Chronic estradiol exposure induces oxidative stress in the hypothalamus to decrease hypothalamic dopamine and cause hyperprolactinemia

Sheba M. J. MohanKumar, Badrinarayanan S. Kasturi, Andrew C. Shin, Priya Balasubramanian, Ebony T. Gilbreath, Madhan Subramanian and Puliyr S. MohanKumar

Neuroendocrine Research Laboratory, Departments of Pathobiology and Diagnostic Investigation & Pharmacology and Toxicology, Neuroscience Graduate Program, College of Veterinary Medicine, Michigan State University, East Lansing, MI 48824

Running title: Oxidative stress mediates estradiol-induced changes in TIDA neurons

Keywords: Chronic estradiol exposure, dopamine, prolactin, nitration, tyrosine hydroxylase

Funding sources: NIH AG 027697, NSF IBN 0236385, and Michigan Agricultural Experimental Station.

Authors have no conflicts to disclose

Precis: This study describes a novel mechanism involved in estradiol-induced hyperprolactinemia. This involves increases in cytokines, nitric oxide and nitration of tyrosine hydroxylase that decreases dopamine synthesis leading to hyperprolactinemia.

Address for correspondence:
P.S. MohanKumar, BVSc, PhD
Associate Professor
Neuroendocrine Research Laboratory
Department of Pathobiology and Diagnostic Investigation
College of Veterinary Medicine
B 336 B Life Sciences Building
Michigan State University
East Lansing, MI 48824
E-mail: mohankumar@cvm.msu.edu
Phone: 517-353-2251
Fax: 517-353-8915
Abstract

Estrogens are known to cause hyperprolactinemia most probably by acting on the tubero-infundibular dopaminergic (TIDA) system of the hypothalamus. Dopamine (DA) produced by TIDA neurons directly inhibits prolactin secretion and therefore, to stimulate prolactin secretion, estrogens inhibit TIDA neurons to decrease DA production. However, the mechanism by which estrogen produces this effect is not clear. In the present study, we used a paradigm involving chronic exposure to low levels of estradiol-17β (E₂) to mimic prolonged exposures to environmental and endogenous estrogens. We hypothesized that chronic exposure to low levels of E₂ induces oxidative stress in the arcuate nucleus (AN) of the hypothalamus that contains TIDA neurons and causes nitration of tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of DA. This results in a significant decrease in DA and consequently, hyperprolactinemia. To investigate this, adult, intact female cycling rats were implanted with slow release E₂ pellets (20 ng/day) for 30, 60 or 90 days and were compared with old (16-18 month old) constant estrous (OCE) rats. Chronic E₂ exposure significantly increased the expression of glial fibrillary acidic protein (GFAP) and the concentrations of interleukin-1β (IL-1β) and nitrate in the AN that contains perikarya of TIDA neurons and increased nitration of TH in the median eminence (ME) that contains the terminals. These levels were comparable to those seen in OCE rats. We observed a significant decrease in DA concentrations in the ME and hyperprolactinemia in an exposure-dependent manner similar to that seen in OCE rats. It was concluded that chronic exposure to low levels of E₂ evokes oxidative stress in the AN to inhibit TIDA neuronal function most probably leading to hyperprolactinemia.
Introduction:

Estrogens are pleiotropic hormones and have been shown to have several beneficial effects: Estrogen therapy can prevent bone loss (27) and decrease the risk of coronary disease (45). Estrogens can also provide neuroprotection during ischemic brain injury and Alzheimer’s disease and are believed to improve memory in postmenopausal women (13, 40, 43). In rats, both acute (5) and subchronic treatment (16, 23) with moderate to high doses of estradiol are known to increase Prolactin (PRL) levels. PRL levels also increase during aging, specifically after rats have been in the constant estrous state for 4-5 months (10). This increase in PRL levels is known to promote mammary and pituitary tumor formation (15, 17). Estrogen is known to act directly on the pituitary gland and also inhibit hypothalamic dopamine (DA) to stimulate PRL release (10). While several studies have examined the mechanism by which estrogen acts on the pituitary gland (9, 38), the possible mechanism by which estrogen decreases hypothalamic DA to inhibit PRL levels is not clear.

The purpose of this study was to understand the mechanisms by which hypothalamic DA levels are reduced after chronic exposure to estradiol at levels comparable to that seen during proestrus. The tuberoinfundibular dopaminergic (TIDA) system is one of the most estrogen-sensitive neuronal systems in the hypothalamus (37) and estrogen’s effects are known to be mediated through estrogen receptor-α (ER-α) (41). The cell bodies of TIDA neurons are located in the arcuate nucleus (AN) and their terminals reach the median eminence (ME). DA released from these terminals acts on the lactotrophs in the anterior pituitary to inhibit PRL secretion (3). A reduction in TIDA neuronal function results in decreased DA synthesis and causes hyperprolactinemia (29). This has been implicated in the development of mammary and pituitary tumors in aging animals (28, 29) and more recently in humans (11).

Although estrogens are known to impact DA levels in the hypothalamus (33), the
mechanism by which chronic estradiol exposure reduces TIDA neuronal activity is unclear. In this study, we propose a novel hypothesis that involves the induction of the cytokine, interleukin-1β (IL-1β) in the AN after chronic estradiol exposure. We chose this cytokine over others because IL-1β levels are known to increase in the brain in response to stressors (12). We propose that IL-1β causes the generation of nitric-oxide related free radicals that promote nitration of tyrosine hydroxylase, a key enzyme involved in DA synthesis. This would result in decreased DA production and elevated PRL levels. To test this, we used female Sprague Dawley rats and treated them for 30, 60 or 90 days with low doses of estradiol-17β (E2) that mimic endogenous levels of estrogen. Old rats that were in constant estrous were used for comparison purposes to determine if a similar phenomenon is in operation in aging animals.

Materials and Methods

Animals: Female Sprague-Dawley rats (4-5 months) were purchased from Harlan Sprague-Dawley, Indianapolis, Indiana and housed in air-conditioned (23 ± 1° C), and light-controlled (lights on from 0500 to 1900 hrs) animal quarters. Rats were provided food and water ad libitum. Estrous cyclicity was determined in young (3-4 months old) and old (16-18 months old) rats by vaginal cytology (24). Young animals showing 4-5 day regular estrous cycles and old animals that exhibited estrous smears for more than 10 consecutive days (old constant estrous-OCE) were used in the experiments. All the animal care and treatment protocols were approved by, and were followed in accordance with, the Michigan State University Institutional Animal Care and Use Committee (IACUC).

Treatment: A large cohort (n=30-40) of regularly cycling adult female rats (4-5 months) were either sham-implanted (control) or implanted with estradiol-17β (E2; 20 ng/day) slow-release pellets (Innovative Research America, Sarasota, FL) for a period of 30 (E-30), 60 (E-60) or 90 (E-90) days. Control animals were age-matched to the E-90 group. A
group of OCE rats (n=8-12) were used for comparison. Animals were checked for the presence of mammary tumors by periodic physical examination. Treatment with E\textsubscript{2} for 30, 60 or 90 days did not induce mammary tumors. About 20\% of the older animals had palpable mammary tumors and were excluded from the study. Vaginal cytology was performed for the large cohort. Most animals in the E-30 (80\%) and control groups (95\%) cycled regularly, they were sacrificed when they were in estrus at 1200 h. Most of the animals in E-60 and E-90 groups were in the state of constant estrous and were also sacrificed at 1200 h. OCE rats were sacrificed at 1200 h for comparison. Upon sacrifice, the brains were quickly removed, frozen on dry ice and stored at -70°C until the time of sectioning. Serum was separated from trunk blood and stored at -70°C until they were used for hormone assays. From the large cohort, tissues and serum from a subset (n=7) were used for measuring the following parameters.

**Radioimmunoassay for hormones:** E\textsubscript{2} and PRL levels in the serum were measured in duplicate by double antibody radioimmunoassay (RIA). The RIA kit for E\textsubscript{2} was obtained from Diagnostic Products Corporation (Los Angeles, CA). The assays were performed according to the manufacturer's instructions. The standards and antibody for rat PRL were obtained from Dr. A.F.Parlow, NHPP, NIDDK. The PRL tracer was obtained from Amersham Pharmacia Biotech (Waukesha, WI, USA). The assay was performed as described previously (24, 30).

**Palkovits’ microdissection:** The brains were mounted and serial sections of 300 µm thickness were obtained using a cryostat (Slee, London, UK) maintained at -10°C as described before (31, 32). The sections were transferred to glass slides placed on a cold stage set at -10°C. The AN and ME were located with the help of a rat brain stereotaxic atlas (35) and microdissected using the Palkovits’ microdissection technique (32, 34). Tissue samples
of the ME were removed first and the AN samples were obtained bilaterally using a 250µm
punch. They were stored at -70°C until they were analyzed for various factors.

**Measurement of glial fibrillary acidic protein (GFAP) and interleukin-1β (IL-1β) in
brain homogenates:** GFAP levels in the AN were measured using a sandwich ELISA based
on an assay developed by O’Callaghan (1991). Briefly, Immulon-2 flat bottomed microtiter
plates were coated with anti-GFAP N-18 (Santa Cruz, Cat # sc-6171, goat polyclonal; 1:100
diluted in I Ab dilution buffer) for 1h at 37°C and overnight at 4°C. After washing with PBS,
the next day, the wells were blocked for an hour at room temperature and washed. Standards
and samples were added in duplicate and incubated for 1 h at 37°C. Premixed anti-GFAP
antibody (DAKO, Carpinteria, CA) was used as the second antibody and incubated for 1 h at
37°C. After washing, an anti rabbit IgG HRP conjugate (Sigma, Cat # A1949) was added at
1:3000 dilution and incubated for 1 h at 37°C. Premixed TMB solution was used as the
developing reagent. The reaction was stopped with 2N HCl and the plate was read at 450 nm
using an ELISA reader. The rat IL-1β ELISA kit was purchased from Biosource
International (Camarillo, CA). The protocol supplied by the manufacturer was used to
perform this assay.

**Measurement of Nitric oxide generation in the AN:** A commercially available kit
(Total nitric oxide assay kit, Assay Designs Inc., Ann Arbor, MI) that involved the Griess
reaction was used to measure total nitrate levels in the AN. NO, by nature, is transient and
volatile and cannot be measured easily. However, its two stable breakdown products, nitrate
and nitrite can be measured colorimetrically. Tissue samples contain both nitrate and nitrite.
To measure the total NO generated, nitrate is enzymatically converted to nitrite by Nitrate
reductase and this is measured as a colored azo dye product. The assay quantifies the total
nitric oxide (NO) produced and has been used to measure NO generation by microglial cells
in mesencephalic cultures (44).

**Immunoprecipitation of Tyrosine Hydroxylase and detection of Nitrotyrosine residues by Western Blotting:** Immunoprecipitation of TH was carried out according as described by others (2). Microdissected ME tissue samples were homogenized in 50 μL cell lysis buffer (composition: 20 mM Tris-HCl, 150 mM NaCl, 4 mM EGTA, 10 % Glycerol, 1% Triton X-100, 1 mM PMSF, 0.2 mM Sodium orthovanadate, pH 7.4) and incubated with 0.5 μg of Anti-TH antibody (Chemicon Intl., Temecula, CA.) for 12 hours at 4°C. Protein-A Agarose slurry (100 μl; KPL, Gaithersburg, MD) 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 30 μL of the elution buffer (0.2 M Glycine, pH 3) was added to the pellet, mixed and left on ice for 5 minutes. The mixture was centrifuged at high speed for 10 minutes and 10 μL of the supernatant containing TH was loaded onto two separate 20% SDS- polyacrylamide gel and electrophoresed at 70 V for 1.5 hours. Gels were electro-blotted onto nitrocellulose membranes for 30 minutes at 22V. Membranes were immersed in blocking solution, probed with primary antibody (anti-TH, 1:1000 dilution, Chemicon Intl. Temecula, CA, or anti-nitrotyrosine, 1:1000 dilution SIGMA, Saint Louis, MO) overnight, and exposed to AntiRabbit- IgG tagged with horseradish peroxidase (SIGMA, Saint Louis, MO), for 4 hours. Bands were visualized using 4-Chloro-1-Naphthol (Biorad, Hercules, CA). Pixel intensities were determined by densitometric scanning using a Kodak Digital Science Image analysis system (Kodak, Rochester, NY).

**Measurement of DA concentrations:** Median eminence homogenates were analyzed for DA concentrations using HPLC-EC as described before (31, 32). Briefly, the HPLC-EC system consisted of the following: 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 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 a temperature of 37°C. At the time of analysis, ME punches were homogenized in 150 µl of 0.1 M perchloric acid. After separating the homogenates for protein analysis, they were centrifuged at 14000 x g for 5 minutes. One hundred µl of the supernatant along with the 30 µl of 0.05 M dihydroxy benzylamine (DHBA; internal standard) was injected into the HPLC system using an autoinjector. DA concentrations were determined using the Class-VP software version 7.2. The sensitivity of the system was <1 pg.

**Protein Assay:** Protein concentrations in the AN and ME homogenates were determined using a micro Bicinchoninic acid assay (Pierce, Rockford, IL). Concentrations of DA, GFAP, IL-1β and NO were expressed in terms of protein concentrations.

**Statistical analysis:** Differences in serum E2 and PRL, DA concentrations in the ME, GFAP, IL-1β and NO concentrations in the AN and the ratio of TH to nitrated TH in the ME were analyzed using one-way ANOVA followed by Fisher's LSD test.

**Results**

**Effects of chronic E2 exposure on estrous cyclicity and serum E2:**

**Estrous cyclicity:** Effects of E2 exposure on estrous cyclicity is shown in Fig. 1A. All the sham-implanted (control) animals had regular 4-5 day estrous cycles. Exposure to 30 days of E2 did not affect estrous cyclicity and 80% of the animals exhibited regular estrous cycles. In contrast, treatment with E2 for 60 or 90 days resulted in 74% and 88% of the animals becoming acyclic and these animals were in the state of constant estrus. All the OCE animals were in the state of constant estrus.
Serum estradiol: Effects of chronic E2 exposure on serum E2 levels are shown in Fig. 1B. Serum E2 levels (pg/ml; Mean ±S.E) in control animals were 32.3±2.3 and exposure to E2 for 30 days did not alter these levels. However, E2 exposure for 60 or 90 days resulted in a significant increase in serum E2 levels (67.2±8.4 and 90.2±9.2 in E-60 and E-90 groups, respectively) (p<0.05). This is most likely due to the presence of cystic follicles in the ovaries of these animals as previously reported (24). Similarly, serum E2 levels in OCE rats (94.2±22) were significantly higher compared to control and E-30 rats (p<0.05).

GFAP and IL-1β levels in the AN:

In control animals, GFAP concentrations in the AN (µg/mg protein; Fig. 2A) remained low at 3±0.7. Treatment with E2 for 30 days did not alter GFAP levels. In contrast, treatment with 60 or 90 days of E2 increased GFAP levels to 6.8±1 and 6.3±1 respectively (p<0.05). GFAP levels in OCE animals were also significantly high (5.6±1.4) compared to the control group (p<0.05).

Chronic exposure to E2 produced a similar effect on IL-1β levels in the AN(Fig. 2B). IL-1β concentrations in 30-day E2-treated rats (9.5±3.0) were similar to that seen in control animals (8.0±2). In contrast, exposure to E2 for 60 or 90 days increased IL-1β concentrations to 17.5±2.1 and 17.3±1.6 respectively (p<0.05) that was similar to that seen in OCE rats (15.8±2; p<0.05).

Total NO levels in the AN and TH and nitrated TH levels in the ME:

Total NO levels in the AN measured in terms of nitrite concentrations (µM/µg protein) of E2-treated and OCE animals are given in Fig. 3A. Exposure to E2 for 30 days did not alter NO levels in the AN compared to control rats. In contrast, E2 treatment for 60 or 90 days increased NO levels in the AN to 3.6±1.1 and 4.8±1.5 respectively compared to control rats (0.7±0.1; p<0.05). NO levels in the AN of OCE rats was also significantly higher compared to control rats (p<0.05).
Fig. 3B shows representative Western blots of TH and nitrated TH in control, E2-treated and OCE rats in the ME. The ratio of densities of nitrated TH to TH is shown in Fig. 3C. As shown in the figure, E2 increases the ratio of nitrated TH to TH in a duration-dependent manner. The ratio of nitrated TH to TH also increased significantly in OCE rats compared to control rats.

**DA concentrations in the ME and serum PRL:**

DA concentrations in the ME (pg/µg protein) in control, E2-treated and OCE animals are shown in Fig. 4A. DA concentrations in the ME were 263.5±72.0 in control animals and treatment with E2 for 30 days did not alter DA levels in the ME. In contrast, exposure to E2 for 60 or 90 days decreased DA concentrations significantly to 114.2±46 and 53.2±28 respectively (p<0.05). DA concentrations in the ME of OCE rats were also significantly lower (49.7±30) compared to control rats (p<0.05).

Serum PRL concentrations (ng/ml; Fig. 4B) in the control group was 3.5±0.4. Exposure to E2 for 30 days did not alter PRL levels. In contrast, exposure to E2 for 60 days produced a modest increase in PRL levels (24.5±11.7) and exposure for 90 days further increased PRL levels to 101.1±34.7 (p<0.05). Similarly, serum PRL levels were elevated in OCE rats (71.9±26.6) compared to animals in the control, E-30 and E-60 groups (p<0.5).

**Discussion**

Estrogens are one of the major factors that are known to promote hyperprolactinemia (6, 7, 8). Several estrogenic preparations including estradiol benzoate (22), estradiol valerate (5) and estradiol 17β (25) are known to stimulate PRL secretion. This is known to be brought about by either a direct stimulatory action on the pituitary gland (1) and through a reduction in hypothalamic DA, especially DA that is produced by TIDA neurons (37). The mechanism by which estradiol-17β affects TIDA neurons to increase serum PRL levels has not been
studied in detail. Results from this study demonstrate a novel possibility that estrogens could increase pro-inflammatory cytokines to stimulate the production of nitric-oxide related free radical production in the AN that would in turn cause nitration of TH and decrease DA synthesis in the ME. In the following paragraphs, we will explain how this cascade of events could take place.

DA synthesized by TIDA neurons is the primary regulator of PRL secretion. As described earlier, cell bodies of TIDA neurons are located in the AN and their terminals extend to the ME. DA released from these terminals passes through the portal vessels and acts on the lactotrophs in the pituitary to inhibit PRL secretion (3). Several studies indicate that TIDA neurons are sensitive to estrogen. E$_2$ has been shown to bind to TIDA neurons (26) most probably through estrogen receptor $\alpha$ (41). Moreover, acute estrogen treatment decreases TH mRNA levels in TIDA neurons suggesting that it can act directly on these neurons to decrease DA synthesis (36). However, this does not explain the reduction in DA levels observed with chronic exposures or aging. The results from this study indicate that E$_2$ produces a duration dependent reduction in DA synthesis and hyperprolactinemia that is very comparable to changes observed in aging and that these changes are accompanied by gradual alterations in glial-neuron interactions as described below.

We hypothesized that chronic exposure to low levels of E$_2$ causes gliosis in the AN that up-regulates the production of pro-inflammatory cytokines and NO. This is in fact supported by a few studies that have described degenerative changes in the AN after animal exposure to various forms of estrogen (5-7). These changes have been marked by the presence of reactive microglial cells and astrocytes (4). In the present study, chronic exposure to E$_2$ increases the levels of GFAP, a marker for astrocytes, in a duration-dependent manner indicating that E$_2$ exposure does activate astrocytes in the AN. The changes, observed in E$_2$-treated young animals, are very similar to what is observed in OCE rats. Aging has been
shown to increase the number of microglia and astrocytic granules in the AN of female rats that could be prevented by ovariectomy (39) suggesting that ovarian steroids, most probably estrogen, play an important role in this phenomenon. Moreover, estrogen receptors are expressed in different types of glial cells (34), suggesting the possibility that estrogen can act on glial cells. Young rats supplemented with estrogen are known to have similar changes in glia in the AN mimicking the glial hyperactivity observed in aging rats (5). The reactivity of astrocytes to estrogen exposure could translate into increased cytokine production as explained below.

Astrocytes and glial cells are capable of producing many cytokines including IL-1β (8). In the present study, activation of astrocytes by E2 exposure was accompanied by an increase in the production of IL-1β. This increase was duration-dependent suggesting that astrocytes continue to react to E2 exposure for extended periods of time. Cytokines, in turn, are known to cause elevation of inducible nitric oxide synthase (iNOS), nitrite and associated free radicals in the hypothalamus (18, 42). Our results demonstrate that chronic exposure to E2 increases the concentrations of nitrate, a stable product of the nitric oxide metabolism, in the AN in a duration-dependent manner. We hypothesize that the increases in IL-1β and nitrate probably play a critical role in decreasing TIDA neuronal activity. A recent study demonstrated that intracerebroventricular injections of lipopolysaccharide increased IL-1β production by microglial cells in the AN. This led to a reduction in TH activity and TH mRNA by 6 hours and increased PRL levels by 12 h (14). A similar phenomenon could be in operation with E2 exposure.

There is evidence to indicate that an increase in NO-related free radicals can increase nitration of tyrosine residues on tyrosine hydroxylase (TH), the rate limiting enzyme in DA biosynthesis (19, 21). Peroxynitrite, one of the products of NO metabolism, facilitates the formation of nitrotyrosine, which results in nitration of tyrosine residues in proteins (19, 21).
Nitration of tyrosine residues is one of the important hallmarks of NO-induced pathology and can lead to inactivation of protein function, particularly of proteins like TH that contain several tyrosine residues in their structure (2). The tyrosine moieties in TH are clustered around the active site of the enzyme (20). Nitration of the tyrosine residues in TH causes steric hindrance and inhibits the activity of this enzyme (20). In the present study, the ratio of nitrated TH to TH increased significantly after chronic E₂ exposure. Thus, it is possible that E₂-induced increase in NO metabolism probably caused nitration of tyrosine residues in TH. This most likely contributed to the reduction in DA levels in the ME and elevations and the increase in serum PRL levels that were observed in this study.

In conclusion, results from this study provide evidence that chronic exposure to E₂ activates astrocytes in the AN resulting in elevated levels of IL-1β and NO-related free radical production. This increase in oxidative stress is probably responsible for the nitration of tyrosine residues of TH observed in these animals. Nitration of TH, as mentioned before, is believed to be one of the hallmarks of NO–induced pathology. Thus, it is possible that estrogen-induced increase in NO production could be responsible for reduction in TIDA neuronal function (fig 5). The changes observed in young animals after prolonged E₂ exposure was similar to what was observed in OCE rats. Hyperprolactinemia and the development of spontaneous mammary and pituitary tumors is commonly observed in aging rats, especially after they have been in constant estrous for 4-6 months (37). Therefore, the results from this study provide important clues about how prolonged exposure to endogenous estrogens could decrease DA levels to produce hyperprolactinemia during aging.

Although this study appears to link independent molecular and cellular changes that occur in the AN after estrogen exposure, it nevertheless suggests a possible mechanism for E₂ effects and aging-induced decreases in TIDA neuronal activity and hyperprolactinemia. Mechanistic studies are in progress to provide a cause and effect relationship between these
different molecular and cellular components of this cascade.

**Perspectives and significance**

Estrogen exposure is known to inhibit TIDA neuronal function but the mechanisms are not clear. Results from this study suggest that chronic exposure to low levels of estradiol can increase IL-1β and nitric oxide-related free radicals in the hypothalamus. There was an associated increase in the nitration of tyrosine hydroxylase, and a reduction in dopamine levels. This could be a possible mechanism by which chronic exposures to low levels of estrogenic compounds can impact the TIDA system.
Acknowledgement:

This work was supported by NIH AG027697, NSF IBN0236385 and the Michigan Agricultural Experiment Station. The authors would like to thank Ms. Katrina Linning for editorial assistance.
References:


Pirolti GG, Grillo CA, Ferrini MG, Lux-Lantos V, and De Nicola AF. Antagonism by progesterone of diethylstilbestrol-induced pituitary tumorigenesis in Fischer 344 rats: effects on sex steroid receptors and tyrosine hydroxylase mRNA. *Neuroendocrinology* 63: 530-539, 1996.


Figure legend:

**Fig. 1:** A. Effects of chronic E₂ exposure on estrous cyclicity indicated as % of animals that showed regular estrous cycles. Adult young (4-5 month old) sham implanted (control) and 30 (E-30), 60 (E-60) or 90 (E-90) day slow release E₂ pellets implanted rats and OCE rats that are 15-19 months old were used. B. Serum E₂ levels (mean±SE; pg/ml) in young (4-5 month old) sham implanted (control) and 30 (E-30), 60 (E-60) or 90 (E-90) day slow release E₂ pellets implanted rats and OCE rats that are 15-19 months old. * indicates significant difference from control animals (p<0.05).

**Fig. 2:** A. Glial Fibrillary Acidic Protein (GFAP) concentrations (mean±SE; µg/mg protein) in the AN of sham implanted (Control) and 30, 60 or 90 day E₂ pellet implanted (E-30, E-60 and E-90) and OCE rats. * indicates significant difference from control animals (p<0.05). B. Interleukin-1β (IL-1β) concentrations (mean±SE; pg/µg protein) in the AN of sham implanted (control) and 30 (E-30), 60 (E-60) or 90 (E-90) day slow release E₂ pellets implanted rats and OCE rats that are 15-19 months old. * indicates significant difference from control animals (p<0.05).

**Fig. 3:** A. Total Nitrate concentrations (mean±SE; µM/µg protein) in the ME of sham (Control) and 30 (E-30), 60 (E-60) or 90 (E-90) day E₂ pellet implanted and OCE rats. * indicates significant difference from control animals (p<0.05). B. Representative western blots of tyrosine hydroxylase (TH) and nitrated TH in the ME of sham implanted (control) and 30 (E-30), 60 (E-60) or 90 (E-90) day slow release E₂ pellets implanted rats and OCE rats that are 15-19 months old. C. Ratio of nitrated TH to TH in the ME of sham implanted (Control) and 30 (E-30), 60 (E-60) or 90 (E-90) day E₂ pellet implanted and OCE rats. The intensities of the bands were calculated by measuring the pixel intensities that was determined using densitometric scanning with a Kodak Digital Science Image analysis system. * indicates significantly different from control animals (p<0.05).
**Fig. 4:** A. Dopamine (DA) concentrations (mean±SE; pg/µg protein) in the ME of sham implanted (Control) and 30, 60 or 90 day E2 pellet implanted (E-30, E-60 and E-90) and OCE rats. * indicates significant difference from control animals (p<0.05). B. Serum prolactin (PRL) concentrations (mean±SE; ng/ml) in sham implanted (control) and 30 (E-30), 60 (E-60) or 90 (E-90) day slow release E2 pellets implanted rats and OCE rats that are 15-19 months old. * indicates significant difference from control animals (p<0.05).

**Fig 5:** Figure depicting the possible mechanism by which chronic E2 exposure decreases TIDA activity to cause hyperprolactinemia. Chronic estradiol exposure activates glial cells in the AN and increases the production of cytokines. This stimulates NO-related free radical production that causes nitration of TH. This leads to a reduction in DA synthesis and an increase in PRL.
Fig 1

A

% animals

Control  E-30  E-60  E-90  OCE

B

Serum Estradiol (pg/ml)

Control  E-30  E-60  E-90  OCE

*
Fig 2

A

IL-1β in the Arc (pg/μg protein)

B

GFAP in the Arc (μg/mg protein)

Control  E-30  E-60  E-90  OCE

Il-1β: *

GFAP: *
Fig 3