Decompensated hemorrhage activates serotonergic neurons in the subependymal parapyramidal region of the rat medulla

NICOLE M. PELAEZ,1 ANN M. SCHREIHOFER,1,2 AND PATRICE G. GUYENET1

1Department of Pharmacology, University of Virginia Health System, Charlottesville, Virginia 22908-0735; and 2Department of Physiology, Medical College of Georgia, Augusta, Georgia 30912-3000

Received 8 March 2002; accepted in final form 28 May 2002

Pelaez, Nicole M., Ann M. Schreihofer, and Patrice G. Guyenet. Decompensated hemorrhage activates serotonergic neurons in the subependymal parapyramidal region of the rat medulla. Am J Physiol Regul Integr Comp Physiol 283: R688–R697, 2002; 10.1152/ajpregu.00154.2002.—According to prior evidence opioid and serotonin release by lower brain stem neurons may contribute to hemorrhage-induced sympathoinhibition (HISI). Here we seek direct evidence for the activation of opioidergic, GABAergic, or serotonergic neurons by severe hemorrhage in the medulla oblongata. Blood was withdrawn from awake rats (40–50% total volume) causing hypotension and profound initial bradycardia. Other rats received the vasodilator hydralazine, causing tachycardia and hypotension. Neuronal activation was gauged by the presence of fos-immunoreactive (ir) nuclei after 2 h. Serotonergic, enkephalinergic, and GABAergic neurons were identified by the presence of a diagnostic enzyme or mRNA. Hemorrhaged rats had 30% fewer non-GABAergic Fos-ir neurons in the rostral ventrolateral medulla (RVLM) than hydralazine-treated rats, but they had six times more Fos-ir neurons within the subependymal parapyramidal nucleus (SEPPN). Fos-labeled SEPPN neurons were serotonergic (40–60%), GABAergic (31%), enkephalinergic (15%), or had mixed phenotypes. The data suggest that a reduced sympathoexcitatory drive from RVLM may contribute to HISI. SEPPN neuronal activation may also contribute to HISI or could mediate defensive thermoregulatory mechanisms triggered by hemorrhage-induced hypothermia.

The rapid loss of blood triggers two successive phases of autonomic responses. Initially arterial pressure (AP) is maintained, principally by an increase in sympathetic outflow to the heart and blood vessels. Beyond 30% blood loss, a second phase (decompensated stage, stage II of hemorrhage) is elicited (21, 49). During that stage, sympathetic tone to most organs except the adrenal medulla is reduced, and the heart rate falls (11, 55). The fall in heart rate combined with hemorrhage-induced sympathoinhibition (HISI) triggers a rapid fall in blood pressure (8). The second stage of hypotensive hemorrhage also heralds a marked reduction in oxygen consumption and a fall in core temperature (25).

The neurophysiological mechanisms underlying HISI are poorly understood. The activation of atrial or ventricular receptors contributes to decompensation (40, 49). Indeed a similar pattern of autonomic responses can be produced without blood loss by artificially reducing venous return (simulated hemorrhage; Ref. 19). However, vagotomy delays rather than abolishes the decompensation phase in conscious rabbits subjected to simulated hemorrhage, suggesting that vagal afferent traffic may not be the only trigger (20).

The cardiac output threshold for phase II induction is elevated by factors that increase baseline sympathetic nerve activity such as reduced blood PO2 and various drugs such as α2-adrenergic antagonists (7, 17).

The rostral ventrolateral medulla (RVLM) probably plays a role in HISI because the resting discharge of many presympathetic neurons is reduced by hypotensive hemorrhage in anesthetized rats (46). Because the activity of RVLM presympathetic neurons is controlled by an extensive central nervous system (CNS) network, the root cause of their inhibition during hypotensive hemorrhage could lie elsewhere within the multiple components of this network. In fact, the contribution of suprapontine regions to HISI, notably the periaqueductal gray matter, has long been suspected (21, 23, 24).

It is probable that many CNS transmitters contribute to HISI although most studies have focused on serotonin and opioids. The notion that serotonin release contributes to HISI originates from the fact that decompensation is delayed in animals treated with a serotonin synthesis inhibitor or with the broad spectrum serotonin receptor antagonist methysergide (18, 36, 48). Further work has suggested that the most critical serotonergic receptors are of the 5-hydroxytryptamine (HT)1A variety (48) and that activation of 5-HT1A receptors specifically within the RVLM makes a notable contribution to HISI (13). However, pharmacological evidence also suggests that the release of opioid peptides in the spinal cord or elsewhere in the CNS contributes to HISI (2, 19, 32).
The present experiments were designed to test whether HISI is associated with the activation of enkephalineric, GABAAergic, or serotoninergic neurons in the region of the rostral medulla of the rat. Stage II hemorrhage was produced in conscious rats, and neuronal activation was gauged by the presence of Fos-related antigens (3, 12, 14, 45). To assess whether neuronal activation was associated specifically with hypotensive hemorrhage rather than with hypotension alone, the hemorrhaged animals were compared with rats treated with the arterial vasodilator hydralazine. Hydralazine causes normovolemic hypotension and a persistent baroreceptor-mediated activation of the sympathetic vasomotor tone.

MATERIALS AND METHODS

Physiological procedures. Experiments were performed on male Sprague-Dawley rats (Hilltop Laboratories, Scottsdale, PA) weighing 250–350 g. On delivery to the animal care facility, the rats were exposed to a 12:12-h light-dark cycle and given free access to food and water. They were allowed to acclimatize to these conditions for at least 48 h. All experiments were designed in compliance with National Institutes of Health and Institutional Animal Care and Use committee guidelines. The University of Virginia Animal Research Committee approved all protocols and procedures.

Rats were anesthetized with halothane (5% in 100% O₂ for induction and 1.8% during surgery) for catheter implantation into the right femoral artery and femoral vein as described previously (52). The catheters were threaded subcutaneously to exit the upper back through a tethering device. An antibiotic (ampicillin, 125 mg/kg im, Bristol-Myers Squibb, Princeton, NJ) and an analgesic (ketorolac, 0.5–0.75 mg/kg ip, Abbott Labs, N. Chicago, IL) were then administered. The next day, rats were subjected to hypotensive hemorrhage (hemorrhage group), received hydralazine intravenously (hydralazine group), or received no further treatment (control group). Each physiological experiment was done using two rats each belonging to a different group. The composition of the pairs was randomized, and the process was repeated until all animals were used. Baseline blood pressure and heart rate measurements were recorded for at least 10 min before experimental treatment.

The hemorrhage group (n = 6) was subjected to a 40% blood withdrawal performed through the arterial line over 3 min. Total blood volume was estimated at 60 ml/kg (56). Further removal of blood, up to 50% of total blood volume, in increments of 0.5 ml was performed to keep blood pressure from rising to baseline levels. The hypotension group (n = 6) received 10 mg/kg of the arterial vasodilator hydralazine (hemorrhage group), or received no further treatment (control group). Each physiological experiment was done using two rats each belonging to a different group. The composition of the pairs was randomized, and the process was repeated until all animals were used. Baseline blood pressure and heart rate measurements were recorded for at least 10 min before experimental treatment.

Two hours after hemorrhage or hydralazine injection the rats were deeply anesthetized with pentobarbital sodium (Nembutal, 50 mg/kg ip). They were perfused transcardially with 200 ml of 0.9% sodium phosphate-buffered saline (pH 7.4) followed by 500 ml of 4% paraformaldehyde solution in 100 mM sodium phosphate buffer (pH 7.4) at −20°C for up to 10 days awaiting histological processing.

Preparation of digoxigenin-labeled RNA probes for histological detection of GAD₆₇ mRNA and preproenkephalin mRNA by in situ hybridization. In situ hybridization was performed using digoxigenin-labeled cRNA probes prepared as described previously (51, 52). The GAD₆₇ riboprobe was transcribed from a 3.2-kb template inserted in the phagemid vector pBluescript SK (Stratagene, La Jolla, CA). This construct was kindly supplied by A. Tobin (16). The antisense cRNA riboprobe for rat preproenkephalin (PPE) was transcribed from a 1,132-bp DNA template inserted into the EcoRl site of Bluescript SK+ (Stratagene) (52). The PPE construct was kindly supplied and previously characterized by Rao and Howells (44). Both antisense riboprobes were synthesized in an in vitro polymerization reaction using T3 RNA polymerase (Promega, Madison, WI) in the presence of digoxigenin-11-UTP (Roche Molecular Biochemicals). The efficiency of digoxigenin-11-UTP incorporation was estimated by direct immunological detection on dot blots using a sheep polyclonal anti-digoxigenin antibody (Roche Molecular Biochemicals).

Histochemistry. All histochemical procedures were done using one-in-six series of sections (sections 180 µm apart) that were kept in order during processing. These procedures were carried out with free-floating sections removed from the cryoprotectant mixture and rinsed three times in Dulbecco's 1× sterile phosphate-buffered saline, pH 7.4. Hybridization histochemistry was performed as previously described (51, 52). Briefly, digoxigenin was revealed with a sheep polyclonal antibody conjugated to alkaline phosphatase (Roche Molecular Biochemicals), and alkaline phosphatase was reacted with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt. Labeling specificity was gauged by the absence of reaction product in cholinergic motor neurons (hypoglossal, facial, vagal, nucleus ambiguus, pars compacta), all fiber tracts, precerebellar nuclei such as the lateral reticular nucleus and inferior olive, and by an overall distribution pattern conforming to prior descriptions (51, 52).

In situ hybridization was always done before immunohistochemistry. On completion of the in situ hybridization protocol, brain sections were rinsed in Tris-buffered saline (TBS, pH 7.4) and placed in blocking solution [10% heat-inactivated normal horse serum (NHS); Life technologies, Frederick, MD] for 30 min at room temperature. They were then incubated in one or two primary antibodies for 1 h at room temperature followed by 24 h at 4°C. c-Fos and Fos-related antigens (Fos B, Fra-1, Fra-2) were detected using a broad-spectrum rabbit polyclonal antibody (antibody K-25; 1:10,000; Santa Cruz, CA, Biotechnology) followed by a biotinylated donkey anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, 1:200 dilution, for 45 min), avidin-biotin solution (Vector Laboratories), and, finally, streptavidin Cy-3 (Jackson, West Grove, PA 1:1,000 dilution). Tryptophan hydroxylase was detected using a mouse monoclonal anti-digoxigenin antibody (Roche Molecular Biochemicals).

Sections were mounted onto glass slides and covered with Vectashield mounting media (Vector Laboratories), and coverslips were affixed.

Microscopy. From each one-in-six series of sections, three coronal brain stem levels were selected for cell counting (Fig. 1). These sections were identified under dark-field illumination by using characteristic landmarks, and they corresponded as closely as possible to bregma levels −12.0, −11.8, −11.2...
and −11.6 mm after Paxinos and Watson (41). These levels were selected because they contain a very high number of presympathetic neurons. Within these sections cell counts were made in the RVLVM, defined as shown in Fig. 1. This region was made to extend medially to within 0.5 mm of the midline so as to include essentially all Fos-ir neurons present in the ventrolateral medulla at these levels except for those present along the ventral surface of the medulla at the lateral edge of the pyramidal tract. Fos-ir neurons located in this second region (subependymal parapyramidal region or SEPPN; Fig. 1, right) were counted separately. In all cases, cell counts were made bilaterally in the three sections from each brain. From these counts a single number of cells per hemisection was derived for each rat. The group mean and SE of these determinations are reported in Tables 1 and 2.

The in situ hybridization reaction product was detected under bright-field illumination. Cy3 and Alexa-488 were visualized under epifluorescence. Section outlines and landmarks were drawn, and cells were plotted with a Lucid camera (Microbrightfield, Colchester, VT), a computer-driven microscope stage (Ludl Electronic Products, Hawthorne, NY), and the Neurolucida software (MicrofieldBrightfield) as described previously (51). Cells were counted under ×25 magnification and checked again at ×40 when necessary.

Photomicrographs were taken with a 12-bit color CCD camera (CoolSnap, Roper Scientific, Tucson, AZ; resolution 1,392 × 1,042 pixels). The resulting tiff files were imported into Adobe Photoshop (version 5.0.1; Adobe Systems, Mountain View, CA) where levels and contrast were adjusted to best represent the original appearance of the material.

Statistics. The blood pressure and heart rate of the hydralazine, hemorrhage, and sham control groups were compared by Kruskal-Wallis one-way ANOVA on ranks, and significance was determined by the all-pairwise multiple comparison procedure (Dunn’s test).

Three-group comparisons (hydralazine, hemorrhage, sham) between cell counts were done by one-way ANOVA followed by Student-Newman-Keuls test. Two-group comparisons were done with the unpaired t-test. All values are expressed as means ± SE. Regardless of the test, we considered differences significant at P < 0.05.

RESULTS

Effect of hemorrhage or hydralazine on blood pressure and heart rate. The first episode of bleeding (removal of an estimated 40% of total blood volume) caused a profound hypotension accompanied by severe bradycardia (Fig. 2A). In most cases including the example shown in Fig. 2A, AP and heart rate began to recover toward baseline levels during the first 5 min. In such cases, rats were kept hypotensive by the additional removal of small amounts of blood (up to three
0.5-ml aliquots). Total blood withdrawal never exceeded 50% of the total estimated blood volume (60 ml/kg; Ref. 56). Each additional bleeding caused a recurrence of the hypotension and bradycardia (Fig. 2A). AP and heart rate eventually stabilized below baseline level for the remainder of the 2-h period. Injection of hydralazine produced hypotension and tachycardia that were sustained during the entire 2-h period (representative example in Fig. 2B). No change in AP and heart rate was observed in control rats (case not illustrated).

In each hemorrhaged rat, blood pressure and heart rate were measured at the times indicated in Fig. 2A. Blood pressure and heart rate measurements were made at the same time points in the hydralazine and control groups. The resulting group data are shown in Fig. 3. Kruskal-Wallis ANOVA on ranks was performed on all time points between /H11002 to 2 h. According to this test the blood pressure of the hemorrhage and hydralazine groups was significantly lower than that of the saline-treated rats, but the test revealed no difference between the hemorrhage and hydralazine groups. However, when the same test was applied to the first three time points after the beginning of the stimulus (5, 10, and 15 min), all three groups were different, indicating that the blood pressure of the hemorrhaged rats was significantly lower than that of the hydralazine-treated group during this initial period. Finally, we also evaluated the overall magnitude of the hypotension experienced by each rat by measuring the area between the curve (AUC) defined by all points from time zero to 120 min and a horizontal line defined by the mean resting AP of the rat before time zero. Expressed in units of millimeters Hg × seconds, the AUC was 142,470 ± 31,722 for rats subjected to hemorrhage and 202,336 ± 28,781 for hydralazine-treated rats. These values were not statistically different, but they indicated that hydralazine-treated rats had experienced a somewhat greater average hypotension than the hemorrhaged rats (28 vs. 20 mmHg) over the course of the 2-h measurement period.

A parallel statistical analysis of the heart rate values was conducted (1-way ANOVA on ranks) on all time points between −10 min and 2 h (Fig. 3B). All three groups were statistically different. Therefore the bradycardia observed in the hemorrhaged group was statistically significant despite its transient nature. Moreover, throughout the experiment, the heart rate of the hemorrhaged rats was significantly lower than that of the hydralazine-treated rats at all time points despite the fact that blood pressure was generally similar in these two groups.

Expression of Fos-related antigens in the RVLM. Consistent with prior results obtained with the same experimental protocol (46), very few Fos-ir neurons were found in the RVLM of control rats (Table 1). In contrast, 8 to 12 times as many Fos-ir neurons were present in the RVLM of rats subjected to either hemorrhage or hydralazine (Fig. 4, Table 1). Although a very small proportion of all GAD67-labeled neurons was Fos-ir (5%), these cells represented about one-third of all Fos-ir neurons present in the RVLM (Table 1). The number of GABAergic cells expressing Fos after
hemorrhage or hydralazine was much greater than the total number of Fos-ir neurons present in the RVLM of control rats, but there was no difference between the two experimental groups. The total number of Fos-ir cells was 30% lower in rats subjected to hemorrhage than in hydralazine-treated rats, but this difference was not statistically significant (Table 1). However, after the Fos-ir neurons with GAD67 mRNA were subtracted, the number of Fos-ir neurons without GAD67 mRNA was significantly higher in hydralazine-treated rats than in hemorrhaged rats (Table 1). To assess the consistency of the counting procedure, we also determined the total number of RVLM neurons that contained GAD67 mRNA. These numbers were the same in both treatment groups (Table 1).

Thus both hypotensive hemorrhage and hypotension cause Fos expression in some GABAergic neurons of the RVLM, but the numbers of activated neurons were the same in both groups. In contrast, hydralazine-induced hypotension produces more Fos expression in non-GABAergic RVLM neurons compared with rats subjected to hemorrhage, suggesting a greater activation of potentially excitatory neurons in the RVLM.

Expression of Fos-related antigens by neurons of the SEPPN. The SEPPN of the hemorrhage group contained large numbers of Fos-ir nuclei whereas Fos immunoreactivity was virtually absent from this region in the hypotension and control groups (Fig. 4, Table 2). As expected, the SEPPN (both the core and lateral wings) contained many serotonergic neurons (examples in Fig. 5, A2, B2, and C2). In the hemorrhage group a large fraction of the Fos-ir nuclei were in serotonergic neurons (40%; Fig. 5, Table 2, set 1).

A second series of sections from the same rats (set 2) was used to detect GAD67 mRNA in addition to Fos and tryptophan hydroxylase immunoreactivities. Control rats were not examined because an absence of Fos immunoreactivity in the SEPPN was observed in set 1 (Table 2). The SEPPN of hemorrhage group contained Fos-ir neurons that expressed GAD67 mRNA, and some of these neurons were serotonergic (example in Fig. 6, A–C, and Table 2). Counts of Fos-ir neurons in the hypotension group were low but also contained a few double-labeled neurons (Fos+/GAD67+ and Fos+/GAD67+/tryptophan hydroxylase+; Table 2, set 2).

In a third series of sections from the same hemorrhage and hypotension groups (set 3), we detected PPE mRNA in addition to Fos and tryptophan hydroxylase

---

Fig. 4. Representative example of the distribution of Fos-ir neurons after hydralazine or hypotensive hemorrhage. Left, hydralazine; right, hemorrhage. The medullary level represented corresponds to bregma −11.8 mm. Insets: enlargements of the SEPPN region. The hemorrhaged rat has fewer Fos-ir cells in the RVLM but a massively increased number of cells in the SEPPN.

Fig. 5. Fos expression by serotonergic neurons within the SEPPN. Pairs of photomicrographs showing Fos-ir (top row, Cy-3 epifluorescence) and tryptophan hydroxylase-ir (bottom row, Alexa 488 epifluorescence). A1 and A2: hemorrhage. B1 and B2: hydralazine. C1 and C2: control. A1 and A2: arrow indicates one of many Fos-ir serotonergic cells; arrowhead indicates a Fos-ir neuron without detectable tryptophan hydroxylase-ir. Calibration bar in C2, 50 μm for all panels.
immunoreactivities (Table 2). The SEPPN of the hemorrhage group contained Fos-ir neurons that expressed PPE mRNA, and some of these neurons were serotonergic (Fig. 6, D–F, and Table 2).

DISCUSSION

The present study contains two findings that shed new light on the neural networks that may be selectively engaged by severe hypotensive hemorrhage. First, despite a comparable severity of hypotension, somewhat fewer RVLM neurons with presumed sympathoexcitatory function expressed Fos-related antigens after hypotensive hemorrhage than after isovolemic hypotension. One interpretation of the data is that HISI could be due to the inhibition of a fraction of the RVLM presympathetic neurons. Second, in contrast to normovolemic hypotension, hypotensive hemorrhage activated a group of subependymal parapyramidal cells that release serotonin as well as inhibitory transmitters (enkephalins, GABA). The discussion considers whether the activation of SEPPN neurons could also contribute to HISI or whether this nucleus mediates reflex thermogenic responses to hemorrhage-induced hypothermia.

Methodological considerations. The limitations inherent in the Fos methodology have been abundantly discussed by others (12). However, two caveats must be reiterated. First, Fos-ir was detected at a single time point of 2 h after the start of the stimulus. Although this optimal interval was based on prior data (12, 52), the absolute or relative numbers of cells of different types could be affected by selecting a different endpoint. Second, results can also be affected by the type of Fos antibody used. In the present case we used a broad-spectrum antibody that also recognizes Fos B, Fra-1 and Fra-2.

The limitations of the physiological preparation must also be acknowledged. Sympathetic nerve recordings were not made. The only objective criteria that allowed us to suggest that the decompensated phase of hemorrhage and, presumably, HISI had been produced was the initial bradycardia and, at later time points, the fact that heart rate was at or slightly below the prehemorrhage level (Figs. 2 and 3). Another limitation of our protocol is that we cannot precisely determine the duration of the inferred HISI. The limited duration of the bradycardia (Fig. 3) suggests that, in contrast to the hypotension itself, HISI may not be sustained, or at least not with the same intensity, during the full 2 h of the experiments. If so, this would tend to attenuate the differences in Fos expression between the hemorrhaged rats and those subjected to isovolemic hypotension.

Hypotensive hemorrhage and Fos activation in the RVLM. Several prior studies have demonstrated that hypotension and hemorrhage cause Fos expression in RVLM neurons, and the present results concur (3, 5, 12, 14, 34, 54). However, other investigators have not examined animals that exhibited sustained bradycardia and long-lasting hypotension, the minimal criteria suggesting that the decompensated stage of hemorrhage may have been reached. In these studies, hemorrhage was more limited in scope (up to 16 ml/kg compared with 24–30 ml/kg in our study), and hypotension, when present, was only of very short duration (3, 5, 12, 14, 34).

Previous investigators have also shown that a large fraction of RVLM neurons that express Fos-related antigens after hypovolemic hypotension or moderate hemorrhage are C1 cells (immunoreactive for tyrosine hydroxylase or phenylethanolamine N-methyltransferase; Refs. 12, 29, 54). Accordingly, we found that, at
the RVLM level investigated, the majority of the Fos-expressing neurons did not contain GAD_{67} mRNA, a diagnostic marker of GABAergic somata that is absent from C1 cells (51). Because the number of GABAergic cells that expressed Fos was the same after hydralazine or hemorrhage, these cells are most likely activated by the common denominator to these two stimuli, namely baroreceptor unloading. Very few if any GAD_{67} mRNA-containing RVLM neurons project to the thoracic spinal cord (51), suggesting that these RVLM GABAergic neurons are interneurons.

Interestingly, the number of non-GABAergic Fos-ir neurons present within the RVLM was significantly lower in the hemorrhaged rats than in the hydralazine-treated group. A majority of these non-GABAergic neurons must be C1 presympathetic cells (12, 22) given that GAD_{67} mRNA is absent from RVLM catecholaminergic neurons (51). At the brain level that we investigated (bregma −11.6 to bregma −12.0 mm) virtually all tyrosine hydroxylase-immunoreactive neurons are C1 cells (42), and ~90% of these cells are bulbo spinal and presympathetic (47). Therefore the present study indicates that fewer RVLM presympathetic vasomotor neurons were activated in the rats subjected to decompensated hemorrhage than in the hydralazine-treated ones. This observation must be interpreted cautiously, however.

At face value the results seem congruent with prior evidence that the discharge rate of many RVLM vasomotor presympathetic neurons decreases in anesthetized rats that exhibit HISI (46). In anesthetized rats every RVLM vasomotor presympathetic neuron is not inhibited, consistent with the fact that HISI is not uniform either. For example, adrenal catecholamine release remains very elevated during uncompensated hemorrhage (55). Since the RVLM is a major source of presympathetic neurons controlling the adrenal medulla (53), these particular presympathetic neurons should be vigorously activated and thus should express Fos after uncompensated hemorrhage. The RVLM presympathetic neurons that expressed Fos after uncompensated hemorrhage in the present experiments may control the adrenal medulla and, possibly, other sympathetic efferents not subject to inhibition during uncompensated hemorrhage. Alternately, the activation of these neurons may be triggered by the brief initial sympathoactivation that usually precedes HISI.

However, the lower number of Fos-ir neurons present in the RVLM of the rats subjected to hypotensive hemorrhage could also be related to the different hypotensive profiles of the hemorrhage and hydralazine groups (Fig. 1). AUC measurements revealed that the hemorrhaged rats experienced somewhat less hypotension over the course of the 2 h preceding euthanasia. Although the difference in AUC value was not significant, the greater overall hypotension caused by hydralazine could conceivably account for a greater recruitment of RVLM premotor neurons and therefore for the higher number of Fos-ir cells found in the RVLM. This interpretation assumes that the stress and greater initial hypotension caused by hypotensive hemorrhage have less influence on Fos expression in RVLM than a slight sustained difference in baroreceptor unloading during the latter part of the experiment. Activation of serotonergic and other SEPPN neurons. The SEPPN defined in the present work is a circumscripted portion of the parapyramidal area outlined by Loewy and colleagues (27). It also overlaps but is not identical to the region previously called nucleus interfacularis hypoglossi (30), also known as the lateral B1 group in reference to the original nomenclature of serotonergic cell groups (for review, see Ref. 6). The region we chose to investigate, the SEPPN, was selected because it can be unambiguously defined by its subependymal location and is quite distinct from the RVLM (Fig. 4). The SEPPN, especially its serotonergic component, projects to the intermediolateral cell column, and thus the SEPPN contains neurons that probably function as presympathetic cells (6, 27, 50) although its sympathetic targets are not precisely defined. There are no published accounts of the pattern of unit activity of these cells, and therefore their function remains speculative at the present time. SEPPN neurons are among the first medullary neurons to be infected after injection of pseudorabies virus (PRV) in the rat’s tail, a strong indication that at least some of these neurons are involved in thermoregulation (50). However, the parapyramidal area, probably including the SEPPN, is also infected rapidly after injection of PRV into both the adrenal gland and the stellate ganglion (26). This evidence suggests that some SEPPN cells may be involved in coactivating several types of sympathetic outflows, possibly including vascular targets other than skin. Still, the available information leaves considerable uncertainty regarding the wiring of the SEPPN, and the possibility that this structure could be very heterogeneous with regard to its targets should not be dismissed.

Fos experiments performed in rats have indicated clearly that the SEPPN is not activated by stimulation of arterial baroreceptors (15). According to the present data, unloading baroreceptors with hydralazine is also ineffective in triggering Fos expression in the SEPPN. As discussed before, baroreceptor unloading may have been even larger overall in hydralazine-treated rats than in hemorrhaged rats, and yet the latter displayed at least six times as many Fos-ir cells in the SEPPN. Thus, contrary to the presympathetic cells of the RVLM, SEPPN neurons are probably not regulated by arterial baroreceptors. In this respect SEPPN neurons resemble the serotonergic neurons of the raphe magnus (33). The apparent absence of baroreceptor influence suggests that SEPPN cells are not primarily involved in buffering blood pressure, but this characteristic does not exclude a role in regulating specialized vascular beds such as the skin (9, 50).

There is considerable evidence that the SEPPN is regulated by peripheral chemoreceptors and perhaps also by tissue hypoxia, independently of conventional chemoreceptors. Electrical stimulation of the carotid sinus nerve produces massive activation of SEPPN neurons (15) as does hypoxia and increases in inhaled
CO\(_2\) (5, 15, 35). We did not measure the blood Po\(_2\) of our rats, and therefore we have no evidence for arterial hypoxia, but even in the absence of arterial hypoxia, a severely lowered hematocrit and a reduced blood flow through the carotid bodies could cause significant chemoreceptor stimulation (1). Tissue hypoxia, produced by inhalation of a dose of carbon monoxide presumed to have little or no effect on peripheral chemoreceptors, also causes massive Fos expression in SEPPN (10).

The SEPPN may also be activated by central chemoreceptors. This nucleus overlaps closely with Schlaecke’s intermediate area, a portion of the ventral medullary surface suspected to contribute to the activation of the respiratory network by hypercapnia (39). Conceivably severe hemorrhage could acidify brain extracellular fluid if an imbalance develops between O\(_2\) delivery and neuronal consumption. However, the SEPPN neurons that express Fos in animals exposed to CO\(_2\)-enriched air may not be the serotonergic ones (35). Other evidence indicates that the serotonergic neurons of the parapyramidal region are excited by extracellular acidification (57).

Finally, Fos expression within the SEPPN could also be related to thermoregulation. Hypotensive hemorrhage causes a profound decrease in oxygen consumption that precedes the hypothermia and therefore presumably contributes to it (25). Unfortunately, it is unknown whether the hypothermia is adaptive or results from a failure of thermoregulatory mechanisms. If the latter is true, defensive thermogenesis and cutaneous vasoconstriction may be maximally triggered by hypotensive hemorrhage in a failed attempt to minimize hypothermia. The fact that SEPPN innervates preganglionic neurons that regulate stellate sympathetic efferents and the adrenal medulla is potentially compatible with a role in sympathetically mediated thermogenic responses (26). This role has been attributed recently to bulbospinal neurons located within the raphe pallidus (e.g., 37, 38), a structure that may be functionally related to the SEPPN despite their anatomic separation by the pyramidal tract. Serotonin could conceivably mediate the thermoregulatory effects of SEPPN because this transmitter excites sympathetic preganglionic neurons (28, 31, 43). However, serotonin can also inhibit preganglionic neurons by activating glycineric interneurons (28), and the hypothesis that SEPPN neurons are uniformly excitatory is not supported by available evidence. For instance, inhibitory transmitters such as enkephalin and GABA are present in many of these cells. Also, only 60% or less of the Fos-labeled nuclei present in the SEPPN after hypotensive hemorrhage could be identified as belonging to serotonergic neurons. These apparent contradictions could be a reflection of the diversity of SEPPN neurons and of their targets.

In summary, hypotensive hemorrhage activates SEPPN neurons. These neurons display a variety of phenotypes compatible with both excitatory (serotonin) and inhibitory (serotonergic, GABAergic, opioidergic) effects on their targets. Although many SEPPN neurons, including serotonergic ones, project to the intermediolateral cell column (27), the rest of their projections is unknown. Their activation by hemorrhage could therefore produce a constellation of effects, including HISI, via the inhibition of selected vasomotor efferents, and cutaneous vasoconstriction, perhaps mediated by the release of serotonin on preganglionic neurons. The activation of opioidergic SEPPN neurons by hypotensive hemorrhage may underlie the recent observation that intrathecal naloxone attenuates HISI (2).

Perspectives

Hypotensive hemorrhage and isovolemic hypotension are both associated with decreased AP, but these two pathological situations produce a divergent spectrum of autonomic responses. Hypotension elicited by direct vasodilation stimulates sympathetic tone to the heart and blood vessels to raise cardiac output and peripheral resistance and maintain circulatory function. In contrast, severe blood loss is associated with a seemingly detrimental reversal of this autonomic response pattern. This pattern has been termed compensatory, a word that implies a failure of normal homeostatic mechanisms, although an adaptive role of the compensatory phase of hemorrhage is not ruled out. The central mechanisms underlying the compensatory phase of the responses to blood loss also remain elusive. The present study provides some additional support to the notion that a reduction in the discharge rate of RVLM presympathetic neurons could be involved in the production of HISI (4, 13, 46). Because the GABAergic interneurons present within the RVLM were no more activated by hypotensive hemorrhage than by hydralazine, their contribution to the inhibition of RVLM presympathetic neurons during hemorrhage remains questionable. By contrast, this negative result reinforces prior evidence that 5-HT\(_{1A}\) receptor activation in the RVLM causes or enables HISI (13). Based on this evidence, it has been proposed that serotonin is released by hemorrhage in the RVLM, but the source of the serotonin release remains unclear and its trigger unknown. The present study shows that severe hemorrhage, but not isovolemic hypotension, activates many serotonergic neurons in the SEPPN. The known anatomy of these cells indicates that their activation should increase serotonin release in the spinal cord, but their contribution to the serotonergic innervation of the RVLM is undocumented. Other possibilities include the raphe magnus, a known source of serotonergic innervation of the RVLM (33).

The present experiments emphasize the phenotypic diversity of the SEPPN and indicate that this nucleus is strongly recruited by hypotensive hemorrhage. Because of the known projections of SEPPN to the intermediolateral cell column, the evidence demonstrates that SEPPN must contribute to some of the changes in sympathetic efferent activity associated with hypotensive hemorrhage. The presence of inhibitory transmitters in SEPPN neurons, especially GABA, a transmitter apparently not made by most other medullary
serotonergic neurons (51), suggests that these cells could be contributing to HISI. However, HISI may be restricted to splanchnic, cardiac, and muscle vasomotor efferents, and it is far from clear that these particular sympathetic efferents are regulated by SEPPN neurons. Further interpretation will require a much more complete understanding of the input-output connections of SEPPN neurons.

This work was supported by National Heart, Lung, and Blood Institute Grant HL-60002 to P. G. Guyenet.

REFERENCES


AJP-Regul Integr Comp Physiol • VOL 283 • SEPTEMBER 2002 • www.ajpregu.org


