Expression of mineralocorticoid and glucocorticoid receptors in preautonomic neurons of the rat paraventricular nucleus

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1Department of Neurobiology and Anatomical Science, University of Mississippi Medical Center, Jackson, Mississippi; 2Department of Medicine, University of Mississippi Medical Center, Jackson, Mississippi; and 3Department of Statistics, University of Mississippi Medical Center, Jackson, Mississippi; and 4G.V. (Sonny) Montgomery VA Medical Center, Jackson, Mississippi
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Chen J, Gomez-Sanchez CE, Penman A, May PJ, Gomez-Sanchez E. Expression of mineralocorticoid and glucocorticoid receptors in preautonomic neurons of the rat paraventricular nucleus. Am J Physiol Regul Integr Comp Physiol 306: R328–R340, 2014. First published December 31, 2013; doi:10.1152/ajpregu.00506.2013.—Activation of mineralocorticoid receptors (MR) of the hypothalamic paraventricular nucleus (PVN) increases sympathetic excitation. To determine whether MR and glucocorticoid receptors (GR) are expressed in preautonomic neurons of the PVN and how they relate to endogenous aldosterone levels in healthy rats, retrograde tracer was injected into the intermediodorsal cell column at T4 to identify preautonomic neurons in the PVN. Expression of MR, GR, 11-β hydroxysteroid dehydrogenase1 and 2 (11β-HSD1, 2), and hexose-6-phosphate dehydrogenase (H6PD) required for 11β-HSD1 reductase activity was assessed by immunohistochemistry. RT-PCR and Western blot analysis were used to determine MR gene and protein expression. Most preautonomic neurons were in the caudal mediocellular region of PVN, and most expressed MR; none expressed GR. 11β-HSD1, but not 11β-HSD2 nor H6PD immunoreactivity, was detected in the PVN. In rats with chronic low or high sodium intakes, the low-sodium diet was associated with significantly higher plasma aldosterone, MR mRNA and protein expression, and c-Fos immunoreactivity within labeled preautonomic neurons. Plasma corticosterone and sodium and expression of toxicity-responsive enhancer binding protein in the PVN did not differ between groups, suggesting osmotic adaptation to the altered sodium intake. These results suggest that MR within preautonomic neurons in the PVN directly participate in the regulation of sympathetic nervous system drive, and aldosterone may be a relevant ligand for MR in preautonomic neurons of the PVN under physiological conditions. Dehydrogenase activity of 11β-HSD1 occurs in the absence of H6PD, which regenerates NADP+ from NADPH and may increase MR gene expression under physiological conditions.

mineralocorticoid receptor; aldosterone; sympathetic nervous system; preautonomic neuron; paraventricular nucleus; glucocorticoid receptor; 11-β hydroxysteroid dehydrogenase; sodium

THE RENIN-ANGIOTENSIN-ALDOSTERONE system (RAAS) and sympathetic nervous system (SNS) interact to maintain fluid, electrolyte, and hemodynamic homeostasis essential for terrestrial life where sodium intake is often limited. Both the RAAS and SNS are activated by a lowering sodium intake, adaptive mechanisms ensuring adequate blood pressure(17, 51, 52, 60, 64, 104). The paraventricular nucleus (PVN), and the SNS were shown by ablation studies to have a crucial role in mineralocorticoid-salt excess models of hypertension (6, 58). Chronic systemic aldosterone excess, as well as the intracerebroventricular infusion of aldosterone administered at concentrations too low to be effective when infused systemically, increase blood pressure and SNS activity (35, 61). In both models, the hypertension and sympathetic activation are prevented by the intracerebroventricular infusion of mineralocorticoid receptor (MR) antagonists at doses lower than the effective dose infused peripherally (35, 39, 61). Xue et al. (108) reproduced these pharmacological results with the intracerebroventricular infusion of MR siRNA (108).

The architecture of the PVN is complex, comprising cell bodies of many types of neurons, including several types of neuroendocrine neurons, interneurons, and preautonomic neurons that project to the intermediodorsal cell column (IML) and rostroventrolateral medulla (RVLM), many of which are still incompletely characterized (21, 88, 98 –100). While a combination of techniques from many laboratories implicates MR in the PVN in modulating SNS activity (7, 20, 108), no studies have determined whether MR are expressed and act within preautonomic neurons directly or influence sympathetic activity indirectly though other neurons of the PVN.

Aldosterone and the primary physiological glucocorticoids have similar affinities to the MR. Cortisol (in species that express 17α-hydroxylase in the zona fasciculata, including the human) and corticosterone have about 1/10th the affinity for the glucocorticoid receptor (GR) as for the MR; aldosterone has even less affinity for the GR. Plasma concentrations of corticosterone and cortisol are 100 to 1,000 times higher than those of aldosterone; thus, MR occupation by glucocorticoids in nonaldosterone target cells is thought to occur at basal glucocorticoid levels, while GR are activated at higher levels. Extrinsic specificity for aldosterone is thought to be conferred to the MR in aldosterone target cells by the presence of the microsomal enzyme 11-β-hydroxysteroid dehydrogenase 2 (11β-HSD2) that converts cortisol and corticosterone to their inactive 11-dehydro metabolites, cortisone and 11-dehydrocorticosterone, using NAD+ as its cofactor, increasing the ratio of aldosterone to corticosterone within the microenvironment of the MR, and allowing aldosterone to activate it (36). Expression of 11β-HSD2 is very limited in the adult brain, including the PVN; thus, glucocorticoids are the primary MR ligand in most neurons (13, 27). 11β-HSD1 is another microsomal hydroxysteroid dehydrogenase that is amply expressed throughout the brain (11, 47, 67, 92). Unlike 11β-HSD2, 11β-HSD1 is bidirectional, depending on cofactor availability. In most cells, 11β-HSD1 is a NADPH-dependent reductase that reduces inactive 11-dehydrocorticosterone and cortisone to active corticosterone and cortisol, respectively, increasing glucocorticoids in the vicinity of MR and GR. However, in the absence of the microsomal enzyme hexose-6-phosphate dehydrogenase.

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(H6PD) required to generate NADPH from NADP\(^+\) within the endoplasmic reticulum. 11\(\beta\)-HSD1 uses NADP\(^+\) and acts as a dehydrogenase-like 11\(\beta\)-HSD2, potentially allowing aldosterone to bind MR (2, 8, 36, 48).

The aims of this study were to determine whether MR and GR were expressed in preautonomic neurons—possibly having direct actions upon sympathetic drive from PVN—and if they were, whether their expression correlated with endogenous aldosterone production altered by sodium intake. Because the 11\(\beta\)-HSD 1 and 2 enzymes are responsible for prereceptor regulation of ligand for the MR, their expression was also assessed. Plasma sodium and the expression of tonicity-responsive enhancer binding protein (TonEBP) in the PVN were measured to assess the level of adaptation of the rats to the chronic diet regimens (103). Neuronal activation within the region of the PVN, where the preautonomic neurons reside was assessed by c-Fos expression. We chose to study preautonomic neurons at the T4 level of the IML because these preautonomic neurons are thought to be involved in anticipatory cardiac regulation; however, the tracer would also be expected to enter and retrogradely label preautonomic neurons projecting caudally to the T4 IML, including those going to the kidneys (80).

**MATERIALS AND METHODS**

**Ethical approval.** All experiments were performed under an approved Veterans Affairs Institutional Animal Care and Use Committee protocol using VA and National Institutes of Health guidelines for animal care and use. Adult female Sprague-Dawley (SD) rats (*Rattus norvegicus*) (Harlan-Sprague-Dawley, Indianapolis, IN), weighing 220–240 g maintained in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited Laboratory Animal Facility at the G.V. (Sonny) Montgomery VA Medical Center were used in this study. Females were chosen because of their more uniform size over the range of 11–15 wk of age compared with males, thus facilitating the matching of the sections at the same levels of the PVN. Animals were housed in a temperature- and humidity-controlled environment on a 12:12-h light-dark cycle and were accustomed to gentle handling to minimize alarm at the end of the study. The colony maintenance diet was Teklad 2016, 0.2% Na\(^+\) and tap water. The high-sodium (HS) diet, Teklad 2016 chow with 0.9% NaCl to drink, and low-sodium diet (LS) diet Teklad 90228 (0.02% Na\(^+\)) and water to drink, ad libitum for 19–21 days were used to suppress and stimulate the endogenous RAAS, respectively. Table 1 summaries the numbers of animals used for the studies herein. Rats on the maintenance diet, \(n = 3\), were used for studies of the colocalization of MR, GR, 11\(\beta\)-HSD1 and 2, and H6PD with the FluoroGold tracer to identify preautonomic neurons. For comparison of MR expression after adaptation to the low- and high-sodium diets, \(n = 16\) for each group, \(n = 5\) for Western blots, \(n = 5\) for real-time PCR, and \(n = 6\) for immunocytochemistry.

**Immunohistochemistry and immunofluorescent staining of mineralocorticoid and glucocorticoid receptors, 11\(\beta\)HSD1, 11\(\beta\)HSD2, H6PD, and c-Fos.** Rats previously acclimatized to handling were rapidly anesthetized by mask with 5% isoflurane, blood was drawn into cold 9 ml EDTA vacuum tubes from the left cardiac ventricle, and the vena cava was sectioned. The rat was then gravity perfused through the left cardiac ventricle with saline containing 1:10,000 heparin, followed by a modified Streck Tissue Fixative [2-bromo-2-nitropropan-1, 3-diol (bronopol), 2.5%; diazolindinyl urea, 5%; zinc sulfate, 5%; formaldehyde, 0.1%]. Because of its lower formaldehyde content, STF is safer for personnel using it, generates less hazardous waste, and forms fewer cross links, which simplifies antigen detection and decreases the need for antigen retrieval (31). The brains were removed, cut coronally into \(~3\)-mm-thick blocks (between the optic chiasm and mammillary bodies) to include the PVN, and were postfixed in STF for 18 h. For frozen sectioning, the brain was immersed in 30% sucrose overnight before embedding in frozen tissue medium (Tissue-Tek OCT, Sakura Finetek, Torrance, CA) and preserved at \(-80{\degree}\)C. Frozen sections (18-\(\mu\)m) were cut on a freezing-stage microtome. Paraffin-embbeded brains were cut into 4-\(\mu\)m sections, as previously described (32).

For immunohistochemistry, sections were blocked with 10% goat serum for an hour at room temperature, then incubated overnight at 4\(\degree\)C in the same blocking solution with the MR mouse monoclonal antibodies [mouse 1:2,000 for immunohistochemistry (IHC), 1:50 for immunofluorescence (IF)] (31, 32), GR antibodies (rabbit 1:200 for IF; GR57, a kind gift from Dr. John Cidlowski) (75), and/or c-Fos antibody (rabbit 1:6,000 for IHC, 1:200 for IF; sc-52 lot no. L1809; Santa Cruz Biotechnology, Santa Cruz, CA). Immunostaining for 11\(\beta\)HSD2 (sheep 1:32,000), 11\(\beta\)HSD1 (rabbit 1:200) (9), and H6PD (sheep 1:200) was as described in our previous work (48). All antibodies had been validated and used in our laboratory previously except for the c-Fos antibody (85). We validated it by Western blot analysis of H293 cell lysates and pilot IHC to ensure its specificity for the correct protein at the molecular weight of \(\sim 60\) kDa before being selected to use in this study. DyLight 488-conjugated goat anti-mouse and DyLight 594 conjugated goat anti-rabbit secondary antibody (Jackson Immunoresearch, West Grove, PA) were added for 1 h at room temperature for fluorescent tagging of the primary antibodies. ImmPRESS anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA) was added for 1 h at room temperature before developing with ImmPACT DAB peroxidase substrate (Vector Laboratories) for light microscopy. Positive control slides, subcommisural organ for 11\(\beta\)HSD2, and adrenal medulla for H6PD, were run simultaneously to ensure that the lack of detection of these two proteins in the PVN was not due to failure of the detection method. Hematoxylin was used as a counterstain in IHC. Slides were analyzed with a Nikon Eclipse E600 microscope equipped with an AmScope camera (San Diego, CA), and images were combined in Photoshop (Adobe).

Table 1. Number of animals used in the study

<table>
<thead>
<tr>
<th>Purpose</th>
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<tr>
<td>Higher Sodium/Lower Sodium</td>
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<tr>
<td>Plasma aldosterone, corticosterone,</td>
<td>32 rats; (n = 16) each for</td>
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<tr>
<td>sodium</td>
<td>LS &amp; HS groups</td>
<td>After 3 wk adaptation</td>
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<td>Real-time PCR</td>
<td>(n = 5) each for LS &amp; HS</td>
<td>for the sodium</td>
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<td>Western blot</td>
<td>(n = 5) each for LS &amp; HS</td>
<td>punches</td>
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<tr>
<td>IHC: MR density c-Fos(+) counting in PVN</td>
<td>(n = 3) each for LS &amp; HS</td>
<td>PVN punches</td>
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<tr>
<td>IF c-Fos activity, neurotracer</td>
<td>(n = 3) each for LS &amp; HS</td>
<td>3 slides/animal</td>
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<td>Maintenance Diet</td>
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<tr>
<td>IHC, IF, neurotracer</td>
<td>(n = 3), MR, GR, H6PD,</td>
<td>4 slides/animal</td>
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<td>11(\beta)HSD1,2</td>
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IHC, immunohistochemistry; MR, mineralocorticoid receptors; PVN, paraventricular nucleus; IF, immunofluorescence; LS, low sodium; HS, high sodium; GR, glucocorticoid receptors; H6PD, hexose-6-phosphate dehydrogenase; 11\(\beta\)-HSD1,2, 11\(\beta\) hydroxysteroid dehydrogenase 1 and 2.
To assess the density of MR immunoreactivity, the relative darkness of DAB-visualized immunostained neurons in the PVN of three sections from each brain from AP approximately −1.7 to −2.0 from bregma (97) in slides with no counterstain using the ImagePro-plus 6.0 (Media Cybernetics, Warrendale, PA) system. 

Identification of PVN preautonomic neurons by neuronal tracer. Anesthesia was induced and maintained with isoflurane in oxygen using an inhalant anesthesia machine, with buprenorphine administered intra- and post-operatively as analgesic. Spinal muscles were separated and a laminectomy was performed to expose the spinal cord at T4, with clear visualization of the posterior spinal artery. Using the coordinates 0.5 mm lateral to the posterior sulcus and 0.5-mm depth from the spinal cord surface (93), 0.08 μl of 4% FluoroGold (FG; Fluorochrome, Denver, CO) was slowly injected into the IML on the right side of the spinal cord with a 1-μl microsyringe (Hamilton, Reno, NV) attached to the microinfusor (Steoelting, Wood Dale, IL). Tissue was harvested and fixed 7 days after tracer injection. For quantitative analyses, four sections (18-μm thickness) from each brain (AP approximately −1.7 to −2.0 from bregma) were used to count preautonomic neurons in which MR, 11β-HSD1, and c-Fos immunoreactivity were expressed (97).

Real time RT-PCR for the MR, GAPDH, and TonEBP mRNA. Tissues were harvested after blood collection and perfusion with saline as described above, without perfusion with fixative. Brains were placed in ice-cold saline and cut into 2-mm-thick slices starting from the mammillary bodies in a coronal slicing mold. Punches containing the PVN from these slices made with a 1-mm ID glass capillary tube were placed in vials and immediately frozen with liquid nitrogen and then stored at −80°C. Tissue samples were homogenized in TRI Reagent RT (Molecular Research Center, Cincinnati, OH) and the RNA extracted. For reverse transcription, 400-ng RNA was incubated with SuperScript III (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol. Real-time PCR primers for the rat MR were designed as follows: sense primer: GCCAAACAGATGATC-CAGG and antisense primer: CAACTCAAAGCGAACGATGA. The primers for TonEBP were sense primer: CCTCTCTCCACCGTCGT-CATC and antisense primer: CTCCCACTTCTCTCTTCTTG (103). The primers for GAPDH as a reference gene (47) were described previously. mRNAs were quantified with 1-μl reverse transcriptions (10 μM each primer, 0.2 mM deoxynucleotide triphosphates, and 1-μl titanium Taq DNA polymerase (CLONTECH Laboratories, Mountain View, CA) in a 1:20,000 dilution SYBR Green (Molecular Probes, Carlsbad, CA). Real-time data were obtained during the extension phase, and critical threshold cycle values were calculated at the log phase of each gene amplification curve. Gene expression levels were analyzed as arbitrary units normalized against GAPDH mRNA expression.

Western blot analysis of MR protein. Tissue was obtained as for RNA, but immediately homogenized in cold 40-μl RIPA buffer with Halt protease and phosphatase inhibitor (1:10; Thermo Scientific, Rockford, IL), and the protein was isolated. The isolated protein was either analyzed immediately or frozen at −80°C (31, 47). After centrifugation for 5 min at 3500 g and 4°C, the supernatant was combined with 1:1 Laemmli sample buffer mix (Bio-Rad Laboratories, Hercules, CA), denatured at 95°C, and then separated by electrophoresis on 12.5% SDS-polyacrylamide gel using a 0.01 M Tris glycine running buffer. The protein solutions were then transferred to polyvinylidene difluoride membranes, incubated with a mouse anti-MR monoclonal antibody (MR-2B7), and then incubated with a horseradish peroxidase-conjugated secondary antibody at room temperature. West Pico reagent (Thermo Fisher-Scientific, Rockford, IL) was used as the chemiluminescence substrate for the peroxidase, and the signal was recorded on autoradiographic film (Fuji film). Tubulin (52 kDa) was used as the reference protein. Results were analyzed using Kodak MI software (Kodak, Rochester, NY) (47).

Plasma aldosterone and corticosterone assays. Steroids were measured in plasma collected, as described above, using previously described combinations of extraction and ELISAs (31, 37). Plasma sodium (n = 6 HS and LS) was measured in the VA Hospital Clinical Laboratory using a Beckman Dxc 600i sodium ion-specific electrode (Brea, CA).

Statsitics. Data are presented as means or proportions ± SE as appropriate. Data were natural log-transformed where necessary. In one case, 1 value ≥ 2 SE was not included (TonEBP RT-PCR, 1 of 5 removed). Differences between groups were tested for statistical significance using independent samples t-tests and nonparametric tests. Figures were pseudocolored with ImagePro-plus 6.0 (Media Cybernetics) and combined by Photoshop (Adobe). Data were analyzed using SPSS v.17.0 (SPSS, Chicago, IL).

RESULTS

Distribution of cell populations in the PVN subdivisions based on somatic size. The schemas in Fig. 1 are based on a representative rat and are provided to orient the photomicrographs and findings presented below. Fig. 1, A and B are rostral to caudal series of chartings starting at AP approximately −0.51 mm from bregma to AP approximately −2.0 mm from bregma, with colored markers representing the location of neurons with FluoroGold labeling traced from the T4 IML injection and MR, GR, and 11β-HSD1 immunoreactivity. These markers do not represent actual numbers of neurons with that immunoreactivity. Figure 1C shows the three-dimensional location of three general types of PVN neurons (AP approximately −1.7 to −2.0 mm from bregma) based upon their general size using the nomenclature of Nunn et al. (65, 80). As has been described previously (56), the majority of parvocellular neurons (D < 10 μm) were located within a region in the medial part of the PVN adjacent to the third ventricle, extending its rostrocaudal length, which, for the purpose of orientation, will be called the parvocellular region (Pa). The majority of the magnocellular neurons (D > 12 μm) were distributed in the lateral part of rostral PVN (Ma), also called the posterior magnocellular lateral area, as described by Swanson (97). Most magnocellular neurons (D = 10–12 μm) were found in the medio cellular region (Me) located in lateral part of caudal PVN (65). The Me region comprises the dorsal parvicellular, medial parvicellular ventral, and paraventricular nucleus hypothalamus lateral parvicellular parts, as described by Swanson (97). We found that GR immunoreactive neurons were largely confined to Pa, while retrogradely labeled preautonomic neurons were primarily found in Me. In contrast, cells with MR and 11β-HSD1 immunoreactivity were distributed throughout all three subdivisions.

Colocalization of MR and GR with tracer-labeled preautonomic neurons using fluorescence immunohistochemistry. Figure 2 is a representative composite of four photomicrographs from two sections of the same brain. Parts A and B of Fig. 2 show the more rostral section corresponding to levels 3 and 4 (AP approximately −1.78 mm from bregma) of Fig. 1. Fig. 2, C and D shows the caudolateral medio cellular region of the PVN at about level 5 (AP approximately −2 mm from bregma) in Fig. 1. Preautonomic neuron cell bodies identified by retrograde tracing from FluoroGold injected into the IML at T4 are pseudocolored red. They were medium-sized neurons found mostly in the caudolateral medio cellular region of the PVN (Fig. 2, C and D), with a few in the magnocellular (Ma) or parvocellular (Pa) region (Fig. 2, A and B). A total of 210 FG-labeled neurons were counted in four sections from each of
three rats, comprising 45 neurons in Ma and 165 neurons in Me. Neurons immunoreactive for MR (Fig. 2, A and C), pseudocolored green, could be found throughout the PVN and comprised neurons of various sizes. Some preautonomic neurons also expressed MR immunoreactivity (Mrir), resulting in a combined yellow color. This can be better appreciated in the higher-magnification insets. GR immunoreactivity (Grir), pseudocolored blue in Fig. 2, B and D, was detected in many small neurons in the parvocellular region (AP approximately −0.5 to −2.0 mm from bregma) near the third ventricle (III), but not within labeled preautonomic neurons (pseudocolored red) or magnocellular neurons in Ma region.

Immunohistochemistry for 11βHSD1, 11βHSD2, and H6PD in the rat PVN. Figure 3 comprises representative photomicrographs of immunostaining for enzymes involved in prereceptor ligand modulation for the MR and GR. In Aa–Dd, the lower-magnification photos are labeled with capital letters, while the areas of higher magnification are designated by the corresponding lower-case letters. Parts A and a of Fig. 3 are representative negative control slides showing the PVN with no primary antibody incubation. Figure 3, B and b demonstrates 11βHSD1 expression in the cytoplasm of many, but not all, neurons throughout the PVN, including mediocellular, magnocellular, and parvocellular neurons. No cytoplasmic ir for H6PD or 11βHSD2 was detected within the PVN (Fig. 3, C and c and 3, D and d, respectively) compared with the negative control (Fig. 3, A and a) or the positive controls Be and Ef. The efficacy of the immunohistochemical procedures for H6PD and 11βHSD2 was ensured by activity in the positive control slides, adrenal medulla for H6PD, 3E and e, and the subcommissural organ (SCO) for 11βHSD2, 3F and f, and run simultaneously with the PVN sections. Fig. 3, E and F are the negative controls for the adrenal and SCO.

Colocalization of MR and 11βHSD1 with tracer-labeled preautonomic neurons using fluorescence immunohistochemistry. Figure 4 shows representative photomicrographs using triple
labeling to demonstrate FluoroGold retrograde tracer from the T4 IML (pseudocolored red), MRir (pseudocolored green), and 11βHSD1 (pseudocolored pale blue). Figure 4, A, C, and E shows the same section through the magnocellular region at about levels 3 and 4 in the schema of Fig. 1; Fig. 4, B, D, and F is at approximately level 5 of Fig. 1. As in Fig. 2, there were only a few labeled preautonomic neurons, at the level of magnocellular region (Ma); most were in the more caudal mediocellular region (Me) (Fig. 4, B, D, and F). MRir was expressed in neurons throughout the PVN, including some labeled preautonomic neurons, primarily in the mediocellular region Fig. 4B, as seen more clearly in the enlarged inset. The proportion of labeled preautonomic cells in which MRir was detected was significantly greater in the mediocellular region (Fig. 4, B, D, and F) than the magnocellular region. In Fig. 4, C and D, 11βHSD1 is pseudocolored pale blue, retrograde-labeled preautonomic neurons are red, and double-labeled, preautonomic neurons expressing 11βHSD1 appear white. Preautonomic neurons that expressed both MR and 11βHSD1 are shown in Fig. 4, E and F as pale golden. These are best seen in the enlarged area in the inset of 4F. 11βHSD1ir was found in parvo cellular, magnocellular, and mediocellular regions of the PVN, including some, but not all, tracer-labeled preautonomic neurons.

Proportion of neurons in which MRir and 11βHSD1ir were found in FluoroGold tracer-stained neurons in the magnocellular and mediocellular regions of the PVN. Figure 5 represents the expression of MR and 11βHSD1, representatives of which are seen in Fig. 4, as a percentage of preautonomic neurons counted as described for Fig. 2. In addition to the consistent finding of more preautonomic neurons in the Me compared with Ma region, a greater percentage of the preautonomic neurons in the Me, 78.46%, was also demonstrated to be immunoreactive for MR, compared with 28.23% of those in the Me region (P < 0.001). The colabeling of 11βHSD1 with FluoroGold-labeled preautonomic neurons in the mediocellular region (69.6 ± 6.5%) was also significantly greater (P < 0.001) than in the magnocellular region (21.96 ± 6.2%), as the proportion of preautonomic neurons that expressed both MR and 11βHSD1 was 17.4 ± 2.3% in the magnocellular region and 63 ± 5% in the mediocellular region of the PVN.

Comparison of plasma corticoids and MR expression in the PVN between rats adapted to HS and LS diets. Having defined the distribution of the MR, GR, HSD1, HSD2, and H6PDH with respect to preautonomic neurons, we determined whether high and low endogenous aldosterone levels physiologically manipulated through dietary salt might modify their expression. Plasma aldosterone (Fig. 6A) was significantly greater in LS (n = 16) compared with HS (n = 16) rats, 528.35 ± 53.06 pg/ml and 59.02 ± 9.6/ml, respectively (P < 0.01); there was no significant difference in plasma corticosterone (Fig. 6B) between the LS and HS rats (175.5 ± 32.9 ng/ml and 170.2 ± 22.8 ng/ml, respectively; P < 0.9). There was no significant difference between rats adapted to the HS and LS diets in plasma sodium; pooled serum from rats on the standard rat chow were run as the first and last sample (134.5 mmol/l) (LS: 135.8 ± 1 mmol/l; n = 6; HS: 135.8 ± 0.60 mmol/l; n = 6; P < 0.05), nor was there a significant difference in mRNA expression of TonEBP relative to GAPDH in the PVN punch samples (LS: 128 ± 0.16; HS: 1.44 ± 0.14; P = 0.434, Fig. 6C). In Fig. 6D, MR mRNA expression in the PVN normalized to GAPDH expression was significantly greater in the LS group, 7.2 ± 0.9, than in the HS group, 0.13 ± 0.04 (P < 0.01). In Fig. 6E, MR protein levels were normalized to those of tubulin, as determined by Western blot analysis, and were also greater in the LS group (0.55 ± 0.05 vs. 0.28 ± 0.02; P < 0.01). Fig. 6F, the relative density of MR immunoreactivity in the PVN under LS conditions was 0.16 ± 0.02 AU, compared
with 0.07 ± 0.01 AU in the HS group (P < 0.01). As expected for a ligand-activated transcription factor, such as the MR, more MRir was found in the nuclei of the rats on the LS, compared with HS diet (4, 23, 24, 54, 79, 83, 84).

Representative c-Fos activity in preautonomic neurons and neurons with MRir. Figure 7 shows results from c-Fos studies in the PVN of rats under LS and HS (n = 3) conditions. For immunofluorescent staining, Fig. 6, A–C is a representative section from a LS rat; row 6, D–F is from a HS rat, at level 5 in Fig. 1, AP approximately 2.0 from bregma. c-Fos is pseudocolored green, preautonomic neurons are in red, and MR appears in light blue. Neurons with triple staining are pale golden and can be seen better in the enlargement (see inset). c-Fos and MR immunoreactive cells were scattered throughout the PVN; triple labeled preautonomic neurons were found in the mediocellular portion of PVN. c-Fos activity was measured by counting the number of c-Fos-stained cell nuclei in the PVN. The number of c-Fos-positive neurons in the PVN mediocellular region (Me) of the LS group (61.7 ± 6.3) was significantly higher (P < 0.01) than in the HS group (9.6 ± 1.5; P < 0.01; Fig. 7G). The proportion of preautonomic neurons expressing c-Fos in the Me was 32.1 ± 3.5% in the LS group, and 11.1 ± 2.5% in the HS group (P < 0.01; Fig. 7H).

DISCUSSION

The mineralocorticoid-salt excess model of hypertension has been a very useful model to study the complex pathophysiology of hypertension and its associated end-organ disease, but it is highly artificial, as most studies of the role of the MR in hypertension have involved the administration of supraphysiological amounts of mineralocorticoids in conjunction with dietary salt loading, obscuring the physiological role of MR, aldosterone, and glucocorticoids in modulating the SNS. We and others have shown that brain tissue levels of aldosterone and corticosterone, the physiological ligands of the MR, reflect physiological concentration in the circulation (37, 109) (reviewed in Ref. 44). Aldosterone synthesis by the adrenal glomerulosa cell is suppressed by a high sodium intake and stimulated by a low-sodium diet and other events that lower
blood pressure and activate the RAAS. Even in severe primary aldosteronism, aldosterone circulates at concentrations of two orders of magnitude less than those of cortisol. Nonetheless, patients with primary aldosteronism present with the hallmarks of the mineralocorticoid-salt excess model, including inappropriately high sympathetic drive to the cardiovascular system. Moreover, patients in heart failure benefit from MR antagonists, even when their plasma aldosterone levels are within the normal range (57, 86). Therefore, it is important to assess the expression of MR in the sympathetic preautonomic neurons during normal adaptation to different levels of sodium intake with physiological ranges of aldosterone.

The 5- to 6-fold increase in plasma aldosterone concentrations in female Sprague-Dawley rats adapted to LS compared with HS intakes in this study is similar to that in Sprague-Dawley males under the same conditions (37, 38). Inappropriate activation of MR in the brain increases blood pressure and SNS activation in both sexes, though cycling females generally have lower blood pressures (46, 49, 107). While the relationship between sodium intake and activation of the RAAS is the same in males and females (17, 40–42, 45, 49, 51, 52), sexual dimorphism must be considered when comparing absolute mineralocorticoid levels between males and females because progesterone is a competitive antagonist of the MR that reaches relevant concentrations for MR occupation during the luteal phase (3). Plasma renin and aldosterone concentrations cycle in
parallel to progesterone throughout the normal estrous cycle and pregnancy to maintain normal MR occupancy by aldosterone in aldosterone target cells, thus ensuring fluid, electrolyte and cardiovascular homeostasis (1, 30, 53). While animal research has tended to favor the use of males because they are thought to be simpler, this bias is not always warranted.

FluoroGold is a stable tracer that persists for at least 1 mo after injection, allowing the clear identification of preautonomic neurons of the PVN by retrograde tracing from the IML (78). Three times more PVN preautonomic neurons project to the IML than to the rostroventrolateral medulla (RVLM), and some PVN neurons project to both the IML and the RVLM, suggesting that presympathetic neurons of the PVN directly and potently regulate cardiovascular tone (88, 93). In the present study, the labeled preautonomic neurons detected in the PVN were those going to the IML at the T4 level of the spinal cord where sympathetic fibers innervate the heart (68) and those projecting caudally past the T4 by fiber-of-passage uptake of the retrograde stain deposited at the T4 level. Injection of other spinal levels of the IML would produce different patterns of preautonomic neuron labeling but would also be located within the same region of PVN (80, 88, 93). Tracer diffusion to axons surrounding the IML would be inconsequential for our analysis of preautonomic neurons of the PVN because these fibers would not be expected to emanate from the PVN. Contralateral traced preautonomic neurons were not counted in this experiment.

In this study, preautonomic neurons start to be labeled from mid-PVN (approximately −1.70 mm from bregma). Most FluoroGold-labeled preautonomic neurons in the PVN in this study were found in the mediocellular region (Me) as expected from the literature (65) and most of these expressed MR. Spinally projecting preautonomic neurons of the PVN are thought to be to be tonically inhibited by GABAergic input and excited by glutaminergic input (10, 21, 80, 106). Among neurons providing such inhibitory input are GABAergic neurons from the sympathetic regulatory center of the anterior hypothalamus, including PVN (15, 94, 106). Pharmacologic inhibition of MR in this anterior hypothalamic area demonstrated that neurons expressing MR are responsible for most of the GABAergic inhibition to the PVN (94). Blocking the GR had little effect on the firing activity of these anterior hypothalamic neurons (94).

MR and GR were coexpressed in neurons of the parvocellular region of the PVN and hippocampus within the same section; however, no GRir was detected in preautonomic neurons. This suggests that the preautonomic neurons do not have a differential response to changing levels of glucocorticoids described for the hippocampus and cortex provided by the coexpression of the high-affinity MR and lower-affinity GR, where the balance of GR and MR is crucial for normal behavior and adaptation to stress (5, 62, 89). We demonstrated that the chronic intracerebroventricular infusion of a synthetic selective GR agonist did not alter the blood pressure in a normotensive rat, but that the concomitant intracerebroventricular infusion of equimolar and twice molar concentrations of corticosterone with aldosterone inhibited the intracerebroventricular aldosterone hypertension in a dose-related fashion (35, 50). Our present results suggest that the higher concentrations of corticosterone produced by the infusion acted indirectly upon preautonomic neurons to decrease their excitability, perhaps through GABAergic parvocellular neurons that express GR, as discussed above (15, 87). In addition, GR activation in neurons of the PVN was shown to dampen the excitability of
other PVN neurons through the endocannabinoid system (101). This would also explain how treatment with GR-specific glucocorticoids that are not agonists for the MR suppresses SNS activation (69, 73) and why the concomitant intracerebroventricular infusion of corticosterone inhibited the hypertension of aldosterone (50).

Soon after the report that 11β-HSD conferred extrinsic ligand specificity to the MR for aldosterone (18) and before it was clear that there were two 11β-HSD enzymes, we reported that the intracerebroventricular infusion of an MR antagonist prevented the hypertension produced by the oral or parental administration of 11β-HSD antagonists (43). Zhang et al. (111) confirmed and extended these studies by documenting that the 11β-HSD antagonists increased SNS activation (111). These results suggested that the mechanisms for prereceptor selectivity for MR in the aldosterone target epithelial cells of the kidney were operant in central structures modulating blood pressure and that blocking 11β-HSD2 activity was allowing inappropriate activation of MR by endogenous glucocorticoids. However, once the 11β-HSD2 was cloned, 11β-HSD2 protein was found to be absent in most areas of the adult brain, including the PVN (26, 91, 95), as confirmed in the present study. Notwithstanding the dearth of evidence for 11β-HSD2 expression, the brain, including the hypothalamus, has significant 11β-hydroxysteroid dehydrogenase capability, as shown by the conversion of both endogenous and tritiated substrates to 11-dehydrocorticosterone following incubation of intact and adrenalectomized rat brain minces (33, 34). While the source of this dehydrogenase has remained elusive (36, 48), our current finding that MR and 11β-HSD1, but not H6PD, are expressed within preautonomic neurons of the PVN suggests that 11β-HSD1 may act as a dehydrogenase and allow aldosterone to successfully compete for binding to these MR (8).

The 11β-HSD antagonists, carbenoxolone, and glycyrrhizic acid inhibit 11β-HSD1, as well as 11β-HSD2, thus providing a mechanism for the activation of MR in the PVN by endogenous glucocorticoids when 11β-HSD antagonists were administered parenterally or centrally (43, 111). We previously reported that 11β-HSD1 and H6PDH were expressed in the rat brain, including the whole hypothalamus (48); however, we did not study their colocalization within individual neurons essential for prereceptor modulation of a ligand and crucial global

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**Fig. 7.** Representative c-Fos activity (green), preautonomic neurons (red), and MRir (blue) in PVN sections at level 5 (in Fig. 1) of rats on lower (LS) or higher sodium (HS) diets. A–C: same section from a LS diet rat. Inset: ×2 magnification. D and E: same section from a HS diet rat. Inset: ×2 magnification. III, third ventricle; Pa, parvocellular region; Me, mediocellular region. Scale bar in D is the same for A–F. The white asterisks mark artifacts on the slides as an aid for comparing plates. G: number of c-Fos-stained nuclei in the PVN at level 5 in Fig. 1. H: percent c-Fos-positive preautonomic neurons at level 5 in Fig. 1. Values are expressed as means ± SE; *P < 0.01.
effects, even when expressed in a small number of neurons, as shown by Geerling et al. (26). It has been postulated that the use of selective 11β-HSD1 inhibitors would alleviate obesity- and age-related maladies from cardiometabolic syndrome to dementia and problems with wound healing (55, 59, 110). However, the present findings suggest caution.

The concept that a microsomal dehydrogenase enzyme can completely reverse the two orders of magnitude stoichiometric advantage of corticosterone concentration over aldosterone concentration has been challenged. Funder (22) has compiled evidence from several types of studies to posit that MR:glucocorticoid complexes in many tissues are normally inactive and proposes that an increase in reactive oxygen species, such as that produced by conversion of NADP⁺ to NADPH when 11β-HSD2 acts as a dehydrogenase alters the conformation and activates the MR:glucocorticoid complexes (22). In this scenario, conversion of NADP⁺ to NADPH when 11β-HSD1 acts as a dehydrogenase would have the similar effect on the redox milieu. Other factors may shift ligand selectivity for aldosterone. In vitro human cell studies of binding and transcriptional behavior using artificial constructs to demonstrate that the MR:aldosterone complex is more stable and has higher transcription efficiency than MR:cortisol (19, 70). In addition, MR, like other steroid receptors, also associate with the plasma membrane and mediate rapid nongenomic effects through various cell signaling cascades, including events that alter membrane excitability (14, 29). There is evidence in hippocampal neurons that the affinity of corticosterone for membrane-associated MRs is significantly less than its affinity for transcriptional MRs that shuttle between the cytosol and nucleus (63). A prominent MR-mediated nongenomic effect is activation of NADPH oxidase (113). Both MR and NADPH oxidase antagonists mitigate the hypertension and SNS activation produced by heart failure, central inflammation, and excessive RAAS-salt administration (20, 108, 112).

The promoter region of the MR gene has not been extensively studied compared with ER or GR (74, 82). Regulatory sequences of the MR gene include MR, GR, GPR48, and TonEBP (77, 82, 103, 105). Activated MR and GR increase MR gene transcription through the P1 and P2 promoters (77, 82). As plasma corticosterone did not change, while aldosterone production by the adrenals was appropriately increased by the LS diet, aldosterone activation of the MR may have contributed to the significantly greater expression of MR message and protein in the whole PVN under LS, high-aldosterone, conditions. This scenario assumes prereceptor inactivation of corticosterone, as even basal levels of corticosterone far exceed those of maximally stimulated endogenous aldosterone. TonEBP is a crucial mediator of adaptive cellular responses to osmotic stress. TonEBP expression is increased and decreased by acute hypertonicity and hypotonicity, respectively (103). In contrast to these findings in acute osmotic stress, TonERB expression in the PVN was not significantly different between our rats on a chronically high- or low-sodium diet to which they had become adapted, as confirmed by normal plasma sodium values that did not differ between groups or from pooled rat serum from the colony on their standard sodium chow.

Activation of the SNS in response to a sodium intake is complex, multifactorial with multiple signals converging upon the preautonomic neurons, including efferents from neurons of the circumventricular organs outside of the blood-brain barrier that sense changes in plasma and cerebrospinal fluid osmolality and/or sodium concentrations, and mediators of the RAAS (76, 96). c-Fos is a transcription factor commonly used as a functional marker of neuron activation. Since its half-life is short, it generally indicates a rapid response to acute and continuing challenges (66). We are interested in the continuing process of adaptation; the early response gene c-Fos provides insight into the continuous current response. In our study, the number of preautonomic neurons that expressed both c-Fos and MR increased with the LS diet; nonetheless, c-Fos was detected in fewer than 30% of T4 preautonomic neurons in the LS diet (Fig. 7H), perhaps because of the chronicity of the diet. Our finding that c-Fos-positive neurons in the PVN were increased by a LS diet is in agreement with a previous report in which the types of activated PVN cells were not differentiated (71). Similarly, c-Fos was increased in aldosterone-sensitive neurons of the NTS of rats on a chronic LS diet and decreased upon sodium repletion with a HS diet (25, 28). Sympathetic drive is greater on a LS diet, compared with HS diet, including in humans (64, 72, 104). Neurons of the subfornical organ and organum vasculosum of the lamina terminalis, circumventricular organs that project to the PVN and modulate SNA activity, are also activated by a chronic LS diet (76), thus probably contributed significantly to the increased activity in the preautonomic neurons in response to a LS diet in our present studies. The moderate activation of preautonomic neurons of the PVN by chronic low salt intake is adaptive for terrestrial animals, most of which do not have ready access to large amounts of sodium and, thus, would experience situations in which the hemodynamic support by the SNS would be required to maintain blood pressure. Our finding that not every tracer-identified preautonomic neuron in the LS diet rats was c-Fos-positive suggests that this dietary condition did not maximally stimulate the SNS.

Perspectives and Significance

The major effectors of sodium and hemodynamic homeostasis—the RAAS, MR, and SNS—are activated when sodium intake is low or loss is high. Most studies of the effects of RAAS and MR activation on SNS activation have used high sodium and supraphysiological amounts of mineralocorticoids that are not produced even by aldosterone-producing carcinomas. The present study was designed to assess MR expression in preautonomic neurons of the PVN under physiological conditions in which endogenous aldosterone was chronically elevated or suppressed in response to a low or high sodium intake to which the animals were adapted. It was inspired by three sets of clinical data: 1) demonstration of the beneficial effects of MR antagonists for chronic heart and kidney failure and the demonstration that diuretics used as first-line treatments for hypertension can induce reflex SNS activation and insulin resistance, which is prevented by use of an MR antagonist (90); 2) recognition of significant detriments of a very low salt diet, particularly in patients with cardiovascular disease (16, 102) and 3) demonstration that MR antagonists in healthy individuals impaired cognition and memory (12, 81). We demonstrate that preautonomic neurons of the PVN express MR, but not GR; thus, MR within these neurons may directly modulate their activity without the counterbalance of
coexpressed GR-mediated activity found in most neurons expressing MR. Glucocorticoids are the primary ligand for MR in most neurons. The significant coexpression of MR and 11βHSD1 within preautonomic neurons in the absence of measurable H6PD suggests that 11βHSD1 acts as a dehydrogenase, allowing aldosterone to be a relevant ligand for the MR. As corticosterone levels did not differ, but aldosterone was increased by low sodium intake, aldosterone may be the activating ligand for MR in sympathetic preautonomic neurons of the PVN. These findings suggest that more information is needed to be able to develop selective MR modulators.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: J.C. and E.P.G.-S. performed experiments; J.C. and E.P.G.-S. analyzed data; J.C., C.E.G.-S., P.J.M., and E.P.G.-S. interpreted results of experiments; J.C. and E.P.G.-S. wrote the manuscript; J.C. and E.P.G.-S. approved the final version of the manuscript.

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