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Chronic estradiol-17 β exposure suppresses hypothalamic norepinephrine release and the steroid-induced luteinizing hormone surge: Role of nitration of tyrosine hydroxylase

Badrinarayanan S. Kasturi^a, Sheba M.J. Mohan Kumar^{b,d}, Madhu P. Sirivelu^a, Andrew C. Shin^d, and P.S. Mohan Kumar^{a,c,d,*}

^aDepartments of Pathobiology & Diagnostic Investigation

^bPharmacology & Toxicology

^cGraduate Programs in Comparative Medicine and Integrative Biology and Neuroscience

^dCollege of Veterinary Medicine, Michigan State University, East Lansing, MI 48824

Abstract

Chronic exposure to estrogens is known to produce a variety of deleterious effects in women including breast and ovarian cancer and anovulation. In female rats, exposure to low levels of estradiol-17 β (E₂) decreases hypothalamic norepinephrine (NE) to suppress luteinizing hormone (LH) secretion and cause failure of ovulation. We hypothesized that E₂ exposure most likely decreases NE release in the medial preoptic area (MPA) of the hypothalamus to produce this effect and that this may be due to E₂-induced inflammatory changes in noradrenergic nuclei leading to nitration of an enzyme involved in NE synthesis. To test this, female Sprague Dawley rats were sham implanted or implanted with slow release E₂ pellets (20 ng/day) for 30, 60 or 90 days (E30, E60 and E90 respectively). At the end of the treatment period, the rats were implanted with a push-pull cannula in the MPA, ovariectomized and subjected to steroid priming to induce a LH surge. Perfusates were analyzed for NE levels using HPLC-EC. Blood samples collected simultaneously were analyzed for LH levels. We measured interleukin-1 β (IL-1 β) and nitrate levels in brainstem noradrenergic nuclei that innervate the MPA. In control animals, there was a marked increase in NE levels in response to steroid priming at 1600h that was reduced in the E30 group, and completely abolished after 60 and 90 days of E₂ exposure. LH profiles were similar to NE release profiles in control and E₂-treated animals. We found that IL-1 β levels increased in all three (A1, A2 and A6) noradrenergic nuclei with chronic E₂ exposure, while nitrate levels increased only in the A6 region. There was an increase in the nitration of the NE synthesizing enzyme in the MPA in this group as well probably contributing to reduced NE synthesis. This could be a possible mechanism by which chronic E₂ exposure decreases NE levels in the MPA to suppress the LH surge.

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*Corresponding author at: Michigan State University, Department of Pathobiology & Diagnostic Investigation, College of Veterinary Medicine, B336 Life Sciences Building, East Lansing, MI 48824, United States. Tel.: +1 517 353 2251; fax: +1 517 353 8915, mohankumar@cvm.msu.edu.

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Keywords

Estrogen; Medial preoptic area; Tyrosine hydroxylase; Nitric Oxide; Interleukin-1 β

1. Introduction

The preovulatory luteinizing hormone (LH) surge that occurs on the afternoon of proestrus is known to be essential for inducing ovulation in rats (Knobil, 1994) and for estrous cyclicity. The LH surge is produced due to the concerted effort of a number of factors including estradiol-17 β (E2) from the ovaries. The gradual increase in serum E2 levels produces a stimulatory effect on the brain stem, the hypothalamus and the pituitary to promote the LH surge on the afternoon of proestrus (Knobil, 1994). Although acute increases in E2 levels, as observed during estrous cycles, stimulates the LH surge, chronic exposure to estrogenic compounds is known to inhibit LH secretion (Armenti et al., 2008; Fernandez et al., 2009; Laws et al., 2000). The mechanism behind this effect is not clear.

During the estrous cycle, E2 influences the secretion of several neurotransmitters in the brain (Mueller and Nistico, 1989). These neurotransmitters in turn, act on gonadotropin releasing hormone (GnRH) neurons in the hypothalamus. When these neurons are activated, GnRH is released from their terminals, enters the portal circulation to stimulate gonadotrophs in the anterior pituitary resulting in an increase in LH secretion (Mueller and Nistico, 1989). Among the many factors that are influenced by E2 to stimulate the LH surge, we selected norepinephrine (NE) for the following reasons. Several studies using NE synthesis blockers (Drouva and Gallo, 1976), NE depletors (Kang et al., 1998), and neurotoxic lesioning (Hancke and Wuttke, 1979; Simpkins et al., 1979) have demonstrated a loss of the LH surge indicating that NE plays an important role in this phenomenon. Moreover, GnRH neurons of the hypothalamus are richly innervated by brainstem noradrenergic neurons (Wright and Jennes, 1993) and these noradrenergic neurons are also sensitive to E2 (Jennes et al., 1992; Kaba et al., 1983; Liaw et al., 1992) and have estrogen receptors (Herbison, 1997). Further, removing the influence of E2 by ovariectomy decreases NE levels in the hypothalamus and abolishes the LH surge (MohanKumar and MohanKumar 2004). Taken together, these studies suggest that E2 most likely acts through NE to stimulate GnRH neurons and thereby LH secretion. In contrast to the acute stimulatory effects of E2 on LH secretion, we have observed that chronic exposure to E2 suppresses LH secretion. This was accompanied by a reduction in NE levels in hypothalamic areas that are rich in GnRH neurons such as the MPA (Kasturi et al., 2009). The reason for the reduction in NE levels is not clear but it is possible that it could be due to reduced NE synthesis.

In a recent study, we provided evidence that chronic E2 exposure significantly decreased dopamine (DA) levels in the tuberoinfundibular dopaminergic (TIDA) system of the hypothalamus. TIDA neurons are located in the arcuate nucleus and their terminals are located in the median eminence. Chronic exposure to E2 induced an inflammatory reaction in the arcuate nucleus that resulted in increased generation of the cytokine interleukin-1 β (IL-1 β) and nitric oxide-related free radicals. This led to the nitration of tyrosine hydroxylase (TH). Since TH is the rate-limiting enzyme in catecholamine biosynthesis, nitration of this enzyme led to reduced TH activity resulting in decreased DA synthesis (MohanKumar et al., 2011). We hypothesized that a similar phenomenon may be in operation in noradrenergic neurons as well since DA and NE share a common biosynthetic pathway and TH is rate-limiting to the synthesis of both DA and NE (Nagatsu et al., 1964) resulting in reduction in NE in the MPA. TH positive neurons that innervate the MPA are localized in three distinct nuclei in the brainstem viz. the A1 (rostral ventrolateral medulla), A2 (nucleus tractus solitarius) and A6 (locus coeruleus) nuclei (Moore and Bloom, 1979).

TH that is synthesized in the brainstem noradrenergic neurons is probably transported to the MPA where it plays an important role in NE synthesis. Thus, chronic estradiol exposure-induced nitration of TH might be responsible for the reduction in NE in the MPA.

To investigate this possibility, young, intact Sprague-Dawley rats were exposed to low levels of E2 for 30, 60 or 90 days. Simultaneous changes in NE levels in the hypothalamus and LH levels in the serum were monitored. IL-1 β and nitrate, a stable end product of nitric oxide (NO) metabolism, were measured in brainstem noradrenergic nuclei that provide noradrenergic innervation to the MPA. Nitrated TH (NO-TH) was measured in the MPA. Results from this study will help us understand the possible molecular mechanisms by which chronic E2 exposure decreases NE biosynthesis to suppress LH secretion in female rats.

2. Results

Effects of chronic E2 treatment on estrous cyclicity

Effects of chronic estradiol exposure on estrous cycles are shown in Fig. 1. More than 95% of the control animals showed 4–5 day regular estrous cycles. Exposure to E2 for 30 days did not have any significant effect on estrous cyclicity (> 85% of the animals showed regular estrous cycles). In contrast, after 60 and 90 days of E2 exposure the percentage of regular cyclers declined to 35% and 20% respectively.

Location of the push-pull cannulae

Fig 2 depicts the histological location of push-pull cannulae in different treatment groups. Animals that did not have their cannula in the MPA were excluded from analysis.

Effects of Chronic E2 exposure on steroid-induced NE release profiles in the MPA

NE release profiles (pg/min; mean $\pm$ SE) in the MPA of sham-implanted animals are shown in Fig. 3a, panel A. In the OVX+oil group, NE release was 1.4 $\pm$ 0.3 at 1300 h and remained at that level during the rest of the observation period. In contrast, in the OVX+EP group, NE release was 2.9 $\pm$ 0.7 at 1300 h and increased gradually to reach a peak at 1500 h (9.2 $\pm$ 3.5; p <0.05) before decreasing to 4.9 $\pm$ 2.5 at 1700 h.

Effects of chronic E2 exposure on NE release profiles in the E30, E60 and E90 groups are shown in Fig 3a, panels B-D. In the E30 group, NE levels after OVX+oil treatment were 1.1 $\pm$ 0.06 at 1300 h and did not change throughout the evening. In the E30 group that was treated with OVX+EP, NE levels were 1.7 $\pm$ 0.1 at 1300 h and increased significantly to 3.5 $\pm$ 0.7 at 1530 h (p <0.05) and reached peak levels of 5.3 $\pm$ 1.3 at 1630 h (p <0.05). The marked difference in NE profiles between sham-implanted and the E30 group after EP treatment was that NE levels reached a peak at 1500 h in the former but reached peak levels only at 1630 h in the latter. In contrast to the sham-implanted and E30 groups, NE release in the MPA of E60 and E90 remained unaffected both in the OVX+oil and OVX+EP groups.

Fig 3b provides a comparison of average NE levels during the entire observation period in all the groups. Average NE levels (Mean $\pm$ SE; pg/min) in the oil-treated sham-implanted group was 1.38 $\pm$ 0.4 and was significantly different from the EP treated group (5.71 $\pm$ 0.69; p <0.0001). Average NE levels in the E30 group treated with EP (2.58 $\pm$ 0.45) was significantly lower than the sham+EP treated group (p <0.01), but was significantly higher than the E60+EP (0.92 $\pm$ 0.2) and the E90+EP (0.62 $\pm$ 0.11) groups (p <0.001). Average NE levels in the E60+oil and E90+oil groups were lower than the Sham+oil group.

Effects of chronic E2 exposure on steroid-induced LH

When sham-implanted animals were ovariectomized and treated with oil, serum LH (Mean $\pm$ S.E; ng/ml; Fig. 4a, panel A) was 2.3 ± 0.6 at 1300 h and remained at that level throughout the observation period. As expected, treatment of sham-implanted animals with OVX+EP increased LH levels markedly from 5.3 ± 3.6 at 1300 h to 252.4 ± 126.0 ($p < 0.05$) at 1600 h and kept it elevated at 1700 h (71.2 ± 42.5 ; $p < 0.05$) but declined at 1800 h. Thus a marked LH surge was evident after OVX+EP treatment in sham-implanted rats.

Effects of chronic E2 exposure on serum LH profiles in the E30, E60 and E90 groups are shown in Fig 4a, panels B-D. Serum LH levels in the E30 group that was treated with OVX +oil was 2.9 ± 0.7 at 1300 h and did not change the rest of the evening. In contrast, there was a marked LH surge in the E30 group treated with OVX+EP. In this group, LH levels were 1.6 ± 0.8 at 1300 h and increased significantly to 89.7 ± 64 at 1600 h ($p < 0.05$) and were 74.8 ± 37 at 1700 h ($p < 0.05$) before declining to 32.5 ± 15 at 1800 h. However, in the E60 and E90 groups, steroid priming did not change LH levels (Figs 4a, panels C and D).

Fig 4b demonstrates the differences in average LH levels between the different treatment groups. Compared to the rest of the groups, treatment of OVX sham-implanted rats with EP produced a robust increase in average LH levels (Mean $\pm$ SE; ng/ml; 61.86 ± 39.4 ; $p < 0.01$). There were no significant differences in LH levels between the sham-implanted and E30 groups after OVX+EP treatment. However, a marked reduction in LH levels were observed in E60 (0.24 ± 0.08) and the E90 (1.08 ± 0.2) groups after OVX+EP treatment when compared to the sham-implanted group ($p < 0.01$).

Effects of E2 treatment on IL-1 β levels in the brainstem noradrenergic nuclei

Since the NE and LH surges were suppressed in the E60 and E90 groups, we measured changes in IL-1 β and nitrate levels in these groups alone. In the A1 region, IL-1 β levels increased only after 90 days of exposure (88.2 ± 3.4) when compared to control levels (75.7 ± 4.6 ; $p < 0.05$). However, in the A2 and A6 regions, IL-1 β levels increased both after 60 days (101.7 ± 12.7 and 73.6 ± 5.5 respectively) and 90 days (106.2 ± 4.9 and 88.2 ± 3.4 respectively) when compared to controls (79.1 ± 8.2 and 43.4 ± 5.1 respectively; Fig 5).

Effects of E2 treatment on NO metabolism in brainstem noradrenergic nuclei

Total NO levels in the brainstem are shown in Fig 6. There were no differences in NO between control and estradiol-treated groups in A1 and A2. However, in the A6 region, NO levels (Mean $\pm$ S.E; μ M/ μ g protein) increased to $16.7 \pm .98$ in the E90 group when compared to the control group (12.3 ± 1.3 ; $p < 0.05$).

Effects of E2 treatment on nitration of TH in the MPA

Fig. 7A-B shows representative Western blots of TH and NO-TH in the MPA of control and E2-treated rats. The ratio of densities of NO-TH to TH is shown in Fig. 5C. As shown in the figure, the ratio of NO-TH to TH increased in a duration-dependent manner. In sham-implanted control animals the ratio was 0.3 ± 0.03 . Treatment with E2 for 30 days did not change the ratio significantly. However, treatment with E2 for 60 days increased the ratio of NO-TH to TH to 0.8 ± 0.2 and increased it further to 1.3 ± 0.3 in animals treated with E2 for 90 days ($p < 0.05$).

3. Discussion

In this study, we demonstrate that exposure to low levels of E2 on a chronic basis suppresses NE release in the MPA of the hypothalamus upon steroid priming. This is accompanied by a suppression of the LH surge in a duration dependent manner. Chronic E2 exposure also

increased IL-1 β and nitrate levels in specific noradrenergic nuclei that innervate the MPA. There was a concurrent increase in the nitration of tyrosine residues in TH, a modification that is known to inhibit the activity of this enzyme. This reduction in TH activity most probably contributes to decreased NE levels leading to a loss of the LH surge. These results suggest that a novel phenomenon involving inflammation, free radicals and TH inactivation is in operation in brainstem noradrenergic neurons after chronic E2 exposure. This could potentially contribute to loss of estrous cyclicity in E2 exposed animals. Although these results do not provide mechanistic evidence for the cascade of events that are presented, it provides insight into a possible sequence of events by which chronic E2 exposure could interfere with the preovulatory LH surge and estrous cyclicity.

E2 is an ovarian hormone that plays an important role in the female reproductive system. Acute increases in serum estrogen are critical for the stimulation of GnRH neurones to trigger the LH surge that is essential for ovulation (Caraty et al., 1995). On the other hand, chronic exposures to estrogens are believed to interfere with reproductive cycles (Biegel et al., 1998; Jesionowska et al., 1990; Kasturi et al., 2009; Rosa et al., 2003; Renner and Luine, 1986). Several studies in the past have examined the effect of chronic estrogen exposure on reproductive cycles and the LH surge. These studies involved various estrogenic preparations such as ethinyl estradiol, estradiol valerate, estradiol benzoate and estradiol-17 β at doses ranging from a few micrograms to milligrams. The duration of exposure ranged from a few days to several weeks (reviewed in (Karsch, 1987)). These studies identified that acute exposure to estrogens initially suppressed LH levels followed by stimulation of LH secretion. On the other hand, long term exposures effectively suppressed LH secretion (Karsch, 1987). In the present study, we used a very low dose of E2 (20ng/day) and exposed animals to varying durations ranging from 30 to 90 days. This dose was chosen because it produced serum estradiol levels (~50pg/ml) that were equivalent to those observed during the morning of prestrous (Lapolt et al., 1986; Kasturi et al., 2009) and it is much lower than the currently available no observed adverse effect level for E2 (5 μ g/kg) (Snyder, 2008). It is also close to the acceptable daily intake levels published by the Joint FAO/WHO committee (50ng/kg) (Joint FAO/WHO expert committee, 2004). Results from our study indicate that even these low levels of estrogen are capable of affecting estrous cycles after 60 days of exposure. We have observed that similar doses and exposures of E2 result in significant increases in serum E2 (Kasturi et al., 2009; Mohankumar et al., 2011). This increase in E2 levels could have a negative impact on LH secretion as described below.

Chronic E2 treatment can interfere with LH secretion in both intact animals and the ovariectomized, steroid-primed rat model (Kasturi et al., 2009; Tsai and Legan, 2001; Tsai and Legan, 2002). The mechanism by which E2 exposure suppresses LH secretion is not clear. Since GnRH neurons are regulated by a variety of neurotransmitters and neuropeptides (Ciechanowska et al. 2010), E2 could affect one or more of these mediators to suppress LH secretion. We chose to look at the involvement of NE in this mechanism because NE is known to be an important player in LH secretion (Barracough, 1983). We have found that similar doses of E2 as used in this study can indeed decrease NE concentrations in the MPA (Kasturi et al., 2009). In the present study, we took this a step further and examined the effect of low dose E2 exposure on NE release using push-pull perfusion of conscious, freely moving animals. We found that E2 exposure not only decreases NE release in response to steroid priming in a duration-dependent manner, it also suppresses basal NE release in the oil-treated groups. While exposure for 30 days had minimal effects on NE release, exposure for 60 and 90 days suppressed the basal and steroid-induced increase in NE release that occurs at the time of the LH surge. These results are supported by other studies that have observed reductions in NE turnover (Hiemke et al., 1983) and release (Legan and Callahan, 1999) after varying durations and doses of estrogen exposure.

The reduction in NE levels observed in the E-60 and E-90 groups correlates well with the lack of a LH surge in these animals. However, the mechanism by which E2 causes a reduction in NE levels in the MPA is not clear. It is very likely that the prolonged exposure to E2 causes de-sensitization or downregulation of E2 receptors in the brainstem and/or hypothalamus. We considered the possibility that chronic E2 exposure may impair NE synthesis. There is evidence to indicate that E2 treatment incites an inflammatory response in the hypothalamus (Brawer et al., 1978). We have also observed an increase in the levels of a pro-inflammatory cytokine, IL-1 β , in the arcuate nucleus of the hypothalamus after similar exposures to E2 (Mohankumar et al., 2011). Therefore, it is possible that the E2 exposure paradigms that we used in this study could increase IL-1 β levels in brainstem noradrenergic nuclei. Our results indicate that indeed, E2 exposure increased IL-1 β levels in all three noradrenergic nuclei. However, this resulted in an increase in nitrate levels only in the A6 region. The reason for this difference is not clear but it could relate to differential IL-1 signaling (Sirivelu et al., 2012) or nitric oxide synthesis in these nuclei. Since the exposures are chronic it is likely that free radical scavenging mechanisms are activated and these could affect the levels of nitrates in the brainstem nuclei. The increase in IL-1 β and nitric oxide-related free radicals could affect noradrenergic activity in the MPA as described below.

NE synthesis is under the tight control of TH which is the rate-limiting enzyme in the synthesis of NE (Nagatsu et al., 1964). Peroxynitrite, one of the products of nitric oxide metabolism facilitates the formation of nitrotyrosine, which, results in nitration of tyrosine residues in proteins (Huie and Padmaja, 1993; Ischiropoulos et al., 1995). This is especially true of proteins like TH that contain several tyrosine residues in their structure (Ara et al., 1998). Since the tyrosine moieties in TH are clustered around the active center of the enzyme, nitration of these tyrosine residues is believed to cause steric hindrance, resulting in reduced activity of this enzyme (Imam et al., 2001) and decreased NE synthesis. Although we did not measure TH activity, measurement of NO-TH could provide insights into the possible reasons for chronic E₂-induced reduction in NE levels in the MPA. The results from the present study clearly indicate that there is a significant increase in the nitration of TH in the MPA of E₂-treated animals in a duration-dependent manner. It is possible that TH gets nitrated in the brainstem and is later transported to the MPA where it remains inactive. Nitration of tyrosine residues is an irreversible process (Drew and Leeuwenburgh, 2002). Since the E₂ exposures were chronic, it is likely that any new enzyme that is synthesized to make up for the loss of enzyme activity will probably be nitrated leading to continued suppression of NE synthesis. This probably accounts for the higher levels of NO-TH observed in the E-90 animals compared to E-60 and E-30 groups. As more NO-TH is generated, specific systems are activated for removal of nitrated proteins. NO-TH is supposed to be selectively degraded in vivo by chymotrypsin (Souza et al., 2000). Besides causing nitration of proteins, the NO that is generated could cause nitration of NE also (Daveu et al., 1997), leading to reduced binding to adrenergic receptors. Chronic E₂ exposures could interfere with these mechanisms as well and these needs to be investigated further.

In conclusion, the present study provides evidence that chronic exposure to low levels of E₂ is capable of suppressing the steroid-induced LH surge in a duration-dependent manner. This was accompanied by a reduction in NE release in the MPA. The confidence in our results is increased by the fact that both NE release and LH levels were measured in the same animals. Our study also provides evidence for a novel mechanism by which E₂ exposure suppresses NE release in the MPA. E₂ exposure increased IL-1 β and nitrate levels in the brainstem noradrenergic nuclei with a concurrent increase in the nitration of TH in the MPA. This could be responsible for the reduction in TH activity, leading to decreased NE

synthesis and LH secretion. Further studies are needed to determine the underlying mechanisms by which nitric oxide metabolism is activated in the brain stem.

4. Experimental Procedure

Animals

Adult female Sprague-Dawley rats, around 3 months of age, were obtained from Harlan Sprague-Dawley, Inc., (Indianapolis, IN, USA) and were housed in temperature ($23\pm 2^{\circ}\text{C}$) and light-controlled (lights on from 0500 to 1900 h) animal rooms. They were given food and water *ad libitum*. All the protocols followed in this study were approved by the IACUC at Michigan State University.

Treatment

Estrous cycles were monitored regularly and those animals showing regular 4- day cycles were chosen for the experiment. The animals were randomly divided into different treatment groups (final $n=6/\text{group}$ for push-pull perfusion studies and $n=7/\text{group}$ for measuring IL-1 β , nitrate and NO-TH). Animals in the control groups were sham-implanted and those in the treatment groups were implanted subcutaneously with slow-release E₂ pellets for 30 (E30 group), 60 (E60 group) or 90 (E90 group) days. The pellets were capable of releasing E₂ at the rate of 20 ng per day (Innovative Research America, Sarasota, FL). We have observed previously that serum E₂ levels after 30, 60 and 90 days of exposure are about 35, 70 and 95 pg/ml respectively (Kasturi et al., 2009). Estrous cyclicity was monitored daily as described before (Mohankumar et al., 1994b). At the end of the treatment period, animals used for IL-1 β , nitrate and NO-TH measurements were sacrificed at 1200 h when they were in the state of oestrus. Animals that were used for push-pull perfusion were treated as described below.

Push-pull cannula implantation and perfusion

At the end of the treatment period (30, 60 or 90 days of E₂/sham treatment), animals were bilaterally ovariectomized under pentobarbital anesthesia (Day-0). Simultaneously, these animals were also implanted with a push-pull cannula in the MPA as described previously (MohanKumar and MohanKumar, 2004). The push-pull cannulae were constructed as described before (Mohankumar et al., 1991). The animals were implanted with a cannula stereotactically in the MPA using the following coordinates: 8.5 mm ventral, 0.3 mm posterior and 0.3 mm lateral to the bregma (MohanKumar and MohanKumar, 2004). The cannula was secured with dental cement and a 29-g stainless steel stylet was introduced to prevent blockage due to gliosis. The animals were allowed to recover for seven days. On the eighth day, the animals were given 0.1 ml of either corn oil (OVX+oil) or E₂ (30 μg , s.c.) at 1000 hrs. On the 9th day, they were implanted with a jugular catheter between 3–5pm. On the 10th day, (the day of perfusion), animals in the OVX+oil group were given 0.1 ml of either corn oil while the remaining animals that had received E₂ earlier, were treated with progesterone (2 mg, s.c.; OVX+EP) at 1000 hrs.

The animals were connected to a peristaltic pump and perfusion was started around 1030 hrs as described previously (Mohankumar et al., 1991; MohanKumar and Quadri, 1993; Mohankumar et al., 1994b). Animals were conscious and freely-moving in the perfusion cages. Artificial cerebrospinal fluid was used as the perfusion medium at a flow rate of 10 $\mu\text{l}/\text{min}$. Perfusates were collected from 1300–1700 hrs at 30 min intervals. Perfusates were mixed with 0.5 M HClO₄ at a ratio of 25:1 v/v and stored at -70°C until analyzed for NE concentrations using HPLC-EC. Blood samples (200 μl) were collected from 1300–1800 hrs, at one-hour intervals through a jugular catheter. Plasma samples were stored at -70°C until they were analyzed for LH concentrations by RIA. At the end of perfusion, animals were

sacrificed, their brains were removed and frozen at -70°C until sectioned for histological verification of cannula location.

HPLC-EC

The following were the components of the HPLC-EC system used: a phase II, 5 μm ODS reverse phase C-18 column (Phenomenex, Torrance, CA, USA), a glassy carbon electrode, a CTO-10 AT/VP column oven, a LC-10 AT/VP pump (Shimadzu, Columbia, MD, USA), and a LC-4C amperometric detector (Bioanalytical Systems, West Lafayette, IN, USA). The mobile phase was made using nanopure water and it contained monochloroacetic acid (14.14 g/l), sodium hydroxide (4.675 g/l), octane sulfonic acid disodium salt (0.3 g/l), ethylenediamine tetraacetic acid (0.25 g/l), acetonitrile (3.5%) and tetrahydrofuran (1.4%). The mobile phase was filtered and degassed through a Milli-Q purification system (Millipore, Bedford, MA, USA) and pumped at a flow rate of 1.8 ml/min. The sensitivity of the detector was 1 nA full scale, and the potential of the working electrode was 0.65 V. The column was maintained at 37°C . The perfusate (90 μl) was mixed with 30 μl of the internal standard (0.05 M dihydroxy benzylamine) and 100 μl of the mixture was injected into the system. The HPLC system was capable of detecting $<1\text{pg}$ of NE. NE levels in the perfusate were expressed as pg/min.

Brain sectioning

A cryostat (Slee, London, UK) was used to obtain serial brain sections. Brains were sectioned at 40 μm thickness and stained using cresyl violet to determine the location of the cannula tip (Mohankumar et al., 1994b). Only those animals with the cannula in the MPA were included in the study. Brains from the groups used for measuring IL-1 β , nitrate and NO-TH were sectioned at 300 μm and the MPA and brainstem noradrenergic nuclei were microdissected using Palkovits' microdissection technique (Palkovits et al., 1975) with a 500 μm punch. Tissue punches were stored at -70°C until analyzed for IL-1 β , nitrate and NO-TH as described below.

IL-1 β ELISA

IL-1 β levels were measured in control, E-60 and E-90 groups. Tissue punches were homogenized in phosphate buffer (pH 7.4). Fifty μl of the homogenate was used in duplicate for measuring IL-1 β using a commercial ELISA kit (Enzo Life Sciences, Farmingdale, NY). Samples were assayed according to the manufacturer's directions. The sensitivity of the assay was $<12\text{pg/ml}$. IL-1 β was expressed as pg/ μg protein.

Measurement of total NO generation in brainstem noradrenergic nuclei

NO levels were measured in control, E-60 and E-90 groups. Total NO levels in the brainstem nuclei were measured using a commercial kit that used the Griess reaction (Total nitric oxide assay kit, Assay Designs Inc., Ann Arbor, MI). Since NO is transient by nature and cannot be measured easily, the Griess reaction uses nitrate reductase to convert NO ultimately to nitrate that is measured photometrically. The sensitivity of the assay was 0.625 $\mu\text{M/L}$. Total NO produced is expressed as $\mu\text{M}/\mu\text{g}$ protein.

Immunoprecipitation of Tyrosine Hydroxylase and detection of Nitrated-TH

TH was immunoprecipitated from tissue samples from the MPA and subjected to western blotting as described previously (Ara et al., 1998; Mohankumar et al.). Briefly, MPA samples were homogenized in cell lysis buffer and incubated with Rabbit Anti-rat Tyrosine Hydroxylase antibody (0.5 μg ; Chemicon Intl., Temecula, CA.) overnight at 4°C . Protein-A Agarose slurry (100 μL) was added to the mixture and incubated at 4°C for 1.5 hours. The mixture containing the antigen:antibody complex was centrifuged at 14000 rpm for 10

minutes and TH was eluted using 30 μ L of the elution buffer (0.2M glycine, pH 3). The supernatant containing TH was divided into two equal halves and loaded onto two separate 20% SDS- polyacrylamide gel. After electrophoresis, the gels were blotted onto nitrocellulose membranes, and one membrane was probed with anti-rat tyrosine hydroxylase (1:1000 dilution, Chemicon Intl. Temecula, CA) and the other with rabbit anti- nitrotyrosine, (1:1000 dilution, SIGMA, St. Louis, MO). Bands were detected using 4-Chloro-1-Naphthol (Biorad, Hercules, CA). TH and NO-TH concentrations were quantified after densitometric scanning using a Kodak Digital Science Image analysis system (Kodak, Rochester, NY). A ratio of pixel intensities was used to express the ratio of TH to NO-TH.

LH-RIA

LH was measured in duplicates in the plasma by RIA using a double antibody method as described before (MohanKumar et al., 1994a). The standards (RP1) and antibody (Anti rLH-S11) were obtained from Dr. A.F.Parlow, NHPP, NIDDK. The LH tracer was obtained from Amersham Pharmacia Biotech (Waukesha, WI, USA). Briefly, 40 μ L of the serum was assayed in duplicate. The first antibody was added at a dilution of 1:758,000. The intra-assay variability of the LH RIA was 5.2%. LH levels were expressed as ng/ml.

Protein measurements for tissue samples

Protein concentrations in tissue homogenates of the MPA and brainstem noradrenergic nuclei were determined using a micro Bicinchoninic acid assay (Pierce, Rockford, IL). Prior to the IL-1 β and nitric oxide assays, tissue samples were homogenized in PBS and 20 μ L aliquots were used for the protein assay according to the manufacturer's protocol.

Statistical Analysis

Differences in NE release, and serum LH, profiles between different treatment groups and across different time points within each group were determined using two-way repeated measures ANOVA followed by post hoc Fisher's LSD test. Differences in nitrate, IL-1 β and the ratio of NO-TH to TH were analyzed by one-way ANOVA followed by post hoc Fisher's LSD test.

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Highlights

- ▶ Chronic estradiol exposure induces inflammatory changes in the brainstem.
- ▶ This results in reduced norepinephrine levels in the hypothalamus.
- ▶ This leads to the suppression of the luteinizing hormone surge.

Fig. 1. Effects of chronic E₂ exposure on estrous cyclicity. Animals were sham-implanted (control) or implanted with 20 ng/day of E₂ pellets subcutaneously for 30 (E-30), 60 (E-60) or 90 (E-90) days.

Fig. 2.

Locations of push-pull cannula in the medial preoptic area in animals from various groups are indicated in the schematic representation. Sham-implanted animals that were injected with either oil or estradiol and progesterone (E+P) are indicated by open and closed circles respectively. E-30 animals that were injected with oil and EP are indicated by open and closed triangles respectively. E-60 animals treated with oil and EP are indicated by open and closed rectangles respectively. E-90 animals treated with oil and EP are indicated by open and closed pentagons respectively. A1-P3 represents coronal sections in reference to the Bregma. MPA: Medial preoptic area; StHy: striohypothalamic nucleus; MPO: medial preoptic nucleus; AVPO: anteroventral preoptic nucleus; Sch: suprachiasmatic nucleus; OX: optic chiasm; SOX: supraoptic decussation; LA: lateroanterior hypothalamic nucleus; AH: Anterior hypothalamus; VMH: ventromedial hypothalamus.

A **B** **C** **D**

Fig. 3.

a Norepinephrine (NE) release (mean $\pm$ SE, pg/min) in the MPA of OVX, sham-implanted animals (Panel A), 30- (Panel B), 60- (Panel C) and 90- days (Panel D) E2- treated animals, that were OVX and treated with oil (n=6) or EP (n=6). * Significant difference ($p<0.05$) compared to 1300 hrs in the same group and at all time points in the oil-treated group. **Fig 3b** Average NE release in all groups during the entire period of observation. * indicates significant difference from rest of the groups. a indicates significant decrease from control EP but significant increase compared to the rest of the groups. b indicates significant reduction when compared to the control-oil group.

Fig. 4.

a Serum luteinizing hormone (LH) levels (mean±SE, ng/ml) in OVX, sham-implanted animals (Panel A), 30- (Panel B), 60- (Panel C) and 90- days (Panel D) E2- treated animals, that were OVX and treated with oil (n=6) or EP (n=6). * Significant difference ($p<0.05$) compared to 1300 and 1400 hrs in the same group and at all time points in the oil-treated group. **Fig 4b** Average LH levels in all groups during the entire period of observation. * indicates significant difference from rest of the groups. a indicates significant decrease compared to control+EP group, but significant increase compared to the rest of the groups.

Fig. 5. Effects of varying durations of E2 exposure on IL-1 β levels in brainstem noradrenergic nuclei. Clear bars represent the control group, grey bars represent the E- 60 group and black bars represent the E-90 group. * indicates $p < 0.05$ when compared to the corresponding control group.

Fig. 6. Effects of chronic E2 exposure on total nitric oxide levels measured as nitrate in the brainstem (Fig B). Clear bars represent the control group, grey bars represent the E-60 group and black bars represent the E-90 group. * indicates $p < 0.05$ when compared to corresponding control group.

Fig. 7. Effects of chronic E₂ exposure on nitration of tyrosine hydroxylase (TH) in the MPA. A-B. Representative Western blot showing immunoprecipitated TH (A) and NO-TH (B) in the MPA of control, E-30, E-60 and E-90 group. C. The ratio of NO-TH to TH in the MPA of control, E-30, E-60 and E-90 group. * Significant difference ($p < 0.05$) compared to control and E-30 groups.