Estrogen Receptor-α in the Medial Amygdala Prevents Stress-Induced Elevations in Blood Pressure in Females

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Abstract—Psychological stress contributes to the development of hypertension in humans. The ovarian hormone, estrogen, has been shown to prevent stress-induced pressor responses in females by unknown mechanisms. Here, we showed that the antihypertensive effects of estrogen during stress were blunted in female mice lacking estrogen receptor-α in the brain medial amygdala. Deletion of estrogen receptor-α in medial amygdala neurons also resulted in increased excitability of these neurons, associated with elevated ionotropic glutamate receptor expression. We further demonstrated that selective activation of medial amygdala neurons mimicked effects of stress to increase blood pressure in mice. Together, our results support a model where estrogen acts on estrogen receptor-α expressed by medial amygdala neurons to prevent stress-induced activation of these neurons, and therefore prevents pressor responses to stress. (Hypertension. 2016;67:1321-1330. DOI: 10.1161/HYPERTENSIONAHA.116.07175.) ● Online Data Supplement

Key Words: amygdala ■ blood pressure ■ estrogen ■ hypertension ■ neurons

The prevalence of hypertension has continued to rise for the past 20 years. In the United States, only 1 of 3 patients with hypertension have their blood pressure (BP) controlled. Interventions that treat and prevent hypertension are urgently needed. The pathogenesis of essential hypertension remains poorly understood. Genetic and behavioral factors do not fully explain the development of hypertension. Increasing evidence suggests that psychological stress leads to the development of hypertension in humans. For example, acute stress exposure triggers rapid increases in BP and heart rate (HR).4,5 Chronic stress and particularly the nonadaptive response to stress are associated with sustained elevation of BP. Dysfunctions of central nervous system and neuroendocrine system are thought to contribute to the pathophysiological process of stress-induced hypertension, but the critical central nervous system networks and the neurohormonal systems that regulate BP during psychological stress remain to be unraveled.

The ovarian hormone, estrogen, is long thought to prevent development of hypertension. Before menopause, BP has been typically lower in women than in age-matched men; after menopause, however, the incidence of hypertension in women increases dramatically. In particular, psychological stress causes greater pressor responses in postmenopausal women than in premenopausal women, and this effect is blunted by 17β-estradiol replacement. Multiple clinical trials have reported antihypertensive benefits of estrogen replacement in postmenopausal women. Unfortunately, higher risk of thromboembolism was also reported in postmenopausal women receiving estrogen replacement, probably because of the procoagulation effects of estrogen. Better understanding specific mechanisms for the antihypertensive effects of 17β-estradiol may facilitate developing new estrogen replacement therapies that only produce cardiovascular benefits with no or fewer side effects.

The amygdala, a neural complex near the temporal pole of the mammalian cerebral hemisphere, plays important roles in mediating the emotional and hormonal responses to stress. Recent evidence indicates that the amygdala is involved in development of stress-induced pressor responses in humans. In particular, neural activities in the medial amygdala (MeA), a subdivision of the amygdala, are positively correlated with stress-induced pressor responses in mice. Thus, increased MeA neural activities may mediate stress-induced pressor responses. MeA neurons express abundant estrogen receptor-α (ERα), one of ERs. Here, we tested a hypothesis that ERα expressed by MeA neurons is a key site where 17β-estradiol acts to prevent stress-induced pressor effects. To this end, we generated 2 mouse models with ERα deleted in the MeA. Using these tools, we examined the physiological functions of MeA ERα on stress-induced pressor responses. We also

Received January 18, 2016; first decision February 9, 2016; revision accepted March 16, 2016.

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Hypertension is available at http://hyper.ahajournals.org DOI: 10.1161/HYPERTENSIONAHA.116.07175

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explored mechanisms by which ERα signals may regulate firing activities of MeA neurons. Finally, we designed the receptor exclusively activated by designer drugs (DREADD) approach to examine effects of MeA neural activity on BP balance in mice.

**Methods**

**Mice**

All mouse strains were backcrossed onto C57BL/6 background for >12 generations. SIM1-Cre transgene was bred onto Esr1f/− mice to generate Esr1f/−/SIM1-Cre and Esr1f/− littermates, which were referred as SIM1-ERα-knockout (KO) and control mice, respectively. These mice were used for BP studies, quantitative polymerase chain reaction, and Western blotting, as described below. In parallel, Esr1f/−/SIM1-Cre mice were crossed to Esr1f/−/rosa26:GFP (SIM1-ERα-KO) mice and Esr1f/+/SIM1-Cre/rosa26GFP (control) mice. These mice were used for histology validation. In addition, the rosa26tdTOMATO allele was crossed onto SIM1-Cre transgenic mice (control) and Esr1f/−/SIM1-Cre mice (SIM1-ERα-KO), which were used for electrophysiological recordings. In addition, Esr1f/−/SIM1-ERα-KO mice were crossed to Esr1f/− to generate Esr1f/− mice, which received AAV-Cre-GFP-AAV-GFP stereotaxic injections into the MeA. SIM1-Cre transgenic mice were crossed to C57BL/6 mice to produce SIM-Cre transgenic mice, which were used for DREADD study.

All mice were weaned on chow diet (6.5% fat, no. 2920, Harlan) at 3 to 4 weeks of age and were grouped housed (2–5 mice per cage). For mice used in BP recording, they were singly housed after surgery; other mice (eg, for electrophysiological studies) were grouped housed till study. All mice were housed in a 12-hour light and 12-hour dark cycle. Care of all animals and procedures were conformed to the Guide for Care and Use of Laboratory Animals of the US National Institutes of Health and approved by the Animal Subjects Committee of Baylor College of Medicine.

**Colocalization of SIM1 and ERα**

Female Esr1f/−/SIM1-Cre/rosa26:GFP and SIM1-Cre/rosa26:GFP mice were perfused with 10% formalin. To avoid influence of estrous cycles on expression pattern, female mice were all perfused at diestrous. Briefly, mice for this study were subjected to daily vaginal smear followed by cytology evaluation. Exclusive leukocytes in the vaginal smear samples were used as a determinant for the stage of diestrous. Brain sections were cut at 25 μm (1.5 series) and the sections were incubated in the primary rabbit anti-ERα antibody (1:10000; no. C1355, Millipore, Billerica, MA) overnight, followed by donkey antirabbit AlexaFluor594 (1:500; no. A21207, Invitrogen, Grand Island, NY) for 1.5 hours. Then, the sections were incubated in primary chicken anti-GFP antibody (1:5000; no. GFP-1020, Aves Laboratories, Inc, Tigard, OR) overnight, followed by the goat antichicken AlexaFluor 488 (1:250; no. A11039, Invitrogen) for 1.5 hours. The slides were cover slipped and images were analyzed using the Leica 5500 fluorescent microscope.

**Effects of Subcutaneous 17β-Estradiol on Stress-Induced Pressor Responses**

To examine the effects of estrogen, 12-week-old female control and SIM1-ERα-KO mice were anesthetized with inhaled isoflurane. As previously described, bilateral ovariectomy was performed, followed by subcutaneous implantations of pellets containing 17β-estradiol (0.5 μg/d lasting for 30 days, OVX+E) or empty pellets (OVX+V). These pellets were purchased from Innovative Research of America. Under the same anesthesia, the left carotid artery was isolated, and the catheter of the telemetry transmitter (TA11PA-C10, Data Sciences International, St. Paul, MN) was inserted into the vessel. The body of the transmitter was slipped in a subcutaneous pocket along the right flank. The mice were allowed to recover for 7 days and then followed by recording for BP and HR using DSI PhysioTel Receivers (Data Sciences International). On the recording day, BP and HR were recorded continuously for 2 phases: the acclimation phase (8 AM–11 AM) and the restraint phase (11 AM–12 AM). Twenty minutes before the restraint phase (10:40 AM), the experimenter entered the room and turned on the telemetry probe with a magnetic switch. At 11 AM, the experimenter re-entered the room to restrain mice with a mouse plastic restraining cone, which immobilized the mice. Importantly, mice were maintained at the normal position and were able to breathe normally; there were no pain or discomfort except that mice could not move. BP and HR data during the past 20 minutes during the acclimation phase were used as the baseline.

Because the stress-induced pressor responses may be influenced by different baseline BP/HR levels, we first performed pilot studies in a small cohort of wild-type OVX+V and OVX+E mice to compare their 24-hour baseline mean arterial pressure (MAP) and HR. We found that OVX+E mice showed significantly reduced MAP compared with OVX+V mice during the entire dark cycle and the early phase of light cycle; however, during 10 AM to 2 PM, there was no significant difference in MAP between the 2 groups (Figure S1A, in the online-only Data Supplement). However, HR was not significantly different between OVX+V and OVX+E mice throughout the entire 24-hour period (Figure S1B). On the basis of these observations, we chose to perform the restraint stress during 11 AM to 12 PM, as described above.

Note that hypertension is often associated with obesity. We chose to perform BP/HR recording 7 days after OVX+V/E treatment, because body weight is comparable within this time point. At the end of recordings, mice were euthanized. Fat pads were weighed to ensure comparable adiposity between the groups (data not shown). Uteruses were weighted to confirm successful 17β-estradiol depletion in OVX+V mice and sufficient 17β-estradiol supplement in OVX+E mice.

**Deletion of ERα in the MeA**

Female Esr1f/− mice were anesthetized with isoflurane and received bilateral stereotaxic injections of AAV-Cre-GFP (AAV9.CMV.HGF-Cre.WPRE.SV40, Penn Vector Core at University of Pennsylvania, Philadelphia, PA) into the MeA (200 nL/side), and these mice were referred as MeA-ERα-KO. Esr1f/− mice that received AAV-GFP in the MeA were used as controls. The coordinates for the MeA were 1.7 mm posterior and 2.5 mm lateral to the Bregma, and 5 mm ventral to the dura. After the recovery from the stereotaxic surgeries (7 days), these mice received the OVX+E treatment and implantation of the telemetry transmitters, followed by recordings of BP and HR under the baseline condition and the restrained condition, as described above.

At the end of the experiment, all mice were perfused with 10% formalin. Brain sections were collected, and expression of GFP was checked in the MeA. Only those with expression of GFP exclusively in both MeA sides were included in data analyses. To further validate the deletion of ERα, some brain sections with accurate GFP expression were also subjected to ERα immunofluorescent staining, as described above.

**DREADD Activation of MeA SIM1 Neurons**

Female SIM1-Cre mice (at 14–16 weeks of age) were anesthetized by isoflurane and received bilateral stereotaxic injections of AAV-hM3Dq-mCherry (AAV-hSyn-DIO-hM3D(Gq)-mCherry in AAV2, UNC Vector Core, Chapel Hill, NC) into the MeA (200 nL/side), followed by a second surgery to receive the implantation of the telemetry transmitters, as described above. After recovery, BP and HR were recorded continuously during 9 AM to 11 AM, with an intraperitoneal injection of saline or clozapin N-oxide (CNO; 3 mg/kg) at 10 AM. Both saline and CNO were injected in the same mice at 2 different trials (at the same time of the day) with a 1-week interval; the order of the 2 injections was randomized to avoid any sequence effect.

At the end of experiment, all mice were perfused with 10% formalin. Brain sections were collected and subjected to mCherry immunohistochemistry, as described before. Briefly, brain sections were first incubated in 0.3% H2O2 in PBS for 30 minutes to abolish endogenous peroxidase activity. After several washes, sections were incubated overnight at room temperature with the primary rabbit Living Colors DsRed polyclonal antibody (1:1000, Clontech Laboratories, Inc, Mountain View, CA). After several washes, sections were incubated
with the biotinylated secondary antibody (1:1000, antirabbit, Jackson Immuno Research, West Grove, PA) for 1 hour. Sections were then washed and visualized by incubation with the VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Sections were washed and treated with diaminobenzidine (Sigma, St. Louis, MO) for 5 minutes, followed by dehydration in a graded ethanol series from 50% to 100% and a final wash in xylene. The slides were cover slipped and images were analyzed using the Leica 5500 microscope under the brightfield. Only those with mCherry immunoreactivity selectively in both MeA sides were included in data analyses.

To prove the concept that CNO does activate hM3Dq-mCherry–expressing MeA SIM1 neurons, some SIM1-Cre mice were used for electrophysiological recordings after the stereotaxic AAV-hM3Dq-mCherry injections, as described below.

Electrophysiology
Whole-cell patch-clamp recordings were performed on TOMATO-labeled SIM1 neurons in the acute MeA slices from SIM1-Cre/rosa26tdUTOMATO mice and Esr1<sup>−/−</sup>/SIM1-Cre/rosa26tdUTOMATO (SIM1-ERα-KO) mice. As we described before, 26,27 6- to 12-week-old female mice were deeply anesthetized with isoflurane and transcardially perfused with a modified ice-cold sucrose-based cutting solution (adjusted to pH 7.3) containing (in mmol/L) 10 NaCl, 25 NaHCO<sub>3</sub>, 195 sucrose, 5 glucose, 2.5 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 2 Na pyruvate, 0.5 CaCl<sub>2</sub>, 7 MgCl<sub>2</sub>, bubbled continuously with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The mice were then decapitated, and the entire brain was removed and immediately submerged in ice-cold sucrose-based cutting solution. Coronal sections containing the MeA (250 μm) were cut with a Microm HM 650V vibratome (Thermo Scientific). The slices were recovered for 1 hour at 34°C in artificial cerebrospinal fluid (adjusted to pH 7.3) containing (in mmol/L) 126 NaCl, 2.5 KCl, 2.4 CaCl<sub>2</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 1.2 MgCl<sub>2</sub>, 11.1 glucose, and 21.4 NaHCO<sub>3</sub> saturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> before recording.

Slices were transferred to the recording chamber and allowed to equilibrate for at least 10 minutes before recording. The slices were perfused at 34°C in oxygenated artificial cerebrospinal fluid at a flow rate of 1.8 to 2 mL/min. tdTOMATO-positive SIM1 neurons in MeA were visualized using epifluorescence and IR-DIC imaging on an upright microscope (Eclipse FN-1, Nikon) equipped with a moveable stage (MP-285, Sutter Instrument). Patch pipettes with resistances of 3 to 5 mol/L·Ω were filled with intracellular solution (adjusted to pH 7.3) containing (in mmol/L) 128 K gluconate, 10 KCl, 10 HEPES, 0.1 EGTA, 2 MgCl<sub>2</sub>, 0.05 Na-GTP, 3 Mg-ATP, and 0.1 Lucifer yellow dye. Recordings were made using a MultiClamp 700B amplifier (Axon Instrument), sampled with Digital/data 140A and analyzed offline with pClamp 10.3 software (Axon Instrument). Series resistance was monitored during the recording, and the values were generally <10 MΩ and were not compensated. The liquid junction potential was +12.5 mV and was corrected after the experiment. Data would be excluded if the series resistance increased >20% during the experiment or without overshoot for action potential.

Neural firing frequency and resting membrane potential were measured under the current-clamp mode. For the miniature excitatory postsynaptic current (mEPSC) recordings, the internal recording solution contained (in mmol/L): CsCH<sub>2</sub>SO<sub>4</sub>, 125, CsCl 10, NaCl 5, MgCl<sub>2</sub>, 2, EGTA 1, HEPES 10, (Mg)ATP 5, and (Na)GTP 0.3 (pH 7.3 with NaOH). mEPSC was measured in the voltage-clamp mode with a holding potential of ~60 mV in the presence of 1 μmol/L tetrodotoxin<sup>28</sup> and 50 μmol/L bicusculine<sup>30</sup>. For the miniature inhibitory postsynaptic current (mIPSC) recordings, patch electrodes were filled with a recording solution that contained (in mmol/L): 153.3 CsCl, 1.0 MgCl<sub>2</sub>, 5.0 EGTA, and 10.0 HEPES, pH of 7.20 with CsOH<sup>31</sup>. CsCl was included to block potassium currents. Mg-ATP (3 mmol/L) was added to the intracellular solution before recording. Glutamate receptor–mediated synaptic currents were blocked by 30 μmol/L D-AP-5 and 30 μmol/L CNQX in the external solution, along with 1 μmol/L tetrodotoxin in the external solution blocking action potentials. Neurons were voltage clamped at ~70 mV during the recording. Slices were fixed with 4% formalin in PBS at 4°C overnight and then subjected to post hoc identification of the anatomic location of the recorded neurons within the MeA.

To validate that hM3Dq-mCherry–expressing MeA SIM1 neurons can be activated by CNO treatment, the MeA-containing brain slices were prepared from SIM1-Cre mice receiving stereotaxic AAV-hM3Dq-mCherry injections. Effects of CNO (10 μmol/L, bath perfusion 6 minutes) on the firing frequency and resting membrane potential were recorded in mCherry-labeled neurons under the current-clamp mode.

Real-Time Reverse Transcription–Polymerase Chain Reaction
Female SIM1-ERα-KO and control littermates were euthanized at diestrus, and the amygdala was quickly microdissected and stored at ~80°C. Real-time reverse transcription–polymerase chain reaction was performed as described previously. 30 Primer sequences were listed in Table S1.

Western Blot
Female SIM1-ERα-KO and control littermates were euthanized at diestrus, and the amygdala was quickly microdissected. Subsequently, the fresh whole amygdala tissue was homogenized in cell lysis buffer that was supplemented with protease inhibitors and phosphatase inhibitors. Then, cell lysates were combined with 1X Laemmli buffer, and boiled for 5 minutes. The precipitated proteins were separated by SDS-8% PAGE and the samples were transferred onto nitrocellulose membranes (BioRad Transfer equipment). Dry powder milk was dissolved into Tris-buffered saline and Tween 20 (50 mmol/L Tris-HCL, 150 mmol/L NaCl [pH 7.5], 0.1% Tween 20) to have a final 3% concentration and added to the nitrocellulose membranes for 1 hour. The membranes were washed in Tris-buffered saline and Tween 20 for 5 minutes 3x. The nitrocellulose membranes were incubated with anti-glutamate receptor N subunit antibody (anti-GluN1; 1:1,000; Cat no. 4204, Cell Signaling Technology) overnight, followed by incubation with the anti-rabbit horseradish peroxidase–conjugated secondary antibody (1:10,000; Cat no. 7074, Cell Signaling Technology) for 1 hour at the room temperature. Similarly, as a loading control, the nitrocellulose membranes were incubated with mouse anti-β actin (1:5,000; Cat no. A3853, Sigma-Aldrich), followed by the antimouse horseradish peroxidase–conjugated secondary antibody (1:10,000; Cat no. 7076P, Cell Signaling Technology). Western blots were visualized with Thermo Fisher Scientific SuperSignal West Pico Chemiluminescence substrate.

Serum Corticosterone
To measure stressed levels of corticosterone, mice were restrained for 15 minutes, and blood was collected from the tail vein; for the measurements of basal corticosterone, mice were rapidly decapitated at 9:00 AM and trunk blood was collected. Blood samples were processed to measure serum corticosterone using a corticosterone EIA kit (900-097, Assay Designs, Ann Arbor, MI).

Statistical Analyses
The data are presented as mean±SEM. Statistical analyses were performed using GraphPad Prism to evaluate normal distribution and variations within and among groups. Methods of statistical analyses were chosen based on the design of each experiment and are indicated in figure legends. P<0.05 was considered to be statistically significant.

Study Approval
Care of all animals and procedures were conformed to the Guide for Care and Use of Laboratory Animals of the US National Institutes of Health and were approved by the Animal Subjects Committee of Baylor College of Medicine.

Results
Deletion of ERα From SIM1 Neurons Blunts Estrogenic Actions to Prevent Stress-Induced Pressor Responses
We have generated an SIM1-ERα-KO (Esr1<sup>−/−</sup>/SIM1-Cre) mouse line, in which ERα (encoded by the Esr1 gene) is selectively

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deleted in single-minded 1 (SIM1) neurons. As we reported previously, SIM1-ERα-KO mice have ERα deleted primarily in the MeA (Figure 1A), with modest deletion in the medial preoptic area (Figure 1B) and the paraventricular nucleus (PVN) of the hypothalamus (Figure 1C). Here, we used female SIM1-ERα-KO mice and their control (Esr1f/f) littermates to examine the role of ERα in SIM1 neurons in the regulation of BP during stress. To this end, we measured BP and HR in conscious control and SIM1-ERα-KO female mice that received either OVX+V or OVX+E treatment, at the unstressed condition (baseline) followed by a 1-hour restraint that caused stress. We found that in OVX+V control mice, the restraint stress provoked strong and sustained increases in MAP compared with the baseline (Figure 2A and 2C). Importantly, these stress-induced pressor responses were significantly blunted in OVX+E control females (Figure 2A and 2C). These findings indicate that 17β-estradiol supplement in female mice prevents pressor responses induced by stress. We then tested the stress-induced pressor effects in female SIM1-ERα-KO mice. Interestingly, regardless whether SIM1-ERα-KO mice were treated with OVX+V or OVX+E, the restraint stress provoked robust increases in MAP, responses that were comparable with those seen in OVX+V control mice (Figure 2B and 2C). Notably, the levels of HR were significantly elevated by the restraint stress at multiple time points in OVX+V control mice, OVX+V or OVX+E SIM1-ERα-KO mice, but not in OVX+E control mice (Figure 2D and 2E); however, the averaged HR at the baseline and during the 60-minute stress period were not significantly different presumably because of big variations among each mouse (Figure 2F). Changes in systolic arterial pressure and in diastolic arterial pressure were similar as MAP (Figure S2A–S2F). Terminal analyses revealed that OVX+V mice displayed expected uterine atrophy, which confirmed successful 17β-estradiol depletion, whereas OVX+E mice showed heavier uterus than OVX+V mice, indicating sufficient 17β-estradiol supplement (Figure S2G). Together, these results indicate that ERα expressed by SIM1 neurons is required to mediate estrogenic actions to prevent stress-induced pressor responses in female mice.

**Deletion of ERα Selectively in the MeA Blunts Estrogenic Actions to Prevent Stress-Induced Pressor Responses**

It is worth noting that in SIM1-ERα-KO mice, ERα was deleted primarily in the MeA, with modest ERα deletions in the medial preoptic area and the PVN. Therefore, BP phenotypes observed in SIM1-ERα-KO mice may result not only from loss of ERα in the MeA but also to some degree from loss of ERα in the medial preoptic area/PVN. To further confirm the physiological functions of ERα in MeA neurons, we used the stereotaxic AAV-Cre-GFP injections to the MeA (bilaterally) of female Esr1f/f mice (Figure 3A). Female Esr1f/f mice receiving AAV-GFP stereotaxic injections into the MeA were used as controls (see Figure 3A for validation). Both MeA-ERα-KO and control mice were treated with OVX+E, and we assessed stress-induced pressor responses in these mice. In OVX+E control mice, the restraint stress failed to induce elevations in MAP.

**Figure 1.** Deletions of estrogen receptor-α (ERα) in SIM1 neurons. Dual immunofluorescence for green fluorescence protein (GFP; green) and ERα (red) in the medial amygdala (MeA, A), medial preoptic area (MPOA, B), and paraventricular nucleus (PVN, C) in control (SIM1-Cre/Rosa26-GFP, top) and SIM1-ERα-knockout (KO; Esr1f/f/SIM1-Cre/Rosa26-GFP, bottom) mice. 3V indicates third ventricle; and opt, optic tract. Scale bars, 50 μm.
β-estradiol supplement attenuates stress-induced c-fos immunoreactivity in the MeA, indicating that estrogen prevents neural activation of MeA neurons during stress. However, mechanisms underlying these estrogenic actions were not known. Here, we tested if ERα expressed by MeA neurons is involved in the excitability of these neurons. To this end, we used the whole-cell patch-clamp electrophysiology to record firing activities of identified MeA SIM1 neurons from female control versus SIM1-ERα-KO mice (Figure 4A). We found that MeA SIM1 neurons in SIM1-ERα-KO mice showed significantly increased firing frequency compared with those in control mice, whereas the resting membrane potential was comparable between the 2 groups (Figure 4B–4D). We further analyzed the mEPSC in these neurons and found that both the amplitude and the frequency of mEPSC in MeA SIM1 neurons were comparable between the 2 groups (Figure 4B–4D). We further analyzed the mEPSC in these neurons and found that both the amplitude and the frequency of mEPSC in MeA SIM1 neurons were significantly increased compared with those from control mice (Figure 4E–4G). In contrast, the amplitude and frequency of miniature inhibitory postsynaptic currents were not significantly different between the 2 groups (Figure 4H–4J). Furthermore, we found that mRNA levels of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunits (GluA1 and GluA2) and the N-methyl-D-aspartate (NMDA) receptor subunit (GluN1) were significantly elevated in the amygdala of female SIM1-ERα-KO mice compared with control littermates, whereas expression of other ERs (including ERβ and G-protein–coupled receptor 30) was significantly different between the 2 groups (Figure 4K). We further confirmed that the protein levels of GluN1 in the amygdala of female SIM1-ERα-KO mice were significantly higher than those from control mice (Figure 4L and 4M). Compared with the ionotropic AMPA and NMDA glutamate receptors, mRNAs of metabotropic glutamate receptors (mGlur1-mGlurR8) were
Selective Activation of MeA SIM1 Neurons Increases BP

It has been shown that lesions of the MeA reduce BP in mice and rats, which suggest that MeA neurons are vasopressors. Here, we used DREADD approach to selectively activate MeA SIM1 neurons in free-moving mice (Figure 5A). To prove the concept that the designers’ drug, CNO, does activate these mCherry-expressing SIM1 neurons, we used the whole-cell patch-clamp electrophysiology to record effects of CNO on the firing activities of mCherry-labeled neurons in brain slices prepared from AAV-hM3Dq-mCherry-infected SIM1-Cre mice (Figure 5B). We showed that CNO (10 μmol/L, bath 6 minutes) increased the firing frequency of all recorded mCherry-labeled MeA SIM1 neurons and caused depolarization (Figure 5C–5E).

Importantly, we showed in vivo that CNO injections (3 mg/kg, IP) significantly increased MAP and HR compared with saline treatment in the same mice (Figure 5F and 5G). Similar changes in systolic arterial pressure and diastolic arterial pressure were observed (data not shown). Together, these data demonstrate that selective activation of MeA SIM1 neurons provokes pressor responses in mice.

Discussion

The MeA has been implicated as a key brain structure that links stress and development of hypertension. First, various physical and psychological stressors (eg, noise, restraint, and forced swim) activate neurons in the MeA, which can lead to increased excitability of these neurons.

In various hypertension animal models, OVX exacerbates the course of hypertension, whereas 17β-estradiol supplementation prevents hypertension. In particular, 17β-estradiol supplementation prevents pressor responses induced by restraint stress in rodents and in postmenopausal women. However, the mechanisms for this protective effect remain unknown. Here, we demonstrated that genetic deletion of ERα selectively in SIM1 neurons blunted the effects of
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17β-estradiol to prevent stress-induced pressor responses in female mice. Given that most of SIM1-Cre–mediated ERα deletion is restricted in the MeA (with minor deletions in the medial preoptic area and PVN), we suggest that this phenotype results primarily from loss of ERα in the MeA. Given that most of SIM1-Cre–mediated ERα deletion is restricted in the MeA (with minor deletions in the medial preoptic area and PVN), we suggest that this phenotype results primarily from loss of ERα in the MeA.

17β-estradiol to prevent stress-induced pressor responses in female mice. Given that most of SIM1-Cre–mediated ERα deletion is restricted in the MeA (with minor deletions in the medial preoptic area and PVN), we suggest that this phenotype results primarily from loss of ERα in the MeA. Consistently, Xue et al52 has reported that selective knockdown of ERα in the PVN does not affect BP in rats. The specific role of MeA ERα was further confirmed in mice lacking ERα only in the MeA (through the stereotaxic AAV-Cre-GFP approach). Thus, we provided compelling evidence to highlight ERα expressed by MeA neurons as the key mediator for the antihypertensive effects of 17β-estradiol during stress. Of course, it is worth noting that other ERs, including ERβ52,53 and G-protein–coupled receptor 30,54 are both implicated in the regulation of BP. Both these receptors are highly expressed in the MeA.19,55 Functions of ERβ and G-protein–coupled receptor 30 in the regulation of stress-induced pressor responses warrant further investigations.

The mechanisms by which estrogen-ERα signals prevent stress-induced pressor responses at least partly involve an inhibition of MeA neurons during stress. Supporting this notion, it has been reported that chronic 17β-estradiol supplement attenuates MeA neural activation (c-fos immunoreactivity) evoked by the restraint stress.34 Consistently, we found that MeA SIM1 neurons lacking ERα showed increased firing frequency associated with increased mEPSC, indicating that ERα-mediated signals in these neurons inhibit their firing activity. Notably, we recently reported that a selective ERα agonist, propyl pyrazole triol, rapidly (in the time scale of mini-seconds) activates MeA neurons in an ERα-dependent manner.26 This propyl pyrazole triol–induced rapid activation seems to be in contrast to the activation of MeA neurons caused by chronic loss of ERα. Similar discrepancy also exists in animals receiving

Figure 4. Effects of estrogen receptor-α (ERα) deletion on excitability of medial amygdala (MeA) SIM1 neurons. A, Electrophysiological recording from an identified MeA SIM1 neuron in the brain slice from a SIM1-Cre/rosa26:tdTOMATO mouse. Illuminations for TOMATO, injected lucifer yellow dye, and the brightfield. Scale bars, 20 μm. B, Representative action potential traces in MeA SIM1 neurons from control and SIM1-ERα-knockout (KO) mice. Averaged firing frequency (C) and resting membrane potential (D). Data are presented as mean±SEM; n=9 or 16 per group. *P<0.05 in t tests. C, Representative miniature excitatory postsynaptic current (mEPSC) traces in MeA SIM1 neurons from control and SIM1-ERα-knockout (KO) mice. Averaged mEPSC amplitude (E) and frequency (F). Data are presented as mean±SEM; n=25 or 29 per group. *P<0.05 and ***P<0.001 in t tests. H, Representative miniature inhibitory postsynaptic current (mIPSC) traces in MeA SIM1 neurons from control and SIM1-ERα-KO mice. Averaged mIPSC amplitude (I) and frequency (J). Data are presented as mean±SEM; n=17 or 14 per group. K, Relative mRNA levels of indicated genes in the amygdala from control and SIM1-ERα-KO mice. Data are presented as mean±SEM; n=7 per group. *P<0.05 in t tests. Note that metabotropic glutamate receptor 6 (mGluR6) were undetectable in all samples and were not shown here. I, Western blot showing protein levels of glutamate receptor N subunit (GluN1) and actin in the amygdala from control and SIM1-ERα-KO mice. M, Summary quantification for the ratios of GluN1/actin. Data are presented as mean±SEM; n=4 or 5 in each group. *P<0.05 in t tests.

A  TOMATO  Lucifer Yellow  Brightfield
B  Control  SIM1-ERα-KO
C  Firing Frequency (Hz)
D  Resting Membrane Potential (mV)
E  Control  SIM1-ERα-KO
F  Averaged mEPSC Amplitude
G  Averaged mEPSC Frequency
H  Control  SIM1-ERα-KO
I  Frequency (Hz)
J  Amplitude (pA)
K  Relative mRNA Expression
L  GluN1
M  GluN1/Actin
N  Control  SIM1-ERα-KO
O  % Increase
P  Control  SIM1-ERα-KO
Q  % Increase
R  Control  SIM1-ERα-KO
S  % Increase
T  Control  SIM1-ERα-KO
U  % Increase
V  Control  SIM1-ERα-KO
W  % Increase
X  Control  SIM1-ERα-KO
Y  % Increase
Z  Control  SIM1-ERα-KO
aa  % Increase
bb  % Increase
cc  % Increase
dd  % Increase
ee  % Increase
ff  % Increase
gg  % Increase
hh  % Increase
ii  % Increase
jj  % Increase
kk  % Increase
ll  % Increase
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nn  % Increase
oo  % Increase
pp  % Increase
qq  % Increase
rr  % Increase
ss  % Increase
tt  % Increase
uu  % Increase
vv  % Increase
ww  % Increase
xx  % Increase
yy  % Increase
zz  % Increase

Figure 4. Effects of estrogen receptor-α (ERα) deletion on excitability of medial amygdala (MeA) SIM1 neurons. A, Electrophysiological recording from an identified MeA SIM1 neuron in the brain slice from a SIM1-Cre/rosa26:tdTOMATO mouse. Illuminations for TOMATO, injected lucifer yellow dye, and the brightfield. Scale bars, 20 μm. B, Representative action potential traces in MeA SIM1 neurons from control and SIM1-ERα-knockout (KO) mice. Averaged firing frequency (C) and resting membrane potential (D). Data are presented as mean±SEM; n=9 or 16 per group. *P<0.05 in t tests. C, Representative miniature excitatory postsynaptic current (mEPSC) traces in MeA SIM1 neurons from control and SIM1-ERα-knockout (KO) mice. Averaged mEPSC amplitude (E) and frequency (F). Data are presented as mean±SEM; n=25 or 29 per group. *P<0.05 and ***P<0.001 in t tests. H, Representative miniature inhibitory postsynaptic current (mIPSC) traces in MeA SIM1 neurons from control and SIM1-ERα-KO mice. Averaged mIPSC amplitude (I) and frequency (J). Data are presented as mean±SEM; n=17 or 14 per group. K, Relative mRNA levels of indicated genes in the amygdala from control and SIM1-ERα-KO mice. Data are presented as mean±SEM; n=7 per group. *P<0.05 in t tests. Note that metabotropic glutamate receptor 6 (mGluR6) were undetectable in all samples and were not shown here. I, Western blot showing protein levels of glutamate receptor N subunit (GluN1) and actin in the amygdala from control and SIM1-ERα-KO mice. M, Summary quantification for the ratios of GluN1/actin. Data are presented as mean±SEM; n=4 or 5 in each group. *P<0.05 in t tests.
acute versus chronic 17β-estradiol treatment. Although acute injections of 17β-estradiol rapidly induce c-fos immunoreactivity in the MeA, chronic 17β-estradiol supplement prevents stress-induced c-fos immunoreactivity in the same brain region. We suggest that these opposite rapid versus chronic effects are mediated by segregated rapid signals versus genomic effects of ERα. On one hand, estrogen can act on ERα to rapidly activate neural activity, phenomena that have been widely observed in multiple neural populations. On the other hand, in a more chronic setting, ERα functions as a classic nuclear receptor to regulate expression of target genes. Indeed, it has been reported that chronic 17β-estradiol treatment in ovariectomized rodents decreases expression of ionotropic glutamate receptors in the amygdala and hypothalamus. Consistently, we showed that deletion of ERα increased levels of ionotropic glutamate receptors in the MeA, which may have contributed to the increased mEPSC and firing frequency. Collectively, we suggest that estrogen inhibits expression of ionotropic glutamate receptors in amygdalar neurons, presumably through genomic ERα actions as a nuclear receptor. Thus, low levels of glutamate receptors decrease the excitability of these MeA neurons and prevent them from stress-induced activation.

Notably, changes in HR were largely correlated with changes in BP in most cases. For example, we showed that DREADD-mediated stimulation of MeA neurons increased BP and HR. Similarly, restraint stress caused increases in both BP and HR. These results are consistent with several previous reports in rats or in mice. Importantly, 17β-estradiol treatment in control mice prevented stress-induced increases in both BP and HR, whereas these effects were both blunted in mice lacking ERα in MeA neurons. This pattern suggests that the regulations on BP are at least partly through modulations on cardiac functions. However, we could not fully exclude the possibility that MeA neurons may also regulate BP via actions on the vascular tone.

How ERα-expressing MeA neurons regulate BP and HR during stress is unknown. Stress is well known to activate the hypothalamus–pituitary–adrenal axis and leads to elevated corticosterone levels in the circulation, which may partly contribute to the stress-induced pressor responses. However, it has been shown that 17β-estradiol supplement potentiates, instead of inhibits, stress-induced corticosterone release in female rats. Similar potentiated corticosterone levels were observed in our OVX+E mice compared with OVX+V mice under the stressed condition, although the corticosterone level at a single time point during the stress may not fully reflect responses of the hypothalamus–pituitary–adrenal axis. Given the antihypertensive effects of ERα-expressing MeA neurons during stress, we suggest that estrogenic actions on the hypothalamus–pituitary–adrenal responses and on BP responses to stress are likely mediated by distinct neural circuits. The neural circuits by which MeA neurons regulate BP balance remain to be elucidated.
Perspectives

In summary, our studies provide evidence that MeA neurons are vasopressors, and activation of these MeA neurons mediates the elevations in BP during restraint stress. Furthermore, we demonstrate that estrogen acts on ERα expressed by MeA neurons to prevent stress-induced pressor responses in female mice. These antihypertensive effects of MeA ERα seem to be mediated through transcription control of ionotropic glutamate receptors in MeA neurons. Thus, we identified MeA ERα as a potential therapeutic target for hypertension, especially in postmenopausal women.

Acknowledgments

We thank the Mouse Phenotyping Core at Baylor College of Medicine for all the measurements of BP and HR in mice.

Sources of Funding

This work was supported by grants from the National Institutes of Health (1F31HL128054, 2T32GM008231, and IMSD R25 GM56929 to A.O. Hinton; R01DK093587 and R01DK101379 to Y. Xu; P01 DK088761 to D.J. Clegg; T32CA059268 to S.A. Khan; and R01DK092605 to Q. Tong), American Diabetes Association (1F31HL128054, 2T32GM008231, and IMSD R25 GM56929 to A.O. Hinton; R01DK093587 and R01DK101379 to Y. Xu; P01 DK088761 to D.J. Clegg; T32CA059268 to S.A. Khan; and R01DK092605 to Q. Tong), American Diabetes Association (1-11-BS-180 to Y. Xu and no. 7-13-JF-61 to Q. Wu), and American Heart Association awards P. Xu and to Q. Tong.

References

18. Merchenthaler I, Lane MV, Numan S, Dellovade TL, and American Heart Association awards P. Xu and to Q. Tong.
Novelty and Significance

What Is New?

- Although the roles of the hypothalamus and brain stem in the regulation of blood pressure (BP) have been well documented, the effects of higher brain structures on BP control are currently less appreciated.
- These higher neural centers (eg, the amygdala) are well positioned to integrate psychological stressors (eg, venous thrombosis and stress-induced cardiovascular responses) in the anteroventral periventricular nucleus of the hypothalamus in social recognition, anxiety and aggression.
- The role of the estrogen receptor alpha in the medial amygdala and ventromedial nucleus of the hypothalamus in social recognition, anxiety and aggression.
- The role of acute physical and psychological stressors elicit distinctive recruitment patterns in the amygdala and in medullary noradrenergic cell groups.

What Is Relevant?

- Because the estrogen receptor-α (ERα) gene variants are associated with hypertension in humans, our studies focusing on the role of medial amygdala ERα in BP control revealed 1 potential mechanism for hypertension in patients with the ERα gene variants.
- Even for women with normal ERα gene, ERα functions will undergo monthly changes (within the menstrual cycles) and eventually diminish (after menopause) because of changes in \( \alpha \)-estradiol levels.
- Thus, our studies are significant in that we may demonstrate the cardiovascular consequences induced by these fluctuations that affect all women.

Summary

\( \alpha \)-estradiol is known to produce antihypertensive benefits in women, but its application has been hampered because of detrimental effects in peripheral tissues (eg, venous thrombosis and breast cancer). Our studies provided the proof of the concept that selective activation of medial amygdala ERα can lower BP. Thus, from a therapeutic perspective, our results may identify a novel drug target for the treatment of hypertension.
Estrogen Receptor-α in the Medial Amygdala Prevents Stress-Induced Elevations in Blood Pressure in Females

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_Hypertension_. 2016;67:1321-1330; originally published online April 18, 2016; doi: 10.1161/HYPERTENSIONAHA.116.07175

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Supplemental Table and Figures

**Estrogen Receptor-α in the Medial Amygdala Prevents Stress-induced Elevations in Blood Pressure in Females**

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**Running Title:** MeA ERα regulates blood pressure
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<tr>
<th>Target gene</th>
<th>Primer sequences</th>
<th>PCR products</th>
<th>GenBank accession</th>
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|            | CYCLO R TGCCGGAGTTCGACAATGAT |
| Grm1       | mGluR1 F AGGGTTTGTGCTGCTTCTG 120bp NM_016976
|            | mGluR1 R ATCTCTGTCTGCCCATCCAT |
| Grm2       | mGluR2 F GCTCCCAAGCTATACCCACG 143bp NM_001160353
|            | mGluR2 R TCATAACGGGACTTGTCGCTC |
| Grm3       | mGluR3 F GTCGGATGAAGAGGACCAAC 130bp NM_181850
|            | mGluR3 R GCTAAAGAGCCCGTCACTG |
| Grm4       | mGluR4 F CCCATACCCATTCAGTTGCGG 115bp NM_001013385
|            | mGluR4 R TGTAGCGCAACAAAGTGACCA |
| Grm5       | mGluR5 F ACGACCATGACGACCCTTCG 90bp NM_001143834
|            | mGluR5 R GGCAGGTGATACCCCTGTC |
| Grm6       | mGluR6 F AGTCATCACCTTCCGGCCTTA 106bp NM_173372
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|            | mGluR7 R GACCACGCAGAGAAGAAGT |
| Grm8       | mGluR8 F GGAGAGAGTGGTGGTTAGGC 94bp NM_008174
|            | mGluR8 R GTCTTTGTCACGTGGAGATT |
| Gria1      | GluA1 F GTCCGGCCTGAGAATCCAG 100bp NM_00113325
|            | GluA1 R CTCGCCCTTTGTCGTACCAC |
| Gria2      | GluA2 F TTCTCTCTTTATGGGACTGA 107bp NM_001039195
|            | GluA2 R CTACCCGAAATGCACTGTATTCCT |
| Grin1      | GluN1 F GCCAGGAGGAGAGACAGAGA 98bp NM_008169
|            | GluN1 R TGGGTTCACATACGCAAG |
Figure S1. 24-hour baseline MAP and HR.
Temporal changes in MAP (A) and HR (B) in OVX+V or OVX+E-treated wild type female mice. Data are presented as mean±SEM. N=3 to 4/group. *, P<0.05 between OVX+V and OVX+E in two-way ANOVA analysis followed by post hoc Sidak tests.
Figure S2. Deletion of ERα in SIM1 neurons blunted 17β-estradiol effects on SAP and DAP.

(A-B) Temporal changes in systolic arterial pressure in OVX+V or OVX+E-treated control (A) and SIM1-ERα-KO (B) mice at the baseline (-20 to 0 min) and the restrained condition (0 to 60 min). Data are presented as mean±SEM. N=6 to 9/group. *, P<0.05 between OVX+V and OVX+E in two-way ANOVA analysis followed by post hoc Sidak tests.

(C) Averaged systolic arterial pressure at the baseline or at the restrained condition in OVX+V or OVX+E-treated control and SIM1-ERα-KO mice. Data are presented as mean±SEM. N=6 to 9/group. **, P<0.01 between baseline and stress condition within the same mice; #, P<0.05 between OVX+V and OVX+E within the same genotype in two-way ANOVA analysis followed by post hoc Sidak tests.

(D-E) Temporal changes in diastolic arterial pressure in OVX+V or OVX+E-treated control (D) and SIM1-ERα-KO (E) mice at the baseline (-20 to 0 min) and the restrained condition (0 to 60 min). Data are presented as mean±SEM. N=6 to 9/group. *, P<0.05 between OVX+V and OVX+E in two-way ANOVA analysis followed by post hoc Sidak tests.

(F) Averaged diastolic arterial pressure at the baseline or at the restrained condition in OVX+V or OVX+E-treated control and SIM1-ERα-KO mice. Data are presented as mean±SEM. N=6 to 9/group. *, P<0.05 between baseline and stress condition within the same mice; #, P<0.05 between OVX+V and OVX+E within the same genotype in two-way ANOVA analysis followed by post hoc Sidak tests.

(G) Uterine weight in OVX+V or OVX+E-treated control and SIM1-ERα-KO mice. Data are presented as mean±SEM. N=6 to 9/group. **, P<0.01 between OVX+V and OVX+E within the same genotype in two-way ANOVA analysis followed by post hoc Sidak tests.
Figure S3. Deletion of ERα in MeA neurons blunted 17β-estradiol effects on SAP and DAP.

(A) Temporal changes in systolic arterial pressure in OVX+E-treated control and MeA-ERα-KO mice at the baseline (-20 to 0 min) and the restrained condition (0 to 60 min). Data are presented as mean±SEM. N=5/group. *, P<0.05 in two-way ANOVA analysis followed by post hoc Sidak tests.

(B) Averaged systolic arterial pressure at the baseline or at the restrained condition in OVX+E-treated control and MeA-ERα-KO mice. Data are presented as mean±SEM. N=5/group. **, P<0.01 between baseline and stress condition within the same mice; #, P<0.05 between control and MeA-ERα-KO mice in two-way ANOVA analysis followed by post hoc Sidak tests.

(C) Temporal changes in diastolic arterial pressure in OVX+E-treated control and MeA-ERα-KO mice at the baseline (-20 to 0 min) and the restrained condition (0 to 60 min). Data are presented as mean±SEM. N=5/group. *, P<0.05 in two-way ANOVA analysis followed by post hoc Sidak tests.

(D) Averaged diastolic arterial pressure at the baseline or at the restrained condition in OVX+E-treated control and MeA-ERα-KO mice. Data are presented as mean±SEM. N=5/group. **, P<0.01 between baseline and stress condition within the same mice; #, P<0.05 between control and MeA-ERα-KO mice in two-way ANOVA analysis followed by post hoc Sidak tests.
Figure S4. 17β-estradiol potentiated stress-induced corticosterone responses.

Averaged plasma corticosterone levels at the baseline or at the stress condition in OVX+V or OVX+E-treated wild type mice. Data are presented as mean±SEM. N=5 to 7/group. ***, P<0.001 between OVX+V and OVX+E; #, P<0.05 between baseline and stress condition within the same mice in two-way ANOVA analysis followed by post hoc Sidak tests.