_0000240 |
| Mouse anti-TH       | MilliporeSigma | Cat#T1299; RRID:AB_477560 |
| Rabbit anti-PMNT    | Bohn et al., 1987, Northwestern University, Illinois | RRID:AB_2315181 |
| AlexaFluor-488 AffiniPure Donkey Anti-Chicken IgY (IgG) | Jackson ImmunoResearch Laboratories | Cat#703-545-155; RRID:AB_2340375 |
| AlexaFluor-488 AffiniPure F(ab')2 Fragment Donkey Anti-Mouse IgG (H+L) | Jackson ImmunoResearch Laboratories | Cat#715-546-150; RRID:AB_2340849 |
| Cy3 AffiniPure F(ab')2 Fragment Donkey Anti-Mouse IgG (H+L) | Jackson ImmunoResearch Laboratories | Cat#715-166-150; RRID:AB_2340816 |
| Cy3 AffiniPure Donkey Anti-Rabbit IgG (H+L) | Jackson ImmunoResearch Laboratories | Cat#711-165-152; RRID: AB_2307443 |
| Bacterial and virus strains |        |            |
| pGP-AAV1-syn-FLEX-jGCaMP7s-WPRE | Dana et al., 2019 | Addgene viral prep # 104491-AAV1 |
| rAAV2-eF1α-DIO-eArch3.0-eYFP | UNC Vector Core | RRID:SCR_002448 |
| rAAV2-eF1α-DIO-hChR2(H134R)-eYFP | UNC Vector Core | RRID:SCR_002448 |
| rAAV2-eF1α-DIO-eYFP | UNC Vector Core | RRID:SCR_002448 |
| Chemicals, peptides, and recombinant proteins |        |            |
| Sodium Nitroprusside | MilliporeSigma | Cat#S0501 |
| Phenylephrine hydrochloride | MilliporeSigma | Cat#P-6126 |
| Hexamethonium | MilliporeSigma | Cat#H087925G |
| Triton-X | MilliporeSigma | Cat#X-100 |
| Experimental models: Organisms/strains |        |            |
| Rat, Sprague Dawley background; SD-THtm1(IRES-Cre) Sage; TGRAS400 | Envigo RMS | RRID:RGD_12905029 |
| Software and algorithms |        |            |
| Spike 2, version 8 | Cambridge Electronic Design | https://ced.co.uk/products/spkovan |
| Prism, version 9 | GraphPad | https://www.graphpad.com/scientific-software/prism/ |
| Neurolucida, version 2021 | MBF Bioscience | https://www.mbfbioscience.com/neurolucida |