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Estrogen inhibits tuberoinfundibular dopaminergic neurons but does not cause irreversible damage

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Abstract

Dopaminergic neurons of the hypothalamic tuberoinfundibular dopaminergic (TIDA) system exert a tonic inhibitory control on prolactin (PRL) secretion whereas estrogen, known to inhibit TIDA neuron function, has been postulated to be toxic to TIDA neurons when it is chronically high. In order to determine whether estrogen in high doses can cause permanent damage to TIDA function, we submitted young female rats to continue high doses of estrogen administered, either centrally (intrahypothalamic estrogen implants) or peripherally (subcutaneous estrogen implants or weekly intramuscular (i.m.) injections for 7 weeks), subsequently withdrawing the steroid and observing the evolution of lactotrophes, serum PRL and TIDA neurons. Serum PRL was measured by radioimmunoassay whereas tyrosine hydroxylase positive (TH+) neurons and PRL cells were morphometrically assessed in sections of fixed hypothalami and pituitaries, respectively. After 30 days, hypothalamic estrogen implants induced a significant increase in serum PRL, whereas TH+ neurons were not detectable in the arcuate-periventricular hypothalamic (ARC) region of estrogen-implanted rats. Removal of implants on day 30 restored TH expression in the ARC and brought serum PRL back to basal levels 30 days after estrogen withdrawal. Subcutaneous or i.m. administration of estrogen for 7 weeks induced a marked hyperprolactinemia. However, 30 weeks after estrogen withdrawal, TH neuron numbers in the ARC were back to normal and serum PRL returned to basal levels. After peripheral but not central estrogen withdrawal, pituitary weight and lactotrophic cell numbers remained slightly increased. Our data suggest that estrogen even at high doses, does not cause permanent damage to TIDA neurons.

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Conflict of interest
The authors declare that they have no competing financial interests.

Keywords

17-β estradiol; Hypothalamus; TIDA neurons; Aging; Reversible inhibition; Prolactin

1. Introduction

The dopaminergic (DA) neurons of the rat hypothalamus are grouped into two main areas, A_{12} and A_{14}, which include different nuclei [7,29]. The DA perikarya of the A_{12} area are located in the arcuate nucleus (ARC) (containing dorsal and ventro-lateral cellular groups) and in the periarcuate and periventricular (PeV) region (Fig. 1), with their axons projecting into the external zone of the median eminence (ME) [17]. There, dopamine is released from the axon terminals into the portal vessels of the ME, where the neurotransmitter is blood-borne to its target cells in the anterior pituitary lobe. The A_{12} area and its corresponding axon terminals constitute the tuberoinfundibular dopaminergic (TIDA) system which regulates prolactin (PRL) secretion by exerting a tonic inhibitory control on both lactotroph proliferation and function [2].

Estrogen exerts a physiological stimulatory action on PRL secretion, affecting lactotroph function by at least two mechanisms, (a) by a direct mitogenic action on the pituitary gland [30] and (b) by depressing the activity of TIDA neurons [9]. In the female, but not male rat, aging is associated with a marked increase in the incidence of pituitary prolactinomas and mammary tumors [8,18]. The prevalence of these pathological alterations begins to rise shortly after the first year of life [12], an age at which most females show irregular estrous cycles [15,19]. They are frequently characterized by extended periods of vaginal cornification, which are associated with sustained estrogen secretion, and low levels of circulating progestagens [16,20]. It has been suggested that in female rats, continuous exposure to moderately increased or medium levels of estrogens unopposed by progesterone leads to irreversible TIDA neuron damage and loss which would be the main mechanism leading initially, to increased PRL secretion and later to the development of PRL-secreting pituitary adenomas [21,26]. As indicated above, estrogen has a direct effect at pituitary level by which it stimulates lactotrophic cell mitogenesis, a fact that makes it difficult to determine to what extent the high incidence of prolactinomas in aging female rats originates from a toxic effect of chronically elevated levels of circulating estrogen on TIDA neurons.

In order to clarify this situation we designed a series of experiments in which we initially submitted young female rats to continued high doses of estrogen, administered either centrally or systemically, subsequently withdrawing the steroid and observing the evolution of serum PRL levels, taken as an index of TIDA neuron function, and TIDA neuron and lactotroph numbers in the hypothalamus and pituitary gland, respectively. The present report describes our findings.

2. Materials and methods

2.1. Animals

Four-month-old female Sprague–Dawley rats, raised in our animal facilities at INIBIOLP were used. Animals were housed in a temperature-controlled room (22 ± 2 °C) on a 12:12 h light/dark cycle. Food and water were available *ad libitum*. All experiments with animals were performed according to the Animal Welfare Guidelines of NIH (INIBIOLP's Animal Welfare Assurance No A5647-01).

2.2. Surgical procedures

Estrogen implants in the hypothalamus—Rats were anesthetized by injection of ketamine hydrochloride (40 mg/kg, i.p.) and xylazine (8 mg/kg, i.m.), and placed in a stereotaxic frame. In order to access the area immediately above the medial PeV-ARC region, stainless steel cannulae (ID, 0.33 mm; OD, 1.78 mm; Small Parts, Miami, FL) were bilaterally implanted through burr holes drilled through the skull over the target sites. The tip of the cannulae was brought to the following coordinates relative to the bregma: 3.0 mm posterior, 8.5 mm ventral and 0.6 mm right and left (Fig. 1). Experimental animals received cannulae filled with crystalline 17β-estradiol (Sigma Chem. Co., St. Louis, MO) whereas control rats received empty cannulae. The upper end of cannulae protruded 2–3 mm from the skull and was fixed to the bone with dental acrylic cement (Subiton, Surrey, UK). The accuracy of cannula placement was checked in the fixed brains by identification of the needle track in brain sections. In general, the tip of the cannulas was above the ARC-PeV region.

Subcutaneous estrogen implants—Rats were subcutaneously implanted with 10-mm long silastic capsules (ID, 1.98 mm; OD, 3.18 mm; Bio-Sil®, SIL-MED, Buenos Aires, Argentina) filled with 17β-estradiol whose ends were sealed with silicone adhesive. Controls were OVX animals with no other treatment, bled every 6 weeks.

Intramuscular estrogen administration—Animals were given seven weekly i.m. injections of 40 μg 17β-estradiol valerate (Sigma Chemical Co.) in 0.2 ml sunflower oil. Controls were OVX animals with no other treatment, bled every week.

Ovariectomy—Ovariectomy (OVX) was performed through a small midline abdominal incision with the animals under ketamine and xylazine anesthesia (see above).

Blood sampling—In order to monitor PRL levels, blood samples were taken under light ether anaesthesia from the tail veins at appropriate intervals.

2.3. Brain immunohistochemistry and morphometry

Animals were placed under deep anesthesia and perfused with phosphate buffered formaldehyde 4% (pH 7.4) fixative. Each brain was removed and trimmed down to a block containing the whole hypothalamus. The block was then serially cut into coronal sections 40 μm thick on a freezing microtome.

In each block, one of every six serial sections was selected in order to obtain a set of non-contiguous serial sections spanning the whole hypothalamus. Typically, a whole hypothalamus comprised about 48 coronal sections, thus yielding 6 sets of 8 non-contiguous serial sections. For counting purposes, each set was considered as representative of the medial basal hypothalamus, which is located between coordinates −2.12 up to −3.30 from the bregma and contains the ARC-PeV region. The PaV region is located between coordinates −1.30 up to −2.30 from the bregma.

For each animal, one set of sections was immunohistochemically processed using an anti-tyrosine hydroxylase (TH) monoclonal antibody (Calbiochem Inc, La Jolla, CA). For detection, the Vectastain® Universal ABC kit (Vector Laboratories, Inc., Burlingame, CA) was used, employing 3,3-diamino benzidine tetrahydrochlo-ride (DAB) as chromogen. In some experiments, $NiCl_2$ was added as color enhancer and sections were counterstained with cresyl violet. The hypothalamic A_{12} and A_{14} areas were captured using an Olympus DP70 digital camera attached to an Olympus BX51 microscope (Tokyo, Japan). Digital images were analyzed using the Image-Pro Plus (IPP™) v5.1 image analysis software (Media Cybernetics, Silver Spring, MA). Images were photographed with a 20× objective, whose space calibration

was 0.625 μm/pixel with a resolution of 1360 × 1024 pixels with a pixels depth of 24 bits RGB for light microscopy. Only the TH immunoreactive perikarya that showed clearly shaped nuclei were counted using the IPP™ semiautomatic command “manual tag”. The number of TH immunoreactive cells per section was determined by the full counting average.

2.4. Pituitary immunohistochemistry and morphometry

Pituitary glands from control and experimental glands were fixed in Bouin's fluid and embedded in paraffin. Serial sections of 4 μm were obtained at different levels of the paraffin blocks following a ventral-to-dorsal sequence. The sections were incubated for 1 h at room temperature with the primary antibody, anti-PRL (murine, Dako, CA, USA), diluted 1:100. Thoroughly washed sections were then treated for 30 min with a ready-to-use EnVision reaction system (Dako, CA, USA). The peroxide-sensitive chromogen was diaminobenzidine.

Measurements of immunostained pituitary cells were made by means of an image-analysis system (Imaging Technology, Optimas 5.2). The number of PRL cells for each gland was obtained on an average of ten micrographs taken from two levels. These measurements were recorded and processed automatically and cell density (CD = number of cells/reference area (RA)) and cell size (CS, expressed in μm^2) was calculated. RA represents the total area throughout which the cells were scored. The number of cells (CD) was calculated by dividing the immunostained area of the pituitary tissue by the mean individual cell area. For estimation of CD and CS, approximately 100 cells were recorded in each field.

2.5. Hormone assays

Serum levels of PRL were measured by a specific radioimmunoassay using the rat materials provided by Dr. A.F. Parlow, Pituitary Hormones and Antisera Center, UCLA Med. Center, USA. Iodination grade PRL was radiolabeled by the Iodo-Gen® method and purified on a PD-10 Sephadex® G-25 M column (Pharmacia, Uppsala, Sweden) equilibrated with 0.01 M phosphosaline, pH 7.6. A 1/10 goat anti-rabbit IgG in 0.9% NaCl was used to separate bound from free hormone. Serum concentrations of PRL were expressed in terms of NHPP rPRL RP-3.

2.6. Statistical analysis

The one-way analysis of the variance (ANOVA) was used to evaluate group differences. Tukey's method was chosen as a post hoc test.

3. Results

3.1. Intrahypothalamic estrogen implants

Estrogen implants in the hypothlamus of 4-month-old females were left for 30 days. After this time, a group of rats was sacrificed whereas in the remainder the implants were removed and the animals sacrificed 30 days later. Immunohistochemistry for TH performed 30 days post-estrogen implant revealed a virtually complete disappearance of TH+ cells in the ARC region of the experimental but not in the control animals (Fig. 2, lower and upper left panels, respectively). Intrahypothalamic estrogen implants did not affect the TH immunoreactivity of DA neurons in the substantia nigra (Fig. 2, lower right panel). Thirty days after cannula removal, the number of TH+ neurons in the tuberoinfundibular region of experimental and control animals was comparable (data not shown).

Serum PRL levels rose steadily after placement of estrogen-filled cannulae but not after placement of empty cannulae (Fig. 3, main panel). Around day 20 post-estrogen implant serum PRL levels reached a plateau. Thirty days after removal of estrogen cannulae, serum PRL was

back to basal levels and pituitary weight was comparable in experimental and control animals (Fig. 3, inset).

3.2. Peripheral estrogen administration

In an initial experiment, subcutaneous estrogen implants in 4-month-old OVX (30 days prior to placement of estrogen capsules) rats induced a very marked increase in serum PRL levels (Fig. 4). Removal of capsules 7 weeks later, led to a rapid fall in PRL levels which by week 17 post-implant reached pre-implantation levels and remained without further changes up to approximately one year of age (longest time tested). As expected, serum PRL in the control rats did not change significantly between experimental day #0, 42 and 238 (13.2 ± 2.9 (5); 17.8 ± 2.6 (5); 16.0 ± 2.8 ng/ml (4), respectively). When animals were sacrificed, 28 weeks after estrogen capsule removal, their pituitary weight was moderately but significantly higher than that of age-matched intact females (Fig. 4, inset).

When estrogen was i.m. injected weekly in 4-month-old OVX (30 days prior to beginning of estrogen injections) animals, serum PRL levels rose but to a lesser extent than in the rats receiving subcutaneous estrogen capsules (Fig. 5). Termination of estrogen treatment after the seventh injection led first to a plateau in PRL levels for 4–5 weeks which was followed by a progressive fall in serum PRL towards pre-treatment levels. Again, serum PRL in the control rats did not change significantly between experimental day #0, 40 and 234 (16.3 ± 3.1 (5); 12.8 ± 2.4 (5); 11.0 ± 3.0 ng/ml (5), respectively). When animals were sacrificed, 31 weeks after termination of estrogen treatment, their pituitary weights were higher than those of intact age-matched females (Fig. 5, inset).

In a second long-term experiment, immunohistochemical and morphometric assessment was performed in the hypothalamus and pituitaries of s.c. E_2 capsule-implanted and i.m. E_2-injected rats. Immunohistochemical assessment of DA neurons during estrogen treatment (experimental week 6) revealed a virtually complete disappearance of TH+ cells in the ARC-PeV region of both of s.c. E_2 capsule implanted and i.m. E_2-injected rats (Fig. 6). Long-term estrogen treatment did not inhibit TH expression in the PaV region or in the substantia nigra (Supplemental Fig. A). Morphometric assessment of hypothalamic TH neurons 30–31 weeks after estrogen withdrawal in animals of the above groups did not reveal differences between control and experimental counterparts (Table 1 and Supplemental Fig. B). In the same animals, pituitary lactotrophic cell number and cell size increased slightly (Table 2 and Supplemental Fig. C).

4. Discussion

Ovarian steroids seem to play an important physiological role in the regulation of PRL secretion during the estrous cycle [9]. In rats, estrogen stimulates PRL secretion by both inhibiting hypothalamic dopaminergic function [9] and directly stimulating PRL secretion at pituitary level [30]. On the other hand, is has been long proposed that chronic exposure to estrogen causes permanent damage and cell loss in the arcuate nucleus [3,4] and that in rodents this steroid is causally involved in histological aging of the arcuate nucleus [27]. The target cell population for the proposed neurotoxic action of estrogen has been a matter of controversy for many years. Thus, based on histofluorescence assessment of catecholaminergic cells in the ARC nucleus and in the ME of young female rats carrying estrogen-induced prolactinomas or old animals carrying spontaneous PRL-secreting adenomas, Sarkar et al. [26] concluded that chronic estrogen is toxic for TIDA neurons. This view was challenged by later studies reporting that in young rats, estrogen administration causes a selective loss of 60% β-endorphin neurons of the ARC nucleus but completely spares somatostatin-, neurotensin- and TH-immunoreactive neurons [10].

Our results do not support the hypothesis that chronic estrogen causes TIDA neuron loss. The experiments with hypothalamic estrogen implants indicate that while estrogen has a powerful inhibitory action on TH expression and DA production by TIDA neurons it does not result in irreversible damage or DA neuron loss. The data also reveal that when estrogen acts on TIDA neurons but not on the pituitary gland, the increase in PRL secretion is significant but much lower than when the steroid is administered peripherally. The levels of serum PRL achieved in our rats by implanting hypothalamic estrogen-filled cannulae are consistent with those reported previously employing the same experimental approach to induce hyperprolactinemia [5,6].

Although long-term peripheral administration of estrogen induced a much higher hyperprolactinemia than estrogen implants in the hypothalamus, the long-term normalization of serum PRL levels (despite a residual increase in lactotroph number) and hypothalamic TH + neuron numbers after estrogen withdrawal is consistent with a major, if not full, recovery of TIDA function. These findings are in line with a study in Fischer 344 female rats submitted to subcutaneous implants of diethylstilbestrol (DES)-filled Silastic capsules. When the capsules were left for 30 days and the animals studied, a 40-fold increase in serum PRL levels, 3-fold pituitary enlargement and an almost complete loss of TH immunoreactive neurons in the ARC was observed. Thirty days after removal of the estrogen capsules, these alterations were reversed [11]. In another study in Fischer 344 females, a single i.m. injection of estradiol valerate to OVX animals markedly increased, after 3 weeks, serum PRL levels and pituitary weight and reduced DA content in the ME. Twenty-four weeks after estrogen administration, PRL levels and pituitary weight had decreased but were still above control values whereas ME DA content had returned to pre-injection levels [1]. These two reports are in general agreement with the present data. In old Sprague–Dawley female rats it was reported that ME catecholamine histofluorescence was low in animals carrying spontaneous PRL-secreting adenomas but not in old rats possessing only moderately enlarged non-adenomatous pituitaries [26].

It is well-established that TIDA neuron function declines with age. Thus there is a marked reduction in hypothalamic, ME and neurointermediate lobe dopamine content in old (24–26 months) as compared with young (4 months) rats [23]. More significant, the rate of dopamine secretion into the hypophysial portal blood of aged (20–26 months) male and female rats declines drastically when compared with young (2–4 months) counterparts [24,13]. This has been ascribed to a functional decline of TIDA neurons rather than to TIDA neuron loss [22], although in extremely old rats (32 months) a moderate but significant reduction in the number of hypothalamic TH+ neurons was reported [25]. Nevertheless, it seems unlikely that this age-related decline in TIDA neuron function and number may be due to a neurotoxic action of estrogen. Not only our present data fail to support this possibility but other documented results are against this hypothesis as well. First, TIDA neuron functional decline with age occurs in both male and female rats (see above); second, since serum estrogen remains elevated in female rats even at very advanced ages [14,16,20], it may keep a continued inhibitory action on TIDA neurons (and a stimulatory effect at pituitary level) without necessarily causing neurodegeneration; third, TIDA neuron function and hypothalamic TH+ numbers can be restored in senile female rats by means of insulin-growth factor-I gene therapy, which strongly suggests that in old rats TIDA neurons are functionally depressed but still viable [14].

We conclude that although estrogen is a powerful inhibitor of TIDA neuron function, in normal rats it does not have toxic effects on this cell population. Whether in highly sensitive rat strains like the Fischer 344, long-term exposure to the steroid can cause TIDA neurodegeneration remains to be clarified.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.brainresbull.2009.08.026.

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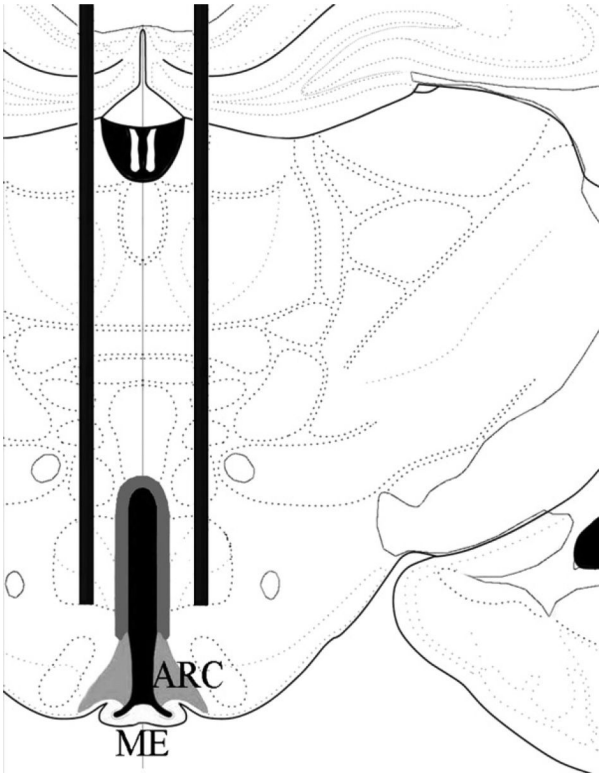

Fig. 1.

Schematic representation of a coronal section of the rat tuberal hypothalamus at the level of the median eminence (ME) showing the distribution of dopaminergic neuron bodies in the arcuate nucleus (ARC) (lower gray area), the main component of the A_{12} area, and in the periventricular (PeVH) nucleus (upper gray area). Adapted from Swanson [28]. Black areas correspond to the cavity of the third ventricle. The estimated position of the bilateral cannulae inserted in some of the experiments reported is indicated by two vertical bars ending above the ARC nucleus.

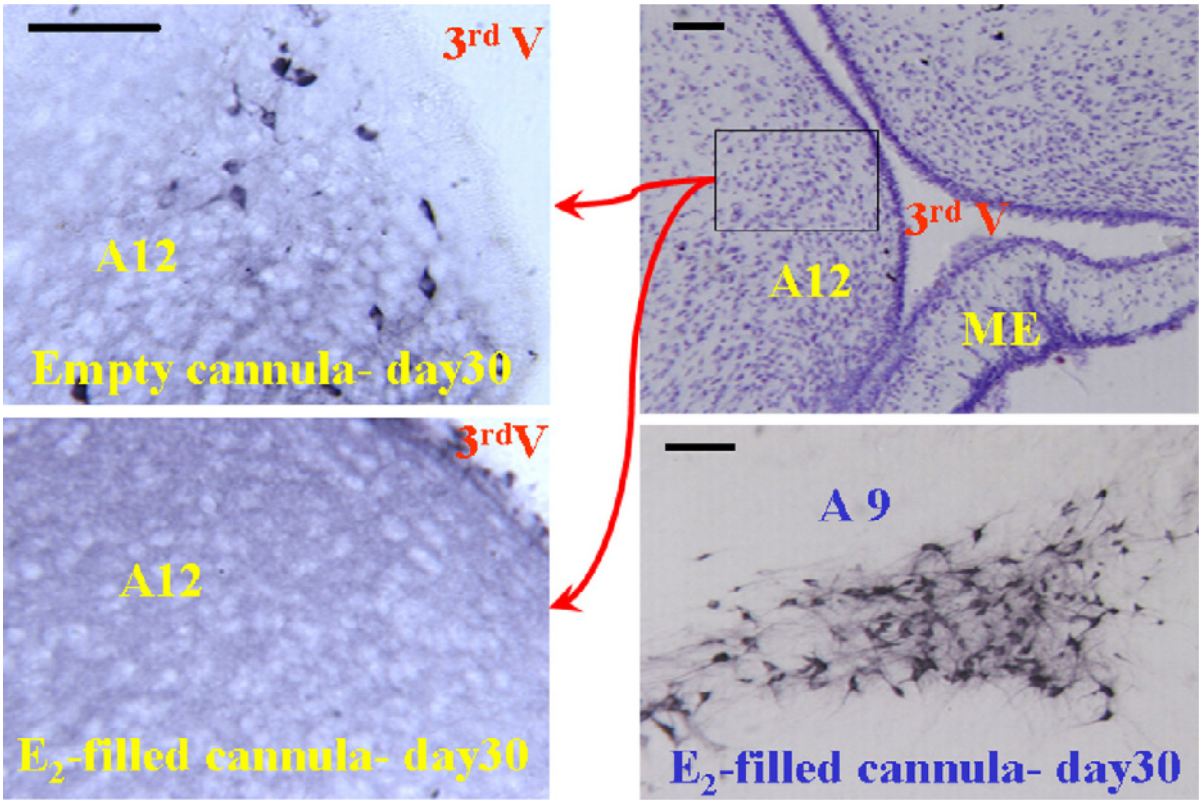

Fig. 2.

Inhibition of the expression of TH in hypothalamic DA neurons of the PeV-ARC area (A_{12}: upper right panel) of young females stereotaxically implanted during 30 days, with empty (upper left panel) or E_2-filled (lower left panel) cannulae. The presence of TH+ neurons was detected by immunohistochemistry. TH expression in DA neurons of the mescencephalic A9 region (susbstantia nigra) was not inhibited in the E_2-implanted animals (lower right panel). Upper right panel: cresyl violet stain of medial hypothalamus. The framed area represents the region shown on the left panels. Scale bars representing 100 μm are indicated.

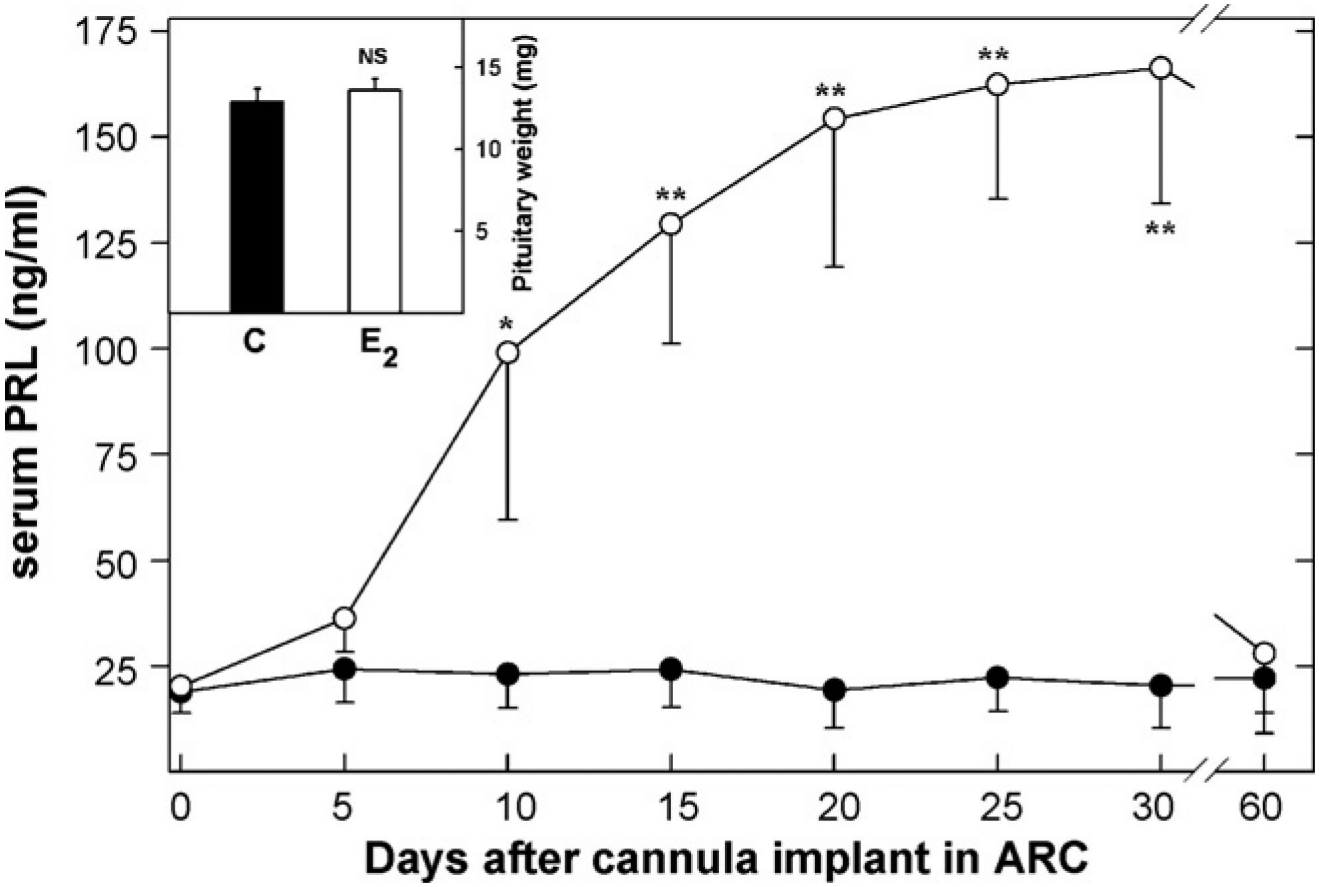

Fig. 3.

Time-course of serum PRL levels in female rats implanted with empty or E_2-filled cannulae (6 rats per group). Cannulae were kept for 30 days and subsequently removed. Animals were studied for a total of 60 days. Two experimental and one control rat were lost between day 30 and 60. Asterisks indicate significant differences from corresponding pre-surgical values. *$P < 0.05$; **$P < 0.01$. *Inset*: Pituitary weight in control and experimental animals at the end of the experiment (experimental day 60).

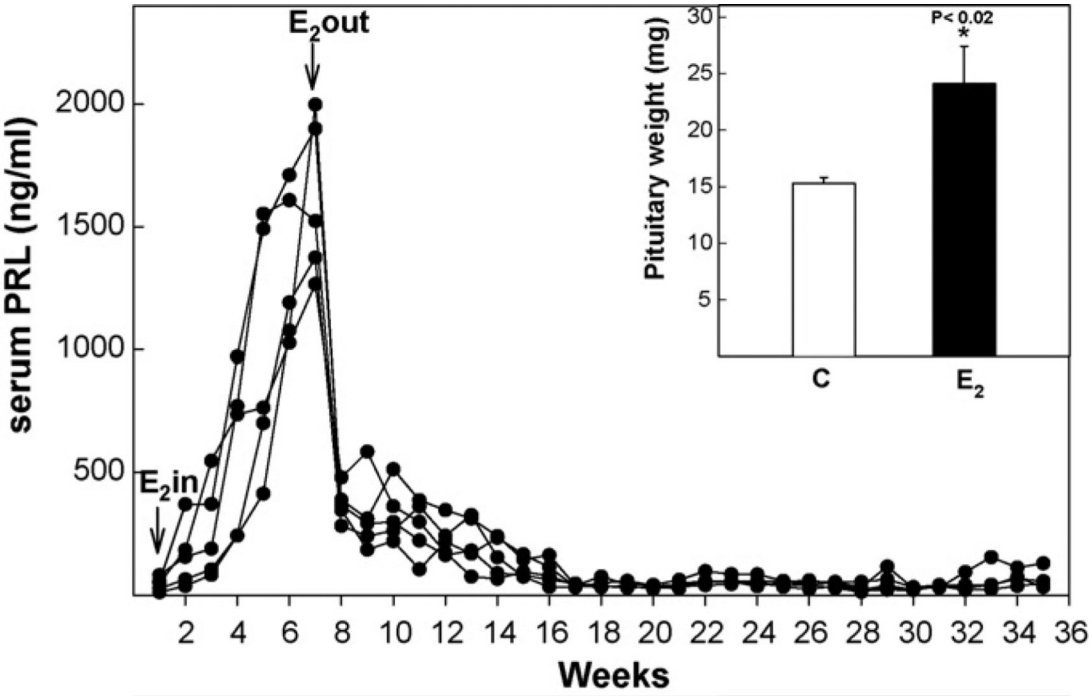

Fig. 4.

Time-course of serum PRL levels in 5 OVX female rats implanted with subcutaneous E_2-filled silastic capsules on experimental week 1 ("E_2 in" arrow). Capsules were kept for 7 weeks and subsequently removed ("E_2 out" arrow). Animals were studied for a total of 35 weeks. At the end of the study they were 51 weeks old. *Inset*: Pituitary weight in experimental (n = 4) and age-matched intact animals (n =5) at the end of the experiment (experimental week 35). Asterisk indicates a significant difference between groups.

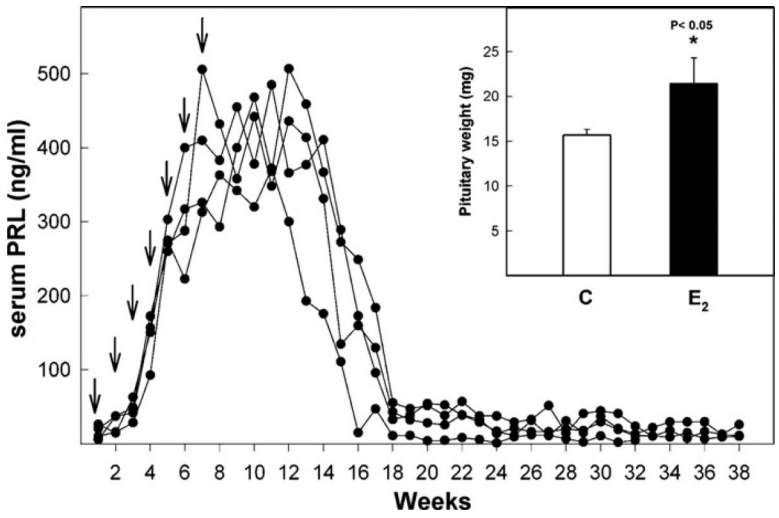

Fig. 5.

Time-course of serum PRL levels in 4 OVX female rats receiving weekly i.m. injections (arrows) of 17β-estradiol valerate for 7 weeks. Animals were studied for a total of 38 weeks. At the end of the study they were 54 weeks old. Notice that i.m. estrogen induced a less marked hyperprolactinemia than estrogen capsules. On the other hand, hyperprolactinemia lasted longer after termination of estrogen injections than when estrogen capsules were removed. *Inset*: Pituitary weight in experimental (n = 3) and control age-matched (n = 5) animals at the end of the experiment (experimental week 38). Asterisk indicates a significant difference between groups.

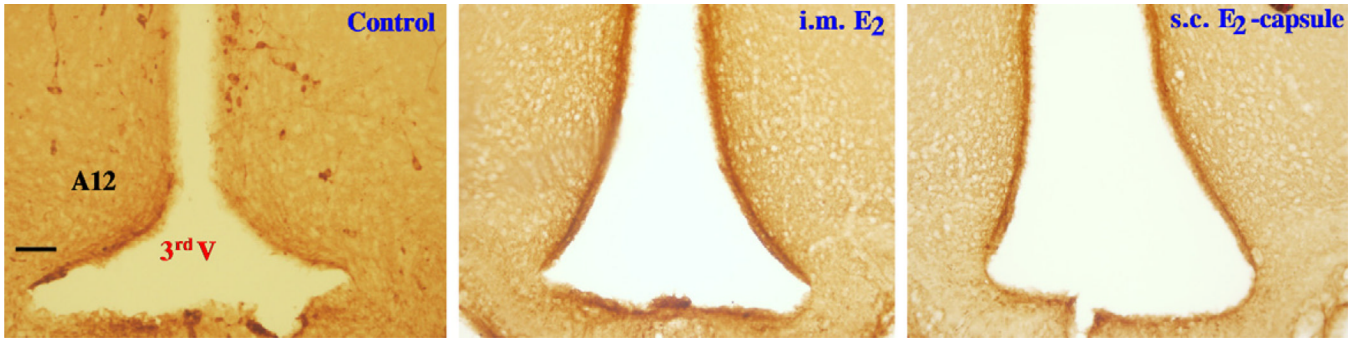

Fig. 6.

Inhibitory effect of peripheral E_2 on TH expression in DA neurons of the PeV-ARC region. Controls were OVX animals with no other treatment whereas experimental animals, which were also OVX, received i.m. injections or s.c. capsules of E_2 for 6 weeks. Notice the strong inhibition of the steroid on TH expression in this region. Hypothalamic sections from representative animals are displayed. Scale bar represents 100 μm.

Table 1

TH neuron number/section in the ARC, PAV and PeV of female rats after long-term E_2 withdrawal.

TH neuron number				
Exptl. group	**ARC nucleus**	**PeV nucleus**	**PaV nucleus**	**ARC + PeV + PaV**
Empty s.c. capsule	39 ± 1 (3)	92 ± 11 (3)	123 ± 8 (3)	243 ± 4 (3)
E_2 s.c. capsule	43 ± 5 (3)	89 ± 15 (3)	120 ± 1 (3)	236 ± 2 (3)
Corn oil i.m.	41 ± 2 (3)	87 ± 15 (3)	124 ± 7 (3)	237 ± 15 (3)
E_2 i.m.	41 ± 3 (3)	102 ± 20 (3)	131 ± 11 (2)	253 ± 18 (2)

Data are expressed as $\bar{x}$ ± SEM. One-way ANOVA was NS.

Table 2

Lactotroph morphometry in the anterior pituitary gland of female rats after long-term E_2 withdrawal.

Exptl. group	Cell density	Cell size (μm^2)
Empty s.c. capsule	23 ± 5 (3)	39.4 ± 3.9 (3)
E_2 s.c. capsule	34 ± 5 (3)	52.3 ± 3.7* (3)
Corn oil i.m.	22 ± 4 (3)	38.4 ± 3.3 (3)
E_2 i.m.	26 ± 4 (3)	43.1 ± 4.0 (3)

Data are expressed as $\bar{x}$ ± SEM. Asterisk indicates significant difference ($P < 0.05$).