Release of Norepinephrine in the Preoptic Area Activates Anteroventral Periventricular Nucleus Neurons and Stimulates the Surge of Luteinizing Hormone

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The role of norepinephrine (NE) in regulation of LH is still controversial. We investigated the role played by NE in the positive feedback of estradiol and progesterone. Ovarian-steroid control over NE release in the preoptic area (POA) was determined using microdialysis. Compared with ovariectomized (OVX) rats, estradiol-treated OVX (OVX+E) rats displayed lower release of NE in the morning but increased release coincident with the afternoon surge of LH. OVX rats treated with estradiol and progesterone (OVX+EP) exhibited markedly greater NE release than OVX+E rats, and amplification of the LH surge. The effect of NE on LH secretion was confirmed using reverse microdialysis. The LH surge and c-Fos expression in anteroventral periventricular nucleus neurons were significantly increased in OVX+E rats dialyzed with 100 nM NE in the POA. After Fluoro-Gold injection in the POA, c-Fos expression in Fluoro-Gold/tyrosine hydroxylase-immunoreactive neurons increased during the afternoon in the A2 of both OVX+E and OVX+EP rats, in the locus coeruleus (LC) of OVX+EP rats, but was unchanged in the A1. The selective lesion of LC terminals, by intracerebroventricular *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine, reduced the surge of LH in OVX+EP but not in OVX+E rats. Thus, estradiol and progesterone activate A2 and LC neurons, respectively, and this is associated with the increased release of NE in the POA and the magnitude of the LH surge. NE stimulates LH secretion, at least in part, through activation of anteroventral periventricular neurons. These findings contribute to elucidation of the role played by NE during the positive feedback of ovarian steroids. **(*Endocrinology* 154: 363–374, 2013)**

The preovulatory surge of LH triggers ovulation in female mammals. The preovulatory surge of LH is generated by an increase in GnRH release in the portal blood, which in rodents results from an interaction between the positive-feedback effect of ovarian steroids and circadian brain signals (1). Immunoneutralization of estradiol pre-

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Abbreviations: aCSF, Artificial cerebrospinal fluid; A/P, anteroposterior; AUC, area under the curve; AVPV, anteroventral periventricular; DAB, 3,3′-diaminobenzidine-HCl; DSP-4, *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine; D/V, dorsoventral; ER, estrogen receptor; FG, Fluoro-Gold; i.c.v., intracerebroventricular; -ir, immunoreactive; LC, locus coeruleus; MBH-ME, medial basal hypothalamus-median eminence; MHPG, 3-methoxy-4-hydroxyphenylglycol; M/L, mediolateral; NE, norepinephrine; OVX, ovariectomized; OVX+E, estradiol-treated OVX; OVX+P, progesterone-treated OVX; OVX+EP, estradiol and progesterone-treated OVX; POA, preoptic area; PR, progesterone receptor; SCN, suprachiasmatic nucleus; TH, tyrosine hydroxylase.

vents the surge of LH (2), whereas estradiol promotes daily surges in ovariectomized (OVX) rats (3). Progesterone is known to modulate the magnitude of the LH surge. Progesterone antagonism reduces the magnitude of the preovulatory surge of LH, whereas progesterone injection in estradiol-treated OVX rats enhances the LH surge and c-Fos expression in GnRH neurons (4).

GnRH neurons express estrogen receptor (ER)β but not ERα or progestin receptor (PR) (5, 6). However, estradiol-positive feedback depends on classical ERα signaling in non-GnRH neurons (7, 8). The preoptic area (POA), particularly the anteroventral periventricular (AVPV) nucleus, is a key brain region in the positive feedback. Discrete lesions (9) or antiestrogen implants (10) in the rostral POA prevent the LH surge. Neurons in the AVPV contain ERα and PR (11) and express c-Fos synchronously with GnRH neurons by the time of the LH surge (7, 12). However, the neural circuits through which estradiol and progesterone activate AVPV and GnRH neurons remain incompletely understood.

Norepinephrine (NE) is an important neurotransmitter for LH regulation (13-15). GnRH neurons express adrenergic receptors (16). Brainstem NE neurons of A1, A2, and locus coeruleus (LC) express ER and PR (17, 18) and project to brain areas containing GnRH perikarya (19, 20). A2 and LC also provide direct inputs to GnRH neurons in the mouse (21). During the positive feedback, NE release increases in the POA at the time of the LH surge on proestrus and in ovarian steroid-treated OVX rats (22-24). The resulting effect of NE is excitatory because its intracerebroventricular (i.c.v.) injection stimulates GnRH and LH release in estradiol-treated OVX rats (25), whereas antagonism of α1-adrenergic receptors and lesion of NE neurons block the LH surge (26, 27). In OVX rats, however, central injection of NE inhibits LH secretion, whereas both adrenergic agonists and antagonists suppress LH release (28-30). NE has been shown to exert a direct inhibitory effect upon GnRH neuron excitability in cycling or OVX mice (31), suggesting that the stimulatory effect of NE on GnRH neurons might be indirect. Thus, although NE neurons are known to be under ovarian-steroid modulation (32, 33), our knowledge of the specific noradrenergic pathways involved in estradiol and progesterone effects on LH secretion remains incomplete. We performed *in vivo* experiments using microdialysis, triple-labeling immunohistochemistry, and *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP-4) chemical lesion to further investigate the role of NE during the positive feedback.

Materials and Methods

Animals

Adult female Wistar rats weighing 250-300 g were used in experiments 1, 2 and 4, approved by the Ethics Committee for Research Involving Animals of the Medical School of Ribeirão Preto, University of São Paulo. Adult female Sprague Dawley rats weighing 250–300 g (Charles River Laboratories, Wilmington, MA) were used in experiment 3, approved by the Florida State University Animal Care and Use Committee. Rats were grouped housed under conditions of controlled lighting (lights on from 0600 h–1800 h) and temperature (22 ± 0.5 C). Standard rat chow and water were provided *ad libitum*.

Experimental design

Experiment 1: Effect of estradiol and progesterone on NE release in the POA

OVX rats were implanted with a microdialysis guide cannula in the right POA. Rats were treated 7-10 d after surgery with corn oil (OVX) or estradiol (OVX+E) daily for consecutive 3 d. The jugular vein was cannulated (34) and a microdialysis probe implanted in the POA 1 d before the experiment. The microdialysis probe was perfused with artificial cerebrospinal fluid (aCSF) at 1 μl/min. At 1010 h on the fourth day, OVX and OVX+E rats received a further injection of oil (OVX, n = 6; OVX+E, n = 6) or progesterone (OVX+P, n = 6; OVX+EP, n = 7). Microdialysates of 60 μl of aCSF were collected at 1000 h and hourly from 1200 h–1900 h for NE determination. Blood samples of 400 μl were withdrawn at 1000 h and hourly from 14:00–19:00 h for measurement of plasma LH. An equal volume of sterile 0.9% NaCl was replaced after removal of each blood sample. After the experiment, probes were perfused with 100 mM KCl to verify the integrity of brain tissue dialyzed (35).

Experiment 2: Effect of reverse dialysis of NE in the POA on the activity of AVPV neurons and LH surge in OVX+E rats

OVX rats were implanted with a microdialysis guide cannula in the right POA and treated with estradiol as described in experiment 1. The jugular vein was cannulated and a microdialysis probe implanted in the POA 1 d before the experiment. The microdialysis probe was continuously perfused with aCSF at 1 μl/min. On the fourth day, the probe was perfused with vehicle (n = 5), 10 nM (n = 6) or 100 nM (n = 4) NE between 1200 h and 1800 h. Blood samples of 400 μl were withdrawn at 1000 h and hourly from 1400 h–1800 h for measurement of plasma LH. At 1800 h rats were transcardially perfused for evaluation of c-Fos expression in the AVPV and suprachiasmatic nucleus (SCN) (36). The SCN is under NE regulation (37) and, because of its anatomical proximity, was used as a control region for NE diffusion and effects outside the POA.

Experiment 3: Effect of estradiol and progesterone on the activity of NE neurons projecting to the POA

OVX rats were injected with 80 nl of 4% Fluoro-Gold (FG) in the right POA. Ten days after FG injection, rats were treated with oil (OVX) or estradiol (OVX+E) daily for 3 d. On the

fourth day, OVX rats received an injection of oil (OVX, n = 5), and OVX+E rats received injection of oil (OVX+E, n = 4) or progesterone (OVX+EP, n = 7) at 1000 h. Rats were transcardially perfused at 1600 h, when blood samples were collected from the left ventricle for LH measurement. The brains were immunohistochemically processed for c-Fos, FG, and tyrosine hydroxylase (TH) triple labeling in the A1, A2, and LC. The percentage of FG/TH-immunoreactive (ir) neurons expressing c-Fos was used as an index of neuronal activation (36).

Experiment 4: Effect of DSP-4 on the surge of LH in OVX+E and OVX+EP rats

DSP-4 causes a rapid and selective lesion of NE terminals from the LC, with nearly maximal effects found 6 h after drug administration (38-41). OVX rats were implanted with a guide cannula into the right lateral cerebral ventricle. Rats were treated 7-10 d after surgery, with estradiol daily for 3 d and received an injection of oil (OVX+E) or progesterone (OVX+EP) at 1000 h on the fourth day. The jugular vein was cannulated 1 d before the experiment. On the fourth day, OVX+E and OVX+EP rats received an i.c.v. injection of 20 μg DSP-4 (42) (n = 5-7 per group) or vehicle (n = 6-7 per group) at 0800 h. Blood samples of 200 μl were withdrawn at 0800 h and at 30-min intervals from 1300 h–1800 h for plasma LH measurement. At 1800 h rats were decapitated, and levels of NE and its metabolite, 3-methoxy-4-hydroxyphenylglycol (MHPG), were determined in microdissections of the POA and medial basal hypothalamus-median eminence (MBH-ME).

Anesthetic and antibiotic treatment

Stereotaxy, ovariectomy, and transcardial perfusion were performed under ketamine (80 mg/kg, ip) and xylazine (10 mg/kg, ip) anesthesia, except for experiment 3, in which pentobarbital sodium anesthesia was used for perfusions. For jugular vein cannulation, rats were anesthetized with tribromoethanol (250 mg/kg, ip). After surgeries, rats were treated with pentabiotic (Fort Dodge, Campinas, Brazil; 0.2 ml/rat, im) and analgesic (Flunixin meglumine; 2.5 mg/Kg, sc).

Hormonal treatment

Ovariectomy was performed immediately after the stereotaxic surgeries. Estradiol cypionate (Pfizer, São Paulo, Brazil; 10 μg/0.2 ml/rat, sc; experiments 1, 2, and 4), 17β-estradiol (Sigma-Aldrich, St. Louis, MO; 10 μg/0.2 ml/rat, sc; experiment 3), and progesterone (Sigma-Aldrich; 2.5 mg/0.2 ml/rat, sc) were diluted in corn oil for hormonal treatments. The regimens of hormonal treatment used yield physiological levels of plasma 17β-estradiol and progesterone and consistently generate preovulatory-like surges of LH (33).

Microdialysis

The microdialysis guide cannulas (CMA 12; CMA/Microdialysis, Stockholm, Sweden) were implanted such that the tips were placed in the rostral portion of the right POA (incisor bar, −3.3 mm, anteroposterior (A/P) + 0.4 mm from bregma, mediolateral (M/L) 0.4 mm from the midline, dorsoventral (D/V) −5.8 mm from dura) (43). Microdialysis was performed in conscious rats (24). The microdialysis probe (CMA 12 Elite, 2 × 0.5 mm) was perfused with aCSF (145 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 2.5 mM Na_2HPO_4, pH 7.3). In experiment 1, microdialysates were collected in tubes containing 3 μl of antioxidant solution (1 M $HClO_4$ and 2 mM EDTA), and NE levels were immediately analyzed. The *in vitro* noradrenaline recovery of the microdialysis probes used was 19.1 ± 0.9%, determined before the first use of each probe. For reverse dialysis, NE (norepinephrine bitartrate, Sigma-Aldrich) solution was prepared in aCSF with 0.1% ascorbic acid (pH 7.3). Perfusions with 10 and 100 nM NE corresponded to infusion rates of approximately 100 and 1000 pg/60min, respectively. Although we do not know the relative recovery of NE *in vivo*, it is expected to be about 2-3 times lower than that *in vitro* (44). Thus, the dose of 100 nM NE was selected to yield extracellular levels of NE similar to those found in OVX+EP rats, whereas 10 nM caused only a slight increment in extracellular NE. The anatomical location of probes was checked in all experiments.

FG microinjection

A 32-gauge stainless steel needle (LASI Needle; Hamilton, Reno, NV) was lowered into the brain toward the rostral portion of the right POA (A/P + 0.1 mm from bregma, M/L 0.4 mm from the midline, D/V −8.0 mm from dura), and 80 nl of 4% FG (Fluorochrome, LLC, Denver, CO), diluted in 0.9% NaCl, was injected with an microinjection pump over a period of 1 min, with the syringe left in place for 10 min after injection before withdrawal of the needle. To determine the anatomical location of FG injection sites, coronal sections of POA were processed for FG single labeling and analyzed under a light microscope (43).

DSP-4 microinjection

Intracerebroventricular cannulation was performed as previously described (45). A 22-gauge stainless steel guide cannula was implanted in the right cerebral lateral ventricle (A/P −1.0 mm from bregma, M/L 1.6 mm from the midline, D/V −3.2 to −3.7 mm from the skull) and attached to the bone with stainless steel screws and acrylic cement. DSP-4 (Sigma-Aldrich) was dissolved in 0.01 M PBS (pH 7.4) and injected at the dose of 20 μg/3 μl via a 30-gauge needle during 1 min.

HPLC-electrochemical detection

Microdialysates were analyzed for NE levels using the previously described method with modifications (24). Separation was performed on a 150 × 2.0-mm C18 column. The mobile phase consisted of 50 mM NaH_2PO_4, 10 mM NaCl, 0.1 mM EDTA, 5.6 mM sodium 1-octanesulfonic acid, and 0.2% acetonitrile (pH 3.5). The pump flow rate was 0.45 ml/min and the detector potential was 0.55 V. Standard calibration curves were made for NE quantification on each experiment day. The intra and interassay coefficients of variation were 1.7 and 7.3%, respectively. The lower limit of detection was 0.5 pg. The concentrations of NE and MHPG in microdissections of the POA and MBH-ME were measured as previously described (46). All samples from each brain area were measured in the same analysis. The intraassay coefficient of variation was 2.5% for NE and 6.5% for MHPG. In microdissections, NE levels represent neurotransmitter stock in synaptic vesicles, whereas MHPG reflects release of NE, and MHPG/NE ratio represents the turnover rate (47).

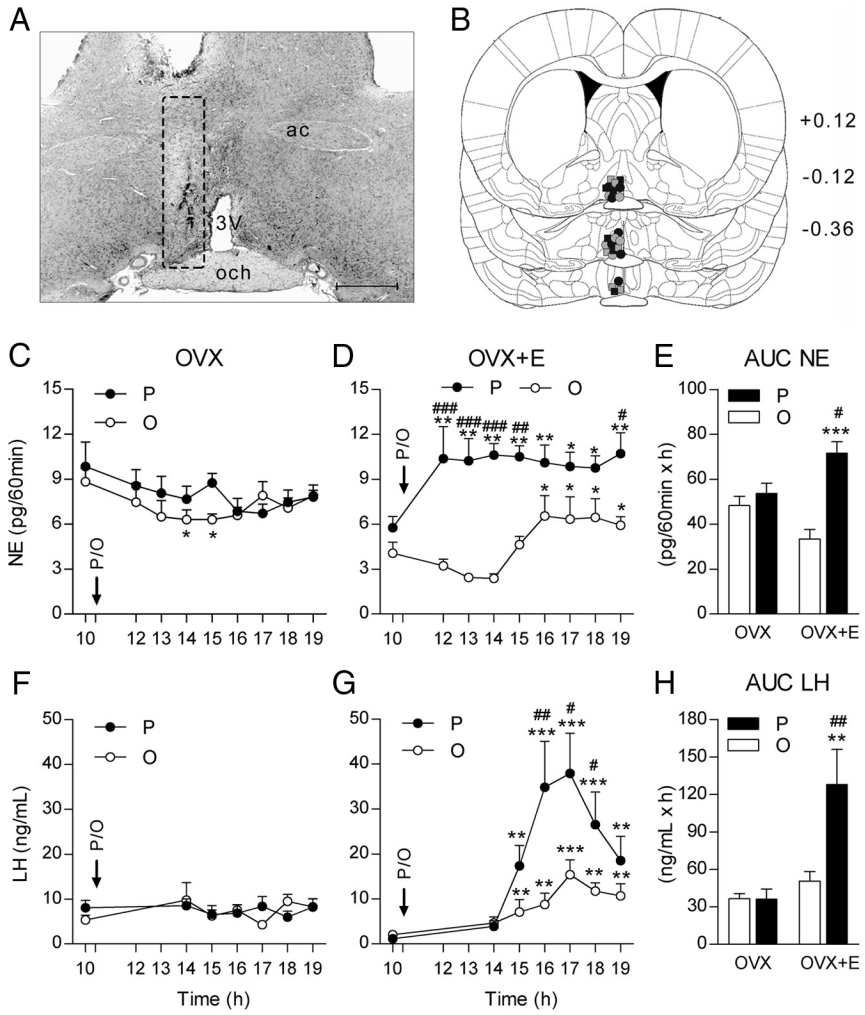

FIG. 1. NE release in the POA is regulated by estradiol and progesterone and correlates with LH surge. OVX rats were treated with oil (OVX) or estradiol (OVX+E) for 3 d. On the fourth day, OVX and OVX+E rats received a further injection of oil (O) (OVX, n = 6; OVX+E, n = 6) or progesterone (P) (OVX+P, n = 6; OVX+EP, n = 7) at 1010 h (*arrow*). A, Photomicrograph of Nissl-stained coronal brain section illustrating the microdialysis probe placement in the POA (*dotted line*). B, Schematic diagram representing location of probe tips in the POA, within +0.12 and −0.36 mm from bregma (43). Symbols represent probes in OVX (*gray square*), OVX+P (*black square*), OVX+E (*gray circle*), and OVX+EP (*black circle*) rats. C and D, Microdialysates of 60 μl were collected at 1000 h and hourly from 1200 h–1900 h for NE determination. OVX: *, $P < 0.05$ compared with 1000 h. OVX+E: *, $P < 0.05$ compared with 1200 h–1400 h. OVX+EP: #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ compared with OVX+E; *, $P < 0.05$; **, $P < 0.01$ compared with 1000 h. E, AUC of NE levels between 1200 h and 1900 h. ***, $P < 0.001$ compared with OVX+E; #, $P < 0.05$ compared with OVX+P. F and G, Plasma LH levels at 1000 h and hourly from 1400 h–1900 h. **, $P < 0.01$; ***, $P < 0.001$ compared with 1000 h; #, $P < 0.05$; ##, $P < 0.01$ compared with OVX+E. H, AUC of LH levels between 1400 h and 1900 h. **, $P < 0.01$ compared with OVX+E; ##, $P < 0.01$ compared with OVX+P. Data shown as mean ± SEM. *Scale bar*, 500 μm. 3V, Third ventricle; ac, anterior commissure; och, optic chiasm.

Immunohistochemistry

Rats were transcardially perfused with PBS followed by 4% paraformaldehyde. Four series of 30-μm frontal sections were obtained for each brain region evaluated (43). Single labeling of c-Fos in sections from the AVPV and SCN was performed as previously described (33). Briefly, sections were incubated with anti-c-Fos rabbit antibody (Ab-5; Calbiochem, EMD Chemicals, Gibbstown, NJ) at 1:15,000, biotinylated antirabbit goat IgG (Vector Laboratories, Burlingame, CA) at 1:600, and Elite ABC kit at 1:100 (Vector Laboratories). A solution of nickel sulfate (Ni; 25 mg/ml) and 3,3′-diaminobenzidine-HCl (DAB; 0.2 mg/ml) was used as chromogen. Omission of the primary antibody resulted in no labeling. Brain sections were blindly analyzed for experimental groups with an image analysis system. The number of c-Fos-ir neurons was quantified bilaterally in the AVPV (three sections between +0.12 and −0.12 mm from bregma) (48) and SCN (two sections between −0.60 and −0.84 mm from bregma). Boxes delimited which neurons were counted (AVPV: width, 80 μm from third ventricle; length, 160, 240, or 360 μm; SCN: 120 × 120 μm). Triple labeling of c-Fos, FG, and TH was performed as previously described (49). Briefly, sections of A1 and A2 were obtained caudally to the obex to evaluate the NE neurons as opposed to those of C1 and C2 (50). Sections were incubated with anti-c-Fos rabbit antibody (Ab-5; Calbiochem) at 1:15,000, biotinylated antirabbit goat IgG (Vector Laboratories) at 1:600, Elite ABC kit, and revealed with Ni-DAB. Sections were then incubated with a cocktail containing anti-FG rabbit antibody (AB 153; Chemicon, Temecula, CA) at 1:15,000 and anti-TH mouse antibody (MAB 318; Chemicon) at 1:80,000, followed by Alexa Fluor 488-coupled antirabbit goat IgG and Alexa Fluor 555-coupled antimouse goat IgG (Molecular Probes, Eugene, OR). Omission of primary antibodies resulted in no labeling, and FG staining was absent in sections from rats not injected with FG. Images were taken with a fluorescent microscope using an image analysis system. The number of TH-, FG/TH- and c-Fos/FG/TH-ir neurons was quantified in the right side (ipsilateral to FG injection) of the A1, A2, and LC (three sections per region) by an experimenter unaware of the experimental groups. Confocal microscopy was used to confirm triple labeling, with stacks of confocal images captured at 1.5-μm intervals. The c-Fos image was obtained using differential interference contrast, inverted, and pseudo-colored to blue for illustration purpose (see Fig. 4). Sections from the POA were incubated with the anti-FG rabbit antibody and revealed using Ni-DAB for analyses of the injection sites.

RIA

LH was assayed by double-antibody RIA with kits provided by the National Hormone and Peptide Program (Harbor-UCLA, Torrance, CA). The antiserum and reference preparation for LH were antirat LH-S10 and LH-RP3, respectively. All samples from

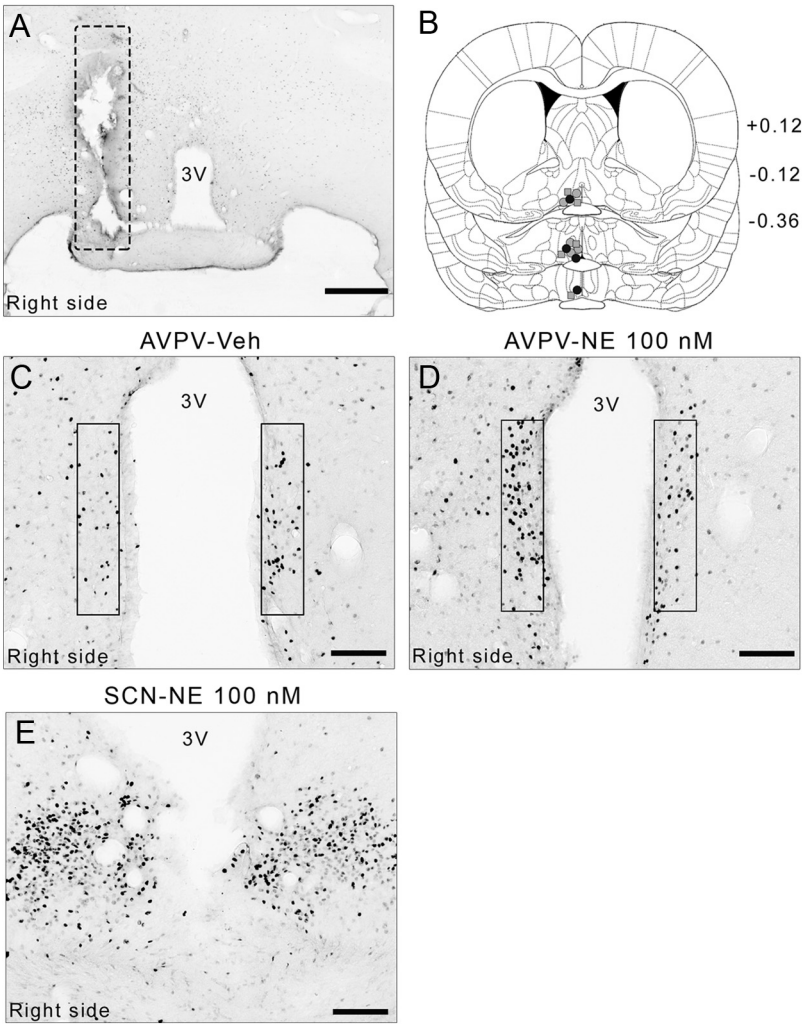

FIG. 2. Reverse microdialysis of NE in the POA and c-Fos expression in the AVPV nucleus. OVX+E rats were dialyzed with vehicle (Veh), 10 nM or 100 nM NE from 1200 h–18:00 h. A, Photomicrograph of coronal brain section illustrating the microdialysis probe placement in the right POA of a Veh rat (*dotted line*). B, Schematic diagram representing location of probe tips in the POA. Coronal sections correspond to +0.12 to −0.36 mm from bregma (43). Symbols represent probes in Veh (*gray square*), 10 nM NE (*gray circle*), and 100 nM NE (*black circle*) rats. c-Fos immunoreactivity in the AVPV of a Veh rat (C), and in the AVPV (D) and SCN (E) of a rat dialyzed with 100 nM NE. Note increased c-Fos expression in the right AVPV of a 100 nM NE rat (ipsilateral to NE dialysis). *Scale bars*, 500 μm (A) and 100 μm (C–E). The right side of the brain (ipsilateral to microdialysis probe) is indicated in the photomicrographs. 3V, Third ventricle.

each experiment were measured in the same assay. The lower limit of detection was 0.08 ng/ml, and the intraassay coefficient of variation was 4.2%.

Statistical analysis

Data are presented as mean ± SEM. NE and LH data were analyzed by two-way or one-way ANOVA. Differences among times within the same experimental group were further determined by one-way ANOVA for repeated measures. Integrated responses, expressed as the area under curve (AUC), and immunohistochemical data were compared by two-way or one-way ANOVA. In all analyses, ANOVA was followed by the Bonferroni *post hoc* test. $P < 0.05$ was considered statistically significant.

Results

Effect of estradiol and progesterone on NE release in the POA

This experiment aimed to determine the specific effects of estradiol and progesterone on NE release in the POA during the positive feedback. Figure 1 shows the effect of ovarian-steroid treatment on NE release and LH secretion. Microdialysis probes were placed in the rostral portion of POA (Fig. 1, A and B). In OVX rats, two-way ANOVA did not detect effect for time or progesterone on extracellular NE levels (Fig. 1C), although they were slightly decreased at 1400 h –1500 h in OVX rats ($P < 0.05$). Estradiol decreased NE levels at 1000 h in OVX+E compared with OVX rats ($P < 0.001$). NE release in OVX+E rats was altered as a function of time ($P < 0.05$) and progesterone ($P < 0.001$; Fig. 1D). NE levels were higher in OVX+EP than in OVX+E rats at 1200 h–1500 h and 1900 h. In OVX+E rats, NE levels started to increase at 1500 h and were higher at 1600 h–1900 h compared with 1200 h–1400 h ($P < 0.001$). In OVX+EP, progesterone treatment increased NE levels from 1200 h–1900 h ($P < 0.01$). As determined by the AUC, integrated release of NE was notably higher in OVX+EP rats compared with the other groups ($P < 0.001$; Fig. 1E). Dialysis with 100 mM KCl elicited a large increase in NE release with no difference among groups (OVX: 69.1 ± 18.1; OVX+P: 56.2 ± 6.1; OVX+E: 72.6 ± 14.4; OVX+EP: 72.2 ± 10.1 pg/60min). Neither time nor progesterone altered plasma levels of LH in OVX rats (Fig. 1F). In OVX+E, LH secretion was modified by time ($P < 0.001$) and progesterone ($P < 0.001$; Fig. 1G). LH levels were higher in OVX+EP than in OVX+E rats at 1600 h–1800 h. Although in different magnitudes, both OVX+E and OVX+EP rats displayed LH surges between 1500 h and 19:00 h. Overall, the integrated secretion of LH was greater in OVX+EP than in the other groups ($P < 0.01$; Fig. 1H). Thus, the dynamics of NE release in the POA and LH secretion into the peripheral circulation were coincident.

Effect of reverse dialysis of NE in the POA on the activity of AVPV neurons and LH surge in OVX+E rats

This experiment evaluated the effect of increased release of NE in the POA on the estradiol-induced LH surge. The reverse dialysis of 100 nM NE in the POA of OVX+E rats produced extracellular levels of NE similar to those found in OVX+EP rats. Figure 2 shows the location of microdialysis probes in the right POA and photomicrographs of c-Fos staining in the AVPV and SCN. Plasma levels of LH were altered as a function of time ($P < 0.001$) and NE dialysis ($P < 0.001$). All experimental groups displayed LH surges between 1500 h and 1800 h, but LH levels were consistently greater between 1600 h and 1800 h in rats dialyzed with 100 nM NE (Fig. 3A). Integrated secretion of LH was also increased by 100 nM NE ($P < 0.05$; Fig. 3B). Moreover, NE at 100 nM markedly increased the number of c-Fos-ir neurons in the right AVPV, ipsilateral to microdialysis probe, compared with the contralateral side ($P < 0.05$) and other experimental groups ($P < 0.05$; Fig. 3C). No effect of NE dialysis was found in the SCN ($P = 0.43$; Fig. 3D).

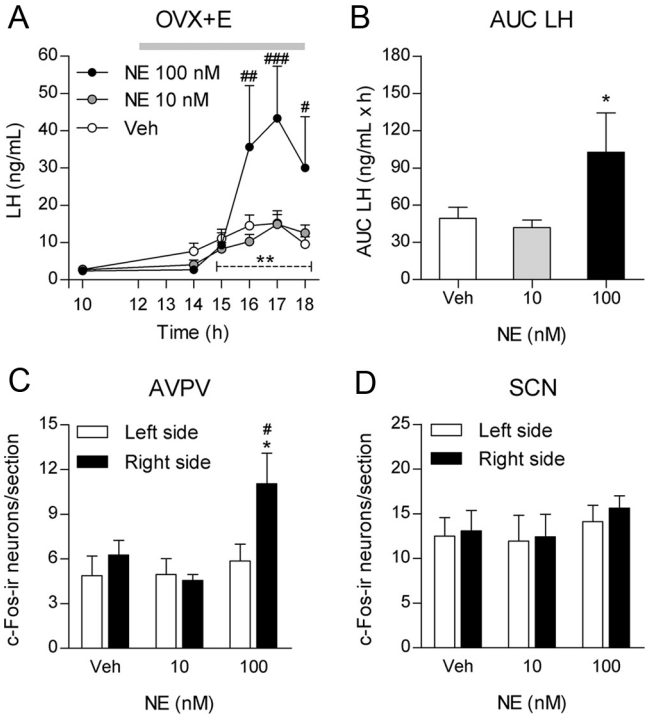

FIG. 3. Reverse dialysis of NE in the POA stimulates the LH surge and increases c-Fos expression in the AVPV nucleus in OVX+E rats. The microdialysis probe placed in the right POA was perfused with vehicle (Veh, n = 5), 10 nM NE (n = 6), or 100 nM NE (n = 4) from 1200 h–1800 h (*gray bar*). A, Plasma LH levels at 1000 h and hourly from 1400 h–1800 h. **, $P < 0.01$ compared with 1000 h for Veh, NE 10 nM, and NE 100 nM. #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ compared with Veh and NE 10 nM. B, AUC of LH levels between 1400 h and 1800 h. *, $P < 0.05$ compared with Veh and 10 nM NE. C and D, Number of c-Fos-immunoreactive (ir) neurons/section in the right and left sides of AVPV and SCN nuclei. *, $P < 0.05$ compared with the *left side* of NE 100 nM. #, $P < 0.05$ compared with the *right side* of Veh and NE 10 nM. Data shown as mean ± SEM.

Effect of estradiol and progesterone on the activity of NE neurons projecting to the POA

We investigate which groups of brainstem NE neurons projecting to the POA were activated by estradiol and progesterone during the positive feedback. Figure 4 shows a representative photomicrograph of FG injection in the right POA (panel A) and the location of injection sites (panel B). Confocal images illustrate the c-Fos/FG/TH triple labeling in the LC, A2, and A1 (Fig. 4, C–O). There was virtually no expression of c-Fos in FG/TH-ir neurons of A1 at 1600 h (Fig. 5A). In the A2, the percentage of FG/TH-ir neurons expressing c-Fos was higher in OVX+E and OVX+EP rats than in OVX rats, the FG/TH-ir neurons of which showed no c-Fos immunoreactivity ($P < 0.05$; Fig. 5B). In the LC, c-Fos expression in FG/TH-ir neurons was significantly increased only in OVX+EP rats ($P < 0.05$; Fig. 5C). The number of TH-ir and FG/TH-ir neurons was not different among groups (Table 1). Approximately 23%, 14%, and 5% of A1, A2, and LC neurons, respectively, expressed FG. c-Fos expression was not significantly detected in non-TH FG-ir neurons of A1 and A2 regions (data not shown). Plasma LH levels at 1600 h were higher in OVX+EP compared with OVX+E and OVX rats (55.3 ± 17.5, 7.8 ± 1.7, and 8.7 ± 3.1 ng/ml, respectively; $P < 0.05$).

Effect of DSP-4 on the surge of LH in OVX+E and OVX+EP rats

This experiment confirmed the role of LC NE on estradiol- and estradiol plus progesterone-induced LH surges. I.c.v. injection of DSP-4 was used to cause a selective lesion of NE terminals from the LC. Plasma LH in OVX+E rats changed as a function of time ($P < 0.001$) and was unaffected by DSP-4 ($P = 0.21$; Fig. 6A). Vehicle and DSP-4 rats displayed similar LH surges between 1600 h and 1800 h. In OVX+EP rats, LH secretion was altered by time ($P < 0.001$) and DSP-4 treatment ($P < 0.001$). LH levels were significantly reduced by DSP-4 between 1430 h and 1600 h. Both DSP-4- and vehicle-treated rats exhibited the afternoon rise in LH levels ($P < 0.01$), but the magnitude of the surge and integrated LH secretion were decreased by DSP-4 to the levels found in OVX+E rats (Fig. 6, B and C). Two-way ANOVA determined a main effect for DSP-4 on NE levels in the POA ($P < 0.05$), reflected by a slight decrease of approximately 10% in both OVX+E and OVX+EP rats. This reduction, however, was significant only for $P < 0.1$ when compared with the respective vehicles by the *post hoc* test (Fig. 6D). On the other hand, DSP-4 significantly decreased POA MHPG

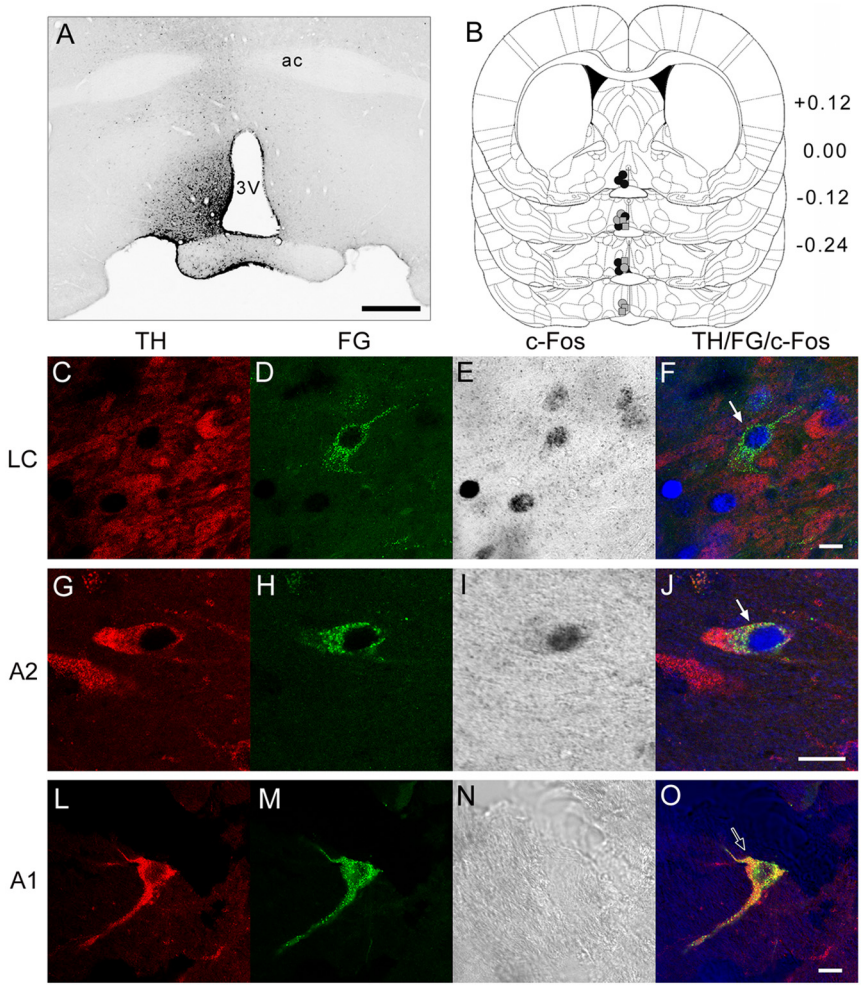

FIG. 4. Triple labeling of c-Fos, FG, and TH in the LC, A1, and A2. A, Photomicrograph of FG injection in the right POA. B, Schematic diagram representing the location of FG injections. Coronal sections correspond to +0.12 to −0.24 mm from bregma (43). Symbols represent the injection sites in OVX (*gray square*), OVX+E (*gray circle*), and OVX+EP (*black circle*) groups. C–O, Confocal images of TH (*red* fluorescence), FG (*green* fluorescence), and c-Fos (bright field) triple labeling in the LC (C–F), A2 (G–J), and A1 (L–O). F, J, and O, Overlapping images. *Solid arrows* indicate triple-labeled neurons. *Open arrow* indicates a FG/TH double-labeled neuron. *Scale bars*, 500 μm (A), 10 μm (F, J, and O). 3V, Third ventricle; ac, anterior commissure.

(25%) and MHPG/NE ratio (21%) in OVX+EP ($P < 0.05$) but not OVX+E rats (Fig. 6, E and F). Additionally, there was no effect of DSP-4 on the MBH-ME (Table 2), consistent with the smaller degree of LC projections to the hypothalamus (51).

Discussion

Before the middle of the last century, NE was implicated in the induction of the ovulation-inducing release of LH (for historical references see Ref. 52). We have provided new findings on the role of NE during the positive feedback of estradiol and progesterone. NE release in the POA was increased coincident with the LH surge in OVX+E rats. Progesterone treatment enhanced the release of NE and the surge of LH in OVX+EP rats. Reverse dialysis of NE in the POA of OVX+E rats stimulated LH release, generating a surge of secretion like that of OVX+EP rats. NE also increased c-Fos expression in AVPV neurons, suggesting this indirect pathway may be implicated in the stimulatory effect of NE on GnRH neurons. The increased release of NE in the POA seems to depend on selective effects of estradiol and progesterone on A2 and LC neurons, respectively. Moreover, the importance of LC NE to the progesterone-induced amplification of LH surge was confirmed with DSP-4 lesion of LC terminals, which resulted in smaller surges of LH in OVX+EP rats, but had no effect in OVX+E rats.

The microdialysis probes and FG injections were placed in the rostral and ventral portion of the POA, so that extracellular NE levels and neuronal projections correspond to the vicinity of nuclei mainly implicated in the positive feedback, such as the AVPV (53). The dialysis of KCl elicited a large increase in NE release, which does not occur in lesioned brain areas (35). The LH surges of OVX+E and OVX+EP rats were very similar to those found in rats not undergoing microdialysis or serial blood sampling, and the LH surge of OVX+EP rats was equivalent in size to the preovulatory surge of proestrus (33). Taken together, these data confirm that the eventual lesion caused by implantation of the microdialysis probe did not alter the function of the POA/AVPV region. Because acute stress alters both POA NE (54) and the surge of LH (55), the physiological patterns of NE release and LH secretion reported also confirm that the studies were performed in unstressed rats. Estradiol exerted a biphasic effect on NE release in the POA. Levels of NE and LH during the morning were lower in OVX+E compared with OVX rats, consistent with our previous report that estradiol rapidly decreases NE release in the POA, whereas i.c.v. NE prevents the estradiol-induced suppression of LH release (46). The increased release of NE by the time of the LH surge is consistent with activation of POA NE by estradiol during the positive feedback (22). Although NE release is known

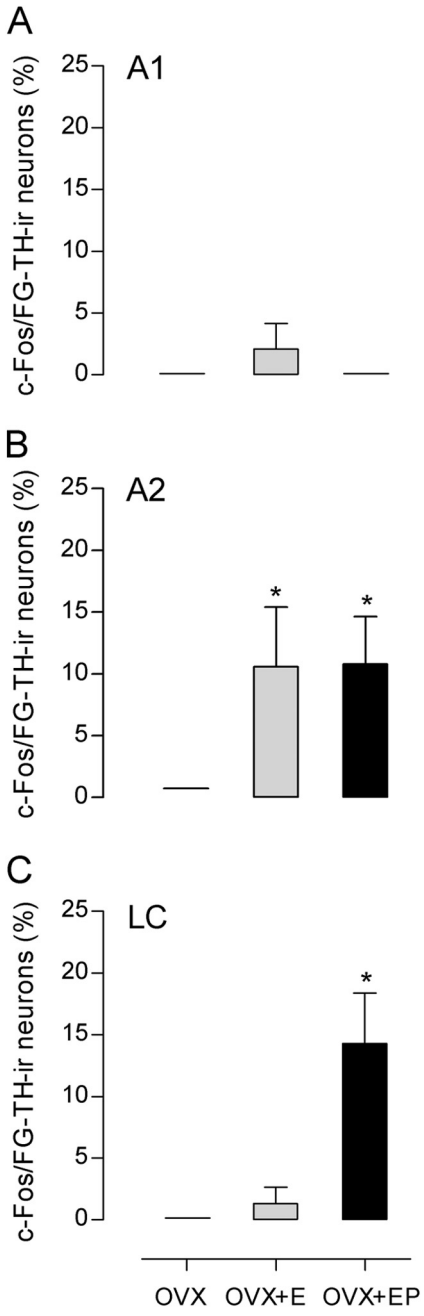

FIG. 5. Estradiol and progesterone selectively activate A2 and LC neurons that project to the POA. Ten days after FG injection in the POA, OVX rats were treated with oil (OVX) or OVX+E for 3 d. On the fourth day, OVX rats received an injection of oil (OVX, n = 5), and OVX+E rats received injection of oil (OVX+E, n = 4) or progesterone (OVX+EP, n = 7) at 1000 h, after which they were perfused at 1600 h. Mean ± SEM percentage of FG/TH-ir neurons expressing c-Fos in the A1 (A), A2 (B), and LC (C). A2: *, $P < 0.05$ compared with OVX. LC: *, $P < 0.05$ compared with OVX and OVX+E.

TABLE 1. Number of TH-ir neurons and TH-ir neurons colocalized with FG in the right side of A1, A2, and LC

		Groups		
Brain area	**Neurons**	**OVX**	**OVX+E**	**OVX+EP**
A1	TH-ir	12.8±1.2	12.8±1.4	12.4±0.8
	FG/TH-ir	2.1±0.4	3.2±0.4	3.3±0.4
A2	TH-ir	21.9±2.8	33.3±4.7	25.4±2.1
	FG/TH-ir	2.3±1.1	5.3±0.8	4.6±1.4
LC	TH-ir	79.5±10.2	96.6±11.1	79.3±16.9
	FG/TH-ir	3.1±0.4	5.1±0.7	4.6±0.7

OVX rats injected with FG in the right POA were treated with oil (OVX, n = 5), estradiol (OVX+E, n = 4), or estradiol plus progesterone (OVX+EP, n = 7) and perfused at 1600 h. Mean ± SEM number of TH-ir neurons and TH-ir neurons colocalized with (FG/TH-ir)/section in the right side of A1, A2, and LC (ipsilateral to the FG injection). Values did not differ significantly among groups.

to increase during the afternoon of proestrus and in OVX+EP rats (23, 24), our data show, for the first time, that estradiol and progesterone have distinct and cumulative effects on brainstem neuronal groups and POA NE. Progesterone treatment in OVX+EP rats consistently increased NE release, which was associated with generation of a physiological surge of LH. This effect seems to depend on the estradiol induction of PR (56), because progesterone has no effect in OVX+P rats.

AVPV neurons send projections to GnRH neurons and are synchronously activated at the time of the LH surge (7, 12). We showed that reverse dialysis of 100 nM NE in the POA of OVX+E rats increased the activation of ipsilateral AVPV neurons associated with stimulation of the LH surge, suggesting that the excitatory effect of NE upon GnRH neurons occurs through AVPV neurons. Furthermore, OVX+E rats dialyzed with 100 nM NE, used to reproduce the NE levels of OVX+EP rats, displayed LH surges like that of OVX+EP rats, which is consistent with a role for POA NE in the progesterone-induced amplification of the LH surge. NE diffusion was probably restricted to the right POA, as evidenced by the lack of effect on c-Fos expression in the contralateral AVPV or in the SCN, which is responsive to NE and activated by the time of LH surge (37, 57). Extracellular concentration of NE evaluated by microdialysis reflects synaptic release (58). However, whether NE released in the POA acts exclusively in the synaptic cleft or extrasynaptically by volume transmission is unknown. Dopamine-β-hydroxylase-ir fibers are found in close apposition to the AVPV kisspeptin neurons (59), suggesting that direct effects on AVPV neurons are possible but, alternatively, NE may also regulate AVPV neurons activating neuronal networks in the POA. This indirect stimulatory effect of NE on GnRH/LH secretion is in contrast to the reported direct inhibitory effect of NE on the excitability of GnRH neurons (31). It seems these opposing effects reflect two different mechanisms through which NE may stimulate or inhibit LH release (25, 28, 29). However, it is unknown how these effects are balanced and which one prevails in different physiological states. Our data suggest that during the positive feedback by ovarian steroids the indirect action through AVPV neu-

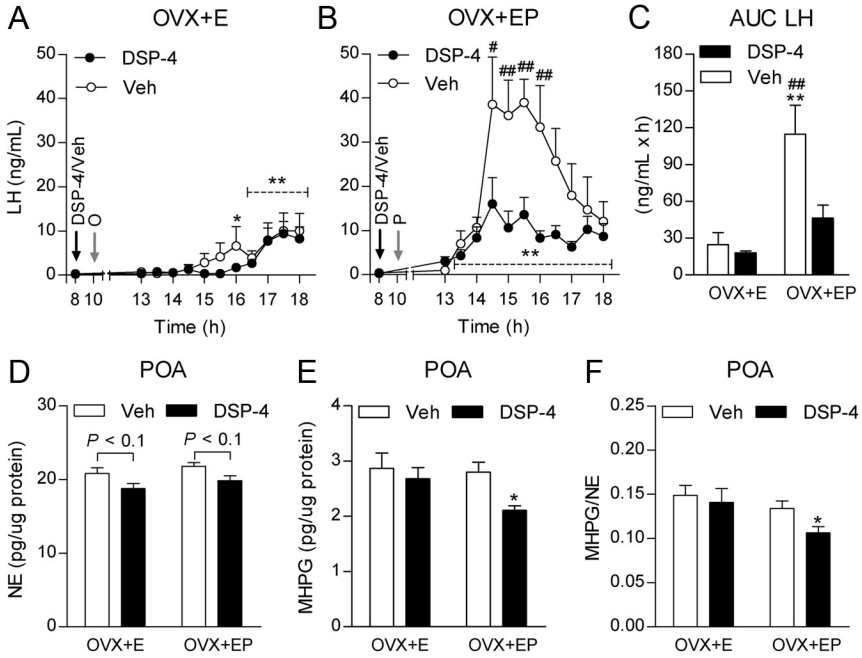

FIG. 6. Lesion of LC NE terminals with DSP-4 reduces the LH surge in OVX+EP but not in OVX+E rats. On the experiment day, OVX+E rats received an i.c.v. injection of 20 μg/3 μl DSP-4 or vehicle (Veh) at 0800 h (*black arrow*) and were injected with oil (O; OVX+E DSP-4, n = 5; OVX+E Veh, n = 7), or progesterone (P; OVX+EP DSP-4, n = 7; OVX+EP Veh, n = 6) at 1000 h (*gray arrow*). A and B, Plasma LH levels determined at 0800 h and at 30-min intervals from 1300 h–1800 h. OVX+E: *, $P < 0.05$ compared with 0800 h for Veh; **, $P < 0.01$ compared with 0800 h for Veh and DSP-4. OVX+EP: **, $P < 0.01$ compared with 0800 h for Veh and DSP-4; #, $P < 0.05$; ##, $P < 0.01$ compared with DSP-4. C, AUC of LH levels between 1300 h and 1800 h. **, $P < 0.01$ compared with OVX+EP DSP-4; ##, $P < 0.01$ compared with OVX+E Veh. (D) NE, (E) MHPG, and (F) MHPG/NE ratio in the POA at 1800 h. *, $P < 0.05$ compared with OVX+EP Veh. Data shown as mean ± SEM.

rons probably determines the stimulatory effect of NE on GnRH/LH release (25-27).

Although progesterone may advance the timing of estradiol-induced LH surge, regardless of when injected, the release of LH induced by progesterone is limited to the afternoon period (60). The reverse dialysis of NE and the DSP-4-induced lesion of LC terminals changed the magnitude but not the timing of the LH rise. It seems therefore that the release of NE induced by progesterone enhances the GnRH/LH secretion, determining a full-size LH surge, but does not alter the timing of the surge, probably governed by circadian neural signals (1). In addition, other neurotransmitters, such as opioid peptides, are possibly involved in the effects of progesterone on the timing and amplitude of the LH surge (61).

Consistent with previous studies (19, 20), A1 displayed a higher percentage of TH-ir neurons projecting to POA than A2 (23% and 14%, respectively). We found a lower percentage of LC neurons (5%) projecting to the POA. The relative number of LC neurons projecting to the POA is controversial, with values ranging between 1–15% reported (19, 20). This discrepancy may be explained by the differing gender of animals, given that the LC is a sexually dimorphic nucleus (62). We found a similar number of FG/TH-ir neurons per section in these three cell groups. As determined by c-Fos expression in FG/TH-ir neurons, A2 neurons projecting to the POA were activated by the time of LH surge in rats treated with estradiol (OVX+E and OVX+EP), whereas no response was found in A1. This finding implicates the A2 as the main cell group responsible for the estradiol-induced release of NE in the POA. Accordingly, neurons from A2 but not A1 that project to the POA express ERα (63), and estradiol stimulates TH mRNA expression in A2 by the time of the LH surge (64). On the other hand, LC neurons projecting to the POA, but not those in A1 or A2, were activated by progesterone treatment in OVX+EP rats, indicating the involvement of LC projections in the progesterone-induced release of NE. This result is consistent with the excitatory effect of progesterone on LC activity in female rats (33). Estradiol treatment failed to alter c-Fos expression in LC neurons. Indeed, estradiol seems to inhibit the electrical activity of LC neurons (33) but induces expression of both TH (15) and PR (18) in the LC. It seems that estradiol primes LC neurons for the stimulatory action of progesterone. Our data suggest therefore that activation of neuronal projections from both A2 and LC are probably responsible for the increased release of NE in the POA by the time of the preovulatory surge of LH in rodents, when circulating levels of estradiol and pro-

TABLE 2. NE and MHPG levels and MHPG/NE ratio in the MBH-ME

		i.c.v. treatment	
MBH-ME	**Groups**	**Veh**	**DSP-4**
NE	OVX+E	15.9 ±2.0	16.8 ±1.0
	OVX+EP	19.5 ±1.8	18.8 ±1.8
MHPG	OVX+E	2.2 ±0.2	2.0 ±0.1
	OVX+EP	2.3 ±0.3	1.9 ±0.1
MHPG/NE	OVX+E	0.15±0.03	0.12±0.01
	OVX+EP	0.11±0.01	0.11±0.02

OVX+E rats received an i.c.v. injection of 20 μg/3 μl DSP-4 or vehicle (Veh) at 0800 h and were treated with oil (OVX+E DSP-4, n = 5; OVX+E Veh, n = 7) or progesterone (OVX+EP DSP-4, n = 7; OVX+EP Veh, n = 6) at 1000 h. Blood samples were withdrawn at 0800 h and from 1300 h–1800 h, and rats were decapitated at 1800 h. Mean ± SEM (pg/μg protein) NE, MHPG, and MHPG/NE ratio in MBH-ME microdissections. Values did not differ significantly among groups.

gesterone are elevated (65). It is worth noting, however, that expression of c-Fos is transitory, peaking 1–2 h after cell activation (66). Thus, although our experimental paradigm revealed the main NE pathways activated during the positive feedback, the maximal degree of c-Fos expression may not have been detected by perfusion of rats at 1600 h, underestimating the actual neuronal response to each hormonal treatment.

According to our findings, the increased release of NE during the LH surge induced by estradiol appears to depend on A2 activation whereas the higher NE output during the surge induced by estradiol and progesterone was probably due to activation of both A2 and LC projections to the POA, resulting in a full, physiological surge of LH. We hypothesized therefore that the blockade of LC function would prevent this further increase in NE release and, consequently, decrease the LH surge proportionally to the contribution of LC NE. The i.c.v. injection of DSP-4, a neurotoxin that causes rapid and selective lesion of LC terminals (38-41), reduced the surge of LH in OVX+EP rats but had no effect in OVX+E rats. Furthermore, the size of the surge in OVX+EP rats treated with DSP-4 was equivalent to that in OVX+E rats. DSP-4 caused a slight decrease of tissue NE levels in the POA, indicating only a small lesion of NE terminals. Importantly, however, DSP-4 significantly reduced POA MHPG levels (25%) and MHPG/NE ratio (21%) only in OVX+EP rats. Thus, DSP-4 selectively impaired NE release in the POA of OVX+EP rats, because MHPG levels reflect NE release in brain microdissections (47). The decreased release of NE is associated with DSP-4-induced reduction of LH secretion, supporting the role of LC NE in the progesterone-induced amplification of the LH surge. Conversely, it has been reported that ip injection of DSP-4 (50 mg/kg) completely blocked the LH and GnRH mRNA increase in a similar model of estradiol plus progesterone-treated OVX rat (67). This blockade of the LH surge might be explained by the nonselective lesion of NE terminals not derived from the LC or other monoamine terminals that may occur with the dose of DSP-4 used (68).

We provide evidence that estradiol and progesterone selectively act upon A2 and LC neurons, respectively, and this is associated with the increased afternoon release of NE in the POA and the magnitude of the LH surge. NE stimulates the LH surge, at least in part, through activation of AVPV neurons. These findings contribute to elucidation of the role played by the NE system during the positive feedback by ovarian steroids on LH secretion and suggest the interaction between NE and AVPV neurons as a functional pathway for ovarian-steroid signaling in the brain.

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