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Angiotensin II-induced Hypertension Differentially Affects Estrogen and Progestin Receptors in Central Autonomic Regulatory Areas of Female Rats

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Abstract

Estrogen receptor (ER) activation in central autonomic nuclei modulates arterial blood pressure (ABP) and counteracts the deleterious effect of hypertension. We tested the hypothesis that hypertension, in turn, influences the expression and trafficking of gonadal steroid receptors in central cardiovascular circuits. Thus, we examined whether ER- and progestin receptor (PR)-immunoreactivity (ir) are altered in medullary and hypothalamic autonomic areas of cycling rats following chronic infusion of the hypertensive agent, angiotensin II (AngII). After 1 week AngII-infusion, systolic ABP was elevated from 103±4 to 172±8 mmHg ($p<0.05$; $N = 8/\text{group}$) and all rats were in diestrus (low estrogen). In AngII-infused rats the number of PR-immunoreactive nuclei was reduced (−72%) compared to saline-infused controls also in diestrus ($p<0.05$). Furthermore, the intensity of ER α -ir in nuclei (16%) and cytoplasm (21%) of cells increased selectively in the commissural nucleus of the solitary tract (cNTS; $p<0.05$) while neither the number nor intensity of ER β -labeled cells changed ($p>0.05$). Following chronic AngII-infusion, electron microscopy showed a higher cytoplasmic-to-nuclear ratio of ER α -labeling selectively in tyrosine hydroxylase (TH)-labeled neurons in the cNTS. Furthermore, AngII-infusion increased ER α -ir in the cytosol of TH- and non-TH neuronal perikarya and increased the amount of ER α -ir associated with endoplasmic reticulum only in TH-containing perikarya. The data suggest that hypertension modulates the expression and subcellular distribution of ER α and PR in central autonomic regions involved in blood pressure control. Considering that ER α counteracts the central and peripheral effects of AngII, these receptor changes may underlie adaptive responses that protect females from the deleterious effects of hypertension.

Keywords

baroreceptor; chemoreceptor; nucleus of the solitary tract; hypothalamus; sex steroids; electron microscopy

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Several lines of evidence suggest that estrogens are responsible for the relative protection from the deleterious effects of hypertension observed in pre-menopausal females [for reviews see (Reckelhoff, 2001;Xue et al., 2007a)]. In particular, studies in rodents have demonstrated that estrogens attenuate the increases in arterial blood pressure (ABP) and sympathetic outflow induced by angiotensin II (AngII), an octapeptide involved in the mechanisms of hypertension (Crofton and Share, 1997;Pamidimukkala et al., 2005). Within the CNS, estrogens are known to act within the well-established medullary sites that regulate peripheral vascular resistance and ABP [for review see (Saleh and Connell, 2007)]. Two such regions are the nucleus of the solitary tract (NTS), which is the site of the first central synapse for baro- and chemo-receptor afferents, and the rostroventrolateral medulla (RVLM), which contains sympathoexcitatory bulbospinal neurons and is critical for tonic (resting) and reflex control of systemic arterial pressure (Aicher et al., 2000;Dampney et al., 2000;Guyenet, 2006). Estrogens also may influence these medullary nuclei indirectly by acting on neurons in the hypothalamus through reciprocal projections with the NTS and RVLM (Hardy, 2001;Hermes et al., 2006;Rahmouni and Morgan, 2007;Vieira et al., 2006).

Estrogens can modulate central autonomic networks through both genomic and nongenomic mechanisms. The genomic or classic mode of estrogen action is on nuclear estrogen receptors (ERs), transforming them into ligand-dependent transcription factors [for review see (Nilsson et al., 2001)]. Estrogens also can act non-genomically on membrane ERs to rapidly elicit the activation of kinases and increase intracellular calcium [for review see (Vasudevan et al., 2005)]. Additionally, estrogens can up-regulate the expression of progesterin receptors (PRs) (Blaustein and Turcotte, 1989;Shughrue et al., 1997). Both nuclear and extranuclear ERs and PR are found in the NTS and RVLM. Specifically, the rodent NTS contains nuclei with ER α -immunoreactivity (ir) and PR-ir in subpopulations of catecholaminergic neurons in rats and in mice (Haywood et al., 1999;Vanderhorst et al., 2005). Moreover, ER β labeled cells are found in the NTS (Shughrue and Merchenthaler, 2001). The RVLM contains nuclei with ER α -ir (Lee et al., 2000;Vanderhorst et al., 2005;Wang et al., 2006) as well as extranuclear ER α - and ER β -ir (Wang et al., 2006). In addition, ER- and PR-ir are present in the hypothalamus; ER α - and PR-immunoreactive nuclei are abundant in the arcuate nucleus (ArH) and present at low levels in the periventricular nucleus (PVH) and paraventricular nucleus (PVN) whereas ER β -labeled cells are primarily in the PVN (Francis et al., 2002;Shughrue and Merchenthaler, 2001;Simonian and Herbison, 1997a).

Recent studies have shown that activation of ER reduces the hypertension and sympathetic activation induced by chronic AngII-infusion (Pamidimukkala et al., 2005;Xue et al., 2007b;Zhu et al., 2002). However, it remains to be established whether hypertension alters the expression and trafficking of gonadal receptors in key medullary and hypothalamic circuits involved in cardiovascular regulation. Changes in ERs and PRs in critical central autonomic neurons could represent an adaptive mechanism aimed at counteracting the deleterious effect of hypertension. Therefore, in this study we tested the hypothesis that AngII-induced hypertension alters the expression and subcellular distribution of ER α -, ER β - and PR-immunoreactivity in cycling female rats. First, we determined whether ER α -, ER β - and PR-immunoreactivity fluctuated across the estrous cycle in brainstem and hypothalamic cardiovascular areas of Sprague-Dawley rats. Subsequently, we examined the effect of chronic infusion of AngII on the levels of ER- and PR-ir in autonomic medullary and hypothalamic areas at the light and electron microscopic level.

EXPERIMENTAL PROCEDURES

Animals

All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Weill-Cornell Medical College Institutional Animal Care and Use Committee. Female (N = 43; 275 – 375 g) adult Sprague-Dawley rats, from Charles River Laboratories (Wilmington, MA) or Taconic Laboratories (Chatham, NY) were used. All rats were housed with 12:12-hr light/dark cycles (lights on 0600–1800). Estrous cycle stage was determined using vaginal smear cytology (Turner and Bagnara, 1971), and females were assessed for at least two full cycles. The proestrus (high estrogen) and diestrus (low estrogen) stages were examined in this study. Rats showing regular estrous cycles were in diestrus (which includes metestrus) for 2–3 days, proestrus (< 1 day) and estrus for 1–2 days. Estrous stages were confirmed by determining uterine weights and by analyzing levels of estrogens and progestins from blood samples collected during the perfusion procedure. Uterine weights were approximately 21% heavier in proestrus rats (0.8 ± 0.07 gm) compared to diestrus rats (0.63 ± 0.04 gm; $p < 0.05$). Estrogen and progesterone levels were determined using Coat-A-Count radioimmunoassay kits (Diagnostic Products Corporation). Although different analysis kits yielded different baseline values, on average the blood levels for estrogens were 10 pg/ml higher at proestrus than diestrus and for progestins were 15 ng/ml lower in proestrus compared to diestrus rats.

Infusion of angiotensin II using osmotic minipumps

As reviewed previously (Glass et al., 2007), peripheral administration of AngII increases systolic ABP and activates central cardiovascular circuits. To minimize stress and accustom rats to the procedure, systolic ABP was measured daily for 7 days with tail-cuff plethysmography prior to the insertion of minipumps. Osmotic minipumps were inserted using previously described procedures (Glass et al., 2007). Briefly, rats were anesthetized with isoflurane (induction 5%; maintenance 1.5 – 2%) and placed on a thermostatically controlled heating pad that maintained body temperature at 37°C. Osmotic minipumps (model #2002, Alzet Corporation, Cupertino, CA) were inserted subcutaneously at the back of the neck. Minipumps contained either AngII (AngII acetate, Sigma, St. Louis, MO; delivery rate 500 ng/kg/min; N = 8 rats) in saline, or saline alone (N = 8). Daily measurements of ABP were made on awake rats for another 7 days using tail-cuff plethysmography. For this, a total of 10 ABP measurements were recorded over a one hour session using a non-invasive tail cuff sphygmomanometer (Kent Scientific Corporation, Torrington CT); the values then were averaged to determine the final systolic ABP measurement for the day. Since the experimental design entailed relative comparisons within groups of rats, the fact that tail-cuff plethysmography can underestimate absolute ABP values (Gross and Luft, 2003) did not compromise the results.

Immunocytochemistry

Antibodies—PR labeling was examined using a well-characterized antiserum. A rabbit polyclonal antiserum to a peptide corresponding to amino acids 533–547 (the N-terminal region present in both the A and B isoforms) of the human PR was purchased from Dako (Carpinteria, CA). This antiserum has been shown to recognize PRs in rat and other species using immunocytochemistry and immunoprecipitation (Molenda et al., 2002; Quadros et al., 2002; Traish and Wotiz, 1990). Specificity has been demonstrated by sucrose density gradients and absence of labeling in absorption controls by immunoprecipitation methods (Traish and Wotiz, 1990) or in thymus and uterus of PR knock-out mice (Kurita et al., 1998; Tibbetts et al., 1999). Additionally, preadsorption of the PR antibody (diluted 1: 500) with cognate peptide resulted in no detectable immunoreactivity in the hypothalamus of acrolein/

paraformaldehyde-fixed rat brain sections (Waters, et al., unpublished). The dilutions used were 1:1000 (immunoperoxidase) and 1:500 (immunofluorescence).

ER α labeling was examined using a polyclonal rabbit antiserum to ER α purchased from Upstate (Lake Placid, NY). This antiserum, C1355, was raised against a peptide corresponding to the last 15 amino acids of the rat ER α . This antiserum was shown by immunoblot analysis to recognize the rat ER α and to yield no labeling when tested on brain sections from adult female ER α knockout mice (Vanderhorst et al., 2005). Moreover, labeling is absent in tissue incubated in antibody preadsorbed with the cognate peptide (Burke et al., 2006). The dilutions used were 1:10,000 (immunoperoxidase) and 1:5000 (immunofluorescence, immunogold).

ER β labeling was examined using a polyclonal antibody generated in rabbit (485; Merck Research Laboratories, Westpoint, PA) against a conserved sequence (rat aa 64–82) of the mouse, human and rat ER β that is located within the A/B domain of ER β (exons 2–3) and is not present in ER α (Mitra et al., 2003). By Western blot, the ER β 485 antibody recognizes a protein that migrates at about 60 kDa on SF9 cells blots, 55 kDa on human ovary and testes blots, and about 70 kDa on whole tissue extracts of rat and mouse brain (Mitra et al., 2003). The antibody labels nuclei in cos-7 cells transfected with ER β and is eliminated in these cells and in rodent hippocampal tissue by preadsorption with the antigenic peptide (Milner et al., 2005; Mitra et al., 2003). Analysis of mouse hippocampal sections fixed with 3.75% acrolein and 2% paraformaldehyde and labeled with the ER β 485 antiserum preadsorbed with the antigenic peptide in the presence of 0.1% Triton-X 100 revealed no detectable nuclear or extranuclear labeling at the light microscopic level (Mitra et al., 2003). The selectivity of this antiserum for individual splice variants of ER β (Price, Jr. et al., 2000) have not been determined.

Catecholaminergic labeling was identified using a monoclonal mouse antibody to tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine synthesis, which was purchased from Incstar (Stillwater, MN). This antibody has been extensively characterized and localized in fixed rat brain (Wang et al., 2006). The dilutions used were 1:20,000 (immunoperoxidase) and 1:2000 (immunofluorescence).

Immunolabeling—Rats (N = 10 for estrous cycle study; N = 6 for light microscopic dual label study; N = 27 for AngII study) were deeply anesthetized with sodium pentobarbital (150 mg/kg, i.p.) and perfused through the ascending aorta sequentially with solutions of: 1) 10 – 15 ml saline (0.9%) containing 1000 units of heparin; 2) 50 ml of 3.75% acrolein (Polysciences, Washington, PA) and 2% paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.4); and 3) 200 ml of 2% paraformaldehyde in PB. Each brain was removed from the skull, cut into 5 mm coronal blocks and post-fixed with 2% paraformaldehyde in PB for 30 min. Blocks containing the RVLM and NTS or hypothalamus were cut into 40- μ m-thick sections on a vibrating microtome (Vibratome; Leica, Wien, Austria). To ensure identical labeling conditions, tissue from each experimental condition was marked with identifying punches and all conditions were pooled during labeling procedures. Moreover, tissue from at least 3 sets of rats was processed together.

For single-label light microscopic experiments examining changes in nuclear ERs and PR over the estrous cycle and in response to chronic AngII-infusion, ERs and PR were labeled with immunoperoxidase to optimally detect labeled nuclei. For this, ER α -, ER β - or PR-ir was visualized using the avidin-biotin complex (ABC)- peroxidase technique as previously described (Wang et al., 2006). Briefly, free-floating sections were treated with 1% sodium borohydride in PB for 30 min and rinsed in PB. Sections were incubated in 0.5% bovine serum albumin (BSA) in 0.1 M Tris-saline (pH 7.6; TS) for 30 min, rinsed in TS and then placed in anti-ER α antiserum (1:10,000), anti-ER β antiserum (1:500) or anti-PR antiserum (1:1,000) in

0.25% Triton X 100 (ER α and PR antisera only) and 0.1% BSA in TS for 1 day at room temperature followed by an additional 4–5 days at 4°C. The sections then were incubated in: (1) biotinylated goat anti-rabbit IgG in 0.1% BSA (1:400, Vector Labs, Burlingame, CA) for 30 min; (2) ABC for 30 min and (3) diaminobenzidine (Aldrich, Milwaukee, WI) and H₂O₂ for 6 min. All incubations were separated by washes in TS. In experiments analyzing changes in PR-ir after AngII-infusion, DAB times were extended until PR-labeled nuclei were visualized in the NTS of control rats (usually 8 minutes). Sections were mounted on gelatin coated slides, air-dried, and coverslipped with DPX mounting medium (Aldrich).

For light microscopic dual localization of TH with ER α or PR labeling, sections were processed using previously described immunofluorescence methods (Drake and Milner, 2002). To maximize expression of the gonadal steroid receptors (see results), tissue from either diestrus (ER α) or proestrus rats (PR) was used. Procedures were the same as described above except that the TH antiserum (1:2,000) was added to the primary antibody diluent one day prior to processing and the primary antibody was more concentrated (ER α , 1:5,000; PR, 1:500). For ER α /TH, sections then were incubated sequentially for 2 hours each in Alexa Fluor 594 donkey anti-rabbit IgG (1:200; Invitrogen-Molecular Probes, Carlsbad, CA) and FITC horse anti-mouse IgG (1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). For PR/TH, sections then were incubated sequentially for 2 hours each in Alexa Fluor 488 goat anti-rabbit IgG (1:200; Invitrogen-Molecular Probes) and Cy5 goat anti-mouse IgG (1:200; Jackson ImmunoResearch Laboratories, Inc.). Sections were mounted on gelatin-coated slides, air-dried and coverslipped with slowFade Gold antifade reagent (Invitrogen-Molecular Probes).

For electron microscopic localization of ER α and TH in NTS, sections were dual-labeled for TH-immunoperoxidase and ER α -immunogold using methods described previously (Wang et al., 2006). Briefly, sections were incubated in anti-ER α antiserum (1:5,000) in 0.025% Triton X 100 and 0.1% BSA in TS for 1 day at room temperature followed by an additional 4–5 days at 4°C. One day before further processing, TH antiserum (1:20,000) was added to the primary antibody diluent. To visualize TH-labeling, sections were incubated in biotinylated horse anti-mouse IgG (Vector) and processed for the ABC peroxidase technique described above. To visualize ER α -labeling, sections were rinsed in TS and incubated in goat anti-rabbit IgG conjugated to 1 nm gold particles (1:50, Electron Microscopy Sciences, Fort Washington, PA) in 0.08% BSA and 0.01% gelatin in 0.01M phosphate-buffered saline (PBS; pH 7.4) for 2 hours at room temperature. Sections then were rinsed in PBS, post-fixed in 2% glutaraldehyde in PBS for 10 minutes, and rinsed in PBS followed by 0.2 M sodium citrate buffer (pH 7.4). The conjugated gold particles were enhanced by treatment with silver solution (EMS) for 5–7 minutes. Sections prepared for EM were post-fixed for 1 hour in 2% osmium tetroxide, dehydrated through alcohols and propylene oxide, and embedded between two sheets of plastic in EMbed 812 (EMS). Ultrathin sections (70 nm thick) were collected from the NTS and cut on an ultratome (Ultracut, Leica). Sections were counter-stained with uranyl acetate and Reynold's lead citrate, and final preparations were analyzed on a FEI Tecnai Biotwin transmission electron microscope.

Immunocytochemical analysis—Coronal sections containing medullary and hypothalamic regions implicated in autonomic regulation (see Introduction) were selected using the Swanson atlas (Swanson, 1992) as a guide. Four regions were examined: A1 noradrenergic area (levels 68–70; –13.60 to –14.16 from bregma; Fig 1B); hypothalamus, arcuate (ArH) region (levels 28–30; –2.45 to –3.25 from bregma), and PVH and PVN regions (levels 24–26; –1.33 to –1.78 from bregma); NTS (levels 69–72; –13.76 to –14.86 from bregma; Fig. 1); RVLM, anterior (aRVLM, levels 61–63; –12.20 to –12.68 from bregma; Fig. 1A) and posterior (pRVLM, levels 64 and 65; –12.88 to –13.15 from bregma).

For light microscopy, immunoperoxidase labeled sections were analyzed and photographed with a Nikon E800 microscope equipped with bright field and DIC optics and a Micropublisher digital camera (Q-imaging, Barnaby, British Columbia). Immunofluorescence images were acquired sequentially using a confocal laser-scanning microscope (Leica, Nussloch, Germany). Z-stack analysis was used to verify if neurons were dually labeled for ER α (or PR) and TH. Alexa Fluor 594 (ER α) and Alexa Fluor 488 (PR) were pseudocolored red while FITC/Cy5 (TH) was pseudocolored green. Electron microscopic images were acquired with a digital camera system (Advanced Microscopy Techniques, software version 3.2). To prepare figures, the levels, brightness and contrast were adjusted in Adobe Photoshop 7.0 on an Apple Power Macintosh G5 computer. Final figures were assembled in Quark Xpress 6.1.

Quantitative analysis—For the experiments comparing influence of estrous stage or chronic AngII on the number of nuclear ER α and PR, sections were coded and analyzed by an investigator unaware of the experimental groups. The region of interest (ROI) from one section per rat was counted. At least 5 rats per experimental condition were examined. Counts were performed on a 0.375 μm^2 area from each ROI using Neurolucida software (MicroBrightField, Inc., Williston, VT) on a Nikon Microphot microscope equipped with a mechanical stage.

The subcellular location of ER α -immunogold labeling was examined using semi-quantitative electron microscopic analysis of 1 block each from 5 rats from each condition (saline and AngII infused). Blocks were selected from sections of the cNTS (see Fig. 1a) that contained both nuclear ER α and TH immunolabeled perikarya identified by light microscopy. To ensure that differences in profile number were not due to differences in antibody penetration and sampling size (Leranth and Pickel, 1989) profiles were sampled from fields near (within 1.5 μm) the plastic-tissue interface and all fields sampled in each block were identical in area ($1.21 \times 10^6 \mu\text{m}^2$). In each field, all cells containing a nucleus and 4 or more immunogold particles for ER α were photographed, and categorized as TH-containing neurons, TH-lacking neurons, or glia. TH immunoperoxidase labeling was identified as a diffuse granular reaction product that rimmed organelles throughout the cytoplasm. In soma profiles labeled for ER α , the subcellular distribution of somatic ER α -immunogold particles to various cytoplasmic organelles, plasma membrane, or the nucleus was assessed using standardized characteristics to identify the cellular components (Peters et al., 1991).

Statistical analysis

Data are expressed as mean $\pm$ standard error. Statistical analyses were conducted using either SPSS for Windows V.11.0.1 (SPSS, Chicago, IL) or Systat for Windows V. 7.0.1 (SPSS, Chicago, IL). Differences between two groups were evaluated by using unpaired Student's t-test. Receptor quantification data was analyzed by ANOVA to control for multiple regions quantification. ER α redistribution in cNTS for TH and non-TH labeled cells was analyzed using repeated measures ANOVA. Bonferroni post hoc analyses followed on significant main effects of group or significant interactions. Differences were considered significant at $p < 0.05$.

RESULTS

The numbers of ER α - and PR-labeled nuclei fluctuated across the estrous cycle in a regionally specific manner

Since a previous study examining the distribution of ER α - or PR-immunoreactive nuclei in cycling rats was performed on a different strain (Wistar) and was limited to the NTS (Haywood et al., 1999), our initial experiments in the Sprague Dawley rats were conducted to determine whether the fluctuation of the number of ER α - and PR-labeled nuclei across the estrous cycle is similar in medullary and hypothalamic autonomic regions. Moreover, we determined whether the numbers of ER β -containing cells fluctuate across the estrous cycle. ER- and PR-

labeling were examined in autonomic medullary and hypothalamic areas from Sprague Dawley rats at two different estrous phases: proestrus (high estrogen) and diestrus (low estrogen).

Overall, ER α -labeled nuclei were more abundant than those with PR or ER β . Within the NTS, nuclei containing ER α -ir were most often detected in the commissural portion (cNTS; Fig. 1C; Fig. 2A; Fig. 5A,B) and less often found in the medial portion (mNTS; Fig. 1B; Fig. 2A; Fig. 5B). At the level of the area postrema, ER α -labeled nuclei were rarely detected in the dorsomedial NTS (dmNTS; Fig. 1B; Fig. 5B). As we observed earlier (Wang et al., 2006), modest numbers of ER α -labeled nuclei were detected in the aRVLM and pRVLM (Fig. 2A; Fig. 5B). Consistent with previous reports (Simonian and Herbison, 1997a), relatively more ER α -labeled nuclei were observed in the hypothalamus, especially the arcuate nucleus (ArH; Fig. 2A). Changes in the number of ER α -labeled nuclei at different stages of estrous were regionally specific. ANOVA analysis revealed a main effect of group difference (proestrus vs. diestrus) ($F_{(1,89)} = 6.41$, $p < 0.05$). In cNTS the number of ER α -immunoreactive nuclei was significantly higher in diestrus than in proestrus (Bonferroni post hoc analyses; $p = 0.002$) whereas the aRVLM showed no differences (Fig. 2A). In hypothalamus the paraventricular nucleus (PVN) showed a marginal elevation ($p = 0.05$) in ER α -labeled nuclei in diestrus (Fig. 2A); no differences between groups were found for the PVH or ArH.

In both the medulla and hypothalamus, PR-labeled nuclei had a similar distribution to ER α -labeled nuclei except that they were not detected in the mNTS and dmNTS. Moreover, in the hypothalamus, relatively fewer PR-immunoreactive nuclei were detected in the PVN and PVH (Fig. 2B). ANOVA revealed a significant 2 way interaction of group by region ($F_{(1,94)} = 3.23$, $p < 0.05$). Post hoc analysis showed that the detectable number of PR-labeled nuclei was significantly higher in proestrus than in diestrus in cNTS (Bonferroni post hoc analyses; $p < 0.001$). Moreover, post hoc analysis showed significantly higher numbers of detectable PR-immunoreactive nuclei in proestrus compared to diestrus in the PVH ($p < 0.01$) and in the ArH ($p < 0.05$) regions of the hypothalamus (Fig. 2B). The numbers of PR-labeled nuclei in the aRVLM and the PVN region of the hypothalamus were not significantly different ($p > 0.05$) at proestrus compared to diestrus, likely because of the low numbers of PR-labeled nuclei in these regions relative to the ArH.

In all regions examined, ER β -immunoreactivity appeared to be primarily extranuclear. Consistent with published reports (Shughrue and Merchenthaler, 2001; Wang et al., 2006), ER β -immunoreactive cells were found in the PVN (primarily the parvocellular division), the aRVLM and the medial region of the NTS (a region primarily associated with gustatory functions (Cunningham, Jr. and Sawchenko, 1990)). Unlike ER α and PR, the number of ER β -labeled cells did not fluctuate over the estrous cycle in the regions examined (PVN: $F_{(1,19)} = 2.54$, $p > 0.05$; mNTS: $F_{(1,19)} = 0.001$, $p > 0.05$; RVLM: $F_{(1,19)} = 0.01$, $p > 0.05$; Fig. 2C).

Chronic AngII-infusion selectively decreases the numbers of PR-labeled nuclei, but not ER α -labeled nuclei, in the cNTS

To determine whether hypertension alters the expression and distribution of gonadal steroid receptors in central autonomic neurons, we examined the effects of sustained ABP elevation induced by chronic AngII-infusion on ER and PR immunoreactivity in autonomic nuclei in the medulla and hypothalamus. After 7 days of infusion with AngII, the female rats showed significantly increased systolic ABP, to 172 ± 8 mmHg, compared to 103 ± 4 mmHg in saline-treated female controls ($p < 0.05$, t-test, $N = 8/\text{group}$). The systolic ABP of saline control rats was not different from values obtained prior to implantation with the minipumps ($p > 0.05$). Prior to implantation of the minipumps, rats had regular estrous cycles. After implantation of the minipumps, both saline and AngII administered rats had estrous cycles; however, most of the rats had shorter diestrus periods (1–2 days vs. 2–3 days) and 2 rats had longer (5–8 days)

diestrus periods. At the time of sacrifice (after 7 days of AngII or saline infusion) all rats were in diestrus, the low-estrogen stage.

In sections labeled for PR, ANOVA revealed a significant 2 way interaction of group (i.e., saline vs. AngII infused) and region ($F_{(1,117)} = 5.24$, $p < 0.01$). The detectable number of PR-labeled nuclei in the cNTS from AngII-infused rats was significantly fewer (Bonferroni post hoc analyses, $p < 0.001$) from those observed in saline-infused rats (Fig. 3B, C). However, the number of PR-labeled nuclei in the RVLM and hypothalamus (PVH, ArH) in AngII-infused rats compared to controls was not significantly different ($p > 0.05$; Fig. 3C).

In contrast to PR, the detectable number of nuclei with ER α -ir in all areas examined was not different in the AngII-infused rats ($F_{(1,110)} = 0.029$, $p > 0.05$; Fig. 3D). Likewise, the numbers of ER β -labeled cells were not significantly different in any of the medullary or hypothalamic regions examined in the AngII-infused rats (PVN: $F_{(1,17)} = 0.61$, $p > 0.05$; mNTS: $F_{(1,19)} = 4.33$, $p > 0.05$; RVLM: $F_{(1,19)} = 0.35$, $p > 0.05$; Fig. 3E). However, on visual inspection of the cNTS, ER α -ir in both the nuclei and cytoplasm of the cNTS appeared to be darker in the AngII-infused rats than in the controls (Fig. 4A, B). Quantification of this using optical density measurements revealed that indeed, AngII-infused rats had significantly more intense ER α labeling in both the nuclear ($t_{(35)} = 2.73$, $p < 0.001$) and cytoplasmic ($t_{(35)} = 2.28$, $p < 0.05$) compartments of labeled neurons (Fig. 4C).

Approximately half of the neurons containing ER α - and PR-labeled nuclei in the Sprague Dawley rat cNTS are catecholaminergic—As shown above, chronic AngII-infusion affected the numbers or levels of ER α - and PR-ir selectively in the cNTS. Since previous studies in the NTS of cycling Wistar rats showed that many ER α - and PR-nuclei are catecholaminergic (Haywood et al., 1999), we examined this issue in cycling Sprague Dawley rats. For comparison, the extent of colocalization of ER α - and PR-labeled nuclei with TH-ir was also examined in other brainstem areas containing populations of catecholaminergic neurons. In these studies, medullary sections dually labeled for TH and ER α or PR were examined by fluorescence confocal microscopy. Since nuclear ER α -ir and PR-ir was maximally expressed in the cNTS at diestrus and proestrus, respectively (see above), medullary sections from these stages were used for the particular receptors.

Relatively more ER α - and PR-labeled nuclei were detected in the cNTS compared to other brainstem catecholaminergic cell groups examined. About half (49%) of the ER α -labeled nuclei in the cNTS were in TH-ir neurons (Fig. 5A,B). At the level of the area postrema, many (38%) of the ER α -labeled nuclei in mNTS were in TH-immunoreactive neurons while only 6% of the dmNTS cells with nuclear ER α contained TH labeling (Fig. 5B). Similar to our previous results (Wang et al., 2006), the aRVLM contained very little co-expression with TH (5%) and the pRVLM contained no detectable ER α -TH co-localization (Fig. 5B). In the A1 region, many (28%) ER α -labeled nuclei were observed in TH-containing neurons (Fig. 5B).

Although a modest number of PR-labeled nuclei were in the cNTS, about half (46%) of these contained TH (Fig. 5C,D). Nuclear PR-ir was in both the aRVLM and pRVLM (Fig. 5D); however, no PR-labeled nuclei were found in cells containing TH-ir. Moreover, no PR-labeled nuclei were detected in the A1 region.

Chronic AngII-infusion alters the subcellular distribution of ER α -ir primarily in the TH-labeled neurons in the cNTS—The light microscopic studies described above suggested that chronic AngII-infusion altered the levels and/or trafficking of nuclear and extranuclear ER α -ir within cells in the cNTS. Moreover, our dual labeling fluorescent studies showed that about half of the neurons containing nuclear ER α -ir in the cNTS were labeled for TH. Thus, dual labeling electron microscopy was used to examine the effect of chronic AngII-

infusion on the subcellular distribution in ER α -ir (identified by immunogold) relative to TH-labeled cells (identified by immunoperoxidase) in the cNTS.

Consistent with our light microscopic results, ER α -ir was detected in the cytoplasm and nuclei of TH- and non-TH-labeled neurons in both control and AngII-infused rats (Fig. 6, Fig. 7 & Fig. 8). Additionally, electron microscopy revealed that ER α labeling was in the nuclei and cytoplasm of glia (Fig. 7). The majority of glial cells with ER α immunoreactive nuclei (22 out of 24) resembled fibrous astrocytes (Peters et al., 1991): their nuclei contained clusters of heterochromatin especially near the nuclear membrane and their cytoplasm was clear with clumps of fibrils (Fig. 6C). The remaining 2 glial cells with ER α -ir resembled oligodendrocytes; their nuclei contained clumped heterochromatin and their cytoplasm was dark (not shown).

In all cell types, varying densities of ER α -immunogold labeling were found in nuclei (Fig. 6A,B; Fig. 7A,C). The majority of ER α -immunogold labeling appeared to be “free” within the cytosol, that is, not associated with any particular organelles in the single planes of section analyzed. The remaining portion of ER α -immunogold labeling was associated with plasma membrane, mitochondria, endoplasmic reticulum and Golgi apparatus (Fig. 7B & Fig. 8) in both control and AngII-infused rats. In some cases, ER α -immunogold labeling was associated with a saccule of endoplasmic reticulum adjacent to mitochondria (Fig. 8B).

In response to chronic AngII-infusion, quantification of the subcellular distribution of ER α in TH- and non-TH containing cells revealed a selective redistribution of ER α in TH-labeled neurons (Fig. 9). ANOVA showed a 2-way interaction of treatment group (control vs. angiotensin) by cell type labeled ($F_{(1,379)} = 6.29$, $p < 0.01$). In AngII-treated rats compared to saline controls, post hoc analysis revealed a significantly higher ratio (Bonferroni post hoc analyses; $p < 0.05$) of ER α -immunogold particles in the cytoplasm relative to the nucleus in the TH labeled cells AngII treated rats relative to controls (Fig. 9A). No differences ($p > 0.05$) were seen in the ratio of cytoplasmic to nuclear ER α -labeling in the TH-lacking neurons or in glia (Fig. 9A).

Chronic AngII-infusion affected the cytosolic levels of ER α in both TH- and non-TH-labeled neurons. Within TH-containing neurons, chronic AngII-infusion resulted in an increased number of ER α -immunogold particles “free” (i.e., unassociated with organelles) in the cytoplasm (Fig. 9B). Repeated measures ANOVA confirmed a cell region by treatment group interaction ($F_{(4,220)} = 4.22$, $p = 0.034$; Bonferroni post hoc analyses $p < 0.001$ for “free” particles). A separate ANOVA on the distribution of ER α -immunogold particles in cytoplasmic organelles revealed a significant 2-way interaction of region by group ($F_{(3,165)} = 3.71$, $p = 0.02$). Significantly more ER α -immunogold particles (Bonferroni post hoc analyses, $p = 0.02$) were associated with the endoplasmic reticulum of TH-labeled neurons from AngII-infused rats (Fig. 9B). The number of ER α -immunogold particles associated with mitochondria was not significant ($p > 0.1$; Fig. 9B). Following AngII-infusion, there was a significant increase in the “free” ER α -ir in the cytosol of non-TH cells (Fig. 9C). Repeated measures ANOVA showed a significant interaction of cell region by treatment group ($F_{(4,1204)} = 3.49$, $p = 0.05$; Bonferroni post hoc analyses, $p < 0.01$ for “free” particles). No significant differences in the distribution of ER α relative to particular organelles were seen in non-TH cells, however.

DISCUSSION

We have investigated the effects of AngII-induced hypertension on gonadal receptors in medullary and hypothalamic centers controlling the systemic circulation. We found that nuclear ER α and PR, but not ER β , fluctuate across the estrous cycle in a regionally selective manner in medullary and hypothalamic cardiovascular areas of female Sprague-Dawley rats. Following chronic AngII infusion, the expression of ER α - and PR-ir was affected exclusively

in the cNTS, where: 1) the detectable number of PR-immunoreactive nuclei was further reduced below diestrus levels; 2) the detectable levels of ER α -ir increased in the nucleus and cytoplasm of cells; 3) the ratio of cytoplasmic to nuclear ER α increased selectively in catecholaminergic neurons and 4) ER α -ir increased in the cytosol of catecholaminergic and non-catecholaminergic neurons but increased in affiliation with specific subcellular organelles only in catecholaminergic neurons. These novel observations indicate that AngII-induced hypertension can modulate the expression and trafficking of ER α and PR in key central autonomic nuclei involved in the control of ABP.

Comparison with previous studies—Although the distribution and catecholamine content of neurons with ER α - and PR-labeled nuclei in the medulla has been examined previously (Haywood et al., 1999; Lee et al., 2000; Simonian and Herbison, 1997b), the present study includes several important differences that complement and extend earlier work. First and most notable is the animal model. Studies by Haywood et al. (Haywood et al., 1999) and Simonian and Herbison (Simonian and Herbison, 1997b) were performed in Wistar rats (normal cycling and ovariectomized, respectively) which differ from Sprague Dawley rats in stress-related behaviors and in catecholaminergic systems (Kinney et al., 1999; Staples and McGregor, 2006; Zamudio et al., 2005). Additionally, from the methods provided, the estrous stages appear to have been determined using time-of-day in the study by Haywood et al. (Haywood et al., 1999); this could lead to misclassification since proestrus can be relatively short and not all rats cycle identically. Moreover, studies by Lee et al. (Lee et al., 2000) were performed on ovariectomized Sprague Dawley rats that were continuously dosed with estradiol via silastic capsules. Second, earlier studies were either limited to the NTS and ventral medulla caudal to the RVLM (Haywood et al., 1999; Simonian and Herbison, 1997a) or were focused on the adrenergic cell groups and did not include the cNTS (Lee et al., 2000). Additionally, earlier studies classified the NTS using rostrocaudal location (Haywood et al., 1999; Simonian and Herbison, 1997a) rather than functional subdivisions as in the present study. Third, all three previous studies used different antibodies to ER α and only one (Haywood et al., 1999) analyzed immunoreactivity for PR. Fourth, light microscopic dual label experiments in two studies (Haywood et al., 1999; Lee et al., 2000) used two peroxidase labels and in one study (Simonian and Herbison) used colchicine-treated rats, all of which can lead to overestimates of the number of dual labeled neurons. Although our study confirms that nuclear ER α and PR are consistently present in subpopulations of catecholaminergic neurons in the female NTS and RVLM, the extent of co-localization varies between studies, suggesting variability due to differences in methodology or animal model. In general, the present study showed fewer PR- and ER α -labeled nuclei in the dmNTS and RVLM and a lower proportion of catecholaminergic neurons containing ER α - and PR-labeled nuclei.

Methodological considerations

We found that some of the female rats implanted with saline or AngII-filled minipumps had a prolonged diestrus phase of the cycle, perhaps due to the stress of the implantation and the subsequent handling during the blood pressure measurements. However, these rats did cycle and were all in diestrus at the time of sacrifice. This could reflect the fact that female rats housed together tend to cycle together (unpublished observation). Given the observations that estrous phase influences ER α - and PR-ir, the fact that all rats were in the same estrous phase eliminated an important variable and made it easier to make the relative comparisons between treatment groups. However, the numbers of ER and PR cells in some brain areas were less in the saline-diestrus group compared to the cycling-diestrus group. While methodological factors (e.g., different experimental dates and DAB times) contributed to these differences, the surgical procedures and subsequent measuring of the blood pressures could also have contributed to the differences in the two cohorts of animals. Thus, caution should be used in comparing the animals with minipumps to normal cycling rats.

When the cNTS of AngII-infused rats was examined at the light microscopic level, the intensity of ER α -ir increased in both the nucleus and the cytoplasm. When examined by electron microscopy, the ratio of cytoplasmic to nuclear ER α -ir increased selectively in the catecholaminergic neuronal subpopulation. At least three methodological differences may contribute to the differences between the light and electron microscopic results. First, light microscopic studies measured the density of ER α -ir from thicker sections (40-micron vs. 70 nm). Second, light microscopic labeling procedures employed high concentrations of detergent, allowing for greater penetration of reagents especially into the nucleus. Third, electron microscopic studies used immunogold to localize ER α which is less sensitive in detecting small amounts of antigen (Leranth and Pickel, 1989). Irrespective of these differences, both light and electron microscopic studies suggest that chronic AngII-infusion up-regulates nuclear and cytoplasmic ER α in the cNTS.

While the specificity of the ER β antibody has been well documented, the selectivity of the antibody for individual splice variants has not been determined. However, previous work using the same ER β -ir antibody showed prominent labeling in the hippocampus, a region which expresses the ER β 1 δ 4 isoform at high levels (Price, Jr. et al., 2000). Since the ER β 1 δ 4 isoform exhibits reduced estradiol binding (Price, Jr. et al., 2000), it is possible that the lack of change in number of ER β -labeled cells over the estrous cycle may have been due to the primary detection of the ER β 1 δ 4 isoform by the antibody. Alternatively, the lack of detectable differences with ER β -ir over the estrous cycle and in response to chronic AngII in the medulla and PVN may be because ER β is a membrane receptor in these regions and involved in rapid, non-genomic functions (Levin, 2001).

Chronic AngII accentuated diestrus levels of PR and ER α nuclei selectively in the cNTS

The number of PR-labeled nuclei was higher in proestrus than in diestrus in most medullary and hypothalamic areas examined, while the number of nuclear ER α s was higher in diestrus only in cNTS and the number of ER β -labeled cells was unchanged over the estrous cycle. Our findings in the NTS are in agreement with previous studies in cycling Wistar rats (Haywood et al., 1999). The lack of cyclical change seen in the number of ER α -labeled nuclei in many of the subregions examined and ER β -labeled cells in the hypothalamus, NTS and RVLM could be due to methodological limitations (see above) or could suggest that ER α in some brain regions may not respond quickly to the estrogen rise of proestrus.

Following chronic AngII-infusion, the number of PR-labeled nuclei in the cNTS significantly decreased and the intensity of nuclear and extranuclear ER α -labeling increased. The AngII-infused animals and the saline-infused controls were all in diestrus, and for each receptor the direction of the AngII-linked changes represents an extension of its relative (and opposite) level in the diestrus phase of the estrous cycle. Thus, the up-regulation of ER α and the concomitant down-regulation of PR following AngII may represent an expansion of the cyclic fluctuations seen in the cNTS, and are consistent with the known opposing expressions of these receptors in other regions (Woolley, 2007). The changes in PR and ER α following chronic AngII-infusion could be due to direct actions on the cNTS and/or could involve secondary effects of other circulating factors (e.g., corticosterone). In support of the first possibility, the cNTS is activated following baroreceptor activation with chronic AngII infusion (Li et al., 1998; Lohmeier et al., 2002; Reagan, 1999). In support of the possibility of indirect effects from circulating factors, gonadal steroid levels can affect hypothalamic-pituitary-adrenal responses to stress (Viau and Meaney, 1991).

Chronic AngII most notably affected ER α in catecholaminergic neurons in the cNTS

Following chronic AngII-infusion ER α -ir was increased in the nuclei and cytoplasm of cells in the cNTS, the majority of which were catecholaminergic (TH) neurons. Closer electron

microscopic examination of the cNTS from AngII-infused rats revealed that the ratio of cytoplasmic to nuclear ER α -ir significantly increased in catecholaminergic neurons. These changes in both nuclear and cytoplasmic levels of the receptor suggest that these compartments are similarly regulated and are consistent with the suggestion that the same ligand can signal at membrane and nuclear sites to achieve integration at the level of transcription (Vasudevan et al., 2005).

Although chronic AngII increased ER α -ir “free” in the cytoplasm of TH-containing and TH-lacking neurons, the increase in ER α -ir associated with endoplasmic reticula was seen only in TH-containing neurons. The increase of ER α -ir in endoplasmic reticula suggests that chronic AngII may increase the synthesis of ER α or that ER α may have relocated to affect the synthesis of other proteins. Moreover, some ER α was associated with a saccule of endoplasmic reticulum adjacent to mitochondria. The affiliation of ER α with mitochondria is in agreement with recent observations in cultured cells (Yager and Chen, 2007). Endoplasmic reticula near mitochondria control multiple cellular processes such as the entry and release of Ca⁺⁺, sterol biosynthesis and apoptosis (Berridge, 2002). Our previous studies have shown that endoplasmic reticula near mitochondria contain subunits for NADPH oxidase, a downstream target of AngII signaling which is important for generating reactive oxygen species (Wang et al., 2004). The selective increase of ER α -ir in TH-containing neurons following AngII infusion may be important for the regulation of TH-expression. Estrogen levels have been shown to modify the expression of TH mRNA in A2 neurons in the NTS (Liaw et al., 1992). TH mRNA increases in the NTS following hypertension induced by aortic coarctation or blood pressure elevations in response to immobilization stress (Maximino et al., 2006; Serova et al., 2005).

Electron microscopy revealed ER α in glia

Our findings demonstrate that ER α is in the nuclei of glia, particularly NTS astrocytes. These findings are consistent with previous studies demonstrating ER α -ir in a moderate number of glial processes in other brain areas (Milner et al., 2001; Wang et al., 2006) and ER mRNA and protein in glia *in vitro* (Langub and Watson, 1992; Santagati et al., 1994; Sierra et al., 2008). Astrocytic ERs may be involved in the regulation of neuronal metabolism [for reviews see (Jordan, 1999; Mhyre and Dorsa, 2006)] and could influence astrocytic Ca⁺⁺ concentration, a variable that plays a key role in neuronal excitability and cerebral blood flow regulation (Iadecola and Nedergaard, 2007). Moreover, ERs in glia are known to regulate the excitotoxic effects of glutamate as well as inflammatory processes (Blurton-Jones and Tuszynski, 2001; Jordan, 1999). Although chronic AngII infusion did not alter the subcellular distribution of ER α -ir in the few glial cells that were detected, these findings suggest that estrogens may influence the functioning of glia in addition to neurons in the cNTS.

Functional Considerations

ER α - and PR-containing neurons in cNTS may be involved in many functions. The cNTS receives primarily chemoreceptive afferents, important for mediating cardiorespiratory responses to hypoxia (Ruggiero et al., 1994). Neurons in the cNTS project directly to the RVLM neurons, including the C1 neurons, that are critical for regulating baroreceptor reflex output to the spinal cord (Aicher et al., 2000; Card et al., 2006; Guyenet, 2006). Moreover, the NTS sends projections, both catecholaminergic and non-catecholaminergic, to a wide variety of brain regions associated with autonomic-endocrine coordination (Hermes et al., 2006; Reyes and Van Bockstaele, 2006; Ruggiero et al., 1994). Some catecholaminergic neurons in the cNTS project to the pontine parabrachial nucleus and periaqueductal gray, which are involved in both cardiovascular and cardiorespiratory responses [reviewed in (Saper, 2002)]. Consistent with a role in synchronizing cardiovascular and stress responses, the NTS projects to PVN (Saper, 2002) and sends a partially-catecholaminergic projection to central nucleus of amygdala (Petrov et al., 1993).

Estrogen activation of ER α , particularly those expressed centrally, is protective against the baroreceptor dysfunction and hypertension induced by AngII in female mice (Pamidimukkala et al., 2005;Xue et al., 2007b). The present studies demonstrate that hypertension modulates the expression and subcellular distribution of ER α s and PRs specifically in cardiovascular regions of the NTS, wherein the changes were most pronounced in TH containing neurons. Considering the protective role of ER α against AngII-induced hypertension (Xue et al., 2007a), the findings raise the possibility that the observed changes in ER α and PR induced by hypertension represent an adaptive response aimed at counteracting the central mechanisms responsible for the elevation in ABP evoked by AngII. Such an adaptive response could represent an additional mechanism by which females are protected from the deleterious effects of hypertension on the brain and other target organs.

Abbreviations

ABC, avidin-biotin complex
 ABP, arterial blood pressure
 AMB, nucleus ambiguous
 AngII, angiotensin II
 AT1, angiotensin type 1 receptors
 ArH, arcuate hypothalamus
 BSA, bovine serum albumin
 CU, cuneate nucleus
 DAB, diaminobenzidine
 DiE, diestrus
 DMX, dorsal motor nucleus vagus nerve
 ECU, external cuneate nucleus
 EM, electron microscopy
 ER, estrogen receptor
 GR, gracile nucleus
 icp, inferior cerebral peduncle
 IO, inferior olivary complex
 LRN, lateral reticular nucleus
 mlf, medial longitudinal fascicle
 ml, medial lemniscus
 MV, medial vestibular nucleus
 NTS, nucleus of the solitary tract
 cNTS, NTS, commissural region
 dmNTS, NTS, dorsomedial region
 PVN, paraventricular nucleus of the hypothalamus
 PVH, periventricular hypothalamus
 PB, phosphate buffer
 PR, progestin receptor
 py, pyramidal tract
 RO, nucleus raphé obscurus
 RVLM, rostral ventrolateral medulla
 aRVLM, RVLM, anterior part
 pRVLM, RVLM, posterior part
 SPV, spinal nucleus of trigeminal
 sptV, spinal tract trigeminal
 TS, tris-saline
 ts, tractus solitarius
 TH, tyrosine hydroxylase
 XII, hypoglossal nucleus.

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Fig. 1. Schematic drawings through the medulla at the levels analyzed for TH and nuclear gonadal steroid receptor dual labeling (adapted from Swanson (Swanson, 1992), plates 61, 69 and 71)

A. The anterior portion of the RVLM (aRVLM; about 12.2mm caudal to bregma) and slightly more posterior portions of RVLM (not shown) were analyzed. **B.** At the level of the area postrema (AP), the medial (mNTS), the dorsomedial NTS (dmNTS), and A1 region (A1) were examined (about 13.76 mm from bregma). **C.** More caudally, the commissural NTS (cNTS) was analyzed (about 14.4 mm from bregma). **Abbreviations:** AMB, nucleus ambiguus; CU, cuneate nucleus; DMX, dorsal motor nucleus vagus nerve; ECU, external cuneate nucleus; GR, gracile nucleus; icp, inferior cerebral peduncle; IO, inferior olivary complex; LRN, lateral reticular nucleus; mlf, medial longitudinal fascicle; ml, medial lemniscus; MV, medial vestibular nuc; py, pyramidal tract; RO, nucleus raphe obscurus; SPV, spinal nucleus of trigeminal; spV spinal tract trigeminal; ts, tractus solitarius; XII, hypoglossal nucleus. Bar, 1 mm.

Fig. 2. Levels of nuclear immunoreactivity for PRs and ERαs fluctuate across the estrous cycle
A. The number of nuclei with detectable ERα-immunoperoxidase labeling is significantly higher in diestrus (DiE) than in proestrus (ProE) in the cNTS, is marginally higher in the paraventricular nucleus of the hypothalamus (PVN), and does not change in the other regions examined. **B.** The number of nuclei with detectable PR-immunoperoxidase labeling is significantly higher in proestrus animals in the cNTS, periventricular hypothalamus (PVH) and arcuate hypothalamus (ArH). The number of PR-labeled nuclei in the aRVLM and PVN tended to be higher at proestrus compared to diestrus, but this was not significant. **C.** The number of cells with ERβ-ir was not significantly different over the estrous cycle in the medial region of

the NTS, aRVLM and the PVN. $n = 10$ sections/region/condition **, $p < 0.01$; *, $p < 0.05$; ^, $p = 0.053$ (ANOVA).

Fig. 3. Chronic AngII treatment decreases the detectable number of PR-immunoreactive nuclei, but not ERα or ERβ, in cNTS

A. In saline-treated controls, PR-labeled nuclei are scattered in cNTS. **B.** Following AngII treatment that increased ABP, PR-labeled nuclei in cNTS appear lighter and fewer than in controls. Bars A & B, 50 μm. **C.** In cNTS, the number of PR-labeled nuclei is significantly lower with AngII administration. **D & E.** In all regions examined, the number of ERα-labeled nuclei (D) or ERβ-labeled cells (E) is not significantly different with AngII administration. **, $p < 0.001$ (ANOVA); $n = 13$ /region/condition for ERα; $n = 10$ /region/condition for ERβ; $n = 18$ /region/condition for PR.

Fig. 4. Chronic Ang II treatment increases the intensity of ER α -labeling in cNTS

A. ER α labeling in cytoplasm and nuclei of neurons in a saline-treated rat in diestrus. **B.** ER α -ir neurons in cNTS of a diestrus rat with Ang-II-induced increases in ABP. In A and B, nuclei are shown with feathered arrows while cytoplasm is designated with plain arrows. **C.** Following Ang II administration, the optical density (OD) of ER α -labeling significantly increased in nuclei and cytoplasm of neurons in cNTS. **, $p < 0.001$; *, $p < 0.05$ (ANOVA) $n = 37$ cells/condition. Bar = 50 μ m.

Fig. 5. About half of the catecholaminergic neurons in the cNTS contain ERα- and PR-immunoreactivities in their nuclei

A. TH (green) and nuclear ERα (red) labeling in cNTS. Some neurons colocalize both labels (arrows) while other only have TH (arrowhead) or only ERα (asterisk). **B.** Nuclear ERα is in varying proportions of TH-ir neurons in NTS and RVLM subfields (n=6–8 sections from 4 rats). The greatest degree of colocalization is in cNTS, while no colocalization is seen in dmNTS or posterior RVLM (pRVLM). **C.** PR-ir (red) and TH-ir (green) are colocalized (arrows) in cNTS cells. A single-labeled TH-ir neuron (arrowhead) is shown for comparison. **D.** About half of the PR-ir nuclei in cNTS are in TH-ir neurons, but other NTS subregions have very little PR-ir and no observed colocalization with TH-ir. In RVLM, no TH-ir neurons have detectable PR-ir, although the rRVLM has relatively more PR-ir than other areas examined (n = 12 sections for NTS, 6 sections for RVLM). Bar A & C, 50 μm.

Fig. 6. At the ultrastructural level, ER α -ir is detected in the nuclei and cytoplasm of TH-labeled neurons in the cNTS

A & B. Examples of two TH-labeled perikarya from control rats containing ER α -immunogold labeling (ER α /TH-P) in their nuclei (black particles) and cytoplasm. The density of ER α -labeled gold particles in the nucleus is less in cell A (0.4 particles/ μm^2) than in cell B (3.3 particles/ μm^2). Within the cytoplasm, ER α -labeled gold particles can be affiliated with the plasma membrane (pm), endoplasmic reticulum (er), mitochondria (m) or lack any organelle affiliations in the plane of section, i.e., “free” (f). Bar, 2 μm .

Fig. 7. In both control and AngII-exposed rats, ER α -ir is found in non-TH labeled neurons and in glial cells in the cNTS

A. In this non-TH labeled perikaryon (Era-P), ER α immunogold particles are found mostly in the nucleus (N; 0.8 particles/ μm^2) and to a lesser extent in the cytoplasm (0.5 particles/ μm^2). A TH-labeled perikaryon (TH-P) is nearby. **B.** High magnification of the cytoplasm of an unlabeled perikaryon showing examples of ER α immunogold particles (arrow) affiliated with a saccule of endoplasmic reticula (er) adjacent to a mitochondrion (m) and near (arrowhead) a Golgi apparatus (G). **C.** ER α immunogold particles (black) are found in the perikaryon (Era-P) of an astrocytic cell body identified by the bundle of fibrils (gf) in its cytoplasm. ER α -labeled

gold particles are found in the nucleus (N) and affiliated with a mitochondrion (m) in the cytoplasm. Bar A & C, 2 μm ; B, 500 nm.

Fig. 8. Chronic AngII-infusion affects the subcellular distribution of ER α -ir in the cytoplasm of TH-labeled neurons in the cNTS

A. Example of a TH-labeled perikaryon from an AngII-infused rat containing ER α -immunogold labeling (ER α /TH-P) in the nucleus (black particles; 2.7 particles/ μm^2) and cytoplasm. In this example ER α -immunogold labeling is affiliated with several mitochondria (m) and endoplasmic reticula (er). **B & C.** High magnification of two different TH-labeled perikarya showing examples of ER α -immunogold labeling affiliated with mitochondria (m), endoplasmic reticula (er) or “free” (f) in the cytosol. Bar A, 2 μm ; Bar B & C, 500 nm.

Fig. 9. Chronic AngII infusion produces re-distribution of ERα in cNTS

A. With Ang II infusion, the ratio of cytoplasmic to nuclear ERα-gold particles significantly increased only in TH-labeled cells and was unchanged in neurons lacking TH or in glia. The number of cells assessed (n) is shown in the bars. **B.** Within TH-labeled neurons of rats chronically infused with AngII, significantly more “free” ERα-gold particles were found in the cytosol. In a separate analysis of ERα-gold particles associated with cytoplasmic organelles, significantly more were associated with the endoplasmic reticulum (er). Although not significant, more ERα-gold particles tended to be associated with mitochondria (mito). **C.** Within non-TH-containing neurons of rats chronically infused with AngII, significantly more ERα-gold particles were free in the cytosol; however, no differences in the subcellular

distribution relative to specific organelles were seen. **, $p < 0.001$; *, $p < 0.05$; ^, $p = 0.02$ (ANOVA).