Sex differences in angiotensin signaling in bulbospinal neurons in the rat rostral ventrolateral medulla

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¹Division of Neurobiology, Department of Neurology and Neuroscience, Weill Cornell Medical College, New York; ²Harold and Margaret Miliken Hatch Laboratory of Neuroendocrinology, The Rockefeller University, New York, New York; ³Department of Pharmacology, University of Mississippi School of Pharmacy, University of Mississippi, University, Mississippi; and ⁴Division of Pharmacology and Toxicology, University of Texas, Austin, Austin Texas

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Wang G, Milner TA, Speth RC, Gore AC, Wu D, Iadecola C, Pierce JP. Sex differences in angiotensin signaling in bulbospinal neurons in the rat rostral ventrolateral medulla. Am J Physiol Regul Integr Comp Physiol 295: R1149–R1157, 2008. First published August 6, 2008; doi:10.1152/ajpregu.09485.2008.——Sex differences may play a significant role in determining the risk of hypertension. Bulbospinal neurons in the rostral ventrolateral medulla (RVLM) are involved in the tonic regulation of arterial pressure and participate in the central mechanisms of hypertension. Angiotensin II (ANG II) acting on angiotensin type 1 (AT1) receptors in RVLM neurons is implicated in the development of hypertension by activating NADPH oxidase and producing reactive oxygen species (ROS). Therefore, we analyzed RVLM bulbospinal neurons to determine whether there are sex differences in: 1) immunolabeling for AT1 receptors and the key NADPH oxidase subunit p47 using dual-label immunoelectron microscopy, and 2) the effects of ANG II on ROS production and Ca²⁺ currents using, respectively, hydroethidine fluoromicrography and patch-clamping. In tyrosine hydroxylase-positive RVLM neurons, female rats displayed significantly more AT1 receptor immunoreactivity and less p47 immunoreactivity than male rats (P < 0.05). Although ANG II (100 nM) induced comparable ROS production in dissociated RVLM bulbospinal neurons of female and male rats (P > 0.05), an effect mediated by AT1 receptors and NADPH oxidase, it triggered significantly larger dihydropyridine-sensitive long-lasting L-type Ca²⁺ currents in female RVLM neurons (P < 0.05). These observations suggest that an increase in AT1 receptors in female RVLM neurons is counterbalanced by a reduction in p47 levels, such that ANG II-induced ROS production does not differ between females and males. Since the Ca²⁺ current activator Bay K 8644 induced larger Ca²⁺ currents in females than in male RVLM neurons, increased ANG II-induced L-type Ca²⁺ currents in females may result from sex differences in calcium channel densities or dynamics.

C1 neurons; reactive oxygen species; NADPH oxidase; calcium channel; estrogen

There is increasing evidence that sex differences contribute to the risk of cardiovascular disease, including hypertension (14, 44). Under the age of 45 yr, fewer women than men die from cardiovascular disease (1), a pattern which is reversed after menopause (52). Similar sex-associated differences exist in animal hypertension models: females develop hypertension later, and less severely, than males (11, 35, 62). Studies in humans and animal models indicate that central nervous system pathways play a critical role in the development and maintenance of hypertension (27). Specifically, increases in sympathetic nerve activity and changes in arterial baroreflex function are strongly implicated in the pathogenesis of the disorder. The rostral ventrolateral medulla (RVLM) contains tonically active presympathetic bulbospinal neurons, most of which express tyrosine hydroxylase (TH) [the C1 cell group (39)]. RVLM bulbospinal neurons are essential for the maintenance of sympathetic vasomotor tone (15, 16). In light of the involvement of tonic sympathoexcitation in hypertension, it is important to determine whether the RVLM bulbospinal pathway displays sex-associated anatomical or functional differences, which could support differences in cardiovascular vulnerability.

Angiotensin II (ANG II) plays a key role in the central regulation of hypertension (38). In particular, injection of ANG II into the RVLM increases sympathetic nerve activity and blood pressure (3, 17), actions that are mediated through angiotensin type 1 (AT1) receptors. AT1 receptors are expressed in the RVLM at high levels in humans (16) and low-to-moderate levels in rats (2, 8, 9). ANG II exerts some of its effects through the modulation of Ca²⁺ currents (57, 58, 64). Furthermore, the production of reactive oxygen species (ROS) by NADPH oxidase has emerged as critical in the mediation of the effects of ANG II (53, 63). Binding of ANG II to AT1 receptors leads to the phosphorylation of the cytoplasmic NADPH oxidase subunit p47, inducing the production of ROS (21). Intracerebroventricular infusion of ANG II significantly increases ROS production in the RVLM (10), and the resulting increased sympathetic activity can be attenuated by NADPH oxidase inhibition (7). Additionally, oxidative stress and ROS production in the RVLM have been shown to directly contribute to the neural mechanisms of hypertension in stroke-prone spontaneously hypertensive rats (26) and two-kidney one-clip rats (34).

Because most studies are conducted on male animals, it is not known whether there are sex differences in AT1 receptor signaling and ROS production in RVLM bulbospinal neurons. Therefore, these neurons were analyzed to determine whether there are sex differences in: 1) immunolabeling for the AT1 receptor and the key NADPH oxidase subunit p47 and 2) the effects of ANG II on ROS production and Ca²⁺ currents.

Materials and Methods

Animals. All experiments were approved by the Institutional Animal Care and Use Committees at Weill Cornell Medical College and the University of Texas at Austin. Postnatal days 20 (P20) and 23 (P23) Sprague-Dawley rats were purchased from Charles

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River Laboratories (Wilmington, MA), and 4- and 12-mo-old Sprague-Dawley rats were purchased from the Animal Resource Center, University of Texas at Austin (originally obtained from Harlan, Indianapolis, IN).

To examine more directly the role of estrogen, all young adult (4 mo) and middle-aged (12 mo) female rats received bilateral ovariocectomies (OVX) under isoflurane anesthesia (2% in oxygen), followed by estrogen replacement using a standard protocol (13). In particular, 4 wk after OVX, rats were subcutaneously implanted with Silastic brand capsules (Dow Corning, Midland, MI) (1.0 cm for young and 1.5 cm for middle-aged animals) under isoflurane anesthesia, containing 17β-estradiol (5% in cholesterol; the OVX+E group) or vehicle (100% cholesterol; the OVX+V group) for 3 days prior to perfusion. Different Silastic capsule implant lengths were used to account for differences in body weights (6). Although serum estradiol levels in these animals could not be measured for technical reasons, other studies from one of our labs show that these capsules result in physiologically relevant circulating estradiol concentrations (13).

**Materials and antibodies.** ANG II, Bay K 8644, nifedipine, prazosin, and thermolysin were purchased from Sigma-Aldrich (St. Louis, MO). Losartan was a gift from Merck and DuPont (Whitehouse Station, NJ). MnIII-tetrakis(4-benzoic acid)porphyrin chloride (MnTBPAP) was purchased from Calbiochem (San Diego, CA). The peptide gp91ds-tat was synthesized by Bio-Synthesis (Lewisville, TX). I-ACSF containing 0.01% BSA was used as a vehicle for peptides, including ANG II and gp91ds-tat. DMSO was used as a vehicle for all other drugs. Rhodamine green was purchased from Lumafuor (Naples, FL). The fluorescent dye dehydroethidium was purchased from Molecular Probes (Eugene, OR). Antibodies to the AT1 receptor [rabbit, No. 92578 (20)], p47 (goat, C-20, sc-7660; Santa Cruz Biotechnology, Santa Cruz, CA), and TH [mouse, No. 22941, Immunostar (12)] have been extensively characterized and localized in fixed rat brain tissue. **Electron microscopic immunocytochemistry.** Methods for dual-label immunoelectron microscopy are described in detail previously (42, 43, 58) and are summarized here. Male and female rats (P23; 4- and 12-mo-old) were anesthetized with pentobarbital sodium (150 mg/kg ip) and transcardially perfused with 3.75% acrolein and 2% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.4). Brains then were blocked and postfixed, and coronal sections were cut on a vibratome. These slices were incubated at 35°C in: (1) l-ACSF (59, 60) with 0.02% pronase for 1 h, and 2) an oxygenated drug-containing buffer by using a custom-designed double-barrel interface was then mapped onto an image of a randomly selected thin section from that grid, and grid squares adjacent to the interface that displayed undamaged morphology (i.e., were complete) were identified and numbered. One of these grid squares was randomly selected, and all TH-ImG-labeled dendritic profiles [identified by being postsynaptic to presynaptic terminals (37)] in it, and five adjacent fields (moving clockwise along the interface) were examined to determine total AT1 or p47 immunogold particle number colabeling the dendritic profiles. This approach limited analysis to the most superficial portions of the tissue (0.1–1 μm from the surface), which displayed the most robust labeling, and ensured that a comparable tissue depth would be examined for all animals (41, 42, 50). Other ultrastructural processes were also identified following the criteria of Peters et al. (37), such as glial profiles, which tend to be long and thin, and are often interposed between pre- and postsynaptic structures, in a manner that conforms to their shape. Since preembedding ImG labeling techniques employing antibody dilutions comparable to those used in this study generate extremely low levels of nonspecific background labeling [estimated to be 3% of all ImG labeling, based on control tests involving omission of the primary antibody (61)], all ImG particles colabeling TH-positive dendrites were counted. Comparisons were only made between experimental groups whose tissue was fixed with the same batch of fixative and processed in parallel in the same run to control for potential differences in fixation, dilutions, and incubation times between runs. Images were captured with a digital camera (AMT, Danvers, MA) and figures were assembled in Photoshop.

**Retrograde labeling of RVLM neurons.** The methods used to retrogradely label RVLM neurons are identical to previous descriptions from our laboratory (59, 60). P20 rats were anesthetized with isoflurane (2% in oxygen), rhodamine-microbeads were microinjected bilaterally into the spinal cord at T3–T4, and 2–3 days later, the P20 rats were killed. The brain stems were removed, immersed in an ice-cold, oxygenized sucrose, artificial cerebrospinal fluid [ACSF (58, 60)] and sectioned coronally into 300–400 μm slices using a Vibratome. These slices were incubated at 35°C in: 1) a lactacid (l)-ACSF (59, 60) with 0.02% pronase for 1 h, and 2) an oxygenated 1-ACSF (22) with 0.02% thermolysin for 1 h. To dissociate RVLM neurons, the region was first identified under an E600 fluorescence microscope by the presence of dye-labeled neurons, was then punched out, and the tissue stirred in 1-ACSF. Dissociated neurons were then immediately moved to petri dishes. Rhodamine-labeled neurons were identified using an inverted fluorescent Nikon Diaphot 300 microscope with brief ultraviolet light exposure.

**Immunolabeling of isolated RVLM neurons.** As described (57), isolated cells were fixed with 4% paraformaldehyde, permeabilized, blocked in 5% donkey serum, and incubated in primary antibodies, or sera, or a secondary antibody cocktail of either: 1) 1:2,000 TH + 1:100 AT1 antisera; or antisera, or 2) 1:2,000 TH + 1:100 p47 antisera for 1 h. Neurons were then incubated in a secondary antibody cocktail of either: 1) 1:50 FITC-conjugated anti-mouse (J60618) + 1:100 Cy5-conjugated anti-rabbit (J71063) antisera, or 2) 1:50 FITC-conjugated anti-mouse (J60618) + 1:100 Cy5-conjugated anti-goat (J72031) antimouse for 1 h (Jackson ImmunoResearch, West Grove, PA), and visualized using the inverted microscope. Since it is not feasible to conduct immunolabeling on isolated cells after the whole cell patch-clamp process, these studies were conducted on a separate group of cells.

**Patch-clamp recordings of isolated RVLM neurons.** Whole cell patch-clamp recording of RVLM neurons was performed with an Axoclamp-2A amplifier with pClamp Window 8.0 (Molecular Devices, Sunnyvale, CA) as previously described (57, 58, 60). To determine whether ANG II affected cell currents, patched neurons were exposed to either vehicle (0.01% BSA in l-ACSF) or other drugs and then ANG II in vehicle (continuously for at least 10 min) or other drug-containing buffers by using a custom-designed double-barrel
perfusion system. Using 2 mmol/l Ca\(^{2+}\) as a charge carrier, the voltage-gated Ca\(^{2+}\) channel currents were elicited by depolarization from a holding potential of \(-60\) mV, which is close to the resting potential, to stepping potentials ranging from \(-50\) mV to \(+20\) mV. The current signals were filtered at 2 kHz, digitized online at a sampling rate of 10 kHz, and stored for later computer analysis using pClamp.

Detection of intracellular ROS. ROS production was assessed using dehydroethidium as an indicator (25, 59). Isolated neurons were incubated in 5 \(\mu\)mol/l dehydroethidium for 30 min and then exposed throughout the measurement to 5 \(\mu\)mol/l dehydroethidium-containing buffer. Time-resolved fluorescence was measured every 30 s by using an ethidium bromide filter and IPlab software (Scanalytics, Fairfax, VA). This sampling rate enabled us to accurately detect the initial phase of ANG II-induced ROS production, since the effects of ANG II on ROS production and Ca\(^{2+}\) currents occurred within 2 min. Recordings commenced when a stable baseline was achieved with vehicle (0.01% BSA in 1-ASC) or other drugs and then ANG II in vehicle. In all experiments, concurrent vehicle recordings were performed until there was no difference in dehydroethidium fluorescence intensity before ANG II application.

ROS and Ca\(^{2+}\) current data analysis. The amplitude of whole cell Ca\(^{2+}\) currents at the end of the 500-ms depolarizing pulse was measured as the amplitude of the L-type Ca\(^{2+}\) current; the amplitude of the transient component of the Ca\(^{2+}\) current was obtained by subtracting the amplitude of L-type Ca\(^{2+}\) currents from that of peak transient Ca\(^{2+}\) currents measured at 30 ms following initiation of depolarization pulses (59). ROS data are expressed as the ratio \(F_t/F_0\), where \(F_t\) is fluorescence following the application of ANG II in a given cell, and \(F_0\) is the baseline fluorescence of the same cell immediately before application of ANG II (57).

Statistical analysis. An EC\(_{50}\) was obtained from the equation \(y = \min + (\max - \min)\left[1/(1 + (x/EC_{50})^\text{Hillslope}\right]\) SigmaPlot software (SPSS Science, Chicago, IL), where \(y\) is the percentage of normalized maximal dehydroethidium intensity at a given ANG II concentration \(x\). The statistical significance between two groups was analyzed by using either the Student’s \(t\)-test, or ANOVAs. \(P\) values \(\leq 0.05\) were considered significant. Values were reported as means \(\pm\) SE, with \(N\) = animal number and \(n\) = data point number or cell number.

RESULTS

Female TH RVLM neurons display significantly more AT\(_1\) and less p47 than males. Similar to previous light microscopic studies (46, 59) the RVLM of P23, 4- and 12-mo-old females and male rats all contained numerous TH-labeled neurons with extensive dendritic processes (Fig. 1A). These TH-ImP-labeled processes also were identifiable at the ultrastructural level (Fig. 1, B and C), and often contained AT\(_1\)-ImG (Fig. 1B) or p47-ImG (Fig. 1C) labeling. AT\(_1\)-ImG labeling was also observed in glial processes (Fig. 1B) and non-TH-positive dendrites, and much less frequently in presynaptic processes and vascular endothelial cells. P47-ImG labeling also was frequently observed in non-TH-positive dendrites, glial processes, presynaptic processes (Fig. 1C), and endothelial cells.

Analysis of all TH-labeled dendrites in randomly selected fields (18,150 \(\mu\)m\(^2\) per animal) of TH-AT\(_1\) dual-labeled P23 material revealed that, on average, the dendrites of P23 females contained significantly higher levels of AT\(_1\)-ImG labeling than those of males [2.6 \(\pm\) 0.1 ImG particles/dendritic profile (ImGP/D) vs. 1.9 \(\pm\) 0.2 ImGP/D, \(t\)-test, \(P = 0.03, N = 6, n = 318\)] (Fig. 2A). Comparable data was obtained from the tissue of both young (Y; 4 mo) and middle-aged (M; 12 mo) animals, processed in parallel to allow the application of a two-way ANOVA with sex and age as factors. TH-labeled RVLM dendrites in females (F) had significantly higher levels of...
AT1-ImG labeling than in males (M) \([F(1,14) = 9.53, P = 0.008]\), while there were no significant differences relating to age \([F(1,14) = 0.003, P > 0.05]\) [YF: 2.0 ± 0.4 ImGP/D, \(n = 319\); YM: 0.8 ± 0.1 ImGP/D, \(n = 181\); MF: 1.8 ± 0.3 ImGP/D, \(n = 347\); MM: 0.97 ± 0.03 ImGP/D, \(n = 187\)] (Fig. 2A). Additionally, OVX+E females had higher levels of AT1-ImG labeling than OVX+V females \([F(1,8) = 5.80, P = 0.04]\), while there was no significant difference relating to age \([F(1,8) = 0.22, P > 0.05]\) [OVX+E: 2.6 ± 0.5 ImGP/D, \(n = 159\); YOVX+E: 1.3 ± 0.1 ImGP/D, \(n = 160\); MOVX+E: 2.0 ± 0.5 ImGP/D, \(n = 178\); MOVX+V: 1.53 ± 0.09 ImGP/D, \(n = 169\)] (Fig. 2B). Examination of TH-labeled dendrites in TH-p47 dual-labeled P23 tissue revealed that the dendrites of females contained significantly lower levels of p47-ImG labeling than those of males \((0.7 ± 0.1 \text{ImGP/D}} vs. 1.3 ± 0.1 \text{ImGP/D}, P = 0.01, N = 6, n = 237)\).

Thus, across the population of TH-labeled RVLM neurons AT1 receptor-ImG labeling is present at higher levels in females than males. This sex difference can be observed in the prepubertal juvenile and persists into young adulthood and midlife. In adult females, estrogen also appears to enhance AT1-ImG labeling than those of males, and ovariectomized estradiol (OVX+E) females had significantly higher levels than ovariectomized vehicle (OVX+V) females, while there were no significant difference relating to age. *\(P = 0.03\), **\(P = 0.008\), +\(P = 0.04\).

ANG II induces ROS production in RVLM bulbospinal neurons of female rats. Next, the effect of ANG II on ROS production was explored. In vehicle-treated neurons, ethidium bromide fluorescence intensity, reflecting ROS production, remained stable during the monitoring period (not shown). ANG II induced an increase in the ROS signal in RVLM bulbospinal female neurons within 2 min of application, in a dose-dependent manner (EC50 = 89.2 nmol/l, Fig. 3A). The effect was first observed at 10 nmol/l \((5 ± 1\%); P < 0.05; n = 5\) and reached a maximal effect at 1,000 nmol/l \((25.3 ± 4.4\%); P < 0.05; n = 5\) (57, 58). A comparable dose response curve was established for male RVLM bulbospinal neurons (EC50 = 98.7 nmol/l, Fig. 3B). Fig. 4A shows a representative image of 100 nmol/l ANG II-induced ROS production in a rhodamine-labeled bulbospinal neuron. The increase in ROS induced by ANG II (100 nmol/l) was blocked \((P > 0.05\) vs. control) by the ROS scavenger MnTBAP (30 \(\mu\)mol/l; \(n = 6\)), the AT1 receptor antagonist losartan (10 \(\mu\)mol/l; \(n = 4\)) and the NADPH oxidase peptide inhibitor gp91ds-tat (1 \(\mu\)mol/l; \(n = 7\)) (Fig. 4B). However, pretreatment with the AT2 receptor antagonist PD123319 (30 \(\mu\)mol/l) did not inhibit ANG II-induced ROS \((P > 0.05; n = 5\).

These data indicate that ANG II induces ROS production in RVLM neurons of female rats via AT1 receptors and NADPH oxidase.

**Fig. 2.** Histograms of the relative density of AT1-ImG particle labeling of TH-positive dendritic profiles in the RVLM of P23 (A), and 4- and 12-mo-old (B) rats. Since the data presented in A and B was obtained from tissue that had been processed separately, values are normalized to the highest value in each, to facilitate comparison (see text for original values). At P23 (A), females displayed significantly higher levels of AT1-ImG labeling than those of males. At 4 and 12 mo (B), females also displayed significantly higher levels of AT1-ImG labeling than those of males, and ovariectomized estradiol (OVX+E) females had significantly higher levels than ovariectomized vehicle (OVX+V) females, while there were no significant difference relating to age. *\(P = 0.03\), **\(P = 0.008\), +\(P = 0.04\).
ANG II increases L-type Ca\(^{2+}\) currents of female RVLM bulbospinal neurons via AT\(_1\) receptors and NADPH oxidase. NADPH oxidase-derived ROS are implicated in the increase of Ca\(^{2+}\) currents elicited by ANG II (21, 53). ANG II (100 nmol/l) enhanced the L-type component of the Ca\(^{2+}\) current in female RVLM bulbospinal neurons (P < 0.05; n = 6). This component was enhanced by the L-type Ca\(^{2+}\) channel activator Bay K 8464 (1 μmol/l; P < 0.05; n = 4) and blocked by nifedipine (1 μmol/l; P < 0.05; n = 4), indicating that it was a dihydropyridine-sensitive L-type current (Fig. 5D). In contrast, ANG II (100 nmol/l) did not affect the amplitude of transient components of the Ca\(^{2+}\) current in rat bulbospinal neurons of females (99.6 ± 3.4%, P > 0.05, n = 6). The increase in the L-type Ca\(^{2+}\) current induced by ANG II (100 nmol/l) was blocked (P > 0.05 vs. vehicle) by either losartan (10 μmol/l; n = 4) or gp91ds-tat (1 μmol/l; n = 4) (Fig. 5, A–C). These observations indicate that the ANG II-mediated increase in L-type Ca\(^{2+}\) currents in female bulbospinal neurons is dependent on AT\(_1\) receptors and NADPH oxidase. Female RVLM neurons not displaying retrograde label, i.e., nonbulbospinal projecting, did not exhibit significant ANG II-induced Ca\(^{2+}\) currents (P > 0.05; n = 4; Fig. 6) as bulbospinal projecting neurons did (P < 0.05; n = 5).

ANG II-induced L-type Ca\(^{2+}\) currents, but not ROS production, are greater in females than in males. We then compared ANG II-induced effects on ROS production and L-type Ca\(^{2+}\) currents between female and male RVLM bulbospinal neurons. The increase in ROS signals induced by ANG II (100 nmol/l) was comparable in bulbospinal neurons of male and female rats (Figs. 3 and 7A) (P > 0.05; n = 5). ANG II increased the ROS signal in male RVLM bulbospinal neurons in a dose-dependent manner, which did not differ from that seen in females (males: EC\(_{50}\) = 98.7 nmol/l; females: EC\(_{50}\) = 89.2 nmol/l, Fig. 3). However, the increase in L-type Ca\(^{2+}\) currents induced by ANG II (100 nmol/l) was significantly larger in females than in males (Fig. 7B; P < 0.05; n = 6). Losartan (10 μmol/l) was equally effective in blocking increases in ANG II-induced ROS and L-type Ca\(^{2+}\) currents in males (L-type Ca\(^{2+}\) currents: control = 100 ± 0%; losartan + ANG II = 102.5 ± 1.5%; P > 0.05; n = 5; ROS: control = 1.0 ± 0.1%; losartan + ANG II = 1.04 ± 0.018; P > 0.05; n = 4) and females (Fig. 5), indicating that the ROS and Ca\(^{2+}\) current changes are AT\(_1\) receptor-dependent in both sexes. Thus, while ANG II induces similar levels of ROS production in female and male bulbospinal neurons, the increase in the L-type Ca\(^{2+}\) current is larger in females than in males (Fig. 7, A and B). To determine whether there were differences in the voltage-gated Ca\(^{2+}\) influx independent of AT\(_1\) receptors in both sexes, losartan was continuously used to block AT\(_1\) receptors, so that the direct effect by Bay K 8644 (1 μmol/l) on L-type Ca\(^{2+}\) currents in RVLM neurons could be revealed. The Bay K 8644-induced Ca\(^{2+}\) current was also significantly larger in females than in males (Fig. 7C; P < 0.05; n = 9). Sex differences in ANG II-induced Ca\(^{2+}\) currents

Fig. 3. Comparison of the dose-response curves of ANG II’s effects on reactive oxygen species (ROS) production in RVLM neurons of female and male rats. A: ANG II dose-dependently induced an increase in ROS production in retrogradely labeled RVLM bulbospinal neurons of female rats (EC\(_{50}\) = 89.2 nmol/l; vehicle = 10, ANG II: 10 nmol/l = 5, 30 nmol/l = 5, 100 nmol/l = 5, 1,000 nmol/l = 9), B: ANG II dose-dependently induced an increase in ROS production in retrogradely labeled RVLM bulbospinal neurons of male rats (EC\(_{50}\) = 98.7 nmol/l; vehicle = 14, ANG II: 10 nmol/l = 4, 30 nmol/l = 4, 100 nmol/l = 5, 1,000 nmol/l = 8). The dotted curves are fitted values calculated from the equation y = min + (max − min)/(1 + (x/EC\(_{50}\))\(^*\)). Ft, fluorescence following the application of ANG II at a given time Fo, baseline fluorescence of the same cell immediately before application of ANG II. *P < 0.05 and **P < 0.01 vs. vehicle.
DISCUSSION

These findings demonstrate that 1) female P23 rats display more AT1 receptor-ImG labeling and less NADPH oxidase subunit p47-ImG labeling in TH-labeled RVLM neurons, compared with male rats, 2) the elevated levels of AT1 receptor-ImG labeling in females persist into adulthood and midlife, and 3) AT1 receptor-ImG labeling was higher in estradiol than in vehicle-treated OVX females. Additionally, in isolated RVLM bulbospinal neurons, ANG II induced comparable ROS production in female and male rats, an effect mediated by AT1 receptors and NADPH oxidase. However, in these neurons ANG II triggered more pronounced L-type Ca\(^{2+}\) currents in females than in males. Female neurons also exhibited larger Ca\(^{2+}\) currents in response to the L-type Ca\(^{2+}\) channel activator Bay K 8644.

Our immunocytochemical studies examined the TH-positive C1 cell group (4) in the RVLM, while our neurophysiological studies examined RVLM bulbospinal neurons. Since C1 neurons represent 50 to 70% of all RVLM bulbospinal neurons (39, 54, 56) these two populations overlap extensively. The C1 cell group was selected for anatomical analysis because TH-ImP labeling can be readily observed in the smallest, most-distal C1 dendritic processes (59), allowing an examination of AT1 and p47 labeling in the full dendritic arbors of these cells.

Some of our neuroanatomical and all of our neurophysiological observations were obtained in juvenile rats (P23). It is therefore appear to result from differences in the density or dynamics of L-type Ca\(^{2+}\) channels.

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**Fig. 5.** ANG II potentiates long-lasting (L-type) Ca\(^{2+}\) currents in female RVLM neurons. 

A: whole-cell Ca\(^{2+}\) currents from the same RVLM neuron in the presence of vehicle and ANG II. B: whole-cell Ca\(^{2+}\) currents from the same RVLM neuron in the presence of losartan and losartan + ANG II. C: whole-cell Ca\(^{2+}\) currents from the same RVLM neuron in the presence of gp91ds-tat and gp91ds-tat + ANG II. D: histogram illustrating the effects of vehicles (n = 6), losartan (10 \(\mu\)mol/l; n = 4), gp91ds-tat (gp91ds, 1 \(\mu\)mol/l; n = 4), Bay K 8644 (Bay K 8644, 1 \(\mu\)mol/l; n = 4), and nifedipine (1 \(\mu\)mol/l; n = 4) on the increase in L-type Ca\(^{2+}\) currents induced by ANG II. *P < 0.05 vs. vehicles. SP, stepping potential; HP, holding potential.

**Fig. 6.** Comparison of the effects of ANG II on Ca\(^{2+}\) currents in retrogradely labeled and nonlabeled neurons of the RVLM in female rats. ANG II (100 nmol/l) induced a significant increase in the L-type Ca\(^{2+}\) current (I\(_{\text{Ca}}\)) in retrogradely labeled RVLM bulbospinal neurons (n = 6). In contrast, ANG II (100 nmol/l) did not have a significant effect on the L-type Ca\(^{2+}\) current in nonlabeled neurons isolated from the RVLM (n = 4). *P < 0.05 vs. vehicle.
neurons from female rats (n = 5), same as vehicle in Fig. 4B) and male (n = 5) rats. B: ANG II (100 nmol/l) induced a greater increase in the L-type Ca\(^{2+}\) current in RVLM neurons from female rats (n = 6), compared with male rats (n = 6). C: ANG II (100 nmol/l)-induced increase in the L-type Ca\(^{2+}\) current was equivalently inhibited by losartan (10 μmol/l) in male and female RVLM neurons (n = 4). In the presence of losartan (10 μmol/l), Bay K 8644 (1 μmol/l) increased the L-type Ca\(^{2+}\) current to a greater extent in female (n = 4) than in male (n = 5) RVLM neurons. *P < 0.05 and **P < 0.01 vs. vehicle; †P < 0.05.

Feasible to patch-clamp dissociated RVLM neurons from young animals. In the adult, one cannot either dissociate RVLM neurons or patch-clamp them in slices, because of more extensive myelination (28). At this age, however, rats are in a prepuberal state and sex hormones have not reached mature levels (45, 51). Therefore, observed sex differences in AT\(_1\) receptors, p47, and Ca\(^{2+}\) currents at P23 cannot be fully ascribed to sex hormones, raising the possibility that genomic organization differences between males and females play a role. But, one cannot rule out that the low levels of sex hormones present at this early age could also be involved (45, 51). Estradiol pretreatment can affect ANG II-induced changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in cultured area postrema neurons isolated at P10 to P16 (36), although the effect observed in this group of cells is a reduction in the ANG II-induced increase in [Ca\(^{2+}\)]\(_i\).

To directly test whether differences in AT\(_1\) receptor-ImG labeling could also be observed at later time points when sex hormones play a greater role, tissue from young adult (4 mo) and middle-aged (12 mo) rats were analyzed. Furthermore, the OVX rat model, with the addition of either estradiol or vehicle, was used to overcome the difficulty of interpreting data in intact females, that are under the influence of fluctuations in estradiol concentrations across the estrous cycle. Comparable sex differences were observed at both ages, suggesting that the characteristics of AT\(_1\) signaling described in this study could persist into adulthood and middle age, when hypertension is more prevalent (32). We have reported similar reductions in p47-ImG labeling in TH-labeled RVLM neurons in adult females (40), indicating that these prepuberal differences can also be observed in adulthood. Additionally, analysis of adult females also allowed an exploration of the potential role of estrogen in modulating AT\(_1\) receptor levels in the RVLM. Consistent with findings in other systems (23), the presence of estrogen (in OVX +E tissue vs. OVX++V tissue) appeared to have a significant effect on AT\(_1\) receptor-ImG labeling. Analysis of p47-ImG labeling in TH-labeled RVLM neurons in adult females (40) did not reveal any significant changes across the estrous cycle.

AT\(_1\) receptors and NADPH oxidase-derived ROS play a key role in the central mechanisms of hypertension (10, 53, 57, 58, 64). The fact that AT\(_1\) receptor-ImG labeling was increased and p47-ImG labeling was decreased in TH-labeled RVLM neurons from female rats raises the possibility of a compensatory balance between AT\(_1\) receptors and NADPH oxidase subunits, aimed at maintaining constant levels of ROS production. Consistent with this hypothesis, ANG II-induced ROS production in dissociated RVLM neurons did not differ between females and males. ROS production was attenuated by the AT\(_1\) receptor inhibitor losartan and by the NADPH oxidase peptide inhibitor gp91ds-tat, attesting to the fact that ROS production was mediated by AT\(_1\) receptors via NADPH oxidase. In addition, other factors could be involved in the apparent compensation observed in females. For example, AT\(_2\) receptors, which have effects on ROS production opposite to those of AT\(_1\) receptors (5) could also be upregulated. Furthermore, the ANG II-derived peptide Ang-(1-7), which can counteract the effects of ANG II, might also be involved (48). These possibilities need to be examined experimentally.

One of the major mechanisms by which ANG II influences neuronal function is by modulating voltage-gated Ca\(^{2+}\) currents (19). Our findings indicate that ANG II increases L-type Ca\(^{2+}\) currents via AT\(_1\) receptors and NADPH oxidase but that the effect is more marked in female than in male rats. The enhancement of the Ca\(^{2+}\) current induced by ANG II in female RVLM bulbospinal neurons is observed only for the dihydropyridine-sensitive L-type Ca\(^{2+}\) current and not for the transient...
Ca\(^{2+}\) current. Several possible explanations can be advanced for the sex difference in the ANG II-induced Ca\(^{2+}\) current in RVLM neurons. First, AT\(_1\) receptor signaling could induce more ROS in female than in male RVLM neurons. This is unlikely, however, since ANG II-induced ROS production is identical in female and male RVLM neurons. Alternatively, ANG II-induced potentiation of Ca\(^{2+}\) currents could be independent of NADPH oxidase-derived ROS, and could be mediated by other signaling pathways initiated by the AT\(_1\) receptor. For example, AT\(_1\) receptor-induced protein kinase C or protein kinase A activation could modulate L-type Ca\(^{2+}\) channels (24). This is also unlikely, because gp91ds-tat blocks all ANG II-induced L-type Ca\(^{2+}\) currents in female RVLM bulbospinal neurons. Finally, the density of L-type Ca\(^{2+}\) channels on RVLM neurons could be greater in females than in males. This possibility is supported by studies in spinal cord motor neurons that exhibit greater L-type Ca\(^{2+}\) currents in females than in males (31) and by our finding that application of the L-type Ca\(^{2+}\) channel activator Bay K 8644 elicits larger Ca\(^{2+}\) currents in female neurons.

**Perspectives and Significance**

We have thus demonstrated that AT\(_1\) receptors in TH-containing neurons of the RVLM are more abundant in female than in male rats, an increase associated with a reduction in the key NADPH oxidase subunit p47. ANG II produces similar increases in ROS production in RVLM bulbospinal neurons of female and male rats, but the associated enhancement in the L-type Ca\(^{2+}\) current is more marked in females than in males. The functional significance of increased L-type Ca\(^{2+}\) currents in females remains to be defined. Potentiated L-type Ca\(^{2+}\) currents could increase neuronal excitability due to the depolarizing effect of Ca\(^{2+}\) influx, potentially affecting synaptic outflow. Alternatively, excitability could be decreased by activation of Ca\(^{2+}\)-activated K\(^+\) channels. In addition, L-type Ca\(^{2+}\) channels participate in gene expression, synaptic plasticity, and cell survival (29). Therefore, increased L-type Ca\(^{2+}\) currents in females could also lead to a greater potential for plasticity within central autonomic networks. These issues need to be explored in future studies. In summary, these findings unveil previously unrecognized sex differences in ANG II receptor expression and signaling in central autonomic neurons that are critically involved in controlling the cardiovascular system.

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