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Chronic exposure to low levels of oestradiol-17 β affects oestrous cyclicity, hypothalamic norepinephrine and serum luteinising hormone in young intact rats

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Abstract

Chronic exposure to oestrogens is known to inhibit the secretion of luteinising hormone (LH) in rats causing anovulation. Hypothalamic catecholamines, norepinephrine (NE) and dopamine (DA) play an important role in LH regulation. However, the effects of chronic exposure to low levels of oestradiol on hypothalamic catecholamines have not been investigated thoroughly. To study this, adult female Sprague-Dawley rats were either sham implanted or implanted with oestradiol-17 β (E2) pellets (20 ng/day) for 30 (E-30), 60 (E-60) or 90 (E-90) days. E2 exposure affected oestrous cyclicity and ovarian morphology in a duration-dependent manner. There was no change in oestrous cyclicity in E-30 rats, however, 75% of E-60 and 95% of E-90 rats were acyclic ($p < 0.05$). Cycling rats from E-30 or control group were sacrificed at different time points on the afternoon of pro-oestrous. E-30 rats in oestrous, constant oestrous rats in the E-60 and E-90 groups and a group of old constant oestrous (OCE) rats were sacrificed at 1200 h. LH was measured in the serum by RIA. Individual hypothalamic nuclei that are involved in LH regulation were microdissected and analysed for NE and DA levels using HPLC-EC. NE levels in the hypothalamic nuclei increased significantly in control and E-30 groups during the afternoon of pro-oestrous which was accompanied by a rise in LH levels ($p < 0.05$). On the day of oestrous, NE concentrations in hypothalamic nuclei and serum LH were significantly lower in E-60, E-90 and OCE rats compared to E-30 and control rats. On the other hand, DA levels declined significantly in one hypothalamic nucleus. These results indicate that chronic E2 exposure affects hypothalamic catecholamine and serum LH levels in a duration-dependent manner. This coincides well with the loss of cyclicity observed in these animals. These results suggest that repeated exposure to endogenous oestrogens could play a role in reproductive senescence.

Keywords

Chronic oestrogen; luteinising hormone; hypothalamus; norepinephrine; aging

Introduction

Reproductive aging in rats is characterised by specific stages that are marked by changes in the levels of circulating gonadal hormones and oestrous cyclicity. When they are young, rats have 4 or 5-day oestrous cycles that are marked by a sharp increase in oestrogen levels during pro-oestrous that results in the luteinising hormone (LH) surge that causes ovulation. Rats continue to cycle regularly from puberty (2 months of age) until they reach middle age (10 -12 months of age) (1). By the time they reach middle age, rats have gone through 50-60 oestrous cycles and have been exposed to higher levels of endogenous oestrogens for 50-60 days. By the end of this period, rats have LH surges that are delayed and lower in amplitude that leads to the constant oestrous state when they are 12-14 months of age (1,2). Failure of ovulation ultimately leads to the constant oestrous state (2). The reasons for the gradual reduction in LH levels have not been clearly understood. It has been suggested that exposure to ovarian oestrogens during normal reproductive cycles may alter the sensitivity of the neuroendocrine system leading to age-related impairments in reproductive function (3).

We hypothesised that repeated exposure to pro-oestrous levels of oestrogen could affect catecholamine levels in the hypothalamus to cause a gradual reduction in LH secretion leading to anovulation. The effects of chronic exposure to low levels of oestrogen for prolonged periods of time on the neuroendocrine system governing LH secretion have not been investigated thoroughly. This is important because the hypothalamus has gonadotrophin releasing hormone (GnRH) neurones that are oestrogen-sensitive and play a critical role in the regulation of LH secretion and reproductive function (4-7). A variety of neurochemicals influence GnRH neurones. Among these, hypothalamic catecholamines play a crucial role (4,8). Using a variety of approaches, it has been shown that the catecholamine norepinephrine (NE) is most probably stimulatory to LH secretion while dopamine (DA) plays a less significant role (9-13). In young cycling animals, increases in endogenous oestrogens during the afternoon of pro-oestrous positively impacts noradrenergic neurones and increases norepinephrine (NE) release in GnRH rich regions including the medial preoptic area (MPA) of the hypothalamus (4). This causes the preovulatory LH surge which results in ovulation (9,13,14). However, repeated exposure to proestrous levels of endogenous oestrogens could lead to a reduction in hypothalamic NE resulting in decreased LH levels and anovulation.

In the present study, we explored the effects of chronic exposure to low levels of oestradiol-17 β (E2) on oestrous cyclicity, LH and hypothalamic catecholamines in young, intact, female rats. We also used a group of old constant oestrous (OCE) rats in order to determine if these effects are comparable to what is observed during normal aging. This would help us determine if chronic exposure to low levels of oestradiol produces aging-like changes in young female rats. This will also help us understand the possible mechanisms that could play a role in reproductive failure caused by chronic exposure to low levels of oestrogens as in birth control pills and other environmental oestrogens.

Materials & Methods

Animals

Adult (3-4 months old) female Sprague-Dawley rats and retired breeders (around 10 months of age) were obtained from Harlan Inc., (Indianapolis, IN, USA). Retired breeders were aged in our animal quarters before experimentation. The animals were housed in temperature-(23 $\pm$ 2°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

Oestrous cyclicity was determined in young (3-4 months old) and old (16-18 months old) rats by vaginal cytology. Young animals showing 4-5 day regular oestrous cycles and old animals that exhibited oestrous smears for more than 10 consecutive days (old constant oestrous-OCE) were used in the experiments. Young animals were either sham-implanted (control) or implanted with oestradiol-17 β (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 (n=8/group).

Body weights were obtained at the beginning of treatment and at the time of sacrifice. Since most animals in the E-30 and the corresponding control groups were cycling regularly, they were sacrificed at 1200, 1400, 1600 and 1800 h on the day of pro-oestrous (n=6-8) to correlate changes in NE, DA in specific hypothalamic areas to LH levels during the afternoon of pro-oestrous. Most of the animals in the E-60 and E-90 groups were in the state of constant oestrous and were sacrificed at 1200 h. A group of E-30 rats and age-matched control groups for the E-30, E-60 and E-90 groups were sacrificed at 1200 h when they were in oestrous. OCE rats were sacrificed at 1200 h for comparison purposes.

Upon sacrifice, the brain was removed, frozen and stored at -70°C until sectioning. The anterior pituitary was weighed and the ovaries were fixed processed for histological examination. Serum was separated from trunk blood for hormone assays.

Histological examination of the ovary

Ovaries were processed for histological examination by the Histology Laboratory, Department of Physiology, Michigan State University. Slides containing the largest sections of the ovaries were examined under a light microscope with image capturing capabilities for identifying and enumerating ovarian structures such as primary, secondary, atretic and unovulated follicles as per Kishi and Greenwald (1999) (15). Care was taken not to count the same structures twice for each ovary.

Hormone Measurements

Oestradiol, progesterone and LH levels in the serum were measured in duplicate by radioimmunoassay (RIA) using the double antibody method as described previously (16). The RIA kits for oestradiol and progesterone were obtained from Diagnostic Products Corporation (Los Angeles, CA). The assays were performed according to the manufacturer's instructions. The standards and antibody for rat LH were obtained from Dr. A.F.Parlow, NHPP, NIDDK. The LH tracer was obtained from Amersham Pharmacia Biotech (Waukesha, WI, USA). The assay was performed as described previously (16).

Microdissection of the brain

At the time of sectioning, the brains were mounted and serial sections of 300 μ m thickness were obtained using a cryostat (Slee, London, UK) maintained at -10°C as described before (10,17). The Diagonal band of Broca (DBB), medial preoptic area (MPA), suprachiasmatic nucleus (SCN), and arcuate nucleus (AN) were microdissected using the Palkovits' microdissection technique (17,18) with the help of a rat brain stereotaxic atlas (19). Tissue samples were obtained bilaterally and included all subdivisions of individual nuclei. They were stored at -70°C until they were analysed for neurotransmitter concentrations by HPLC-EC.

HPLC-EC

Tissue homogenates were analysed for determining the concentrations of NE and DA using HPLC-EC as described before (10,17). Briefly, the following were the components of the HPLC-EC system used: a phase II, 5 μ m 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, tissue punches were homogenised in 200 μ l of 0.1 M perchloric acid. Samples of the homogenate were aliquoted for protein analysis. The rest of the homogenate was centrifuged at $14000 \times g$ for 5 minutes. A mixture of the sample (120 μ l) and the internal standard (0.05 M dihydroxy benzylamine; 30 μ l) were loaded in an auto-injector that was maintained at 4°C. Catecholamine 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 tissue homogenates were determined using a micro Bicinchoninic acid assay (Pierce, Rockford, IL). Neurotransmitter concentrations were expressed as pg/ μ g protein.

Statistical analysis

Differences in body weight, pituitary weight, serum oestradiol, progesterone and ovarian structures were analysed using one-way ANOVA followed by Fisher's LSD test. The differences in serum LH levels and NE concentrations on pro-oestrous were analysed by two-way ANOVA. LH levels and catecholamine concentrations measured on the day of oestrous were analysed by one-way ANOVA followed by Fisher's LSD test. Differences in oestrous cyclicity were analysed using Kruskal Wallis non-parametric test.

Results

Effects of E2 treatment on Body Weight and Pituitary Weight

The body weights and pituitary weights of control and E2-treated rats are shown in Table 1. E2 treatment for 30, 60 or 90 days did not produce any significant change in body weight when compared to the corresponding age-matched controls. The weight of the anterior pituitary (mg; Mean $\pm$ S.E.) in the E-60 group increased to 13.9 ± 0.4 compared to the corresponding, age-matched sham-implanted rats (10.9 ± 0.9 ; $p < 0.05$). There was no change in anterior pituitary weight in the rest of the E2-treated groups.

Effects of E2 exposure on oestrous cyclicity

The effects of E2 exposure on oestrous cyclicity are shown in Fig. 1. All the control animals from the different age groups exhibited regular oestrous cycles. Exposure to E2 for 30 days did not affect oestrous cyclicity in most of the animals. In contrast, E2 exposure for 60 or 90 days resulted in constant oestrous condition in 70 and 95% of the rats, respectively. In comparison, 100% of the OCE rats were acyclic and were in a state of constant oestrous.

Effects of Chronic E2 exposure on ovarian histology

Photomicrographs of representative ovaries from each group are shown in fig 2. The ovaries of control rats and those from the E-30 group had large numbers of fresh corpora lutea (Mean $\pm$ S.E.) since these animals were sacrificed on the day of oestrous (13.6 ± 1.1 and

10±0.7 for control and E-30 respectively) (Figs 2A-B). The number of corpora lutea decreased in E-60 and E-90 rats (4.4±1.6 and 2.2±0.9, respectively) compared to control rats ($p<0.05$; Fig. 2 C-E). On the other hand, E2 treatment resulted in a significant increase in the number of unovulated follicles in E-60, E-90 rats (2.6±0.5 and 2.4±0.6, respectively) compared to control (1±0.2) and E-30 (0.7±0.2) rats ($p<0.05$). There was a significant reduction in the number of developing follicles such as primary and secondary follicles in E-90 and OCE rats compared to control, E-30 and E-60 rats ($p<0.05$). On the contrary, there was a significant increase in atretic follicles undergoing degeneration in E-90 (3±0.3) and OCE (3±0.8) rats compared to control (0.8±0.3), E-30 (0.2±0.1) and E-60 rats (1.9±0.7; $p<0.05$) (Table 2). This suggests that E2 treatment decreased follicular development but increased follicular degeneration.

Effects of chronic E2 exposure on gonadal steroids

Effects of chronic E2 exposure on serum oestradiol and progesterone are shown in Fig. 3. There were no significant differences among the different control groups and therefore, these values were pooled for ease of presentation. Serum oestradiol levels (pg/ml; Mean ±S.E) in control animals were 35.2±2.1 and were unaltered in the E-30 group. However, exposure for 60 or 90 days resulted in a significant increase in serum oestradiol levels (69.4±10.0 and 94.5±3.8 respectively) ($p<0.05$). Serum oestradiol levels in OCE rats (101.9±26.2) were also significantly higher compared to control and E-30 rats ($p<0.05$).

In contrast to serum oestradiol, serum progesterone levels (ng/ml; Mean±SE) were higher in E-30 (54.6±6.8) rats compared to the control group (20.5±2.9; $p<0.05$). Progesterone levels in the E-60 group declined to 34.44±5.57, and declined further in the E-90 group (13.2±1.3; $p<0.05$) which were comparable to levels observed in OCE rats (11.3±2.9).

Effects of chronic E2 exposure on serum LH levels

A. Changes in pro-oestrous—LH levels were measured in cycling animals (control and E-30 group) when the animals were in pro-oestrous. In control rats, serum LH (Mean±S.E.; ng/ml) levels were 2.7±0.4 at 1200 h, 3.4±0.3 at 1400 h, were 4.7±1.7 at 1600 h and reached peak levels at 1800 h (12.6±4.7). In E-30 rats, serum LH levels were 3.3±0.2 at 1200 h, were 3.1±0.1 at 1400 h and remained at that level at 1600 h and increased to 8.7±2.4 at 1800 h ($p<0.05$). There were no significant differences in peak LH levels between E-30 and control animals (Fig. 4A).

B. Changes in oestrous—LH levels were also measured at 1200 h on the day of oestrous in the control, E2-treated and OCE rats. Again, there were no significant differences in LH levels between the age-matched control groups and therefore, these values were pooled together. LH levels in E-60 (2.6 ± 0.3) and E-90 (2.4 ± 0.3) groups were lower than that in E-30 (4.2±0.8) and control animals (4.6±1.0) and were comparable to that seen in OCE rats (2.1±0.04) (Fig 4B).

Effects of E2 exposure on hypothalamic catecholamines

A. Changes in pro-oestrous—The effects of chronic E2 exposure on NE and DA concentrations in various brain areas during pro-oestrous are shown in Fig. 5 and Table 3, respectively. There were no significant differences in treatment or treatment x time interactions. However, the effect of time in each of the areas was found to be significant ($p<0.05$).

In the **DBB**, NE levels (pg/μg protein; Mean±SE) were 17.2±2.3 at 1200 h in control rats and increased by more than two fold to 46.4±7.4 at 1400 h and remained elevated until 1800 h ($p<0.05$; Fig 5A). In the **MPA** however, NE levels increased significantly only by 1600 h

in control animals. NE levels were (10.7 ± 0.7) at 1200 h and increased to 28.3 ± 3.1 at 1600 h and declined to 20.4 ± 1.2 at 1800 h (Fig 5B). In the **SCN**, the increase in NE levels in the control group was delayed further (Fig 5C). NE levels were low at 1200 h (8.8 ± 2) and increased significantly to 16 ± 2 at 1800 h ($p < 0.05$). In the **AN**, NE levels in control rats were 19.1 ± 4.1 at 1200 h, remained at about that level at 1400 h (20.3 ± 1.7) and increased to 36.3 ± 4.7 only at 1800 h ($p < 0.05$; Fig 5D). NE levels in E-30 rats were comparable to that observed in control animals and there were no significant differences between these two groups in the different areas examined.

DA levels on the other hand, decreased significantly in the **MPA** from 11.8 ± 3.8 and 14.9 ± 4 at 1200 h to 2.2 ± 0.9 and 2 ± 1 at 1600 h in control and E-30 rats, respectively ($p < 0.05$; Table 3). DA levels also declined significantly in the **SCN** in both control (1.8 ± 0.4) and E-30-treated rats (4.1 ± 0.9) at 1600 h compared to levels at 1200 h and remained suppressed until 1800 h ($p < 0.05$). However, there were no changes in DA levels in the **DBB** and the **AN**.

B. Changes in oestrous—In the **DBB** and the **AN**, there were no changes in NE levels between control, E2-treated and OCE rats (Figs 6 A and D). In the **MPA** however, NE levels in the E-30 (3.1 ± 0.3), E-60 (2.5 ± 0.4), E-90 (3.1 ± 0.6) and OCE (3.8 ± 0.7) groups were significantly lower compared to control rats in oestrous (10.7 ± 4.5 ; $p < 0.05$; Fig. 6B). In the **SCN**, NE levels declined significantly in E-90 (3.9 ± 1.4) and in OCE (3.4 ± 0.8) rats compared to control rats (11.4 ± 1.4 ; $p < 0.05$; Fig. 6C). There were no significant differences in DA levels in the **DBB**, **AN** and **MPA** (Figs. 7A,B and D). DA levels were also reduced in the **SCN** in the E-60 (2.1 ± 0.8), E-90 (2.2 ± 0.8) and OCE groups (0.6 ± 0.1) when compared to control rats (9.3 ± 3.3 ; $p < 0.05$) (Fig. 7C).

Discussion

This study demonstrates that exposing young intact cycling rats to low levels of E2 on a chronic basis, disrupts oestrous cycles and results in polycystic ovarian morphology. While exposure to 20 ng of E2 for a period of 30 days did not affect oestrous cycles, E2 exposure for 60 or 90 days caused 75% and 95% of the animals respectively to reach constant oestrous. At the end of the 60 or 90-day exposure period, animals were only 5-6 months old, yet, they demonstrated all signs of reproductive senescence similar to 10-12 months old rats. Although earlier studies using different oestrogen exposure regimens demonstrated that chronic oestrogen exposure can decrease LH levels and result in cystic ovaries, they have not examined the underlying mechanisms of this phenomenon. Results from this study suggest that alterations in hypothalamic catecholamine levels may play a role in decreasing LH and causing acyclicity after chronic oestrogen exposure.

Previous studies have reported pro-oestrous levels of serum oestradiol to be 30-55 pg/ml (20,21). In the present study, oestrogen levels in E-30 rats were about this level but increased gradually with duration of exposure. This could be due to oestradiol contribution from the pellets and the unovulated follicles. A recent study indicates that extragonadal sources of E2 could possibly contribute to the increase in serum E2 levels (22). In contrast to oestrogen, progesterone levels declined with increasing duration of oestrogen exposure which could be attributed to the lack of corpus luteum formation. Data from ovarian morphology indicate that E2 exposure decreases the number of corpora lutea and increases the number of unovulated follicles. A similar increase in follicular apoptosis, cystogenesis and decreased follicular growth was observed in a recent study which involved administration of a high dose of oestradiol valerate (23,24).

A number of earlier studies have demonstrated that prolonged exposure to higher doses of E2 for a period of 1 week to a month, or a single dose of oestradiol valerate produce a

decrease in LH levels (25-28). A similar effect was observed in ovariectomised steroid-primed rats as well ((29). The present study uses exposure to very low levels of oestradiol-17 β (20 ng) for 30, 60 or 90 days. Exposure to E2 for 30 days did not significantly affect oestrous cycles and therefore, we studied the LH surge in the animals that were still cycling. In these animals, the LH surge was not significantly different from that in control rats (Fig 4). However, most of the animals in the E-60 and E-90 groups were in a state of constant oestrous, which could be attributed to a reduction in LH levels leading to anovulation. It is therefore important to monitor LH levels in E2-treated animals between 30 and 60 days of exposure to gain a better understanding of the effect of E2 on the LH surge.

The mechanism by which chronic E2 exposure affects LH secretion and causes animals to become acyclic is unclear. Earlier studies suggest that it decreases the activation of GnRH neurones (30). However, this was not associated with any change in the expression of oestrogen receptors on these neurones (31) indicating that E2 could also act through other mechanisms. Another possibility is that E2-induced suppression of LH could be mediated through neurochemicals that are known to be involved in its regulation. Hypothalamic catecholamines, especially NE, is known to play an important role in LH regulation (8). A number of earlier studies have demonstrated the importance of NE in LH regulation using noradrenergic antagonists, NE depletors and lesioning of noradrenergic fibres that innervate the hypothalamus (4,5,9,32). Moreover, their levels decrease with aging and this is known to be one of the main causes for reproductive senescence (9,10,33). We have previously shown that both the release and steady-state concentrations of NE increase during pro-oestrous in young animals and decline with age. This was accompanied by age-associated changes in LH secretion as well (9,10)). Treatment with the catecholamine precursor, L-DOPA has successfully reinitiated oestrous cyclicity in aging female rats (34) indicating that reductions in brain catecholamine levels most probably causes a decline in LH secretion leading to reproductive aging. Therefore, we measured changes in NE and DA levels in discrete brain nuclei such as the DBB, MPA, SCN and the AN which are known to be involved in the regulation of LH surge (35). The increase in NE levels was apparent earlier (1400 h) in rostrally located areas such as the DBB compared to caudally placed nuclei such as the AN. Previous studies have observed activation of anteriorly located GnRH neurones from 1400 h onwards (36,37). Perhaps more numbers of caudally placed GnRH neurones are recruited with time to increase GnRH secretion to cause the LH surge during the afternoon of pro-oestrous.

Two other studies that have attempted to measure catecholamine levels after chronic E2 exposure, did so in different areas of the brain not particularly involved in LH regulation (38) and in gross divisions of the hypothalamus (24). These studies reported an increase or no change in NE levels. The difference in results from the present study can be attributed to the measurement of catecholamines in larger tissue blocks. In the present study, catecholamine levels were measured in specific nuclei that are involved in LH regulation. Although NE levels were not affected to a great extent in E-30 rats, there is a definite decline in NE levels in the MPA in E-60 and E-90 rats on the day of oestrous. This is very similar to what is observed in aging animals. On the other hand, DA concentrations declined significantly in the MPA in E-30 and control rats at the time of the LH surge. We have observed a similar decrease in DA release in the MPA at the time of the LH surge (9). DA levels were also significantly lower in the SCN, but the reason for this is not clear. Since the rat oestrous cycles are strongly affected by lighting cues (39), it may be possible for DA levels in the SCN to play an important role in the modulation of temporal activity of neurones.

The mechanism by which chronic E2 exposure affects NE levels is not clear. Oestrogen receptors are present on noradrenergic neurones in the brain stem (40). These neurones provide innervation to hypothalamic areas containing GnRH neurones and are crucial for the LH surge (41,42). Therefore, this could be a potential site for oestrogen to act and decrease NE levels. The pituitary could be another site at which E2 could directly act to affect LH secretion. Low levels of oestradiol are known to induce prolactinomas (43) and higher prolactin levels are known to suppress LH secretion (44). Although we did not observe any prolactinomas in our animals, this could be another possible mechanism by which E2 affects LH secretion, especially with prolonged exposure as in the E-60 and E-90 groups.

In summary, we conclude that chronic exposure to low-dose E2 in intact, adult, female rats causes anovulation and cessation of oestrous cycles. This is accompanied by changes in catecholamine levels in specific areas of the hypothalamus that regulate LH secretion. This phenomenon could also be relevant in chronic exposures to oestrogens in the form of birth control pills or exposure to environmental oestrogenic compounds that severely impair reproductive functions.

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Fig. 1. Effects of chronic E2 exposure on oestrous cyclicity

E-30, E-60 and E-90 indicate groups that were implanted with 20ng/day slow-release oestrogen pellets for 30, 60 and 90 days respectively. Control rats were sham-implanted. These are compared to old constant oestrous rats (OCE).

Fig. 2. Effects of chronic E2 exposure and aging on ovarian morphology (40 x magnification). Representative sections of the ovary from a control (A), E-30 (B), E-60 (C), E-90 (D) and old constant oestrous rat (E) are shown. Animals in the control and E-30 groups were sacrificed at 1200 h on the day of oestrous. Animals in the E-60, E-90 and OCE groups were in constant oestrous and sacrificed at 1200 h. '1' indicates corpus luteum, 'X' indicates unovulated follicle. Primary and secondary follicles are not marked in these micrographs because of the low magnification.

Fig. 3. Effects of chronic E2 exposure on gonadal hormones

Oestrogen (open bars) and progesterone (closed bars) levels in E-30, E-60 and E-90 groups that were implanted with slow-release E2 pellets (20 ng/day) for 30, 60 and 90 days respectively. These are compared to old constant oestrous rats (OCE). Control group was sham-implanted. Animals in the control and E-30 groups were sacrificed at 1200 h on the day of oestrous. Animals in the E-60, E-90 and OCE groups were in constant oestrous and sacrificed at 1200 h. * indicates significant ($p < 0.05$) difference in oestrogen levels from the control and E-30 groups. # indicates significant differences ($p < 0.05$) in progesterone levels from control, E-90 and OCE groups.

Fig 4. Serum LH levels

A. LH levels during the afternoon of pro-oestrous in control and E-30 groups. Animals were sacrificed in groups (n=6-8) at various time points during the afternoon of pro-oestrous. * indicates significant difference from values at 1200 h. **B. LH levels during oestrous.** LH levels in control, and E-30 animals at 1200 h on the day of oestrous. Animals in the E-60, E-90 and old constant oestrous (OCE) groups were in constant oestrous and were also sacrificed at 1200 h. * indicates significant difference ($p < 0.05$) from the control and E-30 groups.

Fig 5. NE levels in control and E-30 groups during the afternoon of pro-oestrous in the Diagonal band of Broca (DBB), Medial preoptic area (MPA), Suprachiasmatic nucleus (SCN), and Arcuate nucleus (AN). Animals were sacrificed in groups (n=6-8) at various time points during the afternoon of pro-oestrous. * indicates significant differences ($p<0.05$) from values at 1200 h.

Fig 6. NE levels in control and E2-treated groups during oestrous

Young female rats were implanted with slow-release E2 pellets for 30 (E-30), 60 (E-60) or 90 (E-90) days. Control rats were sham-implanted. Old constant oestrous (OCE) rats were used for comparison. Animals in the control and E-30 groups were sacrificed at 1200 h on the day of oestrous. Animals in the E-60, E-90 and OCE groups were in constant oestrous and sacrificed at 1200 h. NE levels (hatched bars) in the Diagonal band of Broca (DBB), Medial preoptic area (MPA), Suprachiasmatic nucleus (SCN), and Arcuate nucleus (AN) are shown. * indicates significant differences from the rest of the groups ($p<0.05$).

Fig 7. DA levels in control and E2-treated groups during oestrous

Young female rats were implanted with slow-release oestrogen pellets for 30 (E-30), 60 (E-60) or 90 (E-90) days. Control rats were sham-implanted. Old constant oestrous (OCE) rats were used for comparison. Animals in the control and E-30 groups were sacrificed at 1200 h on the day of oestrous. Animals in the E-60, E-90 and OCE groups were in constant oestrous and sacrificed at 1200 h. DA levels in the Diagonal band of Broca (DBB), Medial preoptic area (MPA), Suprachiasmatic nucleus (SCN), and Arcuate nucleus (AN) are shown. * indicates significant differences from the control and E-30 groups ($p < 0.05$).

Table 1

Changes in body weight and pituitary weight in control and E2-treated rats

Parameters	C-30	E-30	C-60	E-60	C-90	E-90
Body weight (g)	265.6±3	264±3	280.3±4	278.9±4	287.1±5	283.5±12
Pituitary weight (mg)	12.34±1	11.57±1	10.93±1	13.89±0.4*	14.475±0.8	13.76±0.4

Body weight and pituitary weight intreated-treated rats and their age-matched sham-implanted rats are shown.

* indicates significant difference(p<0.05) from corresponding age-matched control group.

Table 2

Ovarian morphology in control, E2-treated and old constant estrous rats

Ovarian structures	Control	E-30	E-60	E-90	OCE
Corpus luteum	13.6±1 ^a	10±0.75 ^b	4.4±1.6	2.2±1	0.9±1.5 ^b
Unovulated follicle	1±0.2	0.7±0.2	2.6±0.5 ^c	2.4±0.6 ^c	2.9±0.8 ^c
Secondary (developing) follicle	8.3±1.4 ^{cd}	9±2.1 ^d	7.25±2 ^d	3.3±0.3	2±0.3
Primary (developing) follicle	2.4±0.3	2.4±0.8	2.5±0.7	1.6±0.4 ^a	4±1.1
Atretic (degenerating) follicle	0.8±0.3	0.2±0.1	1.8±0.7	3±0.3 ^e	3±0.8 ^e

Ovarian morphology in control, E2-treated and old constant oestrous rats (n=8). Unovulated follicles, developing follicles (primary and secondary), corpora lutea and atretic follicles were counted manually and compared statistically

^a significantly different (p<0.05) compared to the rest of the groups

^b significant difference (p<0.05) from E-60 and E-90 groups.

^c significantly higher (p<0.05) than the control and E-30 groups

^d significantly higher than the E-90 and OCE groups

^e significantly higher (p<0.05) than the Control, E-30 and E-60 groups.

Table 3

Dopamine concentrations (pg/ μ g protein; mean $\pm$ S.E.) in control and E2-treated rats during the afternoon of proestrus

Area	Groups	1200 h	1400 h	1600 h	1800 h
DBB	Control	17.4 $\pm$ 1.1	11.1 $\pm$ 1.3	11.9 $\pm$ 1	13.9 $\pm$ 2.6
	E-30	11.9 $\pm$ 1.6	10 $\pm$ 2.6	11.4 $\pm$ 2.2	16.4 $\pm$ 2.4
MPA	Control	11.8 $\pm$ 3.8	6.9 $\pm$ 3	2.2 $\pm$ 0.9	0.7 $\pm$ 0.1
	E-30	14.9 $\pm$ 4	7.5 $\pm$ 2.3	2 $\pm$ 1*	1 $\pm$ 0.3*
SCN	Control	9 $\pm$ 1.5	6.2 $\pm$ 2.4	1.8 $\pm$ 0.4	3.4 $\pm$ 0.8
	E-30	8.4 $\pm$ 1.6	3.1 $\pm$ 1.4	4.1 $\pm$ 0.9	4.2 $\pm$ 0.5
AN	Control	12.7 $\pm$ 3	10.8 $\pm$ 3.1	5.7 $\pm$ 2.2	6.2 $\pm$ 3.3
	E-30	8.6 $\pm$ 1.7	8.7 $\pm$ 0.6	8 $\pm$ 1.2	8.6 $\pm$ 1.6

Dopamine levels in control and E2-treated rats during pro-oestrous (n=6-8). Young rats were either sham-implanted (control) or implanted with 30 day oestradiol slow-release pellets (20ng/day) (E-30). Animals were sacrificed in groups at different time points during the afternoon of pro-oestrous.

* indicates significant difference (p<0.05) from levels at 1200 h in the corresponding group.