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Effects of chronic estrogen exposure on catecholaminergic activity in the preoptico-suprachiasmatic-tuberoinfundibular system governing the regulation of luteinizing hormone surge in rats : an inquiry into the mechanisms of action and comparison with aging

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EFFECTS OF CHRONIC ESTROGEN EXPOSURE ON CATECHOLAMINERGIC ACTIVITY IN THE PREOPTICO-SUPRACHIASMATIC-TUBEROINFUNDIBULAR SYSTEM GOVERNING THE REGULATION OF LUTEINIZING HORMONE SURGE IN RATS: AN INQUIRY INTO MECHANISM OF ACTION AND COMPARISON WITH AGING

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ABSTRACT

EFFECTS OF CHRONIC ESTROGEN EXPOSURE ON CATECHOLAMINERGIC ACTIVITY IN THE PREOPTICO-SUPRACHIASMATIC-TUBEROINFUNDIBULAR SYSTEM GOVERNING THE REGULATION OF LUTEINIZING HORMONE SURGE IN RATS: AN INQUIRY INTO MECHANISM OF ACTION AND COMPARISON WITH AGING

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Reproductive aging in rats is characterized by progressive alterations in estrous cycles which are accompanied by transition into different states of acyclicity namely, constant estrus, persistent diestrus and finally anestrus. These are marked by changes in the neuroendocrine circuitry with reference to catecholamines in discrete areas of the hypothalamus and changes in the serum hormonal profile, namely that of luteinizing hormone (LH).

It is well known that reproductive decline in rats is a direct consequence of aging of the neuroendocrine apparatus, namely the hypothalamo-pituitary (HP) axis. Evidence for the role of estrogen in promoting the biological aging of the HP axis exists. In this series of studies, we test the hypothesis that chronic estrogen exposure may be responsible for the reproductive decline in rats and this is accompanied by alterations in the catecholamine profile in the hypothalamic areas governing the LH surge. An attempt is also made to investigate the possible role of nitric oxide (NO) and IL-1 beta in estrogen-mediated changes in the neuroendocrine system.

The results of these studies strongly support the idea of chronic estrogen exposure playing a major role in the reproductive decline in female rats. Chronic

estrogen exposure induced a constant estrous condition in a duration dependent manner very similar to that observed in aging animals. This effect persisted even after withdrawal of estrogen. This was accompanied by marked changes in the serum levels of estrogen (E2), progesterone (P4), their ratio (E2/P4), luteinizing hormone, ovarian histology and hypothalamic catecholaminergic activity. Marked changes were also observed in the positive feed back response to steroid priming. Strong evidence for the role of nitric oxide and proinflammatory cytokine, interleukin-1 beta in the estrogen-dependent reproductive aging process was observed.

In summary, it is concluded that the phenomenon of reproductive aging is an estrogen-dependent process and is probably mediated through suppression in the activity of norepinephrine (NE) in the hypothalamic areas governing the LH surge secretion. This is probably responsible for the alterations in the responsiveness of the neuroendocrine system to steroid priming. Results from this series of experiments suggests that chronic estrogen exposure could increase the level of interleukin-1 beta at the level of brain stem and an increase in nitric oxide metabolism resulting in the nitration of tyrosine hydroxylase in the medial preoptic area of the hypothalamus. This could be responsible for the reduction in NE levels leading to a decrease in LH levels leading to the constant estrus state.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AE	Anestrus
AN	Arcuate Nucleus
CEE	Chronic Estrogen Exposure
COMT	Catechol-O-Methyl Transferase
CX	Cortex
DA	Dopamine
D-1	Diestrus-1
D-2	Diestrus-2
DAS	Delayed Anovulatory syndrome
DBB	Diagonal Band of Broca
DBH	Dopamine Beta Hydroxylase
DHBA	Dihydroxy Benzylamine
DOPAC	Dihydroxy phenylacetic acid
E	Estrus
ER	Estrogen Receptor
E2	Estrogen
FSH	Follicle Stimulating Hormone
GABA	Gamma Amino Butyric Acid
GnRH	Gonadotropin Releasing Hormone
5-HIAA	5-hydroxy Indole Acetic acid

HPLC-EC	High Performance Liquid Chromatography with ElectroChemical Detection
5-HT	5-Hydroxy Tryptamine or Serotonin
IL-1β	Interleukin-1 beta
LH	Luteinizing Hormone
LHRH	Luteinizing Hormone Releasing Hormone
MAO	Monoamine Oxidase
MBH	Medial Basal Hypothalamus
ME	Median Eminence
MPA or MPOA	Medial Preoptic Area
MPN	Medial Preoptic Nucleus
NE	Norepinephrine
NO	Nitric Oxide
iNOS	inducible Nitric Oxide Synthase
nNOS	neuronal Nitric Oxide Synthase
OCE	Old constant estrus
O-M	Osborne-Mendel
OVX	Ovariectomy
P4	Progesterone
PBS	Phosphate Buffered Saline
PD	Persistent diestrus
PE	Proestrus
PRL	Prolactin

PSTS	Preoptico suprachiasmatic tuberoinfundibular system
RIA	Radio Immunoassay
SCN	Suprachiasmatic Nucleus
SPE	Spontaneous persistent estrus
TH	Tyrosine Hydroxylase
VMA	Vanilyl Mandeleic Acid

INTRODUCTION

Reproductive aging is characterized by progressive decline in reproductive capacity with age. This phenomenon has been of much interest to scientists for decades, and is well studied in rodents. Yet slow progress is being made because of the intrinsic difficulties associated with aging research. The following few paragraphs (pages 1-4) will briefly introduce the reader to the issue of reproductive aging in female rodents. This would be followed by a detailed background pertinent to this work.

Reproductive aging in female rats is characterized by progressive loss of reproductive cycles or estrous cycles [1] (as it is called in rodents) leading to a non-cyclic or acyclic state wherein there is complete cessation of estrous cyclicity. The Estrous cycle in rats was first characterized by Long and Evans (1922)[2]. It is predominantly a 4 day cycle and is divided into 4 stages. Each day is characterized by different patterns of cells exfoliating in the vaginal epithelium. Hence it is divided into 4 main stages namely, proestrus (PE), characterized by predominantly nucleated epithelial cells, followed by estrus (E), when large amounts of anucleated, cornified cells are found. This is followed by 2 days of diestrus, diestrus-1 (D-1), when the smears are predominantly leukocytic and diestrus-2 (D-2) where there is a mixed leukocytic epithelial cell population [2]. Each of these stages in turn is characterized by varying hormonal milieu in the blood (the details of how estrous cycles would be monitored are explained later in the methods section of chapter-1 and the associated hormonal changes are explained in later paragraphs).

This 4 day cyclicity is maintained in young rats from the time they reach puberty up to the age of around 9-12 months (middle age). When the rats reach this time point, middle age, their estrous cycles are prolonged or lengthened. Thus the 4-day estrous cycles gradually become a 5 or 6-day cycle [2]. During these lengthened cycles, the rats will go through more than one day of one or more of the following stages, namely, E, D-1 and D-2. A new cycle would be initiated with the onset of proestrus. As the rats age further and reach around 12-15 months of age, they enter a state of persistent (or constant) estrus (CE) wherein the animal is in the estrous state for 6-8 months (from 15 to 22 months of age) [1, 3]. This stage is characterized by the presence of very high estrogen levels in the blood and vaginal smear pattern consistent with estrus. Further aging is characterized by transition into the state of persistent diestrus (or pseudo pregnancy) (PD) for a few months [3], wherein the animal is in the D-1 stage on a daily basis throughout this period. This is followed by the entry into the state of anestrus (AE) [1], wherein the animal ceases to have any change in the estrus cycle pattern consistent with any of the above mentioned stages is observed.

In short, at around middle age (8-12 months) the regularly cycling rats enter a state of irregular cyclicity leading to the constant estrous state, followed by persistent diestrus and anestrus. An animal is said to be an "irregular cycler" when they exhibit lengthened cycles. An animal is considered "acyclic" when they exhibit the state of constant estrus (CE), persistent diestrus (PD) or anestrus. The age-dependent changes that are observed in estrous cycles leading ultimately to the constant estrous state are depicted in figure-1. The PD and

anestrus is not shown for simplicity, since this dissertation work does not deal with the issues beyond the constant estrous state.

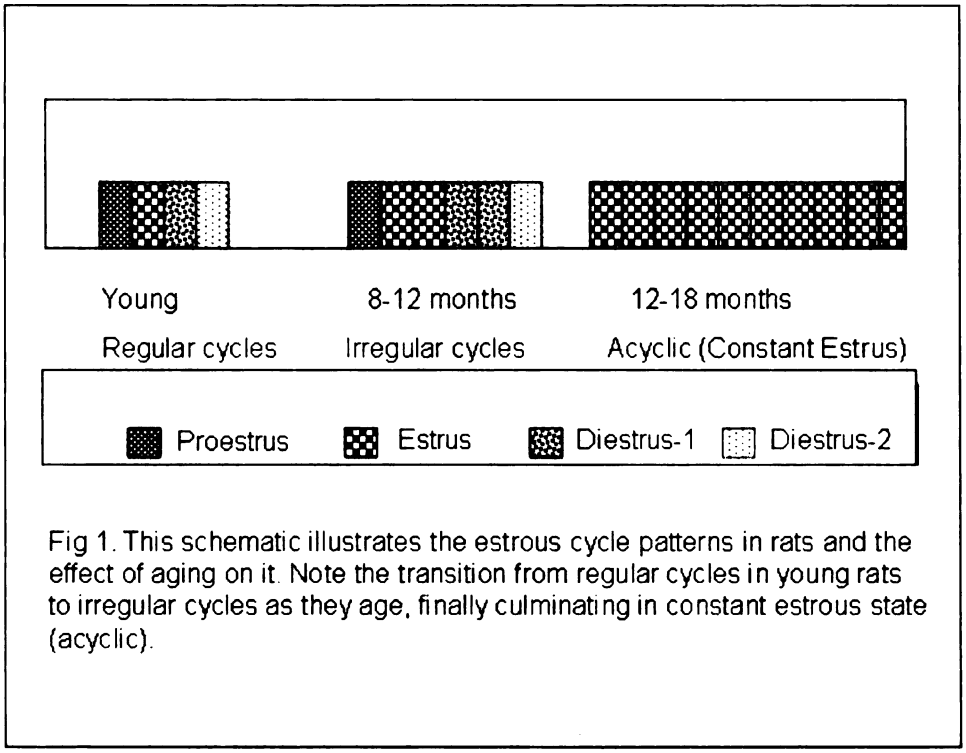

Fig 1. This schematic illustrates the estrous cycle patterns in rats and the effect of aging on it. Note the transition from regular cycles in young rats to irregular cycles as they age, finally culminating in constant estrous state (acyclic).

The reason for the gradual lengthening of the estrous cycle with ageing is not clearly understood. Similarly, the factors responsible for the transition from cyclic to an acyclic state, namely entry into CE or PD, are not known, not to mention their significance. The results of many classic studies clearly support the view that reproductive senescence in rats is a neuroendocrine event wherein aging of the neuroendocrine apparatus [4-6] cause cessation of reproductive activity. Also, there are studies which strongly support the view that ovarian factors are solely responsible for such decline in neuroendocrine functions [4-7], with estrogen being thought of as one of the main players in promoting reproductive aging [8-10]. But not much is known about these events.

This body of work is aimed at understanding the above described reproductive aging process in much detail from the neuroendocrine perspective. Here, the role estrogen play in bringing about cessation of estrous cycle is directly tested. Also, the associated neuroendocrine changes that happen during this process are documented.

The following paragraphs will introduce the reader to the background pertinent to the neuroendocrine control of reproduction in female rats, the classic works in the field of reproductive aging in rodents, and discuss the hypothesis that is being tested, followed by a brief overview of the chapters.

A. Estrous cycle in rats

Vaginal cytology

Since all the work described in this dissertation involved monitoring of estrous cycles in rat, I start with a brief introduction as to how that was performed. Estrous cycle in the rat is monitored by observing cell types present in the smears taken from the vagina each day. The vaginal smears are taken during the morning period on a daily basis at around 8-10 am. The detail of the methodology are given in the methods section of Chapter-1. The vaginal smears are prepared by lavage with lukewarm distilled water thinly spread on a glass slide. It is allowed to dry. The smears are then stained using methylene blue, rinsed with water and dried. The smears are read using the light microscope with a 10X objective and the cell types and their relative number are recorded. This information is then used to determine the stage of the estrous cycle.

Long and Evans (1922) [2](describes 5 stages in estrous cycles including the metestrus. This term is omitted here, as it is a very brief transition period between estrus and diestrus, and is here considered as the initial stage of diestrus. Diestrus is the period between estrus and the oncoming proestrus, and the vaginal cytology reveals the presence of large to moderate number of leukocytes. It is usually 2 days in rats with 4 day estrous cycles, and sometimes is 3 days long as in the case of 5 day cyclers, which is considered to be a normal variant. The rats used in the studies mentioned in this dissertation work were 4 day cyclers only. Diestrus is also the phase during which there is a gradual rise in the levels of estrogen in the blood. This rising estrogen is known to act on the vaginal epithelium leading to proliferation of epithelial cells as seen on proestrus and to the cornification of the same as observed on the day of estrus. The peak level of estrogen is reached on the afternoon of proestrus and then reaches the nadir on the morning of estrus [11, 12].

For the purpose of determination of the stage of estrous cycle based on vaginal cytology, the following guidelines were used:

Proestrus: Presence of large numbers (++ or +++) of small, rounded, epithelial cells with a hyperchromatic nucleus, either as loose cells or in clusters and presence of some mucus and minimal number of leukocytes & cornified cells.

Estrus: Presence of large numbers of cornified cells (++ or +++) (isolated or as sheets, and stains light blue), with minimal number of nucleated epithelial cells as seen on proestrus, and absence of leukocytes.

Diestrus-1: Presence in small or large numbers of cornified cells (+ or ++ or +++) as seen on estrus together with large numbers of leukocytes (++ or +++). The diestrus-1 stage described here includes the early metestrus. For practical purposes, both these stages are described as diestrus-1. There are minimal number of nucleated, epithelial cells.

Diestrus-2: Presence of relatively few numbers of leukocytes (+ or ++), with or without very few numbers of cornified cells (+) and newly forming epithelial cells (+).

Cell Type	Proestrus	Estrus	Diestrus-1	Diestrus-2
Epithelial cells (E)	++ or +++	None or +	None or +	None or +
Cornified cells (C)	None or +	++ or +++	+,++ or +++	None or +
Leukocytes (L)	None or +	None or +	++ or +++	+

Table-1: This table shows the numbers of different cell types that may be observed in the vaginal smears on a given stage of estrous cycle. The relative numbers of different types of cells is important in determining the stage of the estrous cycle.

As shown above, the relative numbers of these cell types are marked as one, two or three "+" signs in the data sheet. No "+" sign is noted if the cells of a given type is not found. In the end, the relative numbers of all the cell types, together with the presence or absence of a given cell type would be used as indicators of the stage of the estrous cycle. Table-1 gives an idea of how the stages would be

arrived at based on the relative cell numbers, and the figures-2A-2D shows the representative pictures of the vaginal smears as seen on each of these stages.

Hormonal changes during estrous cycle

As is now known, the estrous cycle is, in fact, driven by both the neural and the endocrine systems. The maintenance of estrus cycles is dependent on the integrity of at least the following organs namely, the hypothalamus, pituitary, the ovaries and the uterus.

Estrous cycle is characterized by the growth of the ovarian follicles across the cycle, under the influence of follicle stimulating hormone (FSH) from the anterior pituitary, culminating in their rupture under the influence of luteinizing hormone (LH) secreted from anterior pituitary, releasing the ova into the oviduct followed by luteinization of the ruptured follicles, on the night of proestrus . The growth of ovarian follicles is accompanied by a gradual increase in estrogen production and secretion. The thecal cells of ovarian follicles produce androgens that are converted to estrogens by aromatases secreted by granulosa cells. Estrogens that are thus formed are stored within the follicular fluid and also enter the general circulation. The level of serum estrogen is basal on the day of estrus, rises significantly on diestrus and reaches a peak and plateau on the afternoon of proestrus. During the early evening shortly before dark, its levels fall rapidly and reach basal levels early on the morning of estrus [11, 12].

In contrast to estrogen, Progesterone levels rise and fall twice during the cycle. The first peak occurs on the afternoon of metestrus and is derived from the newly formed corpora lutea. The second peak occurs on the proestrous

afternoon where progesterone is secreted by the granulosa cells of the preovulatory follicle [13-15].

The serum level of Luteinizing Hormone (LH) is low shortly after ovulation and this level is maintained till the early afternoon of proestrus. On the proestrous afternoon, a surge of LH occurs [16, 17] under the influence of the positive feed back effect of rising estrogen acting at the level of hypothalamo-hypophysial axis. This preovulatory LH surge is needed for ovulation to occur. The prolactin profile is similar to that of LH. Its levels are low during the evening of estrus and early in the morning of proestrus. On the afternoon of proestrus, there is a surge of prolactin release similar in timing to that of the LH surge [11, 16, 17].

The rising estrogen levels are associated with vaginal cornification as seen on the day of estrus and rise in progesterone is associated with leukocytic smears as seen on diestrus-1.

Ovulation and Luteinization: Role of LH

Luteinizing Hormone (LH) is a peptide hormone produced and secreted by the gonadotrophs of the anterior pituitary. The secretion of this hormone is under the nervous control of the hypothalamic peptide, gonadotrophin releasing hormone (GnRH) [18]. In females, LH is responsible for ovulation by its action on the ovarian follicles. There are two patterns of LH secretion, namely the pulsatile secretion and the surge secretion. The basal LH secretion is generally pulsatile in nature and occurs through out the estrus cycle. But, on the afternoon of proestrus, a surge in the levels of LH occurs [18]. This preovulatory LH surge is

responsible for the ovulation and is generally in excess of the minimal ovulatory quota of LH needed for ovulation. It is suggested that these two modes of LH secretion are governed by two distinct neuronal networks, the identity of which is still not known [18].

The occurrence of the LH surge in turn is dependent on estradiol. Estradiol-17 beta, the natural estrogen secreted from ovarian follicles, serves as the signal for the onset of the LH surge [19]. Estradiol levels in the blood rise gradually from the day of D-1 and is released from the growing ovarian follicles. Increasing levels of estradiol secreted from the growing follicles reaches a threshold when it is presumed to act on the neural systems and, by positive feedback action, initiates the series of events leading to the onset of the LH surge [19-21].

The Constant estrus condition in rats

As explained in the previous introductory paragraphs, constant estrus or constant estrus (CE) is a condition wherein the rat exhibits continuous cornification of the vaginal epithelium for few or more days [22]. This so called "vaginal estrus" condition is due to presence of high circulating estrogen levels in the blood [1] and is usually known to be accompanied by the presence, in the ovaries, of follicles and absence of corpora lutea [3]. This condition could be due to many factors, intrinsic and extrinsic, and is known to occur in aging animals..

Spontaneous Constant estrus (SPE)

The constant estrus condition is known to occur spontaneously in aging rats at some point in their life, and was first recognized by Evans and Long (1922) .[2]

The age of occurrence is known to be a function of the genetic background in rats. Thus, some strains like the Albany A-S strain was shown to exhibit this CE condition within the first year while the Vanderbilt V-S strain has been shown to exhibit this condition during their second year of life [23]. Everett has shown that DA rats exhibit the CE condition during their 4^{th} or 5^{th} month of age and he compared 2 breeding lines of this strain to find out that 50% of the DA_c strain exhibited this condition by 135 days of age while those of DA_f strain showed CE by 210 days. The same was not true with Wistar and O-M rats who showed regular cycles at comparable ages and the O-M rats were not found to display CE condition before the first year. Everett has reported that when the O-M strains and DA_c strains were crossed, their first and second generation hybrids were found to exhibit CE condition at an intermediate age than either of their parental strains [22].

Thus it becomes apparent that the use of a proper strain of rats is important for a work such as this one, where such reproductive aging condition does not spontaneously occur when the rats are young. Also it is convincing that the genetic background does play a role in the determination of the onset of such condition. The rats used in this dissertation were Sprague-Dawley (Harlan), and they exhibited spontaneous CE at around 11-13 months or later. This strain of rat has been used extensively in reproductive aging studies both in our laboratory [24-30] and by others [9, 10, 31, 32].

Induced constant estrus

The occurrence of CE is not only dependent upon the genetic background as explained above, but is also known to be influenced by factors such as the duration of the daily light-dark cycle, exposure to estrogenic compounds during adult life and by perinatal steroid (androgen) exposure.

Constant illumination-induced constant estrus

The duration of illumination is known to be a determining factor as to whether an animal will exhibit estrous cycle or not. Hemmingsen and Krarup (1937) (reference not included), and Browman (1937) [33] demonstrated that rats exposed to constant illumination exhibited persistent cornification of vaginal epithelium. Everett has shown that when rats were exposed to natural illumination, the length of the day/night cycle affected the estrous cycle pattern [22]. When the days were short as seen during the months of December- March, regularly cycling females tended to exhibit estrous cycles with lengthened diestrus phase and some became anestrus, while aged rats which initially were in constant estrus, appeared to resume cyclicity. But once the day length increased during the following months, the rats which showed estrous cycles with prolonged diestrus tended to show regular cycles while the older rats once again became constant estrus. When this was experimentally tested by using artificial illumination, he could clearly establish that by decreasing day light he could increase the percentage of animals exhibiting persistent diestrus and decrease the animals showing constant estrus. But, when the day light was increased, he could increase the percentage of animals showing constant estrus and decrease the percentage of animals showing PD. He concluded that "the critical factor was

evidently some particular duration of illumination (or darkness) and not the rate of increase or decrease. Above a critical ratio of light : darkness, regular cycles or constant estrus prevailed, while below that ratio, cyclic diestrus lengthened in the younger animals and constant estrus gave way to cycles in the older rats"[23].

Estrogen-induced constant estrus

Both chronic and acute estrogen(E2) administration studies show induction of constant estrous state. Administration of 2.5 mg of estradiol-benzoate to young rats has been shown to cause constant estrous condition for months, and maintenance of E2 in the physiological range for 1.5 months has been shown to cause permanent anovulatory syndrome in mice when they become adults [6]. Constant estrous condition could also be induced in young cycling rodents by chronic treatment with estradiol as shown in mice by Caleb Finch et al. [8]. Administration of E2 through drinking water for both short (2 weeks) and longer duration, has been shown to produce the state of acyclicity. Longer treatment has been shown to cause functional deficits at the neuroendocrine level. There are also reports that sheep grazing on clover leaves develop a similar state of acyclicity which has been thought to be due to the concentration of high estrogenic compounds in those leaves [34]. Similar studies in ovariectomized rats have shown that chronic exposure to estradiol can cause loss of responsiveness of the neuroendocrine apparatus to steroids in inducing LH surge [9], similar to what is seen in aging animals.

Perinatal exposure to steroids (Delayed Anovulatory Syndrome)

Exposing rats at 1 week of age to testosterone causes premature cessation of estrous cycles when they are young [35]. This phenomenon is called as Delayed Anovulatory Syndrome or DAS [35]. This DAS is known to involve severe alterations in the neuroendocrine system affecting the normal regulation of LH secretion. The neuroendocrine alteration in the DAS model is dependent upon the presence of ovaries. This was proved by ovariectomizing these rats immediately after weaning and testing the occurrence of LH surge. Removal of ovaries reduces these neuroendocrine alterations seen in the DAS strongly suggesting the dependency on ovaries for DAS to fully manifest. Similar study was carried out by Mobbs, et al., (1985) [36] where they induced DAS in mice through neonatal administration of estradiol and found similar neuroendocrine changes as seen in aging animals. These studies support the idea of ovarian impairments caused by neuroendocrine changes as seen in normal aging rats.

B. Neural control of reproduction

The fact that most of the endocrine functions, and more specifically those of the pituitary, are under the direct control of hypothalamic structures comes from its demonstration by Geoffrey Harris [37]. Since then it has become clear through various studies that many hypothalamic and other non-hypothalamic areas do contribute to the governance of various endocrine functions, one of which is reproduction. Reproductive function in female rats is specifically more complex with the occurrence of regular estrous cycles and its associated hormonal changes. Specific areas of the hypothalamus were found to be involved in the

control of the estrous cycle through studies using various approaches, which involved examining at the effects of specific lesions in hypothalamic structures on reproduction, use of pharmacologic agents to block or enhance neurotransmission, deafferentation studies, etc., all of which helped in understanding of the control of reproductive cycles and more specifically, the control of LH secretion. Yet, complete understanding of the regulation of LH secretion has not been possible due to the involvement of a variety of factors.

Evidence for the neural control of spontaneous ovulation

The first evidence for the neural control of spontaneous ovulation in rats was provided by the studies of Everett et al., where they showed that administration of an alpha-adrenergic blocker, dibenamine, at different time points on the morning of proestrus was able to block spontaneous ovulation, where as administration of the same at 1600 hours on proestrus failed to block ovulation [38]. This was also proved using atropine, an anti-cholinergic agent. The results of this study helped in the development of the idea of the presence of a "critical period" on the proestrus afternoon during or before which the neural stimulus from the hypothalamus is transmitted to the anterior pituitary gland, and hence this could be blocked by administration of specific drugs if given before this critical period, after which the stimulus has already been transmitted, and hence ovulation could not be blocked. Later studies also revealed that other drugs were equally effective in the blockade of ovulation. These included morphine [39, 40], chlorpromazine [39, 40], reserpine [39, 40], and cannabinoids [41]. Through

various studies, they were able to limit the critical period to a two hour time frame from 1400h to 1600h on the day of proestrus.

Results from electrochemical stimulation and lesioning studies

The evidence for the role of the rostral hypothalamic areas in the control of gonadotrophin secretion comes from the work of Hillarp (1949) [42] where he induced persistent vaginal cornification through bilateral destruction of anterior hypothalamic areas. Specifically, bilateral destruction of the medial preoptic nucleus (MPN) has been shown to cause constant estrus condition [43], and similar destruction of the medial preoptic area (MPA) without destruction of MPN, has been shown to cause repeated pseudopregnancies [44].

Knife-cut studies by Halasz and Gorski (1967) [45], and those of Tejasen and Everett (1967) [46] also support the idea of the involvement of rostral hypothalamic structures in the control of ovulation. In their studies, Tejasen and Everett (1967) [46] showed that the unilateral MPOA-Electrochemical stimulation-induced ovulation in pentobarbital-blocked proestrus rats and this could be prevented by knife cuts behind the stimulation site. They showed that while contra-lateral cuts failed to block ovulation, ipsilateral cuts were able to block the ovulation. They could also map the extent of the cut required to block ovulation. Similarly, Everett (1965) [47] found that stimulation of the medial preoptic area was able to induce ovulation in most of the rats as compared to stimulation of the lateral preoptic area, and that MPA stimulation were not that effective (had higher stimulation threshold) in inducing ovulation as compared to stimulation of the tuberal regions. Induction of ovulation through electrochemical stimulation of the

preoptic area in old constant estrus rats have also been reported by a few groups [44, 48, 49]. Many such studies as described in the preceding paragraphs support the view of rostral hypothalamic areas playing an important role in ovulation and that they contain the elements required for gonadotrophin secretion (also see [18, 50]).

The various neural structures that are considered to be important for the control of normal gonadotrophin secretion include, the anterior hypothalamic areas specifically the diagonal band of broca (DBB), the medial preoptic nucleus (MPN) the medial preoptic area (MPA), the suprachiasmatic nucleus (SCN), the arcuate nucleus (AN) and the median eminence (ME) [50]. Several studies have shown the distribution of GnRH cell bodies in these areas, specifically in the anterior hypothalamic areas namely the DBB and MPA, and is known to extend from the DBB to the premamillary region [51-54]. Though these hypothalamic structures are known to be important for the secretion of GnRH, specifically how the neural signals are transmitted for the initiation of GnRH production and release, and how steroids are able to influence the same are still not clear. The functional relationships between these areas are still under investigation.

It was shown that lesioning of the SCN would render the rats into a state of constant estrus [11]. Progesterone treatment of the SCN lesioned rats was able to reinstate the release of LH and hence ovulation if other hypothalamic structures were not destroyed [43, 55, 56]. The same was possible with the electrical stimulation methods [57]. It was reasoned by Kalra SP [50], based on this and other evidence that "SCN ablation either deprived the rats of the driving

signal or that their expression was impaired due to an inappropriate steroid environment, thereby leading to the anovulatory state".

Medial Preoptic Nucleus (MPN) (or antero-ventral periventricular nucleus) is an area which is considered to be very important in the control of ovulation/gonadotrophin secretion. MPN is known to contain steroid concentrating neurons [58]. As was mentioned earlier, careful destruction of the MPN caused constant estrus condition which was not reversible even with the administration of steroids or electrical stimulation [43]. This is in contrast with the destruction of SCN, where steroid treatment or electrical stimulation of the bed nucleus of stria terminalis was able to reinstate the GnRH surge [43, 55-57]. It is thought that the action of ovarian steroids on the initiation of the LH surge (positive feedback) could be brought about through the involvement of the MPN and the MPA, where the majority of GnRH cell bodies are situated.

The MPA is known to contain the cell bodies of GnRH neurons [51-54] and those of other neurons which are known to produce other neurotransmitters/modulators. Specifically, GnRH neurons have been found in close apposition to the terminals of noradrenergic fibers originating from the brain stem [59] and these noradrenergic fibers are also known to synapse with GABAergic neurons [60]. Also, GABAergic neurons are known to synapse with the GnRH cell bodies [61].

The arcuate nucleus (AN), which is located in the ventral hypothalamic area close to the base of the third ventricle, is known to contain steroid concentrating

neurons [58], many neurons are known to project to the median eminence (ME), and produce neuropeptides involved in the steroidal feed back regulation.

The Median Eminence (ME) is the region richly supplied with capillary networks in the base of the hypothalamus and serves as the connecting link between the hypothalamus and the pituitary. This structure is innervated by hypothalamic neuronal systems releasing neurotransmitters/neuromodulators and the peptides like the GnRH [62], into the circulation which are directly taken to the anterior pituitary where they act on the corresponding cell population (like gonadotrophs) leading to the secretion ofLH into circulation.

GnRH producing neurons have been found to be located in the DBB, septal complex and the MPA [51-54]. The fibers from these regions are known to innervate the ME [63, 64]. The GnRH content in the ME is known to increase before the occurrence of the LH surge in cycling rats [31, 65]. This proestrus type response is also seen in overeictomized rats given estrogen and progesterone [66]. The rate of secretion of GnRH increases on the afternoon of proestrus [67, 68] as compared to that of other stages of estrus cycle, and this increase is believed to contribute to the preovulatory surge of LH.

Catecholamines and LH secretion

Various studies have attested to the role of the catecholamines in the secretion of LH. Specifically they include norepinephrine (NE) and epinephrine (E). Inhibitors of dopamine-beta-hydroxylase (DBH), which will decrease the activity of NE and E have been shown to inhibit the proestrus LH surge [69]. This

has also been tested in ovariectomized animals [70]. Alpha-1b adrenergic receptors have been shown to be located on the GnRH neurons in the preoptic area and DBB [71] and that alpha 1-adrenergic receptor blockers were able to inhibit the proestrus LH surge in intact animals[69, 72] and the LH surge in the ovariectomized-steroid primed animals [70]. Administration of NE has been shown to reverse the effect of both DBH blockers [69, 70], and to elicit the LH surge in proestrus and gonadectomized steroid primed animals. Increase in the catecholamine activity (content, turnover and release) has been shown to occur in the rostral hypothalamic structures [28, 29, 73-75] implicated in the governance of the LH surge regulation . DBH inhibitors which block the production of NE and E have been shown to inhibit the rise in the GnRH content in the medial basal hypothalamus (MBH) [76, 77]. Blockade of electrochemical stimulation induced-LH release, by the administration of adrenergic blockers again favors the role of catecholamines in the generation of LH surge.

Further supportive evidence in favor of the role played by catecholamines comes from studies in aged constant estrus rats where administration of the precursors of catecholamine (L-DOPA) [78], or inhibitors (L-deprenyl) [79] of the monoamine oxidase (MAO), an enzyme which degrades catecholamines, have been shown to reinitiate estrus cycles suggesting the decline in the catecholamine activity in the hypothalamus as the cause of reproductive aging. All these facts taken together support the idea of the role played by catecholamines in the events associated with the LH surge.

Models used for the study of Hypothalamo-pituitary-gonadal(HPG) function:

In this series of studies we used both intact animals and ovariectomized subjects as models in order to study the effects of chronic estrogen exposure on the NE and LH system. In those studies where monitoring of estrous cycles were needed, for obvious reasons, intact animals were used. But since chronic treatment with estrogen interfered with estrous cyclicity, in order to assess the lesion at a functional level with respect to the HPG axis we resorted to the use of the acutely ovariectomized animal model, as this will allow us to test the responsiveness of the neural system to steroid priming, in the absence of endogenous steroids.

Use of ovariectomized animals in the generation of the LH surge

Since rising estradiol levels in the serum during diestrus and the morning of proestrus, serves as the stimulator of LH surge secretion [20] on the afternoon of proestrus , removal of ovaries do abolish the further occurrence of the LH surge. But, administration of estradiol to the ovariectomized rats is known to produce daily LH surges [20] (though the amplitude is much lower than that observed on the day of proestrus) and further administration of progesterone to the estrogen primed animals is known to enhance (positive feedback) the LH surge similar to what is seen on the day of proestrus [66, 76]. Also, it is known to prevent the occurrences of LH surges on the following days (Negative feedback) and hence is known to have biphasic effects similar to those of estrogen. Hence, ovariectomized, steroid-primed (Estrogen + Progesterone) animals are used as models to study the GnRH system after various experimental manipulations.

C. Reproductive Aging in Rodents

Changes in vaginal cytology with age

As the animals age reach 10-12 months of age, estrus cycles tend to become lengthened and irregular [3]. A 4-day cycler will typically become a 5-day cycler and enter the state of constant estrus characterized by the presence of cornified cells in vaginal smears [22]. Older rats have been shown to remain in the state of constant estrus from 12-16 months of age [3] followed by persistent diestrus (or repeated psudopregnancy) after 24 months and then proceed to a state of anestrus [1].

Changes in hormonal profile with aging

Serum LH profile has been shown to change with age where the amplitude of the LH surge is known to decrease compared to that seen in young females [31, 80]. LH levels are very low during the constant estrus state and there is a lack of cyclic increases in circulating LH levels [1, 3]. Similarly, ovariectomy-induced increase in the basal levels of LH is reduced in constant estrus animals compared to young cycling females [81]. The responsiveness to steroid priming has been to shown to be decreased with age which is manifested as a decline in the amplitude of the LH surge with age in ovariectomized subjects [30].

Aging and Hypothalamic catecholamine activity

Decline in the NE content of the hypothalamus of the rats and greater reduction specifically in discrete areas of the hypothalamus (anterior hypothalamus, arcuate nucleus and SCN) during aging is well known [28, 82].

NE turnover is found to be reduced in aging animals [82]. In constant estrous rats (CE) ovariectomy-induced-increased NE turnover is not appreciable. NE release also declines with age in discrete areas of hypothalamus [29] involved in reproduction along with altered release of NE in response to steroid priming [30].

Aging and anterior pituitary

The LH,FSH content of the anterior pituitary is not altered in constant estrous animals as compared with the young ones [83], and the GnRH induced LH release is not altered in constant estrous animals [84], though there are some alterations in the responsiveness to estradiol administration in constant estrous (CE) animals compared to the pseudopregnant animals [85]. The responsiveness to ovarian steroids are shown to be affected in CE animals, where steroid priming was not found to elicit a normal LH surge both in intact and acutely ovariectomized animals [7, 85]. When the CE rats were chronically ovariectomized and tested, the steroid priming was able to cause an increase in the LH release [85].

Reinstatement of estrous cycles in old rats

Though estrous cycles tend to get irregular as a function of age eventually leading to a state of constant estrus (OCE), characterized by complete lack of ovulation, it is possible to reinitiate estrus cycles in these old rats. This is possible with the injection of progesterone to these animals [86]. Once the animals started to exhibit estrous cycles, repetitive progesterone injections on the day of proestrus and estrus were able to maintain estrous cycles for a while. Similarly, administration of catecholamine precursors [78], ergot drugs [78] and the

monoamine oxidase inhibitor L-deprenyl [79], to these OCE rats were shown to induce cyclicity. All of the above studies not only support the fact that estrous cycles can be reinitiated in older animals, but also highlights the role of catecholamines in the maintenance of estrous cycles and supports the idea that decline in the brain catecholamines with age as one of the main causes of reproductive aging in female rats.

Neuroendocrine aging & Reproductive senescence

One of the classic studies which initiated the concept of hypothalamic aging was done by Pierre Aschheim [4, 87]. He observed that when older, acyclic rats were grafted with ovaries from either young or old donors, it failed to maintain estrous cycles. Whereas, if the older acyclic recipients were ovariectomized when young, and then grafted(when they were older) with ovaries from young or older animals, the estrous cycles resumed and was maintained. He concluded that the presence of ovaries somehow caused aging of the hypothalamo-pituitary unit, thereby causing decline in their functions with age, leading to cessation of estrous cycles. When the animals were ovariectomized while young, thereby preventing the neuroendocrine system from "seeing" ovarian secretions, their integrity could be preserved. This idea was again supported by his experiment with hypophysectomized subjects.

Similar studies done by the group of Caleb Finch [5, 6] support the conclusion that, neuroendocrine aging is dependent upon the presence of ovarian factors with estrogen being one of the most sought after candidates. There are many

lines of evidence to indicate that estrogen plays serious role in the aging of the neuroendocrine system governing reproduction. Injection of estradiol valerate can cause gliosis-like reactions in the arcuate nucleus [88] and this is dependent upon the presence of ovaries [89]. Similar evidence of inflammation could be found in older constant estrus animals and this could be prevented by ovariectomy when the animals are young [89]. Administration of estrogen to young animals has been shown to cause anovulatory syndrome when they are adults [36, 88] and chronic E2 exposure has been shown to suppress the GnRH system by abolishing the responsiveness to ovarian steroids [9]. In this series of studies we plan to investigate the possible mechanisms that may be involved in mediating the effects of chronic estrogen exposure on the HPG axis.

D. Overall Objective of this study

This dissertation focuses on the issues related to the above described reproductive aging process. As explained before, there is a progressive decline in the reproductive capacity with age manifested as occurrence of irregular or lengthened estrous cycles accompanied by alterations in the LH surge amplitude and timing eventually progressing to the constant estrous state, where there is complete absence of the LH surge and hence ovulation. There is strong evidence to point to the fact that this reproductive decline with age is a neuroendocrine phenomenon and is dependent at least partially upon the presence of ovaries. There are supportive lines of evidence to suggest that estrogen could play a role in the sequential suppression of reproductive cycles and that the hypothesis that

this reproductive decline could be due to the cumulative effects of estradiol exposure across the entire period till the occurrence of reproductive decline in middle age.

We test the hypothesis that *"the reproductive decline in female rats is due to the cumulative effect of estradiol exposure during normal reproductive cycle. This alteration in the estrous cycle is brought about by the action of estradiol on the neuroendocrine circuitry, at the level of hypothalamus and/or brain stem noradrenergic neurons, thereby leading to a reduction in the noradrenergic tone in the hypothalamus, eventually causing a progressive decline in the LH surge amplitude. These series of events manifests as alterations in the estrous cycles with age with the onset of constant estrous condition".*

To test this hypothesis we used the following specific aims:

Specific Aim-1:

Chronic proestrus-levels of estradiol treatment of young intact rats cause irreversible alterations in estrous cycles.

Specific Aim-2:

Chronic E2 exposure alters serum LH levels and catecholaminergic activity in discrete areas of the hypothalamus involved in the control of LH secretion (surge).

Specific Aim-3:

Chronic E2 exposure-induced alterations in the estrous cycle are mediated through alterations in the responsiveness of the neuroendocrine system to ovarian steroids.

Specific Aim-4:

The alterations in noradrenergic activity in the hypothalamus and/or the responsiveness to steroid priming are a consequence of inactivation of tyrosine hydroxylase, the rate limiting enzyme in the synthesis of norepinephrine.

Though the above specific aims tend to test the overall hypothesis in a holistic sense, giving us a broad understanding of the problem at hand, they are in no way considered complete and more mechanistic experiments would be needed to answer more specific questions and to find out the true cause-effect relationships between the various events that take place.

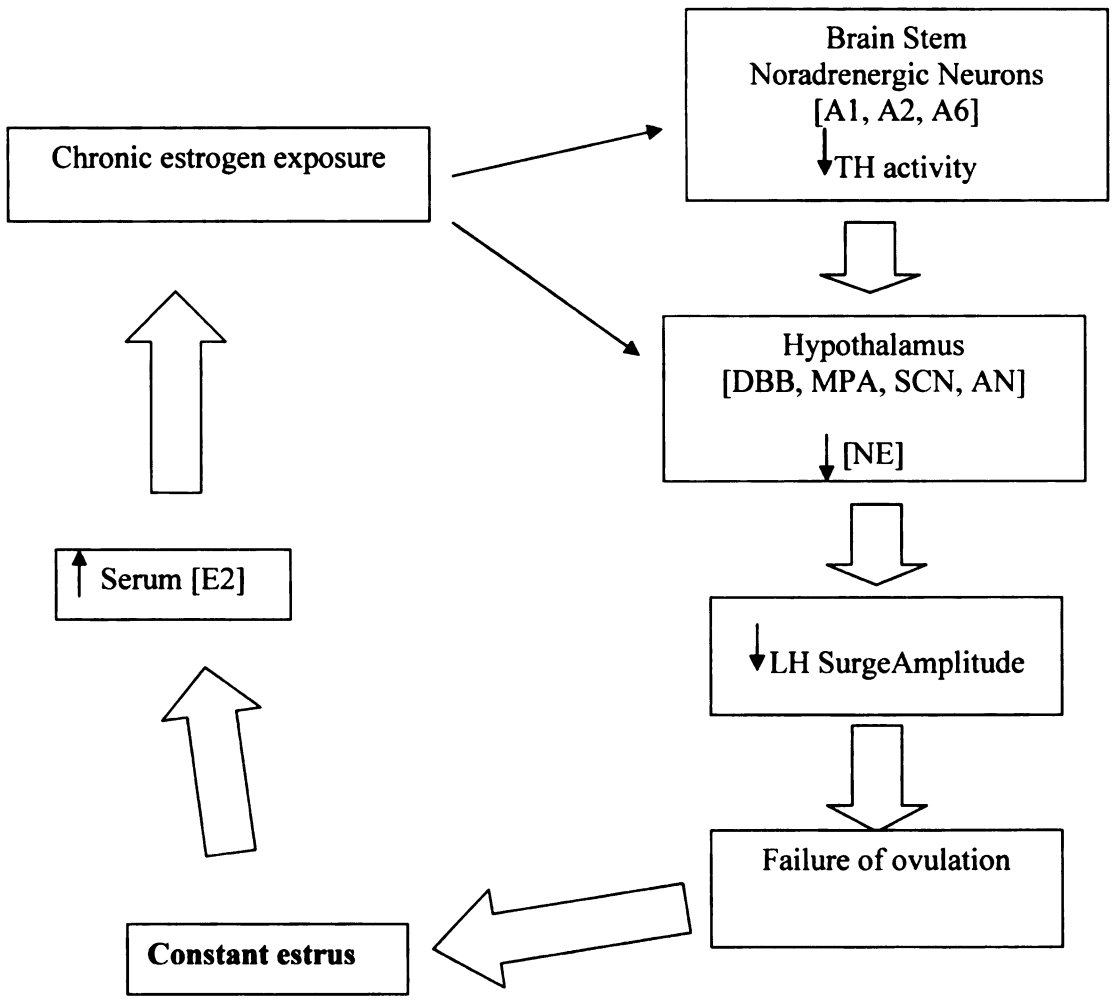

Figure 2: **Schematic of the Hypothesis:** Chronic exposure to estradiol causes decline in the hypothalamic noradrenergic activity through the action of E2 on brain stem and/or hypothalamus, leading to suppression of LH surge which leads to constant estrous condition. The more estradiol secreted from the unovulated follicles during constant estrus in turn contributes to more severe alterations in the neuroendocrine apparatus setting up a vicious cycle.

E. Brief overview of this dissertation

In chapter-1, we tested the hypothesis that chronic E2 exposure causes reproductive aging in rats and that this is an "irreversible" process. This we studied by following estrous cycles both during the E2 exposure period and during the withdrawal periods. If chronic E2 exposure does interfere with reproductive cycles, then we expect to find disruption of regular estrous cycles, presumably leading to the constant estrous condition. If so, then the question of whether this could be reversed or not is investigated by following the estrous cycles after the treatment period of 60 and 90 days. If it is reversible we expect to find the occurrence of regular estrous cycles as seen in controls. Demonstration of constant estrous condition, irregular cycles or pseudopregnancy would be considered to be the result of permanent alterations in the neuroendocrine system due to chronic estrogen exposure.

In chapter-2 the hypotheses that, chronic E2 exposure-induced constant estrous condition is accompanied and probably caused by alterations in the neuroendocrine system with reference to both LH and NE. We expect to observe a reduction in LH secretion similar to what is seen in older constant estrous animals (OCE) and that this would be accompanied by alterations in the NE activity (content) in discrete nuclei of the hypothalamus involved in the control of LH surge. This, again, should reflect those seen in OCE animals.

Since chronic E2 exposure did cause a constant estrous-like condition, and these animals had suppressed LH and NE levels, in chapter-3, we further tested

the hypothesis that chronic E2-induced constant estrous condition is mediated through alterations in the responsiveness of neuroendocrine system to ovarian steroids there by leading to a suppression of the LH surge. This we tested using an ovariectomized, steroid-primed animal model. The outcome was followed by measuring the levels of serum LH and NE release in the MPA. This experiment mechanistically tested the cause of chronic estrogen induced acyclicity, though the relationship between the NE, LH and estrous cycle still remains correlative. (chapter-3).

Since we did find suppression in the NE activity, and NE activity is well known to be important for the occurrence of the LH surge, we attempted to investigate the cause of such decline in NE activity in 60 and 90 days E2 treated animals (chapter-4). We hypothesizes that the reduction in NE levels could be due to a reduction in tyrosine hydroxylase activity since tyrosine hyroxylase is the rate limiting enzyme in the synthesis of NE.

CHAPTER-1

EFFECTS OF CHRONIC ESTROGEN EXPOSURE ON ESTROUS CYCLES, OVARIES AND SERUM ESTROGEN AND PROGESTERONE IN RATS

A. Introduction

Reproductive aging is characterized by progressive alterations in estrous cycle patterns during middle age [1, 3, 90], followed by entry in to the state of persistent estrus [1, 3]. This state is characterized by persistent vaginal cornification and higher levels of estrogen in the blood. The reason for the entry into the persistent estrous state is not clearly understood. Several lines of evidence support for the role of ovarian factors in the phenomenon of reproductive aging which is believed to be the direct consequence of the biological aging of the neuroendocrine apparatus (hypothalamus and pituitary) [4-7]. Specifically, estrogen has been shown to cause alterations in the neuroendocrine system leading to aging-like changes with respect to the estrous cycles and to the responsiveness of the LHRH-LH system to steroid-priming [8-10]. In this study we test the hypothesis that 'cumulative exposure to high levels of endogenous estrogen during the proestrus of successive estrous cycle is responsible for the entry of rats into persistent estrous state probably by causing irreversible changes in the neuroendocrine system controlling reproduction'. To do this, we monitored the estrous cycles both during and after (withdrawal phase)

the estrogen treatment regimen. Also, we attempted to create a model for the reproductive aging process using young rats.

B. Methods

Animals:

Adult female Sprague-Dawley rats, around 3-4 months of age, were obtained from Harlan Sprague-Dawley, Inc., (Indianapolis, IN, USA) and were housed in groups of 3 per cage in temperature (23±2°C) and light-controlled (lights on from 0500 to 1900 h) rooms. They were given food and water *ad libitum*. The animals were used for the experiment after 2 weeks of arrival. All the protocols followed in this study were approved by the University Committee for Animal Care and Use at Michigan State University.

Chemicals and Reagents:

Slow Release 17β-Estradiol pellets were obtained from Innovative Research of America (Sarasota, FL, USA).. The RIA kit for estradiol-17β was obtained from Diagnostic Product Corporation (LosAngeles, CA, USA).

Treatment:

All the female rats were around 3-4 months of age at the beginning of the experiment. Vaginal smears were taken every day between 0800-1000 h. Those animals showing regular 4 day cycles were included in the experiment. The animals were randomly divided into either control or treatment groups. Animals in the treatment groups were implanted subcutaneously with slow release estrogen pellets under mild halothane anesthesia. The pellets would release estradiol 17 beta estrogen at the rate of 20 ng per day for a period of 30, 60 or 90 days

(Innovative Research America, Sarasota, FL). Control animals were sham implanted. Vaginal cytology was monitored throughout the experimental period and withdrawal period, when the relative number of epithelial cells, cornified cells, and leukocytes were recorded for all the groups. This was used to determine the stage of the estrus cycle.

Vaginal Cytology:

Vaginal lavage was performed daily between 0800-1000 hours for all the rats. We used lukewarm, autoclaved, nanopure water for obtaining vaginal smears. Briefly, the vagina was washed with the water 3 or 4 times using a small medicine dropper. A thin film of the lavage was spread on a plain glass slide (previously numbered for each rat). The smears were allowed to dry at 60 degree C for 30 minutes and then stained with methylene blue for 30 seconds. The slides were rinsed in tap water and allowed to dry. The smears were studied under a light microscope using the 10X objective.

Animals were sacrificed by decapitation at the end of the treatment period (30, 60 or 90 days) along with appropriate controls. Controls were matched for age and for the stage of estrus cycle. Animals were sacrificed on the day of proestrus (cycling) or on the day of estrus (non-cycling). The majority of the animals in the E-30 group along with controls were sacrificed on the afternoon of proestrus at 1200, 1400, 1600 and 1800 h in groups of 8. Some animals in E-30 group exhibited constant estrus and were sacrificed at 1200 h. Animals from the E-60 and E-90 groups exhibited constant estrus and were sacrificed at 1200 h along

with controls. Few animals from E-60 and E-90 groups were followed up for vaginal cytology beyond the treatment period, to study the effects of estrogen withdrawal. These were designated as the E-60W and E-90W groups respectively.

Body weights, were obtained at the beginning of treatment and at the time of sacrifice. Upon sacrifice, trunk blood was collected. Brain, anterior pituitaries and ovaries were harvested as mentioned below. Serum was separated from trunk blood and stored at -70°C until they were used for hormone assays. The brain and brain stem were removed and quickly frozen on dry ice. They were then stored at -70°C until the time of sectioning. Ovaries were removed bilaterally and fixed in paraformaldehyde (10%) overnight and then in 70% ethanol. They were stored in the refrigerator until the time of histological examination.

Hormone Measurements:

All the hormones estrogen (E2), and LH were measured in duplicates in the serum by radioimmuno assay (RIA) using a gamma counter (Beckman). Competitive binding assay using double antibody method was used for the LH assay. The radioactive LH label was obtained from Amersham Pharmacia Biotech. The standards for LH and the Anti-LH (anti rLH-S11) antibody were all purchased from Dr. Parlow, NIDDK. The secondary antibody used for LH assay was indigenous.

C.Results

Effect of estrogen treatment on Body Weight:

Administration of estrogen has been shown to modulate body weight [91, 92] in rat models. The body weights of control and estrogen treated rats are shown in fig. 3. Body weights (Mean±S.E.; g) of control rats at the end of 30, 60 and 90 day treatment were 265.6±3.75, 280.3±4.47 and 287.13±5.215 respectively. Estrogen treatment for 30, 60 or 90 days did not produce any significant change in body weight (264±3.81, 278.9±4.32 and 283.5±12.95 respectively) compared to the corresponding age-matched controls.

Interestingly, the body weight decreased significantly ($F(7,77)=2.279, P<0.05$) upon withdrawal of estrogen after the treatment periods of 60 (276.85±4.36), 90 days(265.26±3.67).

Effect of chronic estrogen exposure on anterior pituitary weight:

Anterior pituitary weight increased both in control and treatment animals with increasing duration of treatment (fig.4). This could be attributed to the differences in ages between the three paired groups. The weight of anterior pituitary was normalized with the body weight for more meaningful comparisons. There was no significant change in normalized pituitary weight (mg/gm; mean±SE) between the control and E2 treated group in both the 30 (0.046±.004 and 0.044±0.004 respectively) and 90 (0.051±0.003 and 0.049±0.002 respectively) day cohorts. However, we did see a significant increase in the 60 day estrogen treated group (0.05±0.001) compared to controls (0.041±0.003) though no gross changes were

observed visually. A significant decrease ($F(7,77)=10.12, P<0.001$) was observed in the groups upon E2 withdrawal after 60 (0.032±0.003) and 90 (0.027±0.002) days respectively.

Estrus cycles: Effects of E2 treatment:

To test how chronic estradiol exposure affects the estrus cycles, we monitored the estrus cyclicity by vaginal cytology. The results indicate that, while all the animals in the control groups were cycling through out the experimental period up to the age of around 6 months, the same was not the case in the estradiol treated groups (Fig 3). Upon 30 days of E2 treatment, around 20 percent of the rats exhibited persistent estrus condition during the last 5 days while the remaining 80 percent of the rats were regular cyclers. Sixty and 90 days of E2 treatment resulted in persistent estrus condition in around 70 and 95 percent of the rats respectively while the remaining 30 and 5 percent of the rats respectively still exhibited estrus cycles. In comparison, 100% of old constant estrus rats were acyclic.

Estrus cycles: Effects of E2 withdrawal

While E2 treatment for 60 and 90 days rendered most of the animal acyclic, its withdrawal caused resumption of cyclicity in about 60 percent of the E-60 exposed group, while none in the E-90 group came back to regular estrus cycles. However, the animals of the E-90 treated groups exhibited irregular estrus cycles (7 to 9-day long cycles).

Effects of chronic estrogen treatment on serum estradiol-17ß levels:

To ascertain whether the estradiol levels in the treatment groups remains fairly stable across the treatment period, and to compare with that of the controls, we measured the serum estradiol levels in the treatment groups at the end of the treatment period of 30, 60 or 90 days (Fig 5a). Measurements were made in serum samples obtained on the day of estrus since most of the 60 and 90 days E2 treated rats were in persistent estrus. These were compared with the control groups on the day of estrus. The following were the findings: 1. All, but the E-30 groups had significantly higher ($F(4,31)=11.722, P<0.0001$) levels of estradiol (Mean ±S.E; pg/ml) compared to that of controls (26.8±2.7). 2. The serum estradiol level tends to increase in a stepwise fashion across the treatment period of 30, 60 and 90 days (48.07±3.205, 58.385±6.557 and 79.5±3.525 respectively). 3. The serum estradiol levels in the treatment groups were in the range of estradiol levels normally seen during the afternoon of proestrus (75 pg/ml). 4. The serum estradiol levels between the treatment groups were significantly from each other ($p<0.05$). 5. The estradiol levels in old constant estrus rats were about 106.905±27.241 and was significantly different from the other groups.

Effects of chronic estrogen treatment on serum progesterone levels:

Upon treatment of intact rats with low dose of estrogen, serum progesterone levels (Mean±S.E.; ng/ml) change dramatically (fig.5b). Treatment with estrogen for 30 days significantly ($F(4,31)=18.738, P<0.0001$) increased serum progesterone levels (109.2±13.1) compared to that of controls (41.06±5.89;

$p<0.05$). Treatment for 90 days lowered serum progesterone levels to 68.87±11.14 although this was still significantly higher compared to the control group. Serum progesterone levels decreased with continued estrogen exposure for 90 days (25.909±2.43) and this was comparable to levels seen in old constant estrus rats (22.568±5.78).

Effects of chronic estrogen treatment on serum Estrogen: Progesterone ratio:

Chronic treatment with estrogen altered serum estrogen: progesterone ratio (Fig. 5c). Treatment for 90 days significantly increased ($(F4,27)=42.304$; $P<0.0001$) the serum E2/P4 ratio(pg/ng; mean±SE) 3.378±0.11 when compared to treatment for 30 days (0.425±0.06) or 60 days (1.064±0.214) . Old constant estrus rats too had elevated levels of E2/P4 ratio (4.044±0.534).

Effects of Chronic estrogen treatment on ovarian histology:

The ovaries of control rats had large numbers of corpora lutea since these animals were sacrificed on the day of estrous. A similar number of corpora lutea were also observed in the E-30 group. However, the E-60, E-90 and old constant estrous rats had more follicles with less or no corpora lutea. Moreover, there was an increase in the proliferation of interstitial tissue with estrogen treatment that was not apparent in the old constant estrus rats (Fig 7).

Body weight

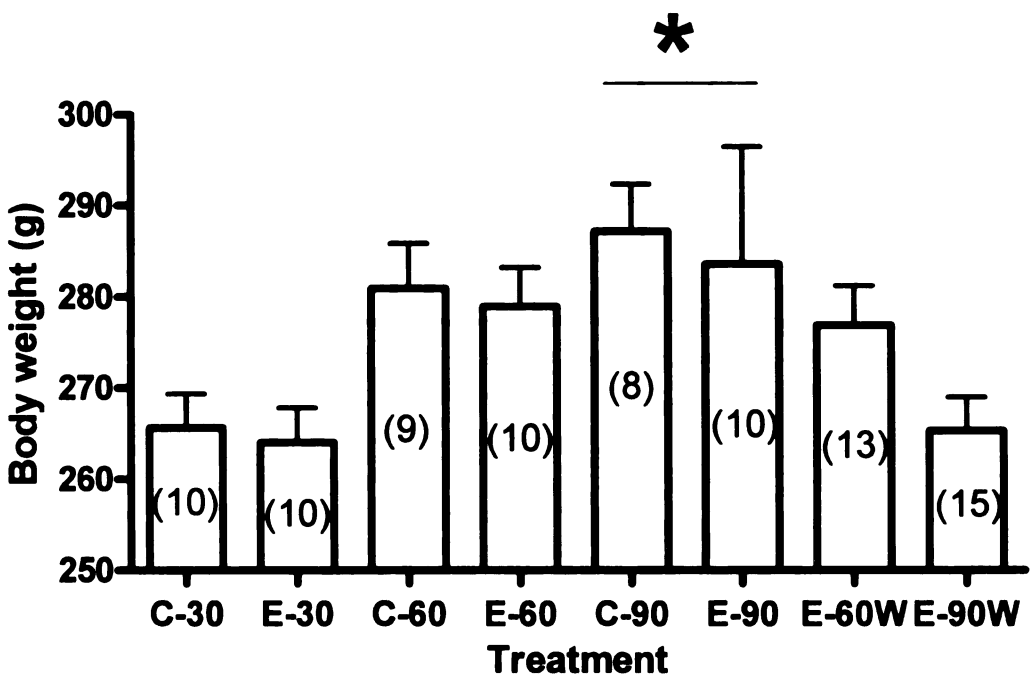

Figure 3: Effects of chronic estrogen exposure (20ng/day) for 30, 60 or 90 days (E30, E60, E90 respectively) on body weight in grams shown along with their respective age-matched controls (C). E60W and E90W indicates the groups that were subjected to a withdrawal period of a 4 weeks prior to measurement. Asterik indicates significant difference from C-30, E-30, E60W and E90W ($p<0.05$)

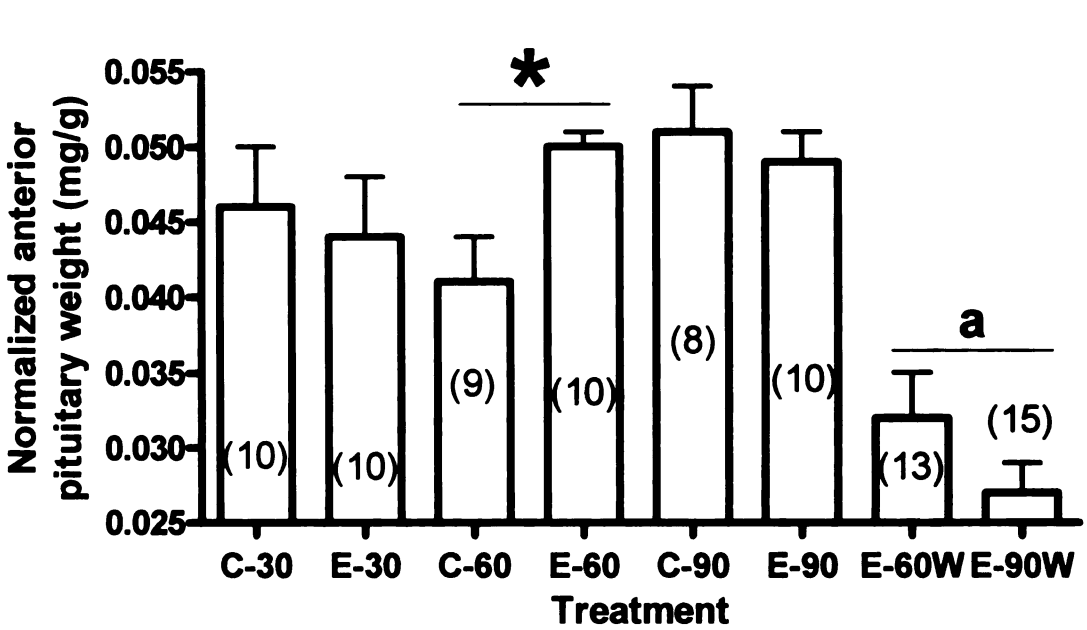

Figure 4: Effects of chronic estrogen exposure (20ng/day) for 30, 60 or 90 days (E30, E60, E90 respectively) on weight of anterior pituitary corrected to their body weight (mg/g) shown along with their respective age-matched controls (C). E60W and E90W indicates the groups that were subjected to a withdrawal period of a 4 weeks prior to measurement. Asterik indicates significant ($p<0.01$) difference between C-60 and E60. 'a' indicates significant difference from rest of the groups ($p<0.01$)

Effect of E2 exposure on serum E2 levels

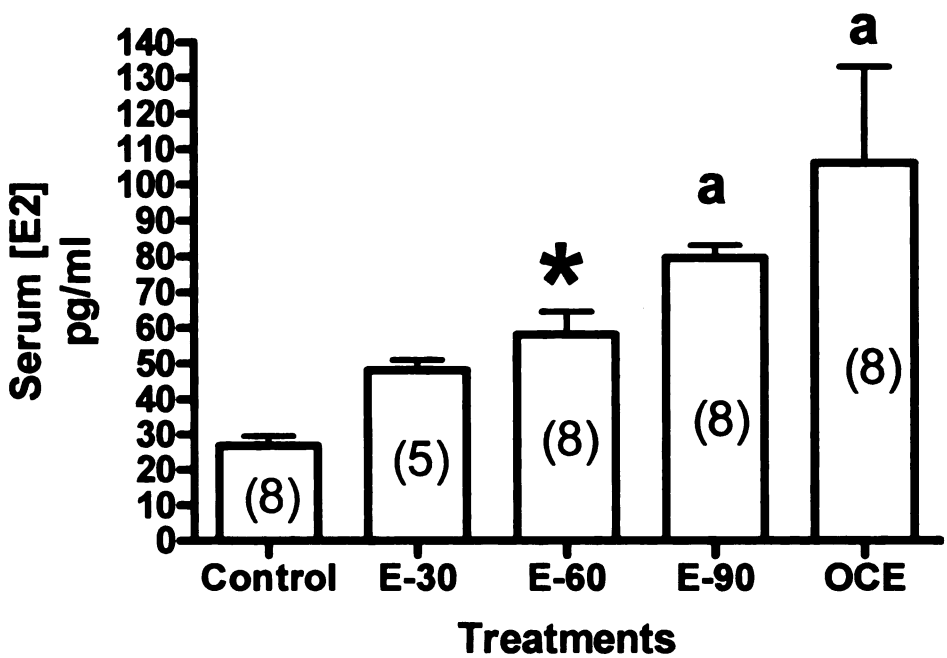

Figure 5a: Significant increase in serum E2 seen upon 60 ($p<0.05$) and 90 ($p<0.001$) days of E2 treatment compared to controlsin estrus age-matched to E2-90. OCE had higher levels ($p<0.001$) compared to controls. The number of animals per group is given in parenthesis. The data shown as mean ± SEM.

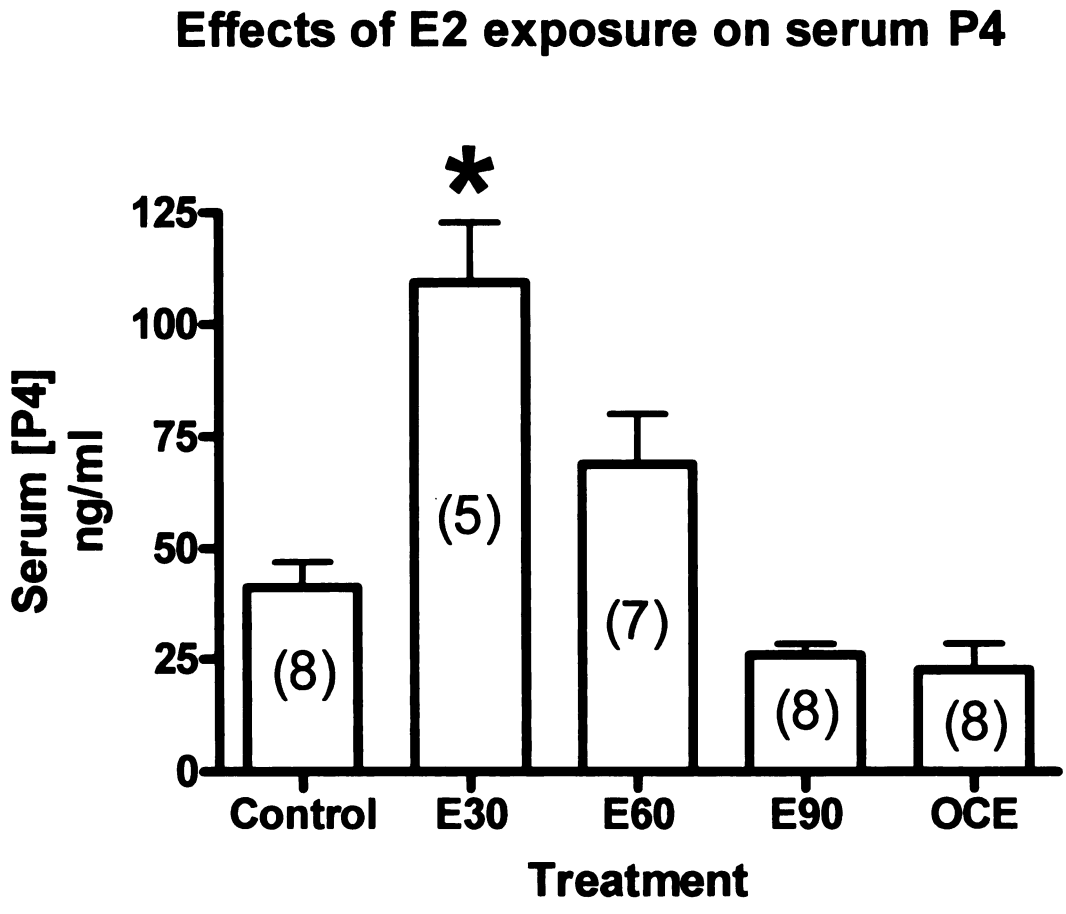

Figure 5b: Significant decrease in serum P4 seen upon 30 ($p<0.001$) days of E2 treatment compared to controls in estrus age-matched to E2-90. Treatment for longer duration brings back the levels to normals. The number of animals per group is given in parenthesis. The data shown as mean ± SEM.

Effects of E2 exposure on serum E2/P4

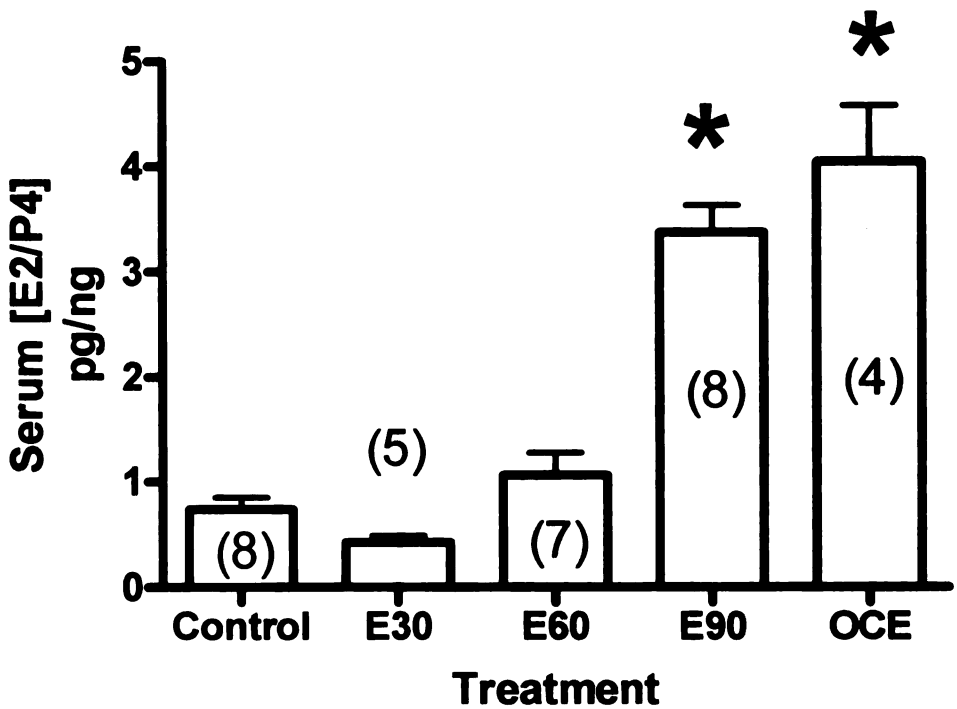

Figure 5c: Significant increase in serum ratio of E2 and P4 seen upon 90($p<0.001$) days of E2 treatment compared to controls in estrus age-matched to E2-90. Aging constant estrous rats had similarly higher levels in E2/P4 ratio. The number of animals per group is given in parenthesis. The data shown as mean ± SEM.

Estrogen Exposure & Estrous cyclicity

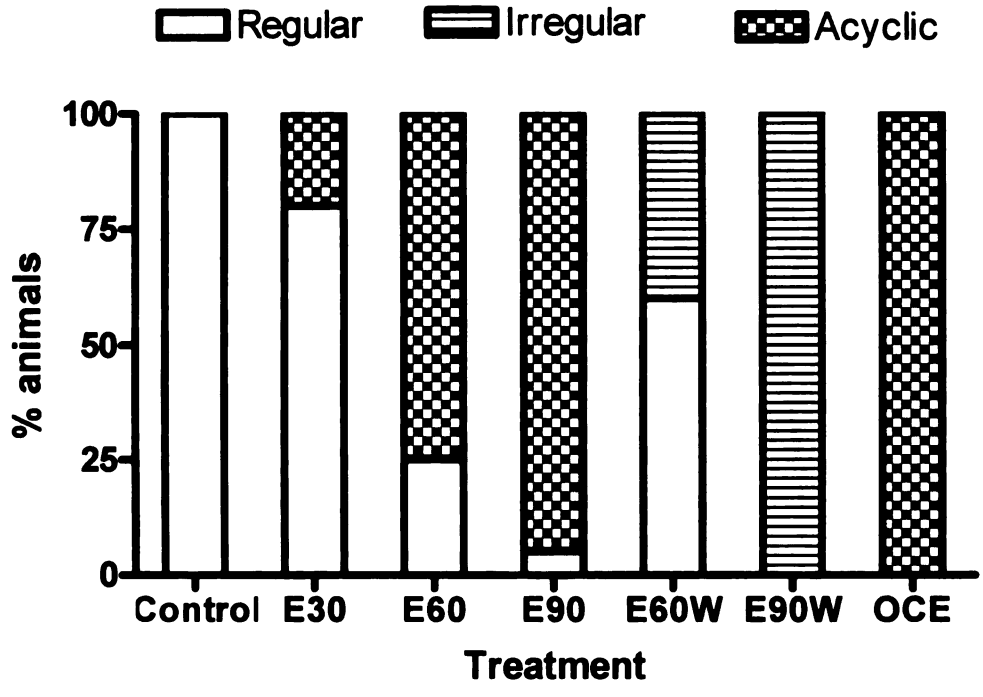

Figure 6: The figure shows the effects of duration of E2 exposure on the estrous cycles. Aging old constant estrous (OCE) rats were used for comparison purposes. The controls include those age-matched for E-90 group. E=estrogen and W=withdrawal. Numbers 30, 60 and 90 denotes duration (days) of treatment. Estrous cycles were followed by vaginal cytology as explained in the text. The E90W animals had prolonged cycles (7-9 days).

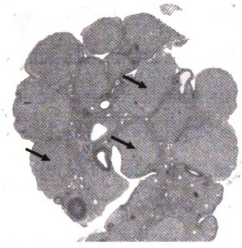

Fig. 7a.

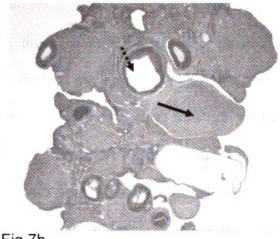

Fig.7b.

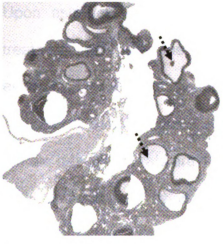

Fig . 7c.

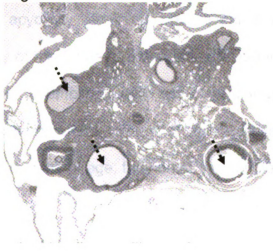

Fig. 7 d.

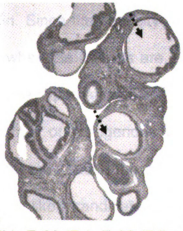

Fig. 7e

Fig 7. Sections of the ovary from a control (7a), E-30 (7b), E-60 (7c), E-90 (7d) and old constant estrus rat (7e). Dashed arrow indicate corpus luteum (CL) and follicles are indicated by stipled arrows.

D. Discussion

This study shows for the first time, in intact rats, that chronic low-dose estrogen treatment disrupts estrus cycles, causing a constant estrus condition, and this may or may not be reversible depending (and strength of E2) on the strength and duration of E2 treatment. Estrogen treatment for 30 days did not interfere with estrus cycles in almost 95 percent of the animals, while treatment for 60 or 90 days caused constant estrus condition in all E2 treated animals. Upon estrogen withdrawal, the acyclicity observed was reversed in 60 day treated animals while the majority of the 90 day treated animals had lengthened estrous cycles of 7-9 days duration, resembling middle aged irregular cyclers.

Loss of estrous cycles is a characteristic feature of reproductive aging in female rats. When rats are 12-14 months of age, they enter the constant estrous state characterized by vaginal smears that have abundant cornified epithelial cells. Although this is an age-associated change, previous studies have attributed this to a failure of ovulation most probably due to a lack of LH secretion. Since the capacity of the anterior pituitary to synthesize LH does not decline when the animals are 12 months old, it is very likely that higher centers such as the hypothalamus are involved in this phenomenon.

A number of endogenous factors produced during the reproductive life of the female rat may cause the age associated loss of LH, we hypothesized that E2 could be a likely candidate. This is because, E2 levels increase gradually during the stage of proestrus to reach a peak which stimulates LH secretion. In

ovariectomized rats where basal LH levels are high, E2 treatment suppresses LH secretion. Therefore there is a direct relationship between E2 and LH. Moreover, cycling rats are exposed to high levels of E2 once with 4 day estrous cycle at regular intervals. Typically, a rat with 4 day estrous cycles would have about 7 days of high estrogen levels in a month and would be exposed to approximately 60 days of high estrogen levels from puberty to middle age. We therefore hypothesized that exposing young rats continuously to 60 days of low E2 levels would render them acyclic and make them enter the state of constant estrous. They would be only 5 months old chronologically, but reproductively, they would be 12 months old. This could potentially be a model for reproductive aging.

The effect of chronic estrogen exposure has been studied previously using ovariectomized rat models. In these animals, chronic estrogen exposure has been shown to suppress the steroid induced LH surge in both young and old constant estrus rats [7]. In the same model, the responsiveness of the anterior pituitary to GnRH is not altered, strongly suggesting the involvement of hypothalamus, and not the anterior pituitary, in the estrogen induced LH suppression. While it is apparent that the effect seen in our study is because of estrogen treatment, the mechanism by which this is brought about is not clear. We believe that, in this experimental paradigm, the chronic estrogen exposure-induced constant estrus condition in 60 and 90 day treated rats is due to suppression of the LH surge and failure of ovulation. This in turn should have caused further production of estrogen from the follicles leading to increase in the levels of estrogen in the serum of 60 and 90 days treated rats as compared to

the 30 days treated animals. Evidence to this effect is shown in the work of Tsai and Legan [9] where chronic elevation of estradiol in ovariectomized rats have been shown to suppress the steroid induced LH surge. They have also suggested a similar mechanism to operate in middle aged rats leading to the constant estrus condition. This mechanism in our model would explain well the gradient increase in the estrogen levels in response to duration of treatment.

Estrogen is known to act on the vaginal epithelium causing their cornification. In our experimental approach it is possible to consider the alternative explanation of the direct action of the exogenous estrogen on the vaginal cells causing the so called "vaginal estrus" and not the true constant estrus condition as seen in aging rats. If this is true, then, the changes in estrus cycles we observed will not be considered to be mediated through the neuroendocrine network. But the results of this study do not seem to convincingly support this alternative, since, the estrus cycles were not affected during the first 30 days of estrogen exposure. Moreover and majority of rats did not come back to regular cycles during the withdrawal period after 90 days of estrogen treatment. Hence, our results support the idea that chronic exposure to low levels of estrogen probably alters the neuroendocrine system and induces constant estrus condition as suggested in the previous paragraph.

The exact mechanism by which estrogen produces acyclicity in young cycling rats is unclear. However, similarities can be drawn from the aging process. Age-related decline in catecholaminergic activity has been reported to occur in areas of the brain known to be involved in the control of reproduction, namely, the

medial preoptic area and medial basal hypothalamus [28, 29]. It is possible that in our animal model of estrogen-induced constant estrus, alterations in catecholamines in these areas could play a major role in the cessation of estrus cycles. But what makes them susceptible to irreversible alterations in estrus cycles is not known.

Reinitiation of estrus cycles in old constant estrus and pseudopregnant rats have been achieved by increasing the levels of central catecholamines. This was attempted successfully by the chronic administration of deprenyl, a monoamine oxidase-B inhibitor, L-DOPA, a catecholamine precursor, or with ergot drugs [78, 86, 93]. Whether administration of any of these drugs to these animals chronically, along with estrogen exposure, would be able to prevent the incidence of constant estrus in estrogen treated animals remain to be explored. Such studies would provide more evidence for the role of catecholamines in the estrogen-induced decline of reproductive functions in this animal model.

It is not clear whether the deleterious effects of estrogen are localized at the level of hypothalamus or pituitary. Lu et al., have shown that the responsiveness of the anterior pituitary to GnRH was not altered after chronic estrogen treatment. Hence they concluded that the changes due to estrogen exposure occurred at the level of the hypothalamus and not the pituitary [7]. Similar studies could be done in this experimental model to test for the responsiveness of the anterior pituitaries to GnRH.

Besides the hypothalamus and the pituitary, the ovary could be another potential site of action for exogenous estrogen. The possibility that chronic

estrogen exposure could cause the ovaries to lose their responsiveness to the LH surge cannot be ruled out.

To summarize, chronic exposure of young cycling rats to low levels of exogenous estrogen produced disruption of estrus cycles. The reversibility of this phenomenon was dependent on the duration of exposure. To probe further into the mechanism of this estrogen-induced alteration in estrus cycles, the next experiment was designed to study the effects of chronic estrogen exposure on the LH surge in 30-day treated proestrus rats, and LH levels in the 60 and 90 days treated acyclic (constant estrus) animals. Changes in LH will be correlated with the catecholamine content in the discrete areas of preoptico-suprachiasmatic tuberoinfundibular system (PSTS) of GnRH neurons.

CHAPTER-2

CHRONIC ESTROGEN EXPOSURE-INDUCED CONSTANT ESTRUS IS PROBABLY MEDIATED THROUGH HYPOTHALAMO-HYPOPHYSEAL AXIS

A. Introduction

Exposure of young cycling rats to low doses of estrogen for chronic periods (60 or 90 days) caused aging-like constant estrous condition, the incidence of which was found to be dependent upon the duration of estrogen exposure (Chapter-1). Likewise, irreversible alterations in the estrous cycles were noted upon estrogen exposure for 90 days as compared to 60 days. The estrous cycle of the 90-day treated rats upon withdrawal was found to be prolonged (7-9 days) indicating aging-like changes upon chronic estrogen exposure as seen in middle age cycling rats. Altogether we found similarities in estrogen treated, constant estrous rats and those of aging spontaneous constant estrous rats, with reference to the state of vaginal cytology, the levels of serum estrogen, the serum E2/P4 ratio, and the ovarian histology, strongly indicating the fact that chronic estrogen exposure indeed caused aging-like changes in the reproductive physiology.

It is common knowledge that age-dependent alterations in estrous cycle in rats [1] are associated with changes in the profile of LH on the day of proestrous [31, 80], and in the LH levels during spontaneous constant estrus [1, 3]. Likewise these changes are known to be accompanied by alterations in the catecholamine activity (content, turnover and release) in discrete areas of the hypothalamus

involved in the generation of LH surge [28, 29, 82]. Here, in this study (chapter-2), we tested the hypothesis that chronic estrogen induced-alterations in estrous cycles are accompanied by changes in the neuroendocrine status with reference to serum LH and hypothalamic catecholamine activity, similar to those of aging rats. Thus we attempted to characterize the profile of serum LH in estrogen (30-days) treated cycling rats on the afternoon of proestrus along with the measurement of catecholamines (NE and DA) in discrete areas of the hypothalamus. Since, majority of the E2 treated rats of the 60 and 90 day treatment group exhibited constant estrous condition similar to those of aging rats, we attempted to compare the levels of serum LH and hypothalamic catecholamine contents at one time point (1200 hours) instead of following their profile across time.

B. Materials and Methods

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 groups of 3 per cage in temperature (23±2°C) and light-controlled (lights on from 0500 to 1900 h) animal rooms. They were given food and water *ad libitum*. The animals were used for the experiment 2 weeks after arrival. All the protocols followed in this study were approved by the University Committee for Animal Care and Use at Michigan State University.

Treatment

Vaginal smears were obtained in the morning hours between 0800-1000 h. Those animals showing regular 4-day cycles were chosen for the experiment. The animals were randomly divided into different treatment groups. Animals in the control group were sham implanted and those in the treatment groups were implanted subcutaneously with slow-release estrogen pellets under mild halothane anesthesia. The pellets were capable of releasing estradiol 17-β at the rate of 20 ng per day for a period of 30, 60 or 90 days (Innovative Research America, Sarasota, FL). The animals receiving estrogen pellets were divided into groups based on the duration of estrogen exposure which was for 30, 60 or 90 days (E-30, E-60 and E-90 respectively). Vaginal cytology was recorded daily as described in the previous chapter for all the animals. At the end of the treatment period (30, 60 or 90 days), animals were sacrificed by decapitation along with

appropriate controls. Control animals were matched for age and for the stage of estrus cycles. The majority of the animals in the E-30 group along with controls were sacrificed on the afternoon of proestrus in groups of 8 at 1200, 1400, 1600 and 1800 h. Some animals in E-30 group (about 15%) exhibited persistent vaginal estrus and were sacrificed at 1200 h. Animals from the E-60 and E-90 groups were in the constant estrus state and were sacrificed at 1200 h along with controls in the state of estrus.

Animals were sacrificed by rapid decapitation under protocols approved by the AUCAUC. Trunk blood was collected. The brain, brain stem and ovaries were harvested as mentioned below. Blood was allowed to stand for 30 minutes in refrigerator and spun at 4000 rpm for 10 minutes. Serum was separated and stored at -70°C until they were used for radioimmunoassay. The brain and brain stem were removed from the skull within 40 seconds of sacrifice and were frozen quickly on dry ice. They were then stored at -70°C until the time of sectioning.

Microdissection of the brain

At the time of sectioning, the brains were mounted on a chuck and serial brain sections of 300 µm thickness were obtained using a cryostat (Slee, London, UK) maintained at -10°C. The sections were transferred to cover slips which were placed on a cold stage set at -10°C. The Cortex (CX) [as non-hypothalamic internal control, not involved in control of LH regulation], Diagonal band of Broca (DBB), medial preoptic area (MPA), suprachiasmatic nucleus (SCN), and arcuate nucleus (AN) [The entire AN were pooled and used for assay] were located with the help of a rat brain stereotaxic atlas [94] and microdissected using the

Palkovits' microdissection technique [28]. Tissue samples were obtained bilaterally and included all subdivisions of individual nuclei. They were stored at -70°C until they were analyzed for neurotransmitter concentrations by HPLC-EC.

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 a temperature of 37°C. At the time of analysis, tissue punches were transferred from –70°C to ice. To each sample, 200 µl of 0.1 M perchloric acid was added and the tissue was immediately homogenized on ice for 10 seconds using a micro-ultrasonic cell disruptor (Kontes, Vineland, NJ, USA). Samples of the homogenate were aliquoted for protein analysis. The rest of the homogenate was spun at 14000Xg for 5 minutes. Sixty microliters of the supernatant was added to 30 µl of the internal standard (0.05 M dihydroxy benzylamine) and 30 µl of 0.1 M

perchloric acid, and were loaded onto the autoinjector, where samples and standards were maintained at 4°C. A mixed standard containing known concentrations of Norepinephrine (NE), dopamine (DA), dihydroxy phenylacetic acid (DOPAC), hihydroxy benzylamine (DHBA), 5-hydroxytryptamine (5HT, serotonin) and 5-hydroxy indoleacetic acid (5-HIAA) was loaded for every 10 samples. DHBA was used as the internal standard.

Protein Assay

Protein concentrations in the tissue homogenates were determined in duplicates using a micro Bicinchoninic acid assay (Pierce, Rockford, IL). Briefly, homogenates (5-10ul) were loaded in duplicate into a 96 well plate. Bovine serum albumin (2mg/ml) was used as a standard. Serial dilutions of the standard were made by adding known volumes of the standard solution into appropriate wells to obtain a standard range from 0-22 µg/well. The standards and samples were diluted with water to obtain a total volume of 100ul. Reagents A, B and C from the kit were mixed at the ratio of 50:48:2 respectively. Hundred micro liters of the reagent solution was added to each well. The plate was gently agitated and left covered in a 37 °C incubator for 2 hours. Then readings were obtained after cooling the plate to room temperature using an ELX 800 ELISA plate reader (Biotek, Winooski, VT) at 562 nm. A standard curve was obtained and protein concentrations in the samples were determined. The neurotransmitter concentrations were expressed as pg/µg protein.

Hormone Measurements

LH was measured in duplicates in the serum by radioimmunoassay (RIA) using a double antibody method as described before [29]. The standards and antibody (Anti rLH-SH) were obtained from Dr. A.F.Parlow, NHPP, NIDDK. The LH tracer (Pre-iodinated radioactive label) was obtained from Amersham Pharmacia Biotech (Waukesha, WI, USA). Briefly, 40 µl of the serum was assayed in duplicate. The volume was made up to 100 ul using PBS-gelatin. 100 µl of the first antibody (rabbit anti-rat LH NHPP, NIDDK) was added to the samples and standards at a dilution of 1:758,000. After overnight incubation at 4 °C, 100 ul of the tracer was added to the total count, non-specific binding (NSB), standard and sample tubes. After overnight incubation, second antibody (sheep anti-rabbit, indigenous) was added at a concentration of 1:12. The final assay volume was 400 µl. Three hours later, a 30% solution of polyethylene glycol (PEG, 100 µl) was added to the tubes followed by 1 ml of PBS. The tubes were vortexed, centrifuged and the supernatant was aspirated. The pellets were counted for radioactivity using a Beckman 5500 B gamma counter. LH concentrations in the samples were determined using a software program (Assayzap, Macintosh). LH levels were expressed as ng/ml.

Statistical Analysis

The LH, NE and DA profile on the day of proestrus in the controls and E-30 rats were analyzed by two-way ANOVA and the post hoc comparisons were made using the QUICKCALCS ($p<0.05$) from GraphPad web site. The LH, NE and DA levels at one time point (1200 hrs) in the acyclic rats were analyzed by one-way ANOVA followed by Tukey's Multiple Comparison Test ($p<0.05$).

C.Results

The following paragraphs gives a detailed analysis of the changes in the profile of serum LH and those of the catecholamine neurotransmitters, NE and DA, in different areas of the hypothalamus and the diagonal band of broca (DBB) implicated to be involved in the estrous cycle/secretion of LH by the anterior pituitary. The various brain areas investigated include, DBB, MPA, SCN, AN and the cortex (CX). We used cortex as an internal control for an area not involved in LH regulation or estrous cycle. We investigated these parameters in the following animals:

1. The animals which were cycling (80%) after 30 days of E2 treatment compared with the untreated animals on the day of proestrus. Since we measured on proestrus, we chose to look at their changes at different time points on that afternoon consistent with the occurrence of LH surge.

2. The animals which exhibited constant estrous condition (percent of animals shown in parenthesis) after treatment with E2 for a period of 30 (20%), 60 (75%) or 90 (95%) days. These were compared with control (untreated) cycling animals on the day of estrus. We also used aged persistent (or constant) estrous (OCE) rats for comparison with the younger rats, which were made constant estrus by estrogen treatment for different duration (comparison between estrogen-induced constant estrous condition in younger animals and spontaneous constant estrous condition in aging animals).

Serum LH

1. Thirty days of estrogen treatment did not cause profound alterations in the LH surge.

Serum LH was measured on the afternoon of proestrus at four different time points of 1200, 1400, 1600 and 1800 hrs. The results were analyzed by two-way ANOVA (Graphpad software) followed by post-hoc test (Quick Calcs from Graphpad website). In control animals, the LH levels were significantly higher ($P<0.05$) at 1800 hrs (125.5 ± 46.5 ng/ml, N=10) as compared to 1200 hrs (27.3 ± 3.9 ng/ml, N=10, t=3.539) and 1400 hrs (34.4 ± 2.7 ng/ml, N=10, t=3.283), indicating a surge. Treatment with E2 for 30 days did not cause any significant suppression of LH levels, when the comparison was made between the estrogen treated animals (86.8 ± 24.2 ng/ml, N=10) with untreated animals (125.5 ± 46.5 ng/ml, N=10) at 1800 hrs (t=1.395). But interestingly, in the E2-30 groups, the LH level at 1800 hrs (86.8 ± 24.2 ng/ml, N=10) was not significantly higher as compared to any other time points. The interaction between the time and treatment was not found to be significant ($F(3,72)=0.47$; $P = 0.7019$) as is the case of the effect of treatment ($F(1,72) =0.91$, $P = 0.3437$). Time was found to contribute to 21.72 % of total variance and this effect is considered extremely significant ($F (3,72) =6.87$, $P = 0.0004$).

2. Chronic E2 treatment-induced constant estrous state is accompanied by suppressed LH levels.

Serum LH levels in the acyclic animals were analyzed and compared with the controls. One-way ANOVA was performed followed by Tukey's Multiple Comparison Test ($p<0.05$). Significant difference between the means were found ($P=0.0024$ and $F=4.907$). We did find significant suppression in the LH levels at 1200 hrs in the E-60 (26.3 ± 3.1 ng/ml), E-90 (24 ± 2.9 ng/ml) and OCE (20 ± 0.4 ng/ml) groups as compared with controls (46.1 ± 10.2 ng/ml) in estrus.

Serum LH profile on proestrus

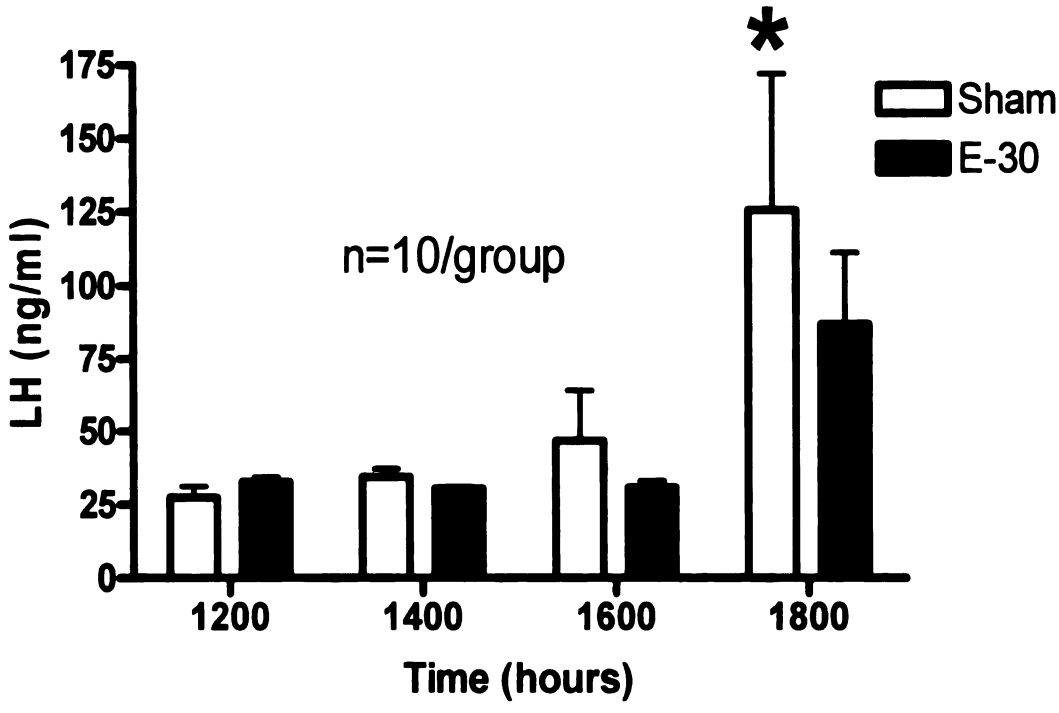

Figure 8: Each bar represents serum LH level as mean ± SEM. The number of animals is 10 per group. Analysis was done at $P<0.05$ level. No significant difference between the control and treatment group was observed at 1800 hours. However the LH levels in the treatment group at 1800 hours were not found to be significantly difference from 1200 hours as found in controls, indicating probable suppression or a shift in the occurrence of LH surge in E-30 animals.

Serum LH levels in Acyclic Animals

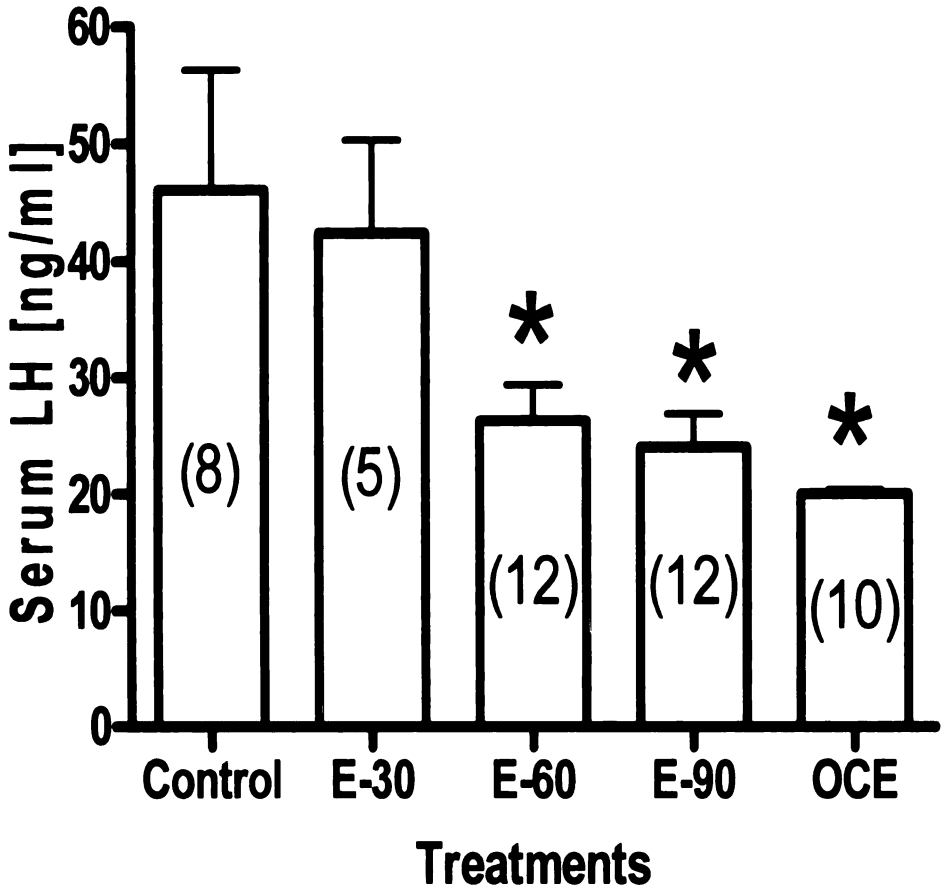

Figure 9: Each bar represents mean ± SEM of serum LH level in persistent estrus animals and those of controls in estrus. The number of animals per group is indicated in parenthesis. The E-60, E-90 and OCE groups all had significantly lower LH levels compared to controls in estrus ($p<0.05$).

Diagonal Band of Broca (DBB)

Norepinephrine

1. Norepinephrine profile was not altered in DBB by 30 days of E2 treatment on proestrus.

Two-way ANOVA was performed to find out the effect of time and treatment on the NE profile at 1200, 1400, 1600 and 1800 hrs in DBB. The results of this analysis showed no significant effect of treatment (3.61% of total variance) ($F(1,35) = 1.85$; $P = 0.1830$). Time was found to contribute to 27.42% of total variance ($F(3,35) = 4.67$; $P = 0.0075$) and this effect is considered very significant. The interaction of treatment at all values of time was not found to be very significant (0.54% of total variance, $F(3,35) = 0.09$; $P = 0.9639$). In the controls, the NE levels rose at 1400 hrs as compared to 1200 hrs, from 17.2 ± 10.7 pg/ug (1200 hrs, N=5) to 46.4 ± 7.4 pg/ug (1400 hrs, N=6)) and remained elevated throughout the experimental period. This increase was not found to be significant. Similar profile was observed in the estrogen treated animals wherein NE levels rose from 13.7 ± 2.7 pg/ug (1200 hrs, N=5) to 35.0 ± 5.1 pg/ug (1400 hrs, N=6) and remained elevated through out the experimental period. This increase was not found to be significant either. No significant difference was observed in the NE content between the controls and the E2 treated groups at any give time point. In short, NE follows the same profile in both E2 treated and untreated rats, with E2 rats showing an overall suppression in NE levels, which was not however statistically significant even at $p<0.1$. It could be observed that

there is however an increase in the NE levels at 1400 hours which stays so till 1800 hours when the LH surge was observed.

2. Norepinephrine content in the DBB of E2-induced constant estrous animals and those of aging, spontaneously constant estrous animals were not significantly different from estrus-controls.

The NE content in the acyclic animals were analyzed by one-way ANOVA followed by Tukey's Multiple Comparison Test (post-hoc) at $p<0.05$ level. The results of the ANOVA indicates no significant differences between the means ($P = 0.2810$, $F = 1.345$). There appears to be a trend towards slight increase in the NE content upon chronic E2 treatment with E2-90 and OCE groups having the highest level of 16.5 ± 1.1 (N=6) and 15.6 ± 2.7 (N=6) pg/ug respectively, compared to the estrus-controls (11.4 ± 2 pg/ug, N=6), though this was not found to be statistically significant.

Dopamine

1. Thirty days of E2 treatment did not alter DA profile in DBB on proestrus.

The DA profile in the control and E2 treated animals at different time points on the day of proestrus was analyzed by two-way ANOVA followed by post test at $P<0.05$ level. The results of the ANOVA showed no effect of treatment, time or their interaction on the outcome. The effect of treatment was found to account for around 1.41% of total variance ($F(1,36) = 0.69$; $P = 0.4128$) and this is not considered significant and time accounting for 16.14% of the total variance

(F(3,36) =2.62; P = 0.0653) which is not considered significant either. Similarly the interaction between treatment and time at all time points was again not found to be significant, with interaction accounting for approximately 8.69% of the total variance (F(3,36) =1.41; P = 0.2548). No significance was observed at P < 0.1 level either. The DA profile in both the control and E2 treated animals follows the same profile at all time points, though there appears to be a mild reduction in its level at 1400 and 1600 hours as compared to rest of the time points.

2. Chronic E2 exposure-induced constant estrous condition is accompanied by a non-significant reduction in DA levels in DBB.

Dopamine profile in the constant estrous animals were compared to the controls in estrus and the aging, constant estrous animals using one-way ANOVA followed by Tukey's Multiple Comparison Test at P < 0.05 level. No significant difference between the means were observed (P = 0.4138, F = 1.025). However, a non-significant reduction in the DA content was observed in animals made constant estrus by E2 treatment with similar levels irrespective of duration of treatment, where as the aging, spontaneously constant estrous animals (OCE) was not found to differ from the estrus-controls.

Norepinephrine Profile in DBB on Proestrus

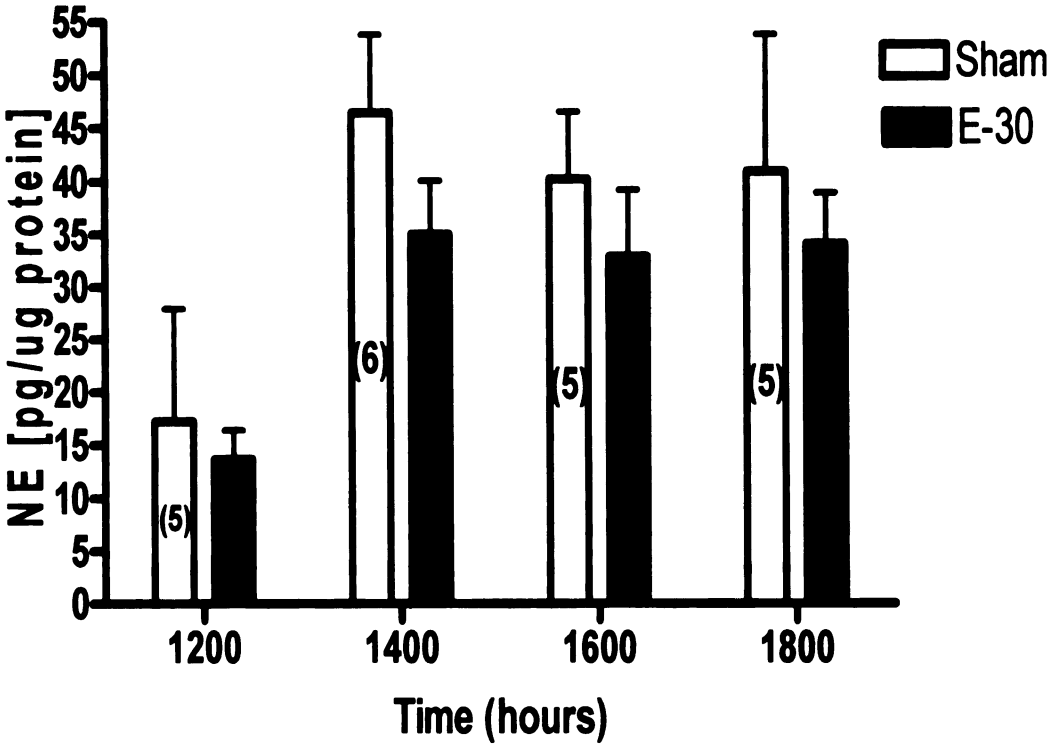

Figure 10: NE levels at different time points on the afternoon of proestrus shows no effects of 30 days of E2 treatment. Number of animals is shown in parenthesis. The data are shown as mean ± SEM. No significance found at $P < 0.05$.

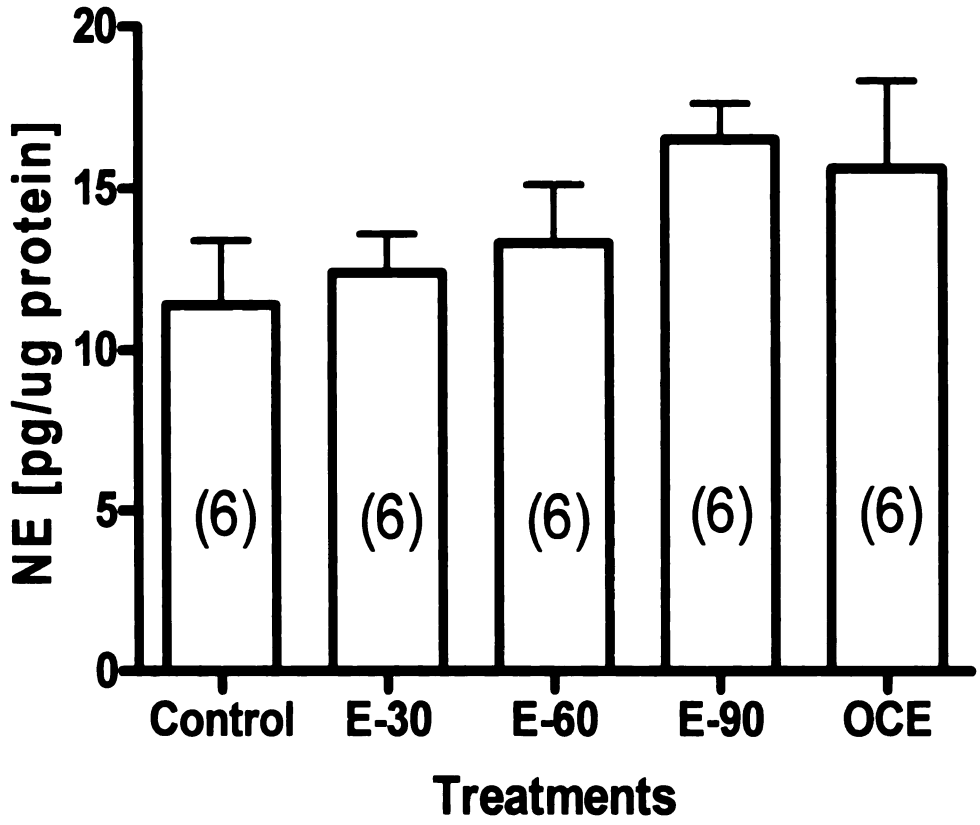

Figure 11: This figure depicts the NE content in the DBB of animals treated with estrogen for 30, 60 or 90 daysand were in the state of constant estrous state. These animals are compared with cycling animals in the estrous stage and with older animals in the constant estrous state (OCE). Though there appears to be a rise in NE content upon E2 treatment it was found to be non-significant. The number of animals per group is indicated in parenthesis. The data shown as mean ± SEM.

Dopamine Profile in DBB on Proestrus

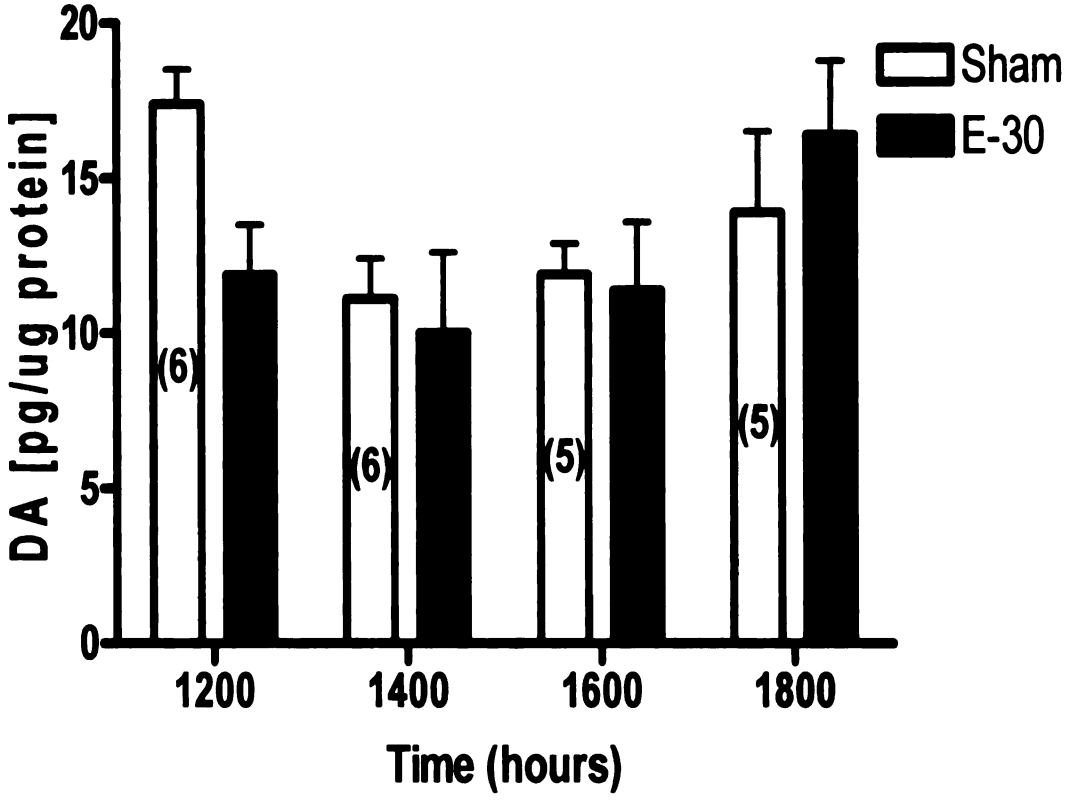

Figure 12: No significant change in the DA profile was observed on proestrus afternoon after 30 days of E2 treatment. The number of animals per group is indicated in parenthesis. The data represent the mean ± SEM.

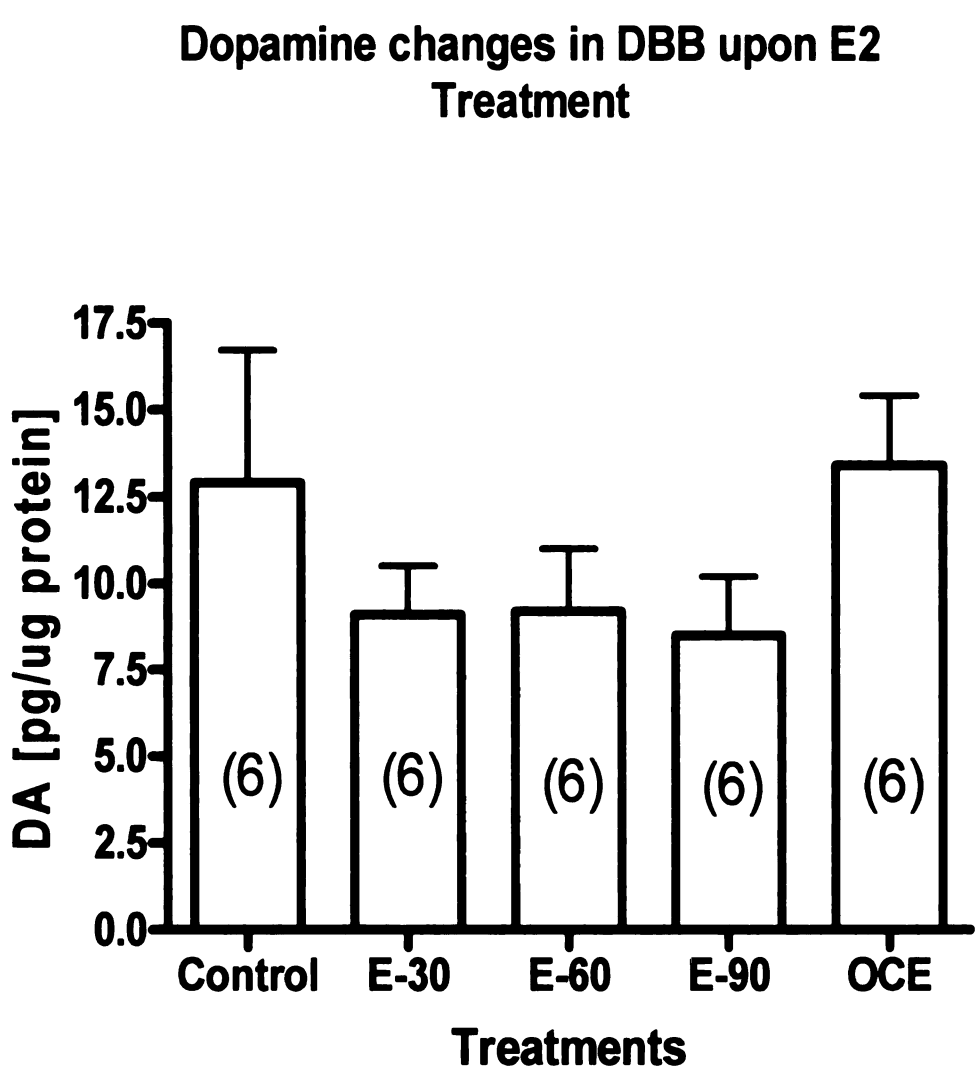

Figure 13: Chronic E2 treatment caused a non-significant suppression in the dopamine content in the DBB with respect to the controls and aged constant estrous (OCE) animals. The number of animals per group is indicated in parenthesis. The data shown as mean ± SEM.

Medial Preoptic Area (MPA)

Norepinephrine

1. Chronic E2 exposure for 30 days does not alter the NE profile in MPA on proestrus.

The NE profile in the E2-30 animals and their respective controls at different time points on the afternoon of proestrus was analyzed by two-way ANOVA followed by post tests at the significance level of $P < 0.05$. We did not find any significant effect of treatment on the outcome measures where it contributes to only 0.39% of total variance ($F(1,37)=0.4$; $P = 0.5310$). The effect of time on the other hand was found to be extremely significant where it accounts for about 60.12% of the total variance ($F(3,37) =20.52$; $P = <0.0001$). Interaction between treatment and time accounted for only 3.35% of total variance and hence does not contribute significantly to the overall outcome ($F(3,37) =1.14$; $P = 0.3441$). Both the control and the E2 treated animals showed the same trend with the controls showing significantly higher levels of NE at 1600 hours (28.3 ± 3.1 pg/ug) compared to 1200 (10.7 ± 0.7, t=3.550)and 1400 (10.8 ± 1, t=3.530) hours followed by a decline at 1800 hours (20.4 ± 1.2). The NE levels in the E2-30 group at 1600 (36.4 ± 8.4 pg/ug) and 1800 (23.8 ± 4.6 pg/ug) hours were both significantly higher than the 1200 (7.4 ± 1 pg/ug) and 1400 (8.8 ± 1.2 pg/ug) hours. It could be observed that there is an increase in the NE content at 1600 hours and 1800 hours in the MPA which precedes the corresponding the increase in the serum LH levels at 1800 hours.

2. Chronic E2 exposure induced-constant estrus in young animals and spontaneous constant estrous condition of aged animals demonstrate significant reduction in NE content in the MPA.

The NE content in the MPA punch samples of all the E2-induced constant estrous animals were compared with those of controls on the day of estrus, and those of the aged, constant estrous rats. The data were analyzed by one-way ANOVA followed by Tukey's post test at $p < 0.05$ level. The MPA of the estrus-controls had significantly higher ($F=4.205$, $P = 0.0106$) NE content as compared with those of the rest of the groups. The NE content in the acyclic rats of all the groups was similar irrespective of the duration of E2 treatment.

Dopamine

1. Dopamine content does not change appreciably in the MPA on proestrus upon 30 days of E2 treatment.

The DA content in the MPA at different time points on the afternoon of proestrus was measured and compared between the E2-30 groups and their corresponding controls at different time points. The results of the two-way ANOVA clearly demonstrates the fact that estrogen treatment for 30 days does not have any significant effect on the outcome with only 0.44% of the total variance being accounted for by such treatment ($F(1,37) =0.32$; $P = 0.5734$). On the other hand the time factor accounts for approximately 47.82% of total variance and the effect is considered extremely significant ($F(3,37) =11.58$; $P < 0.0001$). Interaction accounted for about 0.80% of the total variance and the

effect is not considered significant (F(3,37); P = 0.9002). Both the controls and the E2-30 groups demonstrate similar trend in the DA profile with a gradual reduction across time. In controls, the DA levels were found to be significantly reduced at 1800 hours (0.7 ± 0.1 pg/ug) as compared to 1200 hours (11.8 ± 3.8 pg/ug). Similarly, the E2-30 animals exhibited significant reduction in DA levels at 1600 (2 ±1 pg/ug) and 1800 (1 ±0.3 pg/ug) hours as compared to 1200 hours (14.9 ± 4 pg/ug). It is to be noted that this reduction in DA inversely correlates with significant rise in the NE levels at the same time points in the MPA and with the rising serum LH level.

2. Constant estrous (induced and spontaneous) animals had a non-significant decline in DA levels compared to controls.

The DA content in MPA from estrogen treated, and aged, constant estrous animals were compared with the controls in the estrous state using one-way ANOVA followed by Tukey's post test at $P < 0.05$. Though there was a reduction the DA levels in all the constant estrous animals as compared to the controls, they were found to be statistically significant ($F=1.296$, $P = 0.3052$).

Norepinephrine profile in MPA on proestrus

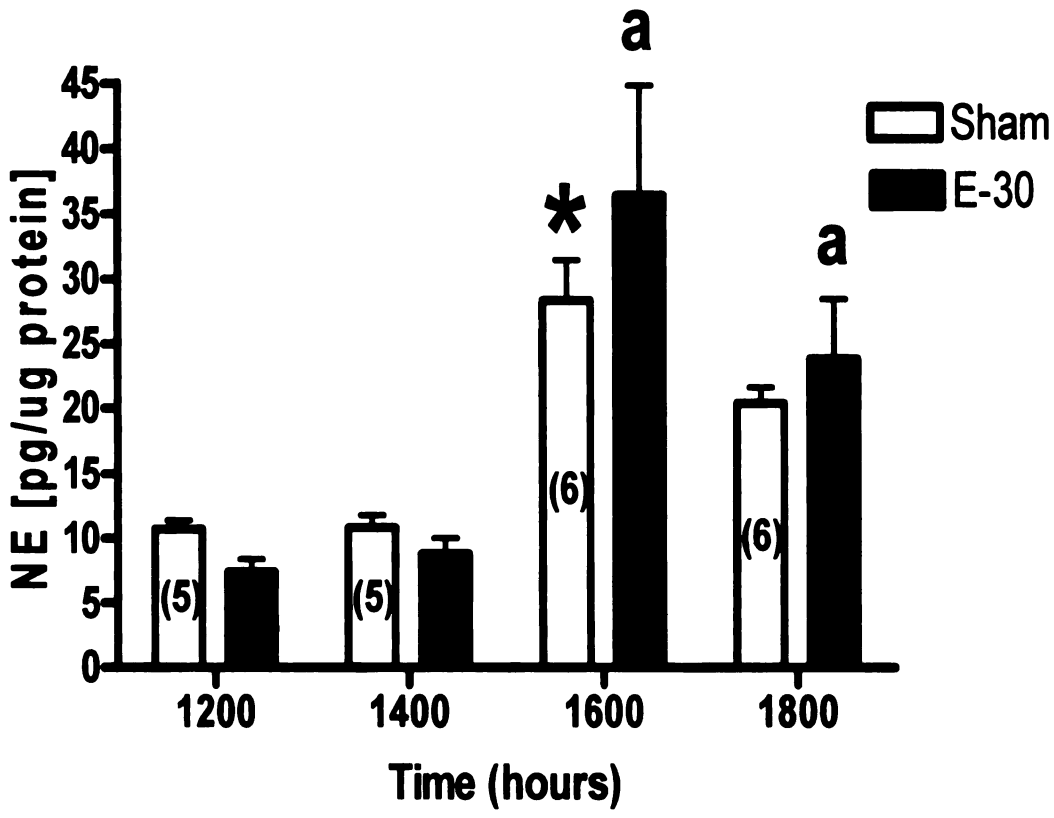

Figure 14:A Significant increase in NE levels is seen across time in both groups. E2 treatment did not have any effect. The number of animals per group is indicated in parenthesis. The data are shown as mean ± SEM, and analysis performed at $P < 0.05$. Both '*' and 'a' indicates significant difference from 1200 and 1400 hours in control and E2-30 groups respectively.

Norepinephrine changes in the MPA

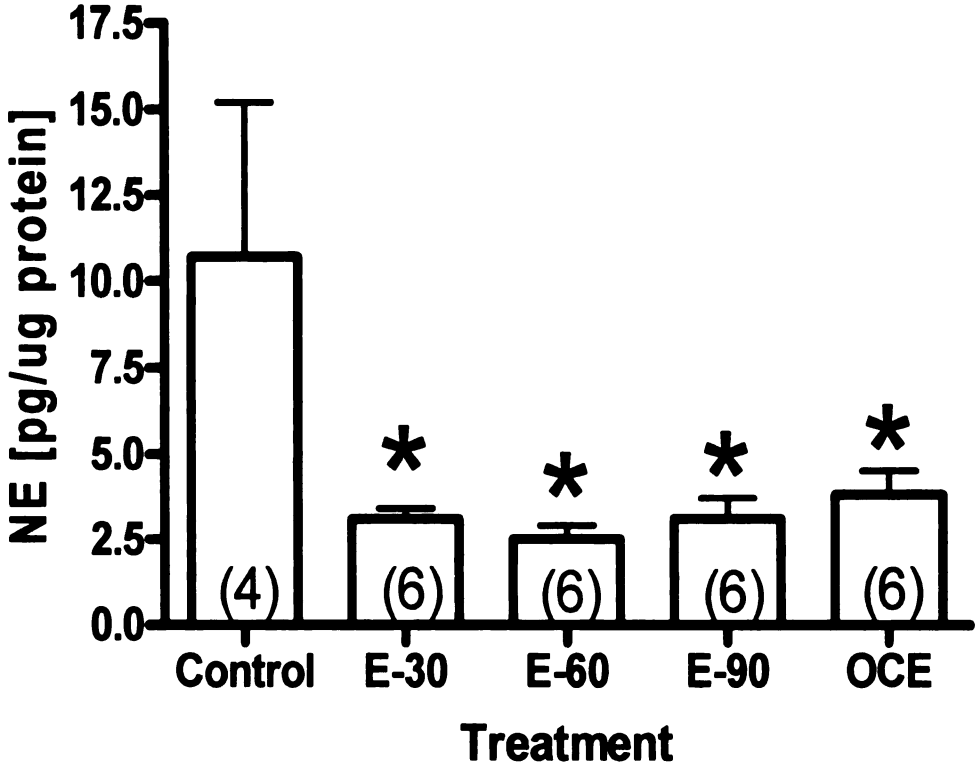

Figure 15: Chronic E2 treatment caused significant suppression in the NE content in the MPA compared to the estrous controls. The levels were comparable to those seen in aged constant estrous animals (OCE). OCE also had significanly lower levels of NE as compared to estrous conrols. The levels of NE in E2 treated rats are similar irrespective of the duration of treatment. The number of animals per group is indicated in parenthesis. The data shown as mean ± SEM. Astericks '*' denotes significance from controls at $p<0.05$.

Dopamine profile in MPA on proestrus

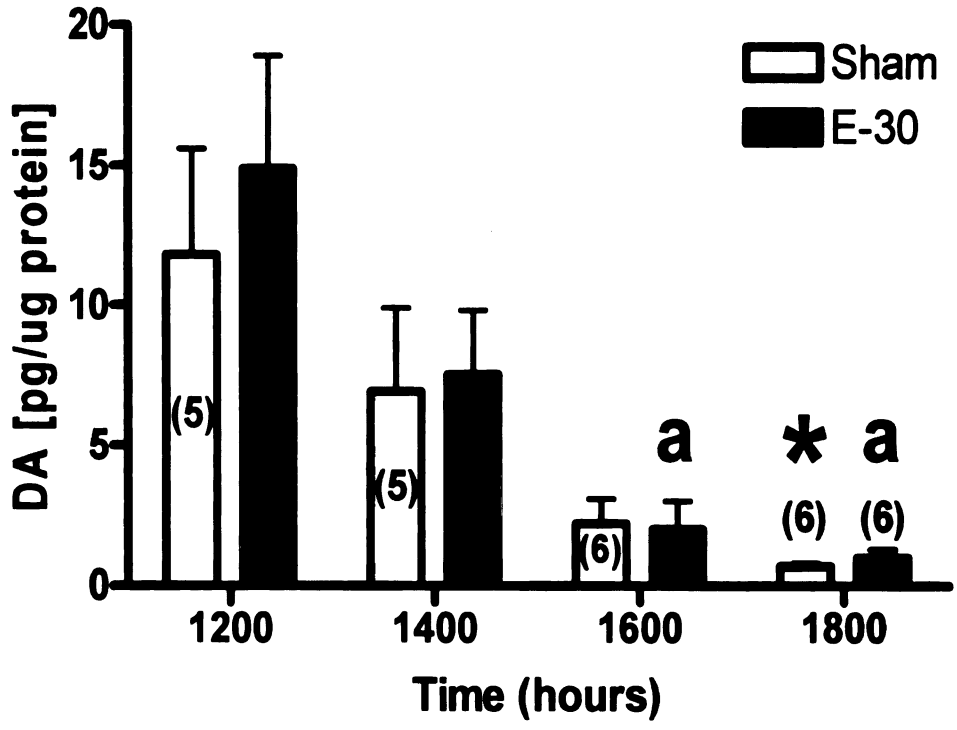

Figure 16: Significant changes in DA levels across time are seen in both controls and E2-30 groups. E2 treatment did not have any effect on the DA levels. Analysis were done at $P<0.05$ level. Number of animals per group is given in parenthesis. The data are shown as mean ± SEM. Both '*' and 'a' represents in control a significant difference from their respective 1200 hour time point.

Dopamine changes in MPA upon chronic estrogen treatment

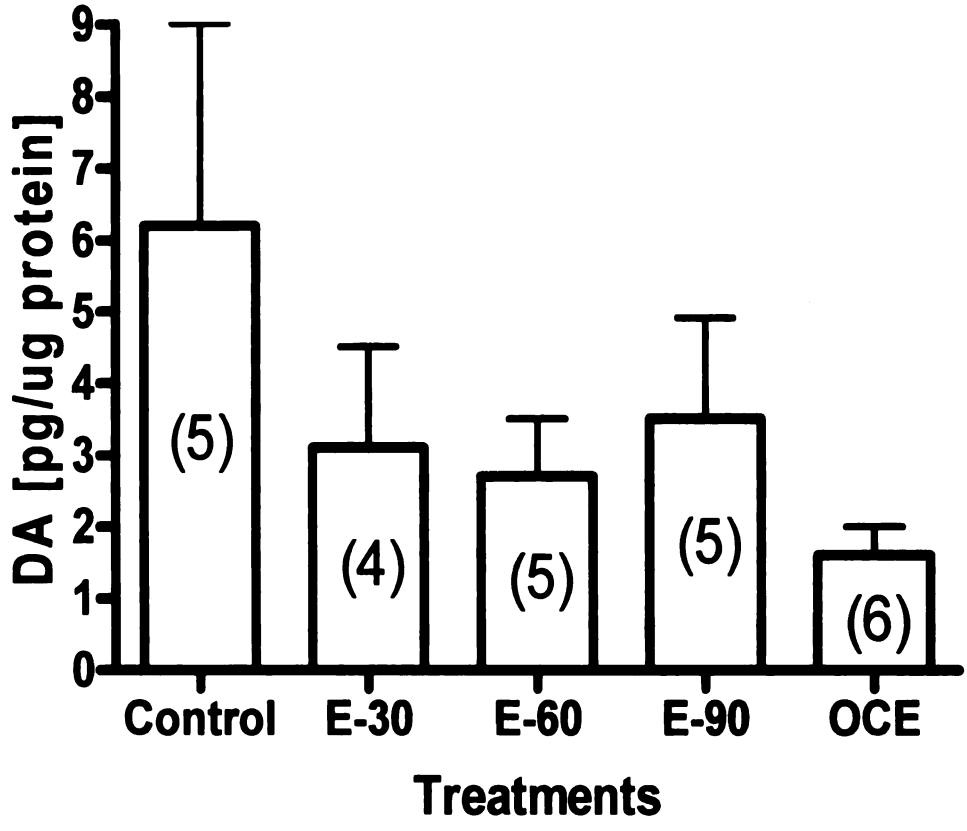

Figure 17: Chronic E2 treatment caused non-significant suppression in the DA levels in MPA of those rats, which were in constant estrous state, as compared with estrous controls at $p<0.05$. Aged constant estrous rats (OCE) too had suppressed DA level which was not found to be significant at $p<0.05$. The number of animals per group is given in parenthesis. The data shown as mean ± SEM.

Suprachiasmatic Nucleus

Norepinephrine

1. Estrogen treatment for 30 days does not cause any alteration in the NE content in SCN on the afternoon of proestrus.

The NE content was analyzed between the E2-30 and control groups, on the afternoon of proestrus at different time points using two-way ANOVA followed by post tests. Treatment with E2 for 30 days did not cause any significant effect in the output, contributing to only 2.35% of the total variance ($F(1,40) = 1.55$; $P = 0.2211$). On the other hand, time seemed to have considerably significant effect on the outcome accounting for 28.73% of the total variance ($F(3,40) = 6.29$; $P = 0.0013$). We did not find any significant effect of the interaction (8.04% of the total variance) on the outcome ($F(3,40) = 1.76$; $P = 0.1700$). Though both the control and E2-30 groups followed the same trend and we did not find any significant difference in the NE content between different time points in both the groups at $P < 0.05$ level, we however could find significance in the E2-30 groups at $P < 0.1$ level between the 1400 hours (9.6 ± 1.4 pg/ug) and the 1600 (17.8 ± 2.5 pg/ug) & 1800 hours (17.8 ± 1.8 pg/ug). It is to be noted that the increase in the NE levels at 1600 and 1800 hours in the SCN may be correlated to the changes in the NE levels happening in the MPA at the same time points and to the occurrence of LH surge.

2. Ninety days of E2 treatment cause significant suppression in NE levels in the SCN similar to that observed in the old constant estrous animals.

When the NE contents were measured in the rats made constant estrus by estrogen treatment for 30, 60 or 90 days and compared them to those of the aging, constant estrous rats and controls on the day of estrus using one-way ANOVA followed by Tukey's Multiple Comparison Test, we found significant suppression in the levels of NE in the SCN of E2-90 (3.85 ± 1.4 pg/ug) and OCE (3.4 ± 0.8 pg/ug) rats as compared to the controls (11.4 ± 1.4 pg/ug). Thirty or 60 days of E2 treatment did not cause such suppression even though these rats exhibited constant estrous condition. The results of ANOVA gave a P of 0.0003 and F value of 8.658, showing a significant difference between means at $P < 0.05$ level.

Dopamine

1. Dopamine levels do not change appreciably in SCN on the day of proestrus in rats treated with estradiol for 30 days.

Two-way ANOVA was used to analyze the data on DA levels in the SCN in E2-30 groups as compared with controls, on the afternoon of proestrus. We did not find any significance in the effect of E2 treatment which accounted for only <0.1% of the total variance ($F(1,40) = 0.03$; $P = 0.8751$) observed. The time factor was found to be extremely significant in contributing 32.75% of the total variance ($F(3,40) = 7.21$; $P = 0.0006$). The effect of interaction of treatment and time was not considered significant, contributing to only 6.65% of the total variance ($F(3,40) = 1.46$; $P = 0.2386$). Post hoc analysis between group means revealed no significant difference between any of the groups at $P < 0.05$ level. However, at

$P < 0.1$ level, DA levels in the control animals at 1200 hrs (9 ± 1.5 pg/ug) were found to be significantly higher compared to 1600 (1.8 ± 0.4 pg/ug) and 1800 (3.4 ± 0.8) hours. The DA levels followed a decreasing trend with time in both the sham and E2 treated groups. DA levels in controls were significantly lower from 1200 hours, during the time of expected LH surge.

2. Significant suppression in the levels of DA seen in SCN of the rats in constant estrus (both induced and spontaneous).

One-way ANOVA of the DA results in the acyclic animals did reveal significant differences in the means. Treatment with estradiol for 60 and 90 days did cause significant suppression in DA levels as compared to those of controls in estrus. The levels seen in these animals were comparable to those seen in the old constant estrous animals (OCE). Treatment for 30 days with E2, however, did not cause any alterations in DA levels, but were similar to control-estrus. It could be seen that though we did find significant suppression in the DA levels in E2-60 groups, we did not find any significant suppression in the NE levels in this group as could be seen from the figure- . The levels were 2.1 ± 0.8 pg/ug, 2.2 ± 0.8 pg/ug and 0.6 ± 0.1 pg/ug in E2-60, E2-90 and OCE respectively, all of which were significantly lesser compared to 9.3 ± 3.3 pg/ug in controls, at $P < 0.05$ level ($P = 0.0022$, $F = 5.916$).

Norepinephrine profile in SCN on proestrus

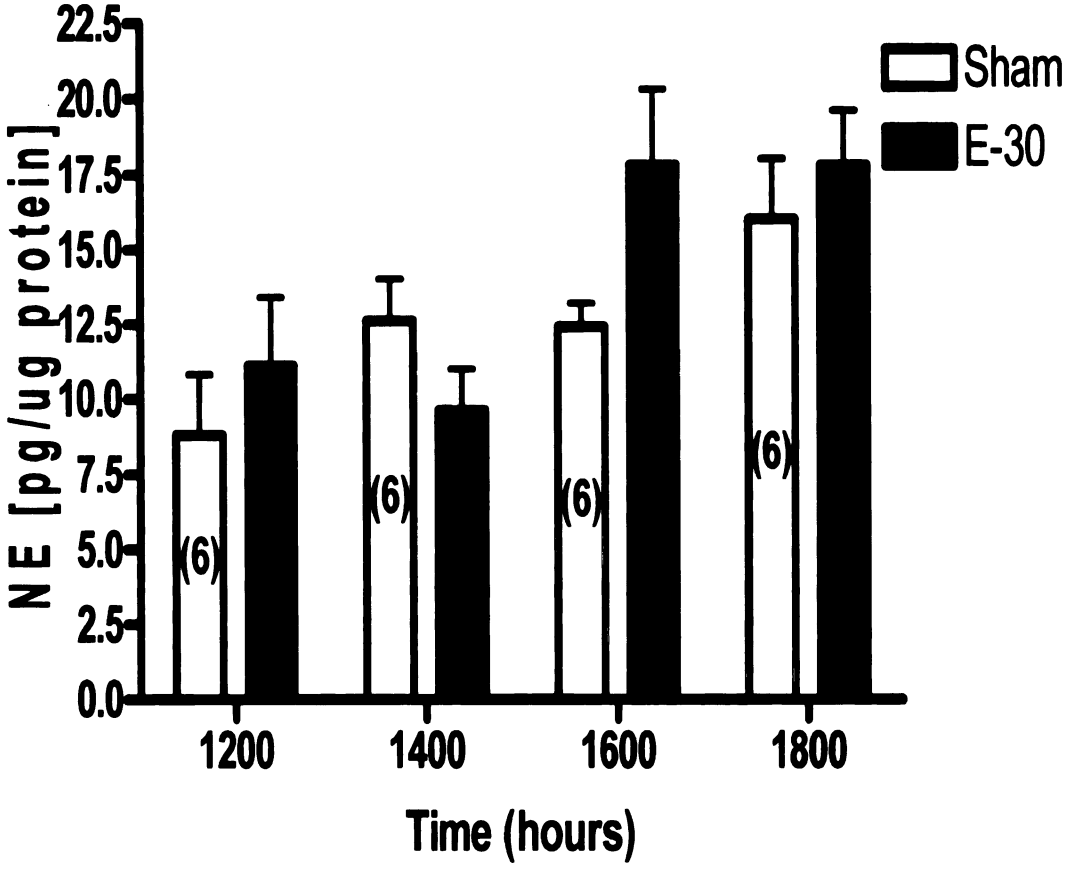

Figure 18: Neither time nor treatment had any significant effect on NE levels at $P < 0.05$. The number of animals per group is shown in parenthesis. The data are shown as mean ± SEM.

Norepinephrine changes in the SCN upon chronic estrogen treatment

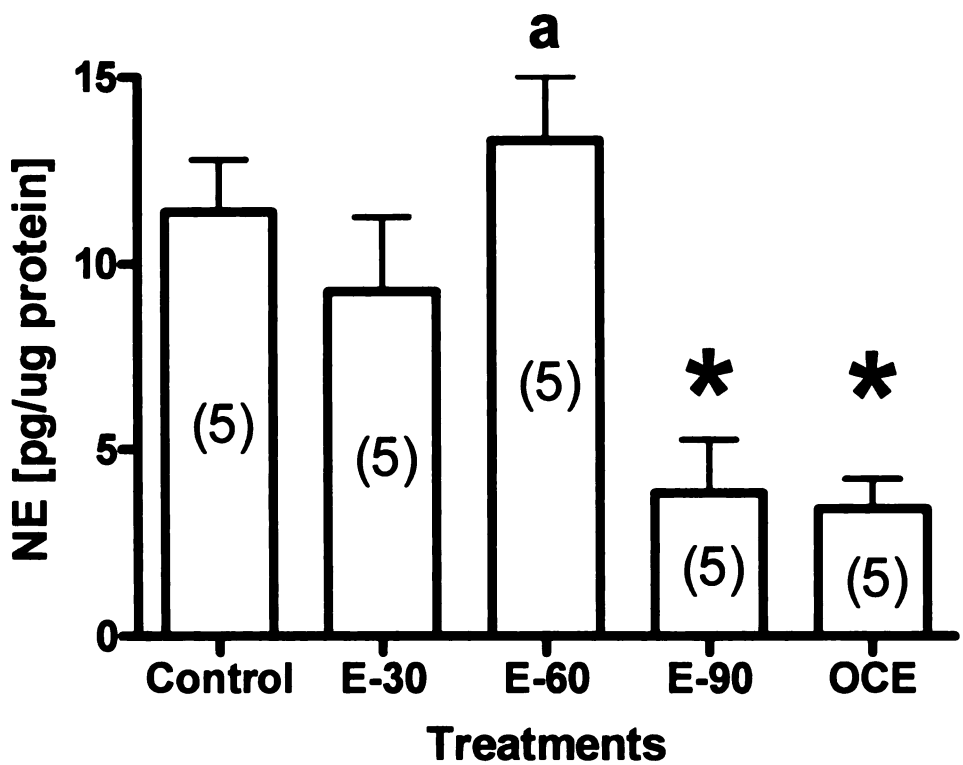

Figure 19: Treatment with estrogen for 90 days caused significant suppression in NE content as compared with estrous control. Old constant estrous animals had significantly lower levels of NE as compared with controls. The levels in E-90 animals were comparable with those of aged constant estrous animals. No significant alterations in NE content is seen in animals treated with E2 for either 30 or 60 days as compared with controls. All comparisons were made at $p<0.05$ level. The number of animals per group is given in parenthesis. Asterix (*) indicates comparison with controls. An 'a' indicates significance with respect to E-90 and OCE. The data shown as mean ± SEM.

Dopamine profile in the SCN on proestrus

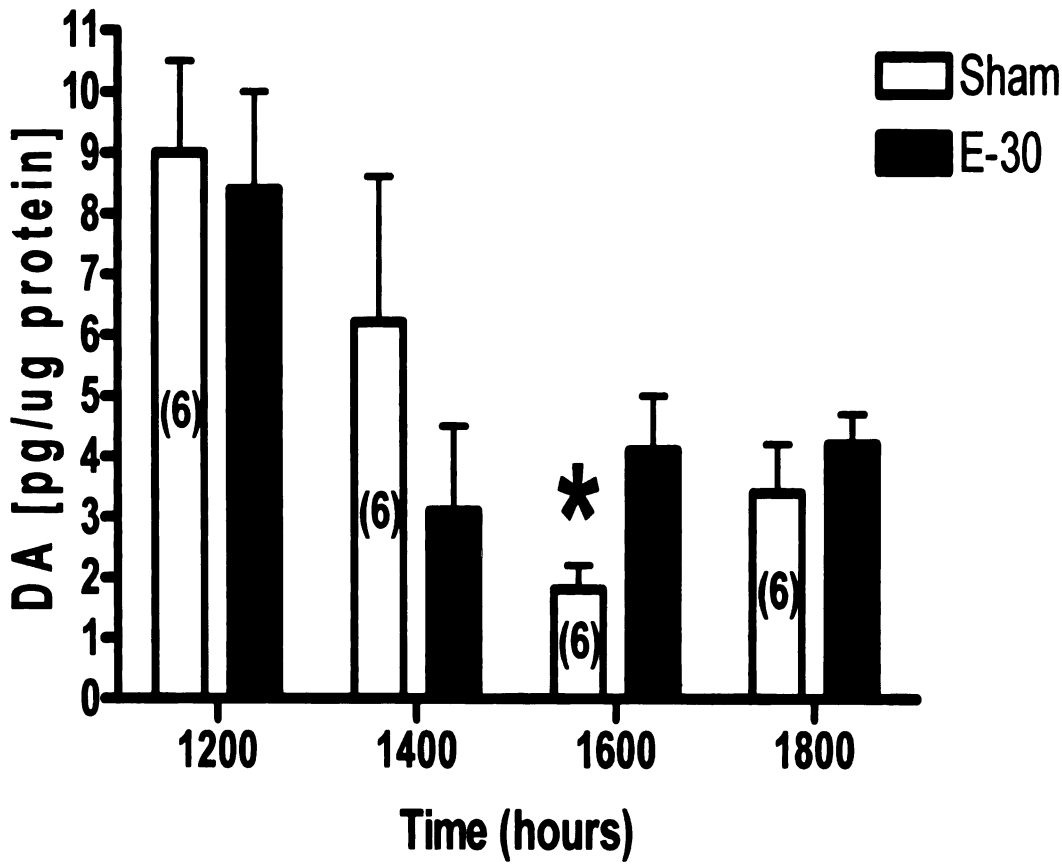

Figure 20: DA levels in both the groups are shown as mean ± SEM. The number of animals per group is indicated in parenthesis. Asterisk (*) denotes significant change ($P<0.05$) from 1200 hrs of control.

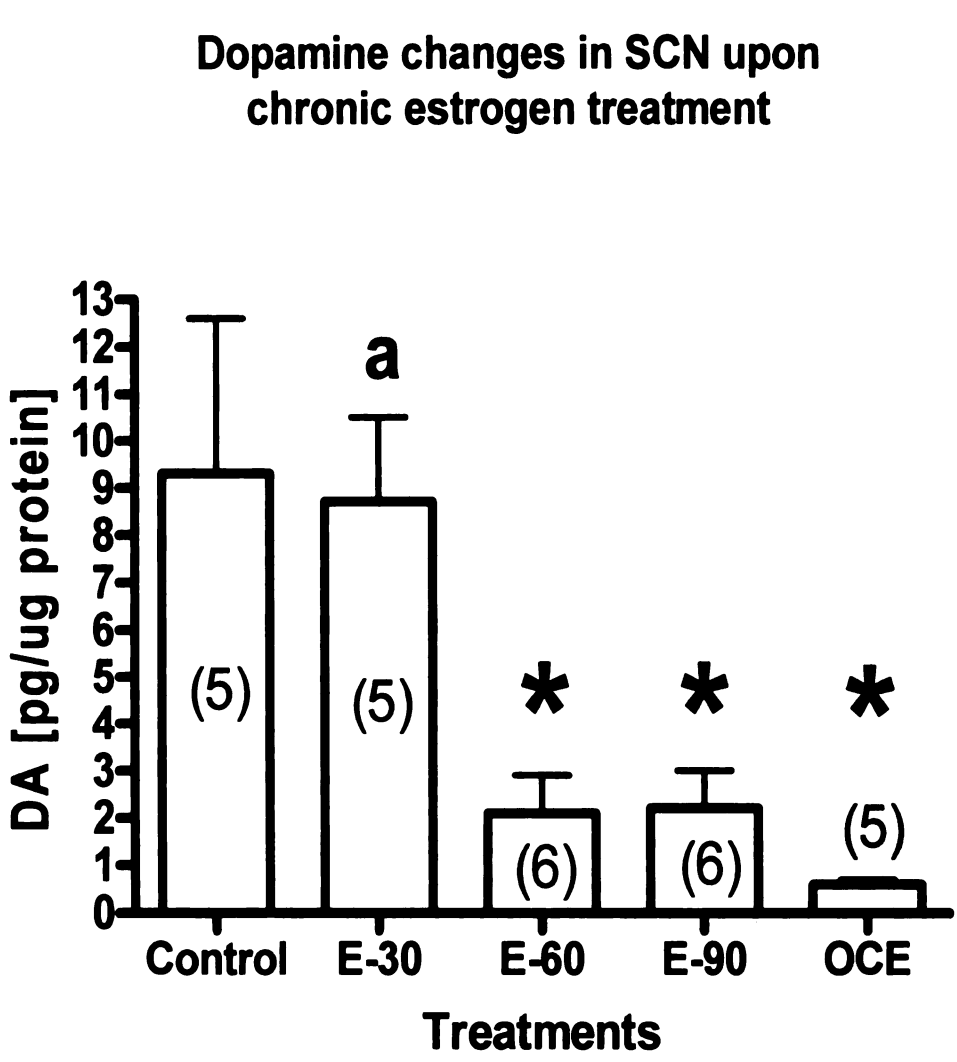

Figure 21: Chronic E2 treatment for 60 and 90 days caused significant (*) suppression in DA content with respect to controlsin estrus. Aged rats in constant estrus (OCE) also had significantly (a) lower DA content as compared with both estrous controls and E-30 animals. No significance observed between OCE and E-60&E-90 groups. The number of animals per group is given in parenthesis. The data shown as mean ± SEM.

Arcuate Nucleus

Norepinephrine

1. Norepinephrine levels not affected by 30 days of E2 treatment in AN.

The effect of chronic E2 treatment for 30 days on the NE profile across time on the afternoon of proestrus was analyzed by two-way ANOVA followed by post test, which revealed the fact that NE activity did not change appreciable with E2 treatment and we found that treatment contributed to only 0.12% of the total variance ($F(1,40) = 0.10$; $P = 0.7585$). We did find significant elevation in the NE levels at 1800 hours, around the time of occurrence of LH surge, as compared to the 1200 hours in both the groups and that time accounted for 49.52% of the total variance ($F(3,40) = 13.74$; $P < 0.0001$) and hence, the effect is considered extremely significant. The interaction between time and treatment was not significant and it accounted for only 2.3% of the total variance ($F(3,40) = 0.64$; $P = 0.5943$). The NE levels in the controls were significantly higher at 1800 hours (36.3 ± 4.7 pg/ug) as compared to both 1200 (19.1 ± 4.1 pg/ug) and 1400 (20.3 ± 1.7 pg/ug) hours, and the E2 treated groups had significantly higher content at 1800 hours (36.2 ± 3.7 pg/ug) as compared to 1200 hours (13.8 ± 2.4 pg/ug). The analysis was done at the level of $P < 0.05$.

2. No appreciable change in NE levels found in animals made constant estrus by chronic E2 treatment.

One-way ANOVA of the NE data from AN, at a level of $P<0.05$ revealed no significant difference between the E2 treated groups and the controls in estrus. We also however did not find any significant change in the NE levels of the old

constant estrus animals (OCE) (16.7 ± 5.8 pg/ug) as compared to control (20 ± 4.6 pg/ug). However, there appeared to be a general suppression in the NE content in the E2 treated groups (12.4 ± 1, 14.6 ± 3.8 and 12 ± 4.4 pg/ug in E-30, E-60 and E-90 respectively), which were not affected by the duration of E2 treatment. The test had a P of 0.6406 and an F value equal to 0.6385.

Dopamine

1. Dopamine levels unaffected in AN on proestrus after 30-days of E2 treatment.

While we found a gradual reduction in the DA levels across time in the controls, which was not found to be significant, we however did not find such changes in the DA levels in E2-30 groups. Rather, the levels in the DA appear to be stable across time in E2-30 group, without any fluctuations. Two-way ANOVA revealed no significant interaction between time and treatment with a contribution of 6.49% towards the total variance ($F(3,40) = 1.02$; $P = 0.3955$). Estrogen treatment contributed to only 0.12% of total variance and hence the effect is considered not significant ($F(1,40) = 0.05$; $P = 0.8173$). Time had significant effect on the outcome and contributed to 8.2% of the total variance ($F(3,40) = 1.28$; $P = 0.2930$).

2. No difference in DA levels observed in E2-treated acyclic animals.

One-way analysis of variance of the DA data from AN revealed no significance in the levels of DA across the treatment groups. The comparison was made at a P of 0.05. The analysis showed a P value of 0.3247 and an F

value of 1.239. Though the old constant estrus animals had a relatively lower DA content (6.4 ± 3.2 pg/ug) as compared to controls (10 ± 1.6pg/ug) in estrus, no significance was observed.

Norepinephrine profile in the AN on proestrus

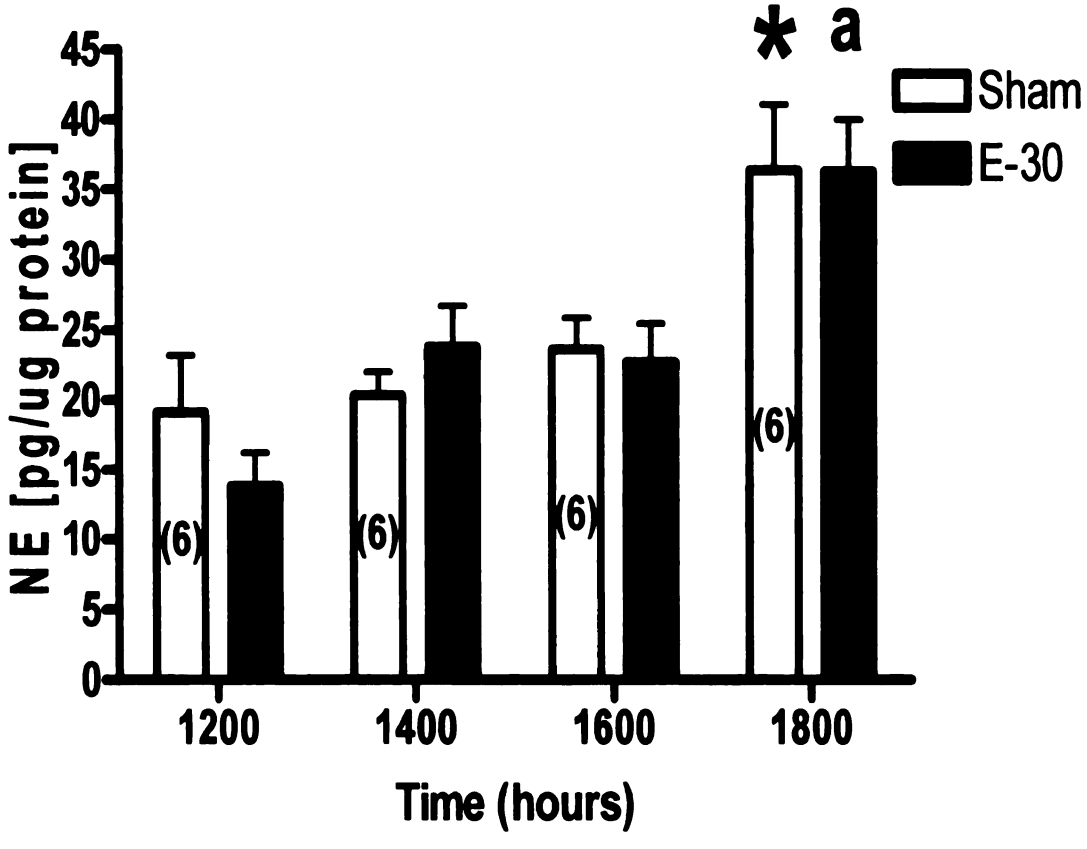

Figure 22: NE profile in the AN is shown as mean ± SEM. Number of animals per group is shown in parenthesis. Asteriks (*) denote significant difference from 1200 and 1400 hours of the corressponding groups and 'a' denotes significant difference from 1200 hours of the E-30 group at a level of $P < 0.05$.

Norepinephrine changes in the AN upon chronic estrogen treatment

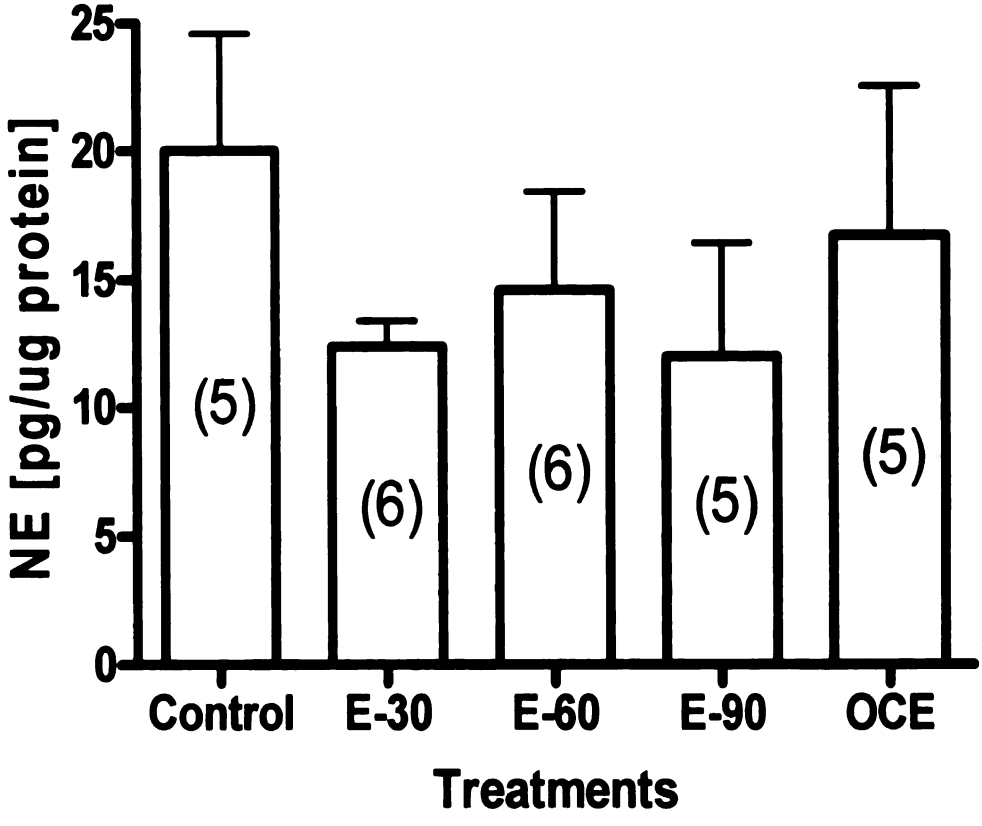

Figure 23: Chronic estrogen treatment did not cause any significant changes in NE content in the AN of these acyclic rats as compared with those of estrous control. There was no significant difference in the levels of NE in the old constant estrous rats as compared with all the other groups at $p<0.05$. The number of animals per group is given in parenthesis. The data shown are mean ± SEM.

Dopamine profile in the AN on proestrus

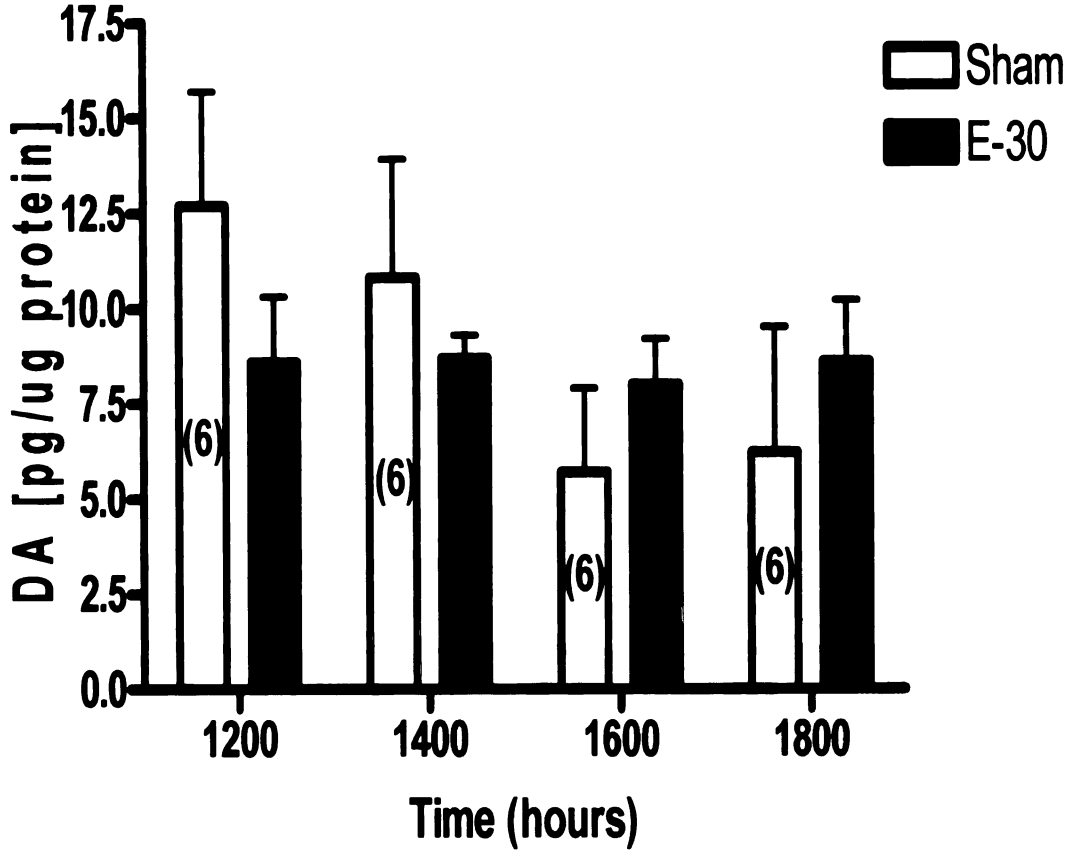

Figure 24: Dopamine levels are indicated as mean ± SEM. Number of animals per group is indicated in parenthesis. Analysis was done at $P < 0.05$ level. A non-significant suppression in DA levels was observed in controls at 1600 hours compared to 1200 hours.

Dopamine changes in the AN upon chronic estrogen treatment

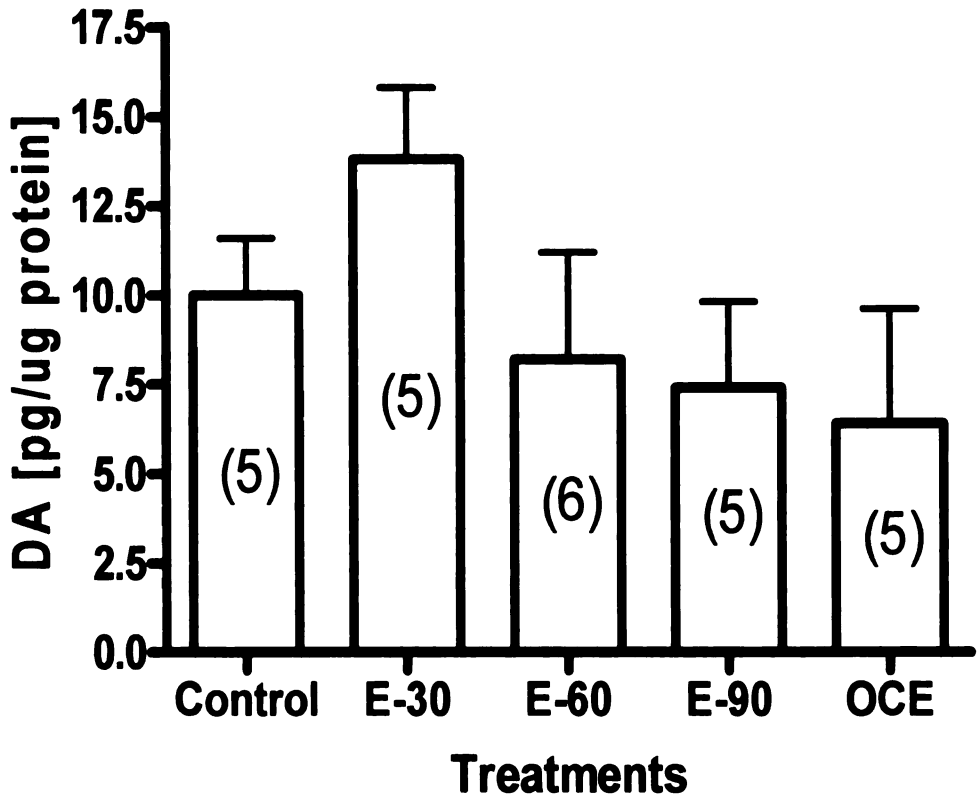

Figure 25: Chronic estrogen treatment did not cause any significant alteration in DA content in the AN in animals which were in the state of constant estrus, as compared with those of controls in estrus and old animals in the state of constant estrus (OCE) at $p<0.05$ level. The number of animals per group is given in parenthesis. The data shown are mean ± SEM.

Cortex

Norepinephrine

1. Estrogen treatment does not profoundly affect the cortical NE content in 30-day E2 treated rats on proestrus.

The two-way ANOVA of the cortical NE content across time on proestrus revealed a significant effect of treatment on the NE levels with a P value of 0.0469 accounting for 8.6% of the total variance ($F(1,40) = 4.21; P = 0.0469$). However subsequent post-hoc test did not find any significant difference between any of the groups tested both across time and treatment, at a P level of <0.05. In contrast to other brain areas tested where we did find significant effect of time on the NE levels, we did not find any significant effect of time on the outcome in the cortex, which accounted for 5.5% of the total variance ($F(3,40) = 0.9$; $P = 0.4510$). Similarly the interaction was not found to be significant either with its contribution of 4.09% towards the total variance ($F(3,40) = 0.67$; $P = 0.5775$). In general, the NE content in the E2 treated groups however appeared to be higher at all time points test when compared with controls though they were not significant.

2. Cortical NE levels were similar in both estrus-controls and E2-treated acyclic animals.

We did not find even mild changes in the NE levels in acyclic rats irrespective of the duration of E2 treatment (30, 60 or 90 days). Though the older constant estrous rats had relatively lower NE content (1.3 ± 0.4) compared to rest of the groups, it was not found to be significant at the level of $P<0.05$. The NE level in

control was 2.6 ± 0.7 pg/ug, and remained similar in all the E2 treated groups. The analysis had a P value of 0.4992 and F = 0.8697.

Dopamine

1. Dopamine levels in 30 day-E2 treated rats remained unaffected.

Two-way ANOVA revealed no significant effect of time ($F(3,40)=2.32$; $P = 0.09$), treatment ($F(1,40) =2.78$; $P = 0.1031$), or their interaction ($F(3,40) =0.23$; $P = 0.8736$) on the outcome.

2. Estrogen-induced constant estrus rats have similar DA content in CX compared to controls in estrus.

Estrogen treatment for 30, 60 or 90 days did not have any effect on the DA content in the cortex and are similar to those of control rats in estrus. Older constant estrus rats had relatively lower DA levels from the rest of the groups which was not found to be significant. The analysis was done at $P < 0.05$ level and had the P value of 0.3602 and an $F = 1.154$.

Norepinephrine profile in the CX on proestrus

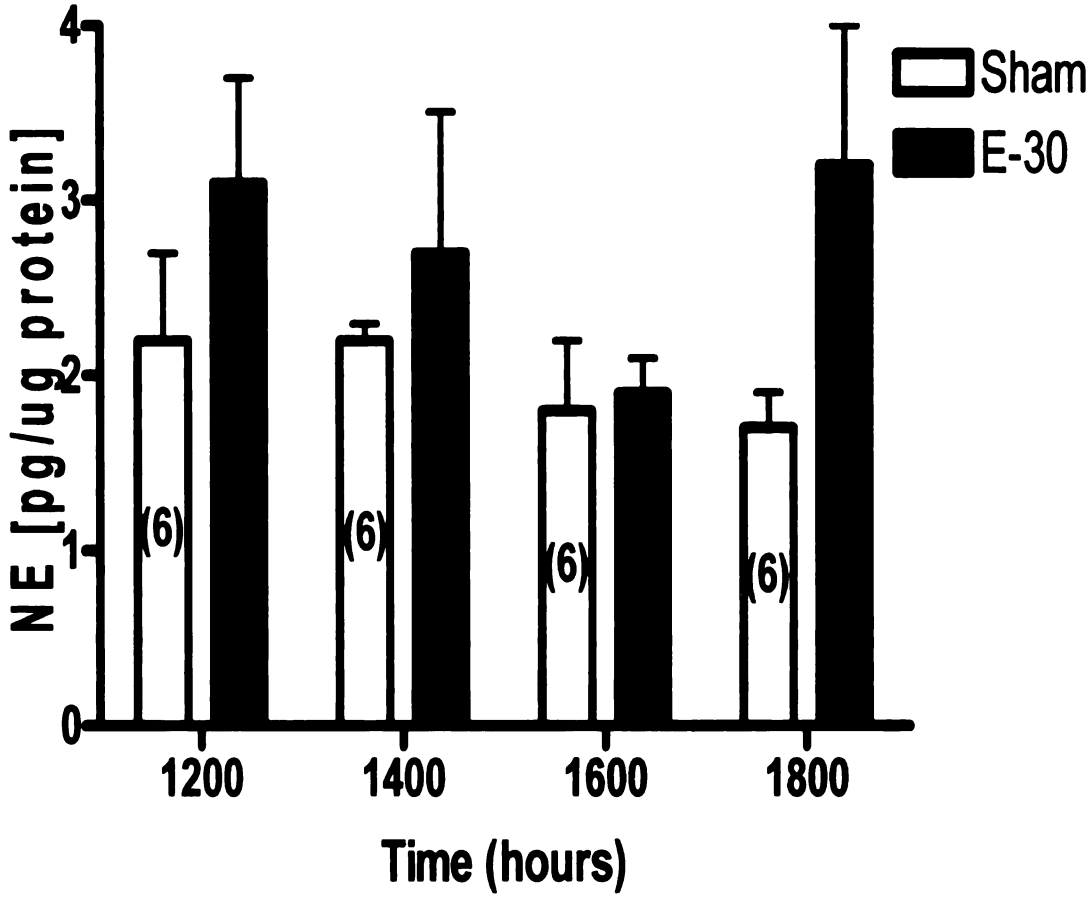

Figure 26: NE levels are shown as mean ± SEM. The number of animals per group is shown in parenthesis. Analysis was performed at $P<0.05$ level. Cortex was used as an internal control for a non-hypothalamic area which is not involved in the control of LH surge.

Norepinephrine changes in the cortex upon chronic estrogen treatment

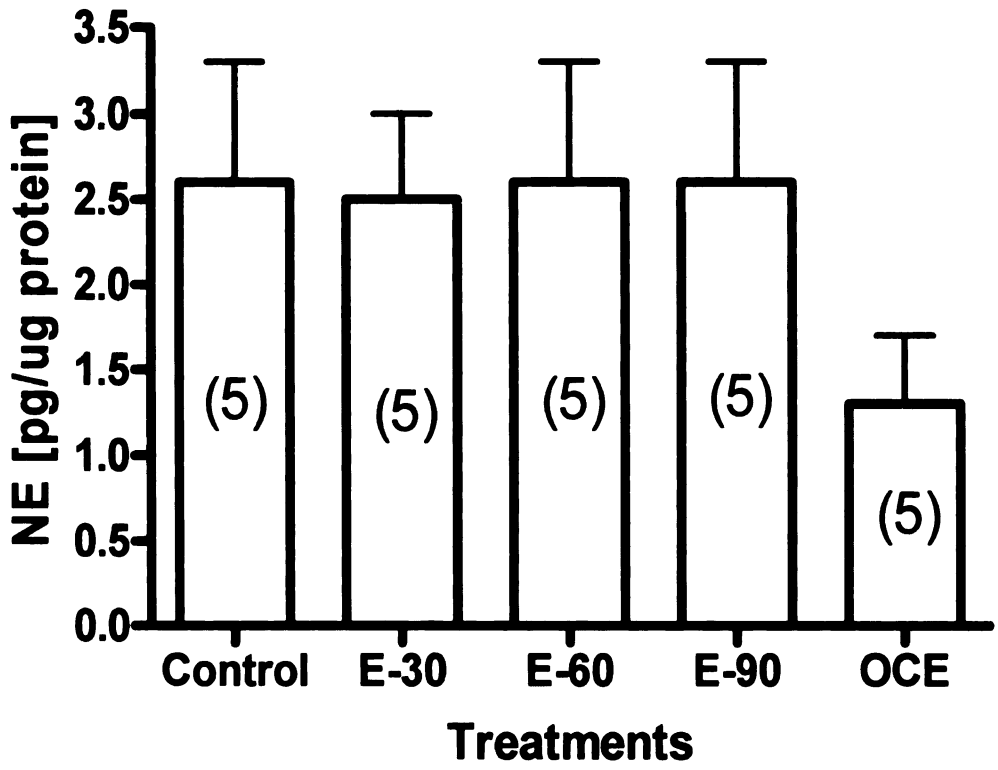

Figure 27: Chronic estrogen treatment did not cause any significant alterations in the NE content in the cortex as compared with those of controls in estrus. Though the old constant estrous animals (OCE) had lower NE content, it was not found to be significant when compared with estrous controls. Analysis was done at $p<0.05$ level. The number of animals per group is given in parenthesis. The data shown are mean ± SEM.

Dopamine profile in the cortex on proestrus

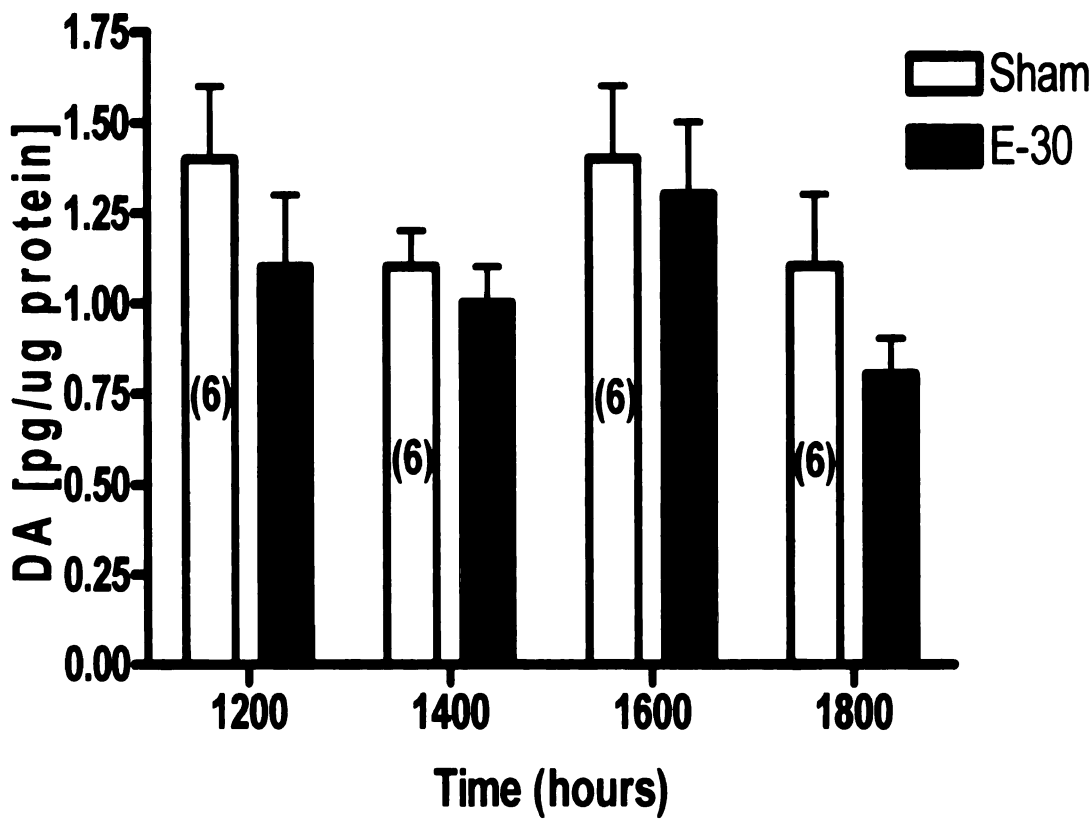

Figure 28: Each bar represents the DA levels as mean ± SEM. Number of animals per group is indicated in parenthesis. No significant differences in means were observed at $P<0.05$ level. The cortex serves as an internal control as an area not involved directly in reproduction and as a non-hypothalamic control.

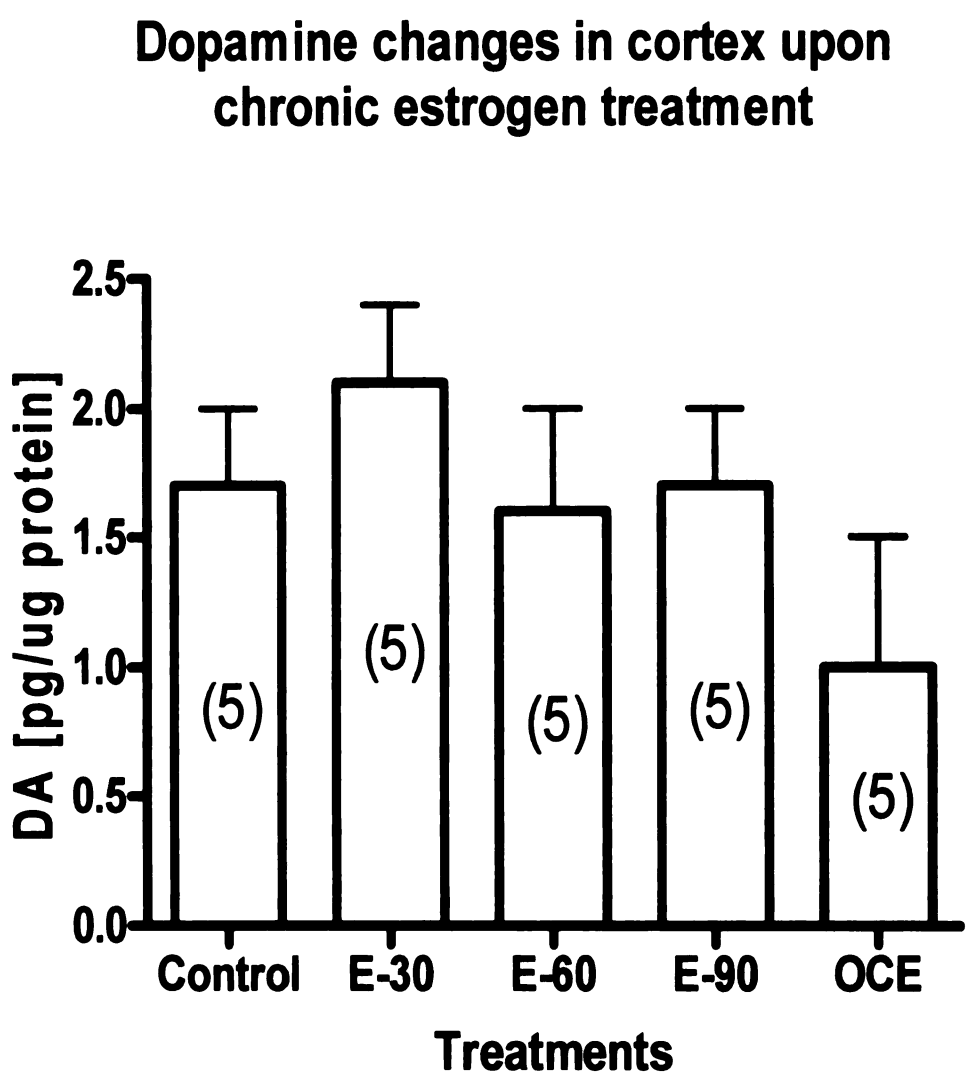

Figure 29: Dopamine content in the cortex of animals treated chronically with estrogen was not significanly different from those of controls in estrus and that of aging constant estrous animals (OCE) at $p<0.05$. The number of animals per group is given is parenthesis. The data shown as mean $\pm$ SEM.

D. Discussion

The results of this study strongly support the hypothesis proposed in our previous study (chapter-1) that chronic estrogen exposure-induced loss of estrous cyclicity is probably mediated through changes at the level of the hypothalamo-hypophyseal axis. Prolonged estrogen exposure not only suppressed the LH surge [levels at 1800 hours were not significantly higher compared to 1200 hours in the E2-30 groups while we did find significant elevation in LH levels in the control group at 1800 hours compared to 1200 hours. While there was an absence of treatment effect] in the E-30 group on the day of proestrus, there was a significant suppression in the levels of LH at 1200 hrs in all the constant estrus animals (acyclic) as compared with age-matched estrus-controls. This was also accompanied by significant suppression in the levels of NE in the MPA and SCN, both of which are components of the Preoptico-Suprachiasmatic-Tuberoinfundibular(PST) system of GnRH neurons. We neither found any effect of estrogen treatment on catecholamines in the E-30 animals across time on the day of proestrus nor did we see any significant alteration in the cortex, a non-hypothalamic, internal control area, which is not known to be involved in the governance of estrus cycles. We are aware of the possible shift in the occurrence of LH surge in the E2-30 groups on proestrus, which could have given rise to the lower LH levels at 1800 hours in that group as compared to 1200 hours. Also, for the first time, we show here the temporal changes in the concentration of NE and DA in different areas of the PSTS on the afternoon of proestrus.

Numerous studies have shown the importance of the PSTS in the generation of LH surge [18, 50, 95] . This system harbors the cell bodies of the GnRH neurons which terminate in the median eminence. It is made up of (from rostral to caudal) DBB-MPA, SCN, AN and ME. The cell bodies of the GnRH neurons located in these nuclei have their terminals in the ME. The two main regions, which are known to contain large number of GnRH neurons includes the DBB and MPA. These areas of the PST system are known to be innervated with catecholaminergic fibers from brain stem nuclei, specifically from the A1, A2 and A6 noradrenergic nuclei [96, 97]. The importance of these catecholaminergic neurons for the generation of LH surge is well established [18, 95, 98]. Monoamine activity in the PSTS specifically that of NE, have been shown to increase just before the onset of the LH surge and is implicated for the same [28, 29]. Decreased monoamine activity is seen in middle aged animals that have a suppressed LH surge on the afternoon of proestrus. The changes in monoamine content and the trend seen in the PST system, in this study, agrees with the previously reported studies on the day of proestrus [28, 29] and with those in old constant estrus rats.

Numerous studies have been done to show the importance of norepinephrine in the generation of the LH surge. These studies include pharmacologic approach (use of adrenergic receptor blocker or drugs that deplete the catecholamine synthesis and other drugs) or lesioning and deafferentation studies and the direct use of norepinephrine in vivo or in vitro, in both intact female rats on proestrus or in the ovariectomized, steroid-primed model. Everett

et al [38] demonstrated the ability of adrenergic blockers on ovulation in intact rats. This was also demonstrated by Sawyer in the same model. They used dibenamine and SKF-501 as blocking agents. Studies have been done using other drugs, namely chlorpromazine[98], Phenoxybenzamine, Haloperidol [99], and by the use of other catecholamine depletors like alpha-MPT [69], a tyrosine hydroxylase(TH) inhibitor, DDC [69], an inhibitor of dopamine beta hydroxylase (DBH) and hence NE synthesis and by the use of Reserpine [98], a drug that has been shown to cause depletion of NE and DA in the synaptic regions [100, 101]. All these treatments block the occurrence of the LH surge and/or ovulation, suggesting the involvement of NE in these events.

There are also other lines of evidence using the ovariectomized steroid primed rat model, where the progesterone induced-LH surge was blocked by alpha-adrenergic receptor blockers, alpha-MPT or with DBH inhibitors [70] and the NE precursor, DOPS (D,L-threodihydrophenylserine), was able to restore the alpha-MPT induced blockade of the LH surge [102].

The possibility of NE playing an important role in the generation of LH surge/ovulation was also examined by deafferentation studies. In one such study, complete deafferentation of the Mediobasal Hypothalamus lead to complete decrease in NE concentrations. This also interrupted estrus cyclicity leading to persistent diestrus conditions. DA concentrations were unaffected in this region by the treatment [103]. However, the studies done by Clifton and Sawyer [104, 105], seem to raise doubts regarding the role of NE in the LH release. Though the animals were subjected to bilateral transection of ascending noradrenergic

bundles, which in turn resulted in severe depletion of NE, all the animals were able to resume estrus cycles. Also, the proestrus levels of theLH surge did not differ significantly in these animals. The non-obligatory role of NE in the neuroendocrine response, and the plasticity of the neuroendocrine systems were suggested [7].

Simpkins et al. [106] showed that implantation of 6-hydroxy dopamine in the SCN was not only able to deplete NE concentrations in the anterior hypothalamus, but also abolished the progesterone-induced LH surge in ovariectomized and estrogen-primed animals. Similar results were obtained with the implantation of 6-OHDA in the preoptic area [107]. Together, almost all the available data point to a stimulatory role of the catecholamine, NE, in the regulation of LH surge. Given this, the changes in the catecholamine profile found in our estrogen treated constant estrus model, strongly reiterates the idea of "perturbations in catecholamine activity plays a leading role in the entry of animals in to constant estrus state". Whether this could be the main reason for the lengthened cycles found in the E-90 withdrawal groups is not known. This information, taken together with the significant suppression of NE in E-60 and E-90 animals in the SCN region, suggests the possibility of E2 causing suppression of HPG activity through the SCN. We do not know whether this could have a direct effect on the SCN or through other routes, viz. brain stem NE neurons.

One possibility for a direct action of E2 on hypothalamic neurons would be through estrogen receptors (ER) in that region of the brain. GnRH neurons have been shown to express estrogen receptors. Studies by Skynner et al. [108] and

Hrabovszky et al. [109] have provided evidence for the presence of ER-beta in GnRH neurons of rats. This was also evidenced by the demonstration of ER-beta immunoreactivity in GnRH neurons [109, 110] and that of the I125-estrogen uptake by the GnRH neurons in vivo [109]. With reference to the idea of the presence of ER-alpha, stronger evidence exists to support its absence in GnRH neurons [109, 111, 112] than its presence [113]. Hence there seems to be a major consensus in ER-beta playing a role in the stimulation of the GnRH system.

Estrogen is known to suppress the expression of ER while P4 has a moderately inducing effect [114]. Its expression pattern in the hypothalamus changes during the estrus cycles with its expression being the lowest in the afternoon of proestrus than at metestrus [114, 115]. While it is interesting to observe the differential expression of ERs across the estrus cycle, much more interesting results have been obtained where they have shown the differential expression of ER-alpha and beta in the right and left side of the preoptic area at the same time point, thereby raising questions regarding different sides playing different roles in the reproductive process or whether this could be part of the regulation of the reproductive process as a whole [116]. The expression of ERs in pituitary gonadotrophs appears to follow a similar pattern as in the case of the preoptic area, with a higher amount during estrus and the lowest amount during diestrus. It has been shown to double (10%) on proestrus as compared to the diestrus values of 5% [117]. Given the above facts, it is possible to presume that chronic estradiol treatment might interfere with the normal changes in ER

expression at the hypothalamic level, where it can suppress the expression of ERs [114] while simultaneously increasing its expression in pituitary gonadotrophs simulating the state of estrus [117]. Though a direct action of estradiol on the anterior pituitary is known to be important for the positive feedback on LH surge secretion [118], it is not known whether chronic E2 treatment can in the long run abolish the same by some compensatory feedback mechanism operating at the level of the pituitary itself.

Though DBB is known to contain large numbers of GnRH neurons, and is thought to be a very important area with reference to the LH surge, we did not find any suppression in NE levels in chronically E2 treated animals. This might mean either that DBB is some how resistant to the effects of chronic E2 treatment, or the specific brain stem noradrenergic neuronal population innervating DBB are resistant to chronic estradiol action. If the temporal changes in NE concentration in different areas of the PST system on proestrus might be due to the temporal activity of the noradrenergic neurons innervating the specific nuclei, then the reason for such changes in the activity of those neurons needs to be investigated. Since the rat estrus cycles are strongly affected by lighting cues, it may be possible for the SCN to play an important role in such modulation of temporal activity of neurons. This might well explain the decline in DA concentrations at the time when NE concentration is significantly increased. The decline in DA per se could also be due to higher conversion of DA to NE in those regions. Hence, it is very much possible for the system to modulate the relative concentrations of NE and DA by regulating the activity of the enzymes involved in

their metabolism, namely, TH, DOPA-decarboxylase, DBH, MAO and Catecho-O-methyl trasferase. Activity measurements of the various enzymes involved in such metabolic processes might be informative to ascertain the cause of alterations in catecholamine content during different states of estrus cycles, and specifically on the day of proestrus. An indirect measure of such activity could be obtained from the measurement of their metabolites, namely, DOPAC and Homo Vanellic Acid (or VMA), though, VMA is a metabolite of both NE and Epinephrine.

Though we did find suppression of serum LH and a corresponding suppression of NE in the MPA and SCN in those rats which have become constant estrus because of chronic E2 treatment for 60 or 90 days, we can not state whether it is a pathologic mechanism (like inflammatory reaction due to E2 exposure leading to decline in the ability to produce enough catecholamines in those areas) or a compensatory feedback mechanism in response to constant E2 signalling, without any pathologic insult as such, where the system perceives the presence of constant E2 stimulus as "not normal" and attempts to prevent the consequences of such constant signaling by suppressing the brain signaling molecules like catecholamines, thereby keeping a check right at the level of the hypothalamus, in the control of neuroendocrine system. There are reports of estrogen causing pathologic manifestations in the arcuate nucleus region of the rat. A single injection of estradiol valerate has been shown to cause constant estrus condition along with polycystic ovaries, in adult rats with regular estrus cycles [88]. Though the amount injected is very large, (2 mg), compared to 20 ng / day treatment according to our regimen, the compound used is also different.

While we used estradiol-17-beta, in our study, they have used estradiol valerate. Nevertheless, they were able to show that this insult is very specific to the arcuate nucleus only and that the other regions are spared. We did not examine the presence of any inflammation in the AN in our studies. Morphological / pathological examination were not done in our study to observe such pathological process if any. Also, this reaction seems to be very specific to beta-endorphin neurons [119]. It is thought that such degeneration of the AN neuronal elements is probably brought about by an increase in free radical production/activity in this region. This in turn is thought to be due to the presence of estrogen 2/4-hydroxylase enzyme, which oxidizes estrogen to o-semiquinones [119]. This reaction pathway appears to be important in the pathogenesis of estrogen-induced toxicity. Future studies will focus on the mechanism of chronic estrogen exposure-induced suppression of the HPG axis in rats.

In summary, based on this and the previous study (chapter-1), we conclude that chronic low-dose estrogen exposure to intact, adult, female rats induces a constant estrus-like condition probably by causing a functional deficit in the HP axis leading to a hyper estrogenic condition. This is probably mediated by profound alterations in the levels of norepinephrine in the PST system, leading to suppressed LH levels (and surge), thereby causing anovulation without polycystic condition. Further evaluation of the responsiveness of the anterior pituitary to GnRH needs to be carried out to localize the lesion. Whether constant maintenance of serum estrogen levels (similar to that found on the day of proestrus) on a daily basis would mimic the same processes as that would

happen during estrus cycle is a question worth pondering, since serum E2 levels tends to increase gradually from diestrus through proestrus with its levels the highest during proestrus afternoon followed by a sudden decline in its level. It still is the fact that serum E2 levels are higher during most part of the estrous cycle, though it is not as high as would be found on the proestrus afternoon. An extention of this is finding is that chronic exposure to estrogenic compounds could severely impair reproductive functions in the long run by acting through hypothalamic pathways. Given the important roles played by the hypothalamus in other homeostatic mechanisms, it is plausible that similar deleterious effects could also be seen in other systems. Hence further investigation into the mechanisms by which estrogen affects hypothalamic catecholamines is warranted.

CHAPTER-3

CHRONIC ESTROGEN EXPOSURE ABOLISHES STEROID INDUCED-RELEASE OF NOREPINEPHRINE IN MEDIAL PREOPTIC AREA IN RATS: A POSSIBLE MECHANISM FOR SUPPRESSION OF LUTEINIZING HORMONE SURGE

A.Introduction

The preovulatory surge of luteinizing hormone (LH) on the afternoon of proestrus is known to be essential for ovulation in rats[120]. The LH surge is generated due to the action of rising serum estradiol levels on the hypothalamo-hypophyseal (HP) system, through a positive feedback mechanism [120]. The mechanism of action of estradiol in the induction of LH surge is not completely understood.

Among the many factors involved in the generation of LH surge at the level of hypothalamus, norepinephrine (NE) is an established candidate [121]. Numerous studies attest to the role of NE in the generation of the LH surge [121]. Moreover, the GnRH neurons of the Preoptico Suprachiasmatic Tuberoinfundibular System (PSTS) are richly innervated by brain stem noradrenergic neurons [59]. A strong correlation between the release of NE in the medial preoptic area (MPA) and the LH surge has been established both in intact proestrus [28, 29] in and ovariectomized steroid primed rats [30] . A similar correlation between the two has been established in aging animals where the corresponding suppression of NE and LH surges is seen [29]. The complete abolishment of the LH surge by

severing the ventral noradrenergic bundle [122] adds strong support to the dependence of the LH surge generator on the noradrenergic neuronal system.

Chronic exposure to estrogen (CEE) has been shown to abolish the LH surge in ovariectomized animals [9] [chapter 2]. A Similar suppression of the LH surge is seen in aging rats [31]. In our previous study, reported in chapter-1, we have shown that chronic exposure of intact rats to low levels of 17-β estradiol causes constant estrous-like condition by 60 days of treatment. Also, we have shown in our next study, reported in chapter-2, that CEE-induced persistent-like estrus condition is accompanied by suppressed levels of LH and NE in the serum and MPA respectively. The reason for this suppression in the NE and LH surges upon chronic estrogen exposure is not known. The data from the previous study strongly supported the hypothesis that CEE-induced constant estrus condition is mediated through the HP axis probably by causing suppression in the levels of NE in the PST system leading to a significant suppression of the LH surge. However the mechanisms which contribute to the reduction in NE levels are not clear.

Since NE neurons have estrogen [121] and progesterone receptors and are sensitive to these hormones [123-125], we hypothesized that chronic estrogen exposure may some how alter the sensitivity of these neurons to these steroids. Since 30 days of estrogen exposure did not cause changes in estrus cycles in a significant number of animals, and since we did not find any changes in the LH surge and NE levels in the PSTS of these animals, we used animals treated with estradiol-17-beta for 60 and 90 days in this study, as we have shown that a

majority of the animals exposed to estrogen for these durations become acyclic. After chronic estrogen exposure, all animals were ovariectomized and primed with estrogen and progesterone to simulate a LH surge. The role of NE in this process was investigated with the help of push-pull perfusion and HPLC-EC.

B. Materials and Methods

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 groups of 3 per cage in temperature (23±2°C) and light-controlled (lights on from 0500 to 1900 h) animal rooms. They were given food and water *ad libitum*. The animals were used in the experiment 2 weeks after arrival. All the protocols followed in this study were approved by the University Committee for Animal Care and Use at Michigan State University.

Treatment

Vaginal smears were obtained in the morning hours between 0800-1000 h. Those animals showing regular 4-day cycles were chosen for the experiment. The animals were randomly divided into different treatment groups. Animals in the control group were sham-implanted and those in the treatment groups were implanted subcutaneously with slow-release estrogen pellets under mild halothane anesthesia. The pellets were capable of releasing 17-β estradiol at the rate of 20 ng per day for a period of 60 or 90 days (Innovative Research America, Sarasota, FL). The animals receiving estrogen pellets were divided into 2 groups based on the duration of estrogen exposure which was for 60 or 90 days (E-60 and E-90 respectively). Vaginal cytology was recorded daily as described in the previous chapter for all the animals. At the end of the treatment period (60 or 90 days), animals were bilaterally ovariectomized under pentobarbital anesthesia. Simultaneously these animals were also implanted with a unilateral push-pull

cannula in the MPA as described below [Based on the observations made from previous experiments (chapter-2), animals implanted with either 60 or 90 day slow release E2 pellets [20ng/day] do not retain the pellet at the end of the treatment period of 60 and 90 days respectively. Hence, we did not make any attempt to check for the presence of pellets at the end of treatment period by opening up the implantation site. Instead palpation was used to identify the same and none was found to retain pellets]. These animals were allowed to recover for seven days. On the eighth day, the animals were given 0.1 ml of either corn oil or estradiol-17beta (30µg/0.1 ml corn oil, s.c.) at 1000 hrs. On the 9^{th} day, the animals were implanted with jugular catheters between 3-5pm. On the 10^{th} day, (the day of experimentation), the animals were given 0.1 ml of either corn oil or progesterone (2 mg/0.1 ml, s.c.) at 1000 hrs. The animals were then connected to the push-pull catheters and the perfusion was started around 1030 hrs. The push pull perfusion procedure is described in detail below. The jugular catheter was flushed with heparin-saline solution at 1130 hrs. Blood samples were collected from 1200 hrs until 1900 hrs, at one hour sampling intervals. Perfusates were collected from 1200 hrs until 1900 hrs, with a sampling interval of 30 minutes. Four hundred microliters of blood was collected every hour, and transferred to heparin-treated eppendorf tubes. These samples were centrifuged at 5000 rpm for 10 minutes yielding around 200 µl of plasma. This was stored in separate eppendorf tubes, pretreated with heparin, and was immediately transferred to -70°C freezer until samples were analyzed for LH by RIA.

Animals were sacrificed by rapid decapitation under protocols approved by the AUCAUC. The brain was carefully removed and immediately frozen on dry ice and was stored in the -70°C freezer until the time of sectioning.

Brain Sectioning

At the time of sectioning, the brains were mounted on a chuck and left inside the cryostat (Slee, London, UK) for 1 hour to attain that temperature (-10°C), before sectioning. Serial brain sections of 40µm thickness were obtained using a cryostat maintained at -10°C. The sections were carefully transferred to slides, which were placed inside the cryostat. Six brain sections were mounted per slide before the slide was transferred to room temperature. The brain sections were stained using cresyl violet as mentioned below, and these were used for determining the position of the cannula tip. Only those animals which had the cannula in the MPA were included in the study.

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 a temperature of 37°C. At the time of analysis, tissue punches were transferred from –70°C to ice. To each sample, 200 µl of 0.1 M perchloric acid was added and the tissue was immediately homogenized on ice for 10 seconds using a micro-ultrasonic cell disruptor (Kontes, Vineland, NJ, USA). Samples of the homogenate were aliquoted for protein analysis. The rest of the homogenate was spun at 14000Xg for 5 minutes. Sixty microliters of the supernatant was added to 30 µl of the internal standard (0.05 M dihydroxy benzylamine) and 30 µl of 0.1 M perchloric acid, and were loaded onto the autoinjector, where samples and standards were maintained at 4°C. A mixed standard containing known concentrations of Norepinephrine (NE), dopamine (DA), dihydroxy phenylacetic acid (DOPAC), hihydroxy benzylamine (DHBA), 5-hydroxytryptamine (5HT, serotonin) and 5-hydroxy indoleacetic acid (5-HIAA) was loaded for every 10 samples. DHBA was used as the internal standard.

LH-RIA

LH was measured in duplicates in the serum by radioimmuno assay (RIA) using a double antibody method as described before [29]. The standards and antibody (Anti rLH-SH) were obtained from Dr. A.F.Parlow, NHPP, NIDDK. The LH tracer was obtained from Amersham Pharmacia Biotech (Waukesha, WI, USA). Briefly, 40 ul of the serum was assayed in duplicate. The volume was made up to 100 ul using PBS-gelatin. 100 ul of the first antibody (rabbit anti-rat

LH NHPP, NIDDK) was added to the samples and standards at a dilution of 1:758,000. After overnight incubation at 4 °C, 100 ul of the tracer was added to the total count, non-specific binding (NSB), standard and sample tubes. After overnight incubation, the second antibody (sheep anti-rabbit, indigenous) was added at a concentration of 1:12. The final assay volume was 400 ul. Three hours later, a 30% solution of polyethylene glycol (PEG, 100 ul) was added to the tubes followed by 1 ml of PBS. The tubes were vortexed, centrifuged and the supernatant was aspirated. The pellets were counted for radioactivity using a Beckman 5500 B gamma counter. LH levels were obtained by using a software program (Assayzap, MacIntosh) and were expressed as ng/ml.

Statistical Analysis

Differences in neurotransmitter concentrations between different treatment groups and across different time periods within each group was calculated for each hypothalamic area individually using two-way repeated measures ANOVA and post hoc Fisher's LSD test. Serum LH levels were analyzed using two-way repeated measures ANOVA and post hoc Fisher's LSD test.

C. Results

Location of the push-pull cannulae:

Fig 30-A and B. shows the histological location of the push-pull cannulae in the different treatment groups. All the animals used had their cannula in the MPA.

Chronic estrogen exposure abolishes the steroid-induced LH surge

When sham-implanted animals were treated with oil, LH levels (Mean±S.E.; ng/ml) in the serum was 2.2908±.5945 at 1300 h and remained at that level throughout the observation period. In contrast, treatment of Sham implanted animals with estrogen and progesterone increased LH levels markedly from 5.291±3.565 at 1300 h to 252.35±126.04 at 1600 h and kept it elevated at 1700 h (71.16±42.5) before declining at 1800 h (Fig 2A). Thus a marked LH surge was evident upon steroid priming in sham-implanted rats. While the time accounted for 17.9% of the total variance ($F(5,40) = 3.44$; $P = 0.0112$) and hence had significant effect on the outcome, the treatment alone did not have significant effect with it contributing to 8.25% of the total variance ($F(1,40) = 4.69$; $P = 0.0623$). The interaction of time and treatment however had significant effect on the outcome with it accounting for 18.11% of the total variance ($F(5,40)=3.48$; $P = 0.0105$).

E2 treatment for 60 and 90 days on the other hand, suppressed the LH surge. When E-60 treated animals were treated with oil, LH levels (mean±SEM ng/ml) were 2.2512±0.694 at 1300 h and remained at that level for the rest of the observation period. Treatment of E-60 and E-90 animals with estrogen and

progesterone left LH levels at .4486±.097 and 1.703±0.914 at 1300 h respectively and they remained around that level throughout the observation period (Fig 2B). Though significant elevation of LH (as a surge) was completely abolished by chronic E2 treatment, we did find significant suppression of the basal LH levels in the E2-60 group, upon steroid priming compared to the E2-60 group treated with oil ($F(2,55) = 7.48$; $P = 0.0089$) and treatment accounted for 44.99% of the total variance. Neither time nor interaction had any significant effect on their outcome with them contributing to 3.39% ($F=1.16$ and $P = 0.3347$) and 2.8% ($F= 1.92$, $P = 0.1061$) of the total variance.

Average LH levels (Mean±S.E.; ng/ml) during the entire period of observation in sham-implanted animals that were treated with oil was 2.271±0.33 and increased dramatically with steroid priming (61.86±46.43). The results of t-test yielded a P value of 0.0267 and $t=2.794$). Steroid priming significantly elevated the average LH released compared to oil treatment. In contrast, steroid priming of E-60 and E-90 rats resulted in a decrease in LH levels (0.2397±0.5457 and 1.0871±0.3630 respectively) compared to E-60 animals that were treated with oil (2.266±0.5457). The one way-ANOVA resulted in a p value of 0.0088 and $F=7.507$. Tukey's Multiple comparison Test revealed significant suppression in average LH level in E2-60 group treated with E2 and progesterone, compared to the E2-60 group treated with oil at $p<0.01$.

The pre-ovulatory surge in NE in the MPA is abolished by chronic estrogen treatment

NE release (Mean±S.E.; pg/min) in the MPA of sham-implanted animals treated with oil was 1.36±0.25 at 1300 h and remained at that level throughout the observation period. In contrast, steroid-priming of sham-implanted rats with estrogen and progesterone increased NE levels from 2.926±0.63 at 1300 h to 5.699±2.22 at 1430, increased it further to 9.159±3.5 at 1500 h, remained elevated at 1600 and 1630 h and declined gradually to 4.896±2.5 at 1700 h (Fig 4A). Overall, the results of repeated measures ANOVA indicate no significant effects of time, treatment or interaction. Time accounts for 4.98% of the total variance ($F(9,72) = 1.5$; $P = 0.1628$), Treatment contributes to 23.32% of the total variance ($F(1,72) = 4.77$; $P = 0.0604$) and interaction between them accounts for 6.13% of total variance ($F(9,72) = 1.85$; $P = 0.0733$), suggesting that none of the variables contributes significantly to the outcome. However, the difference at 1500 hours between the estrogen and progesterone group and the oil treated one is significant at a $P<0.05$.

On the other hand, oil treatment of E-60 rats did not produce any change in NE release in the MPA. Steroid priming of both the E-60 and E-90 groups kept NE levels low and did not produce any significant changes in NE release (Fig 4B).

The average NE release (Mean±S.E.; pg/min) in sham implanted rats treated with oil was 1.4665±0.286 and it increased significantly to 5.5602±2.059 ($P = 0.0302$, $t=2.184$) with steroid-priming ($p<0.05$; Fig 5A). Steroid priming of E-60 rats did not produce any increase in average NE levels (0.3964±.1227)

compared to oil treated E-60 rats (0.4375±0.1762; $p<0.05$). However, E-90 treated animals did not respond to steroid priming (0.618±0.1218) ($P = 0.5276$, $F=0.6747$) (Fig 5B).

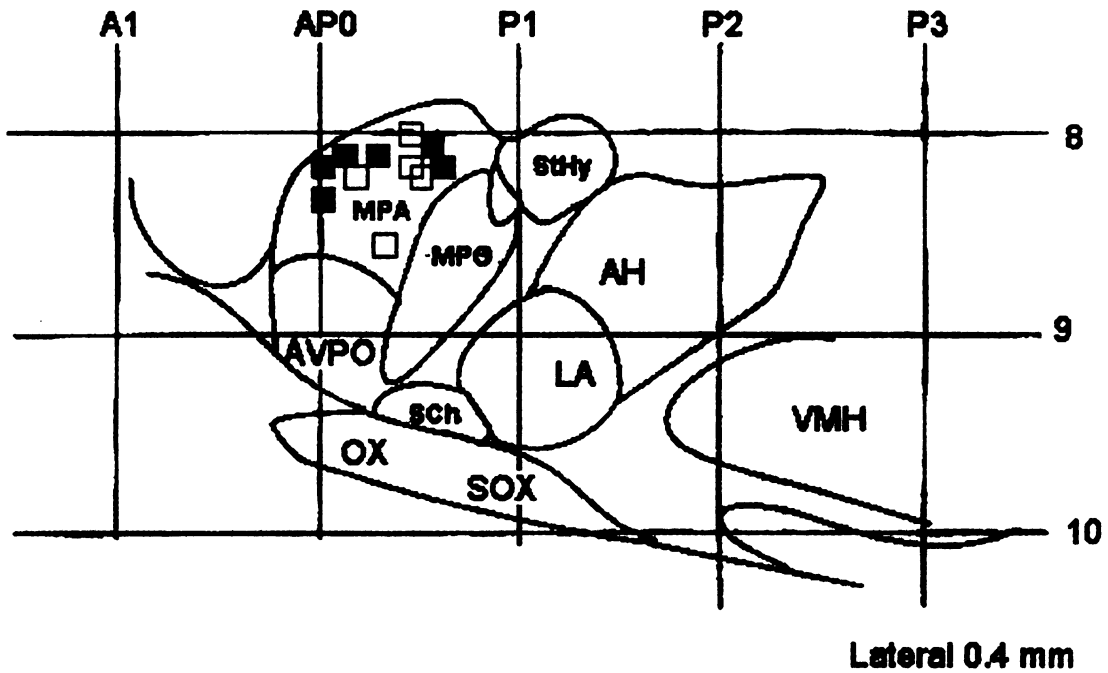

Figure 30-A: Location of push-pull canulla in the medial preoptic area in the sham implanted rats. Open squares indicate oil treated animals. Closed squares indicate steroid primed animals.

AvPO	Anteroventral Preoptic area
MPA	Medial preoptic area
MPO	Medial Preoptic nucleus
StHy	Striohypothalamic area
AH	Anterior hypothalamus
LA	Lateroanterior hypothalamic nucleus
VMH	Ventromedial hypothalamus
OX	Optic chiasm
SOX	Supra optic decussation
Sch	Suprachiasmatic nucleus

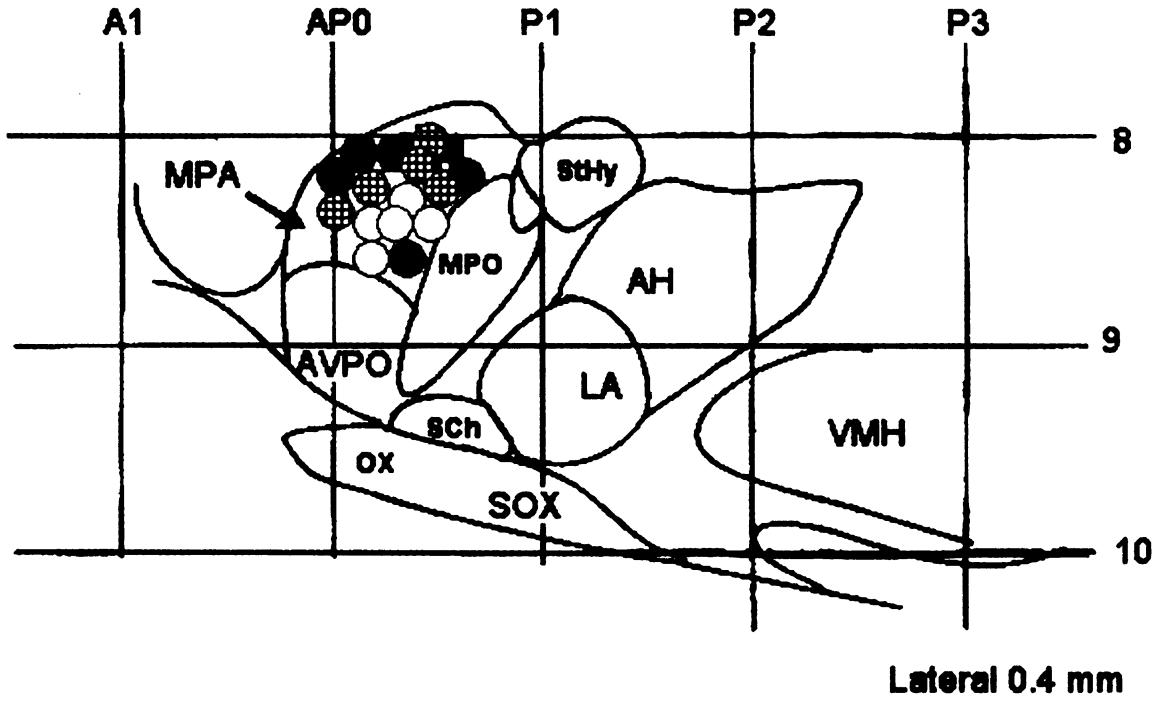

Figure 30B: Location of push-pull cannulae in the medial preoptic area in rats implated with estradiol pellets. Open circles indicate oil treated rats of E2-60 group. Black circles and checked circles indicate steroid-primed rats of E2-60 and E2-90 groups respectively.

AvPO	Anteroventral Preoptic area
MPA	Medial preoptic area
MPO	Medial Preoptic nucleus
StHy	Striohypothalamic area
AH	Anterior hypothalamus
LA	Lateroanterior hypothalamic nucleus
VMH	Ventromedial hypothalamus
OX	Optic chiasm
SOX	Supra optic decussation
Sch	Suprachiasmatic nucleus

Steroid priming-induced LH surge in sham implanted rats

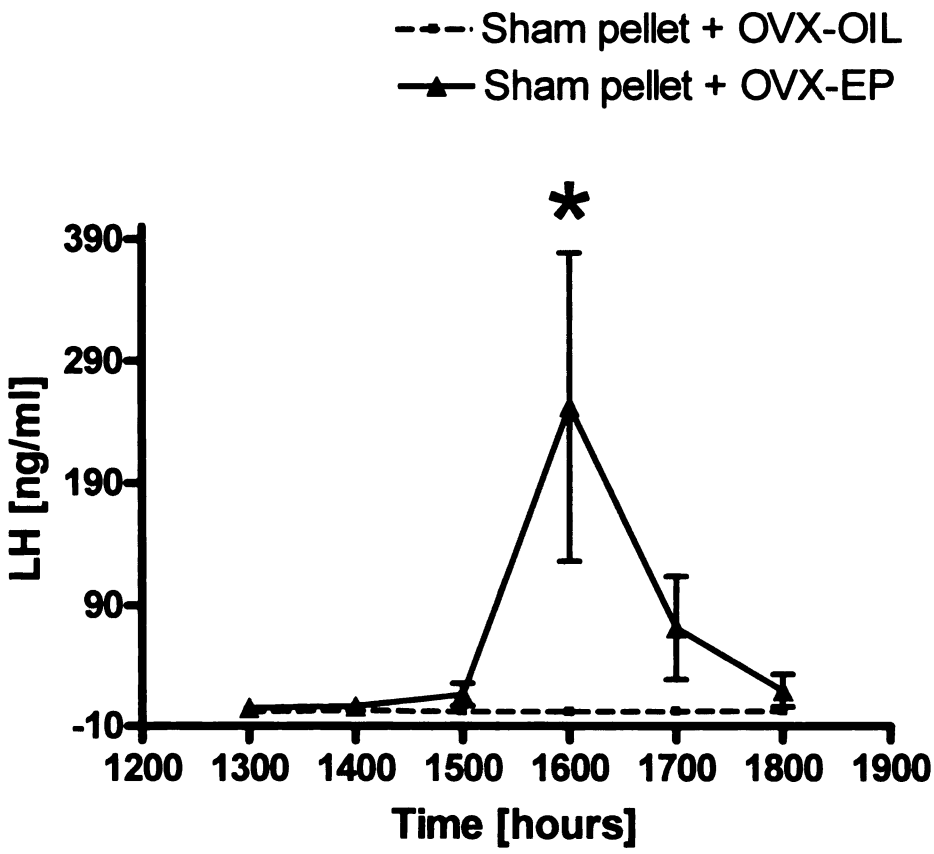

Figure 31: Steroid priming of the sham implanted rats shows an LH surge compared to oil treated rats. Repeated measures ANOVA was performed followed by bonferoni post test at $p<0.05$. '*' indicates significant difference from the oil treated controls at the same time point. The number of animals is 5 per group. NOTE: The basal LH levels in the oil treated animals should be higher than that of the steroid primed animals, which is not what is seen here. The reason for this discrepancy is not known.

Effects of chronic E2 exposure on steroid-priming induced LH surge

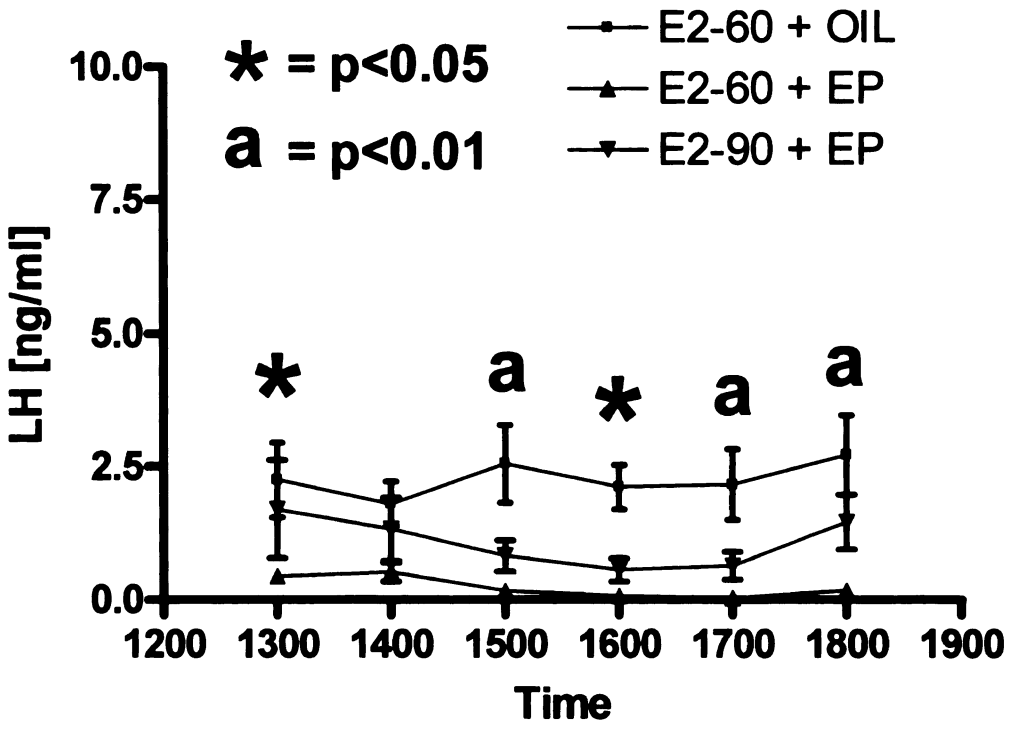

Figure 32: Effect of steroid priming on the LH levels in chronically E2 treated animals. We did not include E2-90 groups treated with oil. Repeated measures ANOVA performed followed by post test. Chronic E2 treatment completely abolished the LH surge and suppressed LH levels in E260 group significantly. The number of animals is 5 per group.

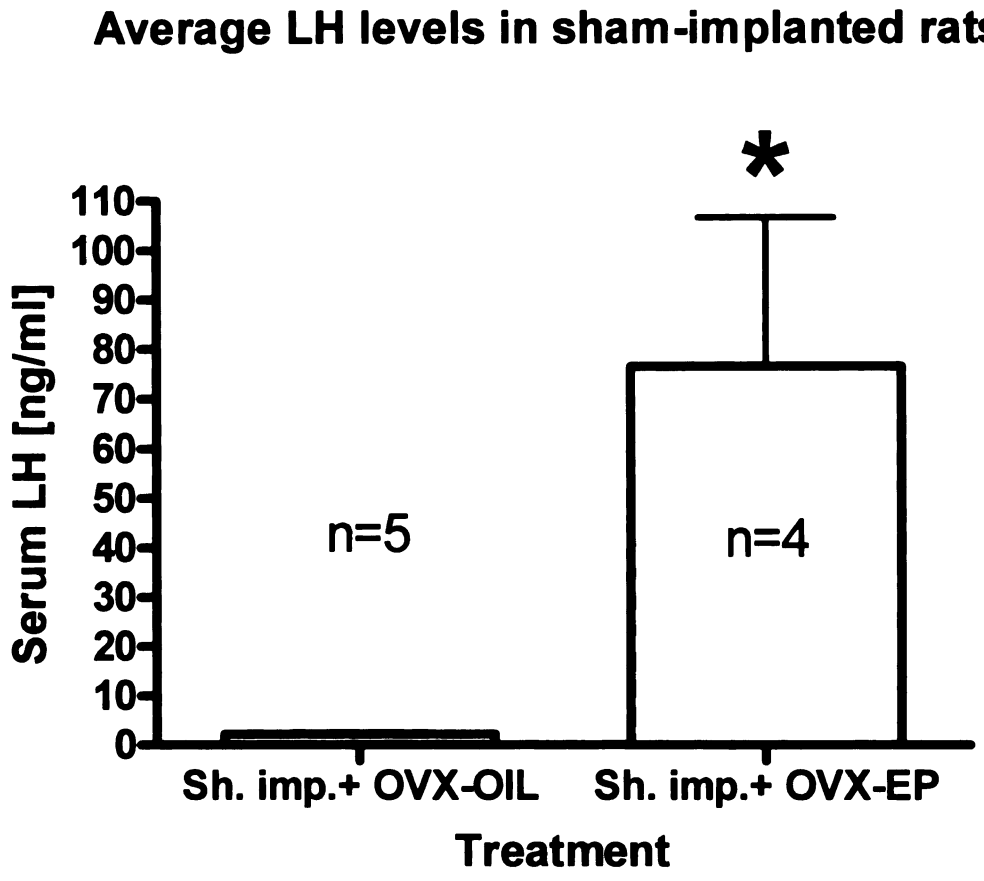

Figure 33: Average LH levels acros the entire treatment period in sham implanted rats. Significantly higher levels seen upon steroid priming in acutely overiectomized rats as compared to oil treatment. Number of animals per group is indicated in the graph. Analysis performed by unpaired t-test at $p<0.05$.

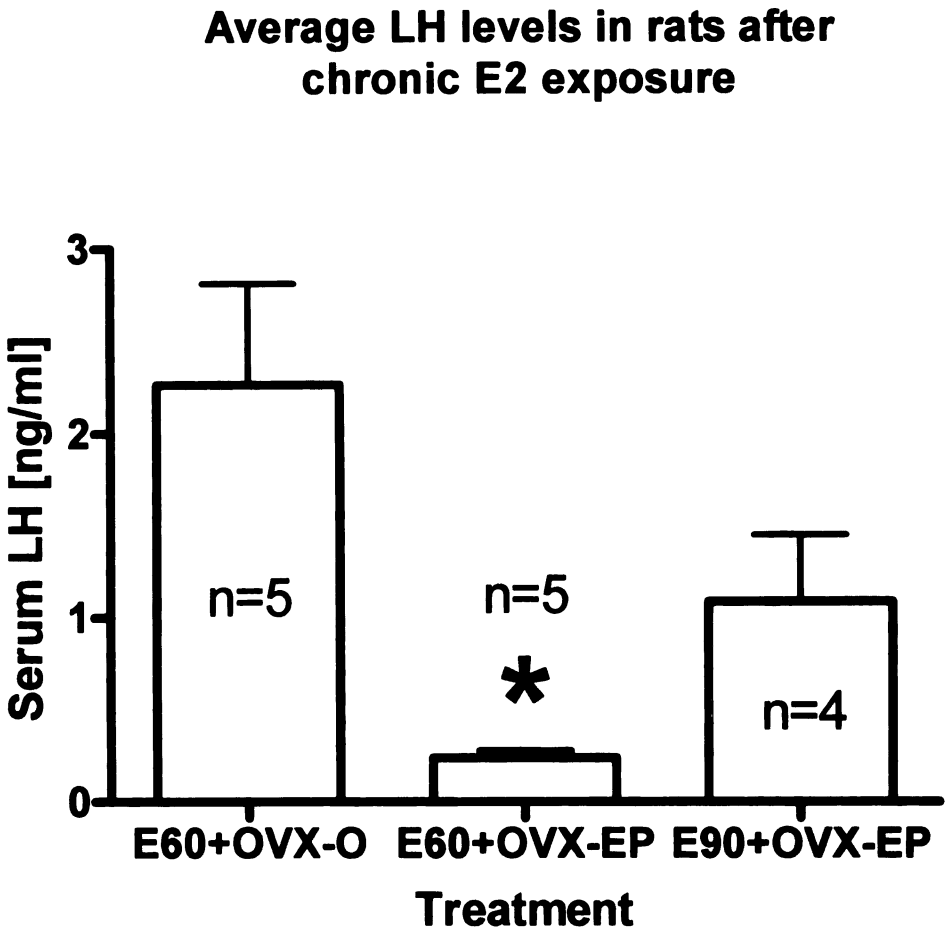

Figure 34: Average LH levels across the entire observation period. Chronic E2 treated rats were acutely overiectomized after the treatment period and tested for the effect of steroid priming on the serum LH surge. Significant suppression in the LH levels seen in E2-60 groups upon steroid priming which is compared to LH levels upon oil treatment in similar E2-60 group. Number of animals per group is indicated in the graph. '*' denotes significant difference from E60+Ovx-O group at $p<0.05$.

NE release profile in steroid primed, sham implated rats

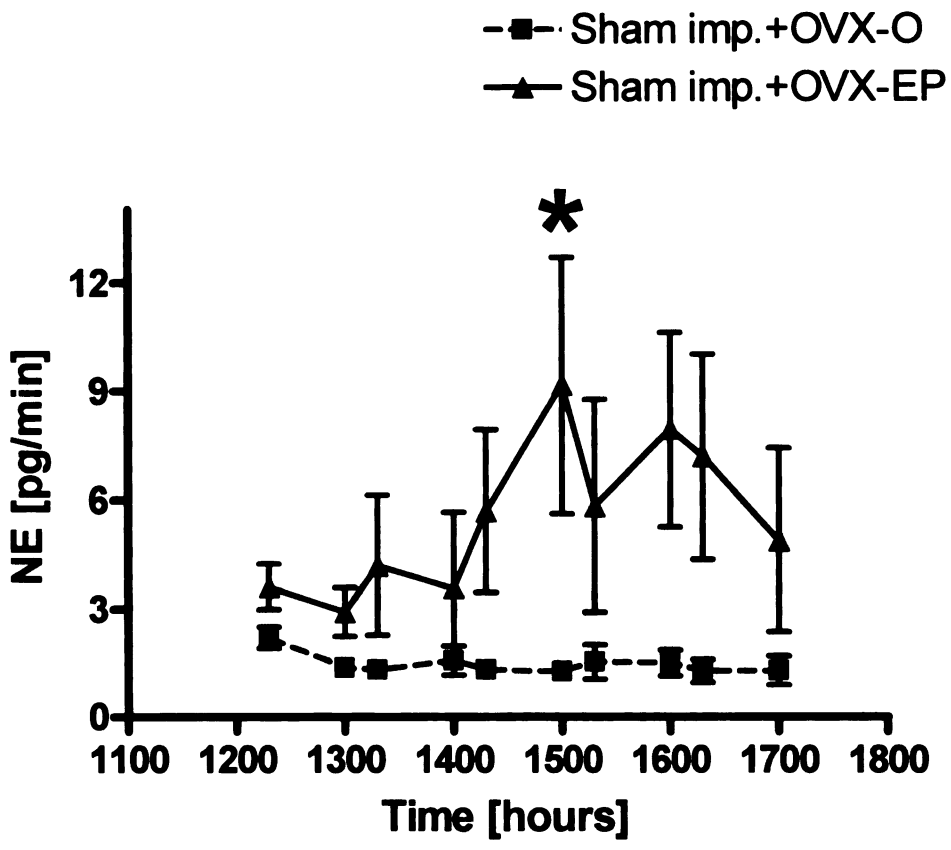

Figure 35: NE release profile in the MPA in sham implanted rats. At the end of treatment period, these rats were ovariectomized and treated with either oil or estrogen and progesterone. Significantly higher NE release is seen in steroid primed animals compared to oil treated controls. Repeated Measures ANOVA performed followed by post hoc test at $p<0.05$. '*' denotes significant difference from controls at the same time point. The number of animals are 5 per each group.

Effect of chronic E2 treatment on NE release profile in response to steroid priming

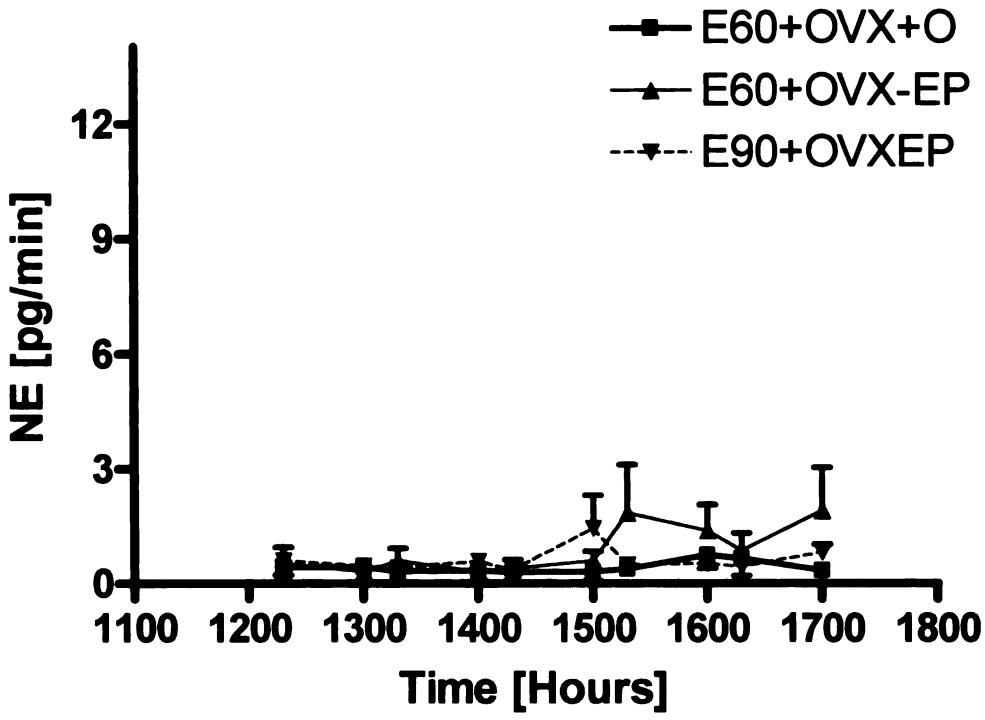

Figure 36: NE release profile in the MPA of chronic E2 treated rats. Comparison is made between the oil treated and estrogen + progesterone treated groups. Repeated Measures ANOVA was used followed by post hoc test and comparison was made at $p<0.05$ level. No significant elevation in the NE release is seen upon steroid treatment as compared to oil treatment. Chronic E2 treatment completely abolished NE release in the MPA as compared to what is seen in the sham implanted rats. We did not have E2-90 rats with oil treatment. Number of animals per group is 5.

Average NE release in sham-implanted rats upon steroid-priming

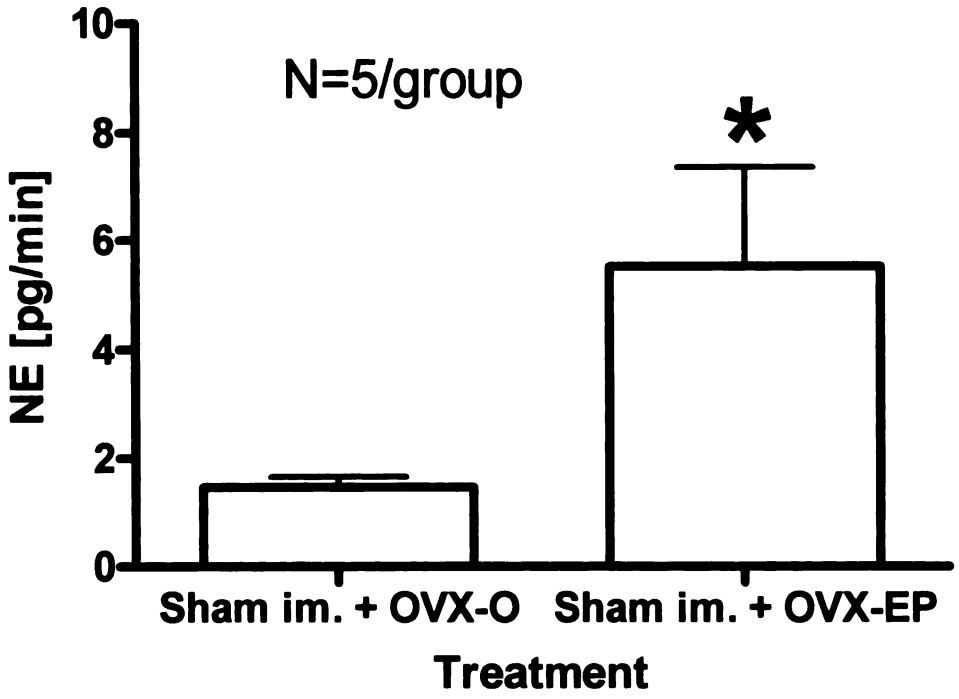

Figure 37: Average NE release during the entire period of observation seen in sham implanted rats. '*' indicates significant difference from the ovx+oil group at $p<0.05$. Comparison made by unpaired t-test. The number of animals per group is 5.

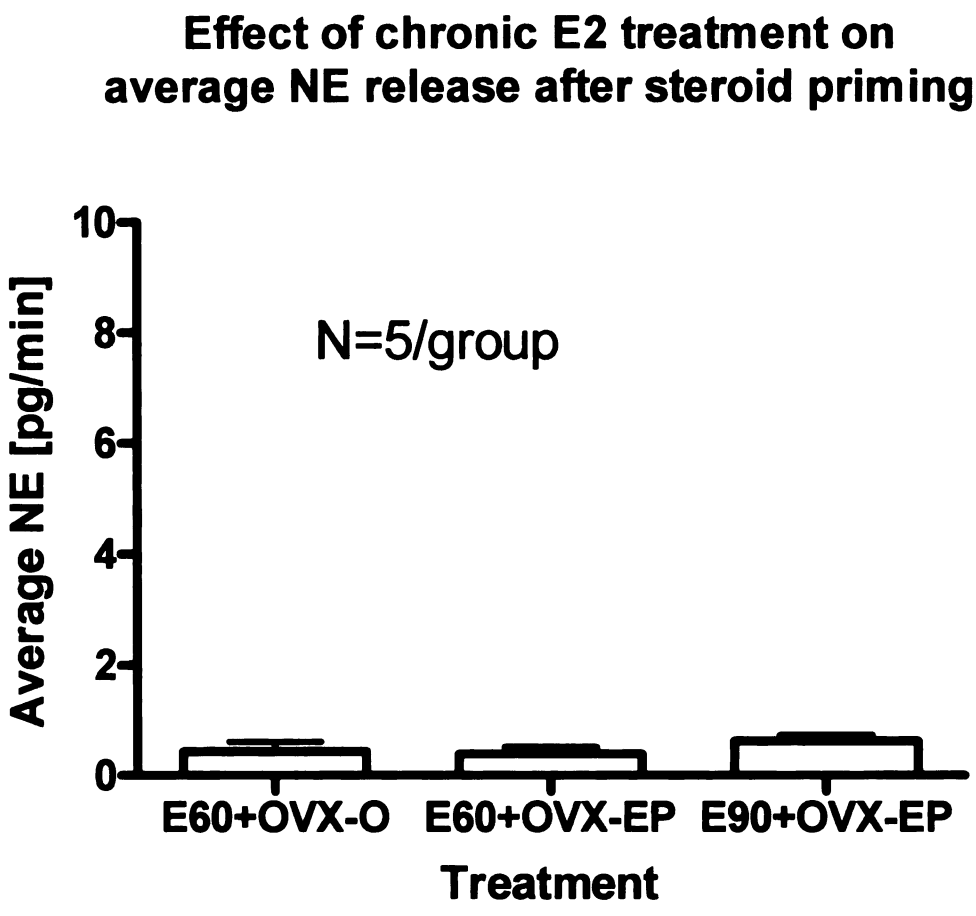

Figure 38: Average NE release during entire period of observation seen in E2 implated rats. No significance between means observed. Comparison by one-way ANOVA at $p<0.05$. Number of animals per group is 5. Chronic E2 treatment abolished the significant increase in the average NE release upon steroid priming as observed in sham implanted rats.

D.Discussion

Chronic estrogen exposure of young, intact rats for 60 and 90 days completely abolished the steroid-induced positive feedback on the LH surge after acute ovariectomy. It also abolished the steroid induced increase in NE release in the medial preoptic area as measured by push-pull perfusion.

This study is in agreement with that of Tsai and Legan [9], where they have shown that chronic estrogen treatment of ovariectomized rats for 6 weeks completely abolishes the steroid-induced LH surge in those animals. However, there are certain points of contrast between the 2 studies. In the present study, the animals used were intact for the duration of estrogen exposure (60 and 90 days) and were ovariectomized only at the end of estrogen treatment. This, in a way, simulates the natural condition. Hence, the compounded effect of such exposure on an intact subject could be effectively monitored. In line with this thought, we have seen an increase in the serum E2/P4 ratio upon 60 and 90 days of estrogen treatment in intact rats. The use of the ovariectomized model might alter the steroidal mileu in the blood [hence the E2/P4 ratio] and might confound the real picture. All these concerns were avoided in the present study by the use of intact animals.

It is possible that in both the studies, ours and that of Tsai and Legan (2001) [9], the E2/P4 ratio could have been the same given the fact that the serum estrogen in our study were in the range of higher proestrus levels during 60 and 90 days of treatment as compared to the initial 30 days of estrogen treatment

where the E2 levels were in the lower proestrus range. It is to be remembered that though we used a 20ng/day dosage of estradiol the level of estradiol in the blood was lower during 30 days of treatment, but its level in intact subjects rises upon 60 and 90 days of treatment to 75 pg/ml and 100 pg/ml respectively. The higher estradiol levels are believed to be derived from the ovaries as shown from our previous study. Hence, it is possible that in both the studies the estrogen and the progesterone levels could have been similar, although this cannot be confirmed. Therefore neither of these studies can really tell us anything about the effects of alterations in the E2/P4 ratio on the GNRH system. But both the studies have shown clearly that exposure of rats to chronic, physiological levels of estrogen do cause suppression of the GNRH system, both at the hypothalamic [norepinephrine] and the pituitary [LH] levels.

What is not known is whether this suppression of the LH surge in these animal models is due to a suppression of NE release in the DBB-MPA region. Here, in this study we find a strong correlation between suppression of NE release in the MPA and a suppression of LH surge. The idea of NE playing an important role in the suppression of estrous cycles is revealed by the studies of Quadri [78], and others where they have shown that administration of the precursors of catecholamines or monoamine oxidase inhibitors [79] to constant estrus rats cause resumption of estrus cycles. Other studies from our lab have also established the positive correlation between NE release in MPA and the proestrus LH surge [29].

The results of this and the previous study [chapter-2] clearly demonstrates the possibility of suppression of GNRH system upon cumulative exposure to proestrus estrogen levels, thereby supporting the hypothesis that cumulative estrogen exposure during every proestrus is responsible for the suppression of LH surges eventually leading to entry into constant estrus state. Our study clearly supports this idea using intact animals as the model and by the use of low doses of estrogen.

Further studies are needed to look at the mechanisms of action of estrogen, in eliminating the steroid-induced positive feed back action on LH surge, when given chronically to either intact or overiectomized subjects. It is possible that this could be mediated by suppression of norepinephrine release from the medial preoptic area, and in other areas of the preoptico-suprachiasmatic tuberoinfundibular system. This study adds support to this hypothesis though it was not tested. This could be tested by the use of L-Dopa, Deprenyl or by the administration of NE agonists in the estrogen-treated animals.

Other possible causes could be in the alterations in the levels of estrogen receptors in the brain stem noradrenergic neurons which are presumed to play an important role in the generation of LH surge [122, 126] thereby leading to decreased levels of tyrosine hydroxylase and hence norepinephrine production. It is possible that all the other receptor dependent functions are normal, but the receptor number per se could have been reduced. First priority to the measurement of estrogen receptor protein levels must be made than to the measurement of its gene transcripts, since the possibility of absence of any

alteration at the level of gene expression but a suppression of translation mechanism there by leading to decreased protein production do exist.

It is not known whether there is alteration in the levels of tyrosine hydroxylase or in its activity in this animal model. The activity of tyrosine hydroxylase could have been suppressed by covalent [127, 128] modifications which in turn could be reversible or irreversible. Such modifications could in turn be mediated through estrogen receptors or other non-receptor mediated mechanisms [128]. Other possible ways by which there could be decreased norepinephrine activity might include the mechanisms involving reactive oxygen or reactive nitrogen species. One such candidate is nitric oxide (NO). It is a reactive oxygen/nitrogen species which has been shown to be produced in large amounts by all the three forms of nitric oxide synthase enzyme upon exposure to estrogen [129]. More specifically, the inducible nitric oxide synthase, which is found in glial cells, has been shown to be up regulated by estrogen. There is evidence to point to the role of estrogen in the induction of gliosis [88], an inflammatory reaction of glial cells. Hence, it is possible for such inflammatory process to occur upon exposure to estrogen chronically, thereby leading to increased production of pro-inflammatory cytokines and/or nitric oxide at the level of either brain stem and/or hypothalamus, thereby leading to alterations in the noradrenergic activity in these regions.

In conclusion, chronic estrogen exposure suppresses the LH surge by blocking the steroid-induced positive feedback on the GnRH/noradrenergic neurons. This supports the idea of the role of cumulative estrogen exposure in the suppression

of LH surge in aging animals leading to constant estrus condition, which was tested in this study mechanistically. This is also accompanied by suppression of norepinephrine release in the medial preoptic area. Whether the suppression of LH surge is a function of decreased NE release, and whether this decline in NE release is a function of alteration in the positive feed back at the brain stem or other hypothalamic level is not known and needs further investigation. There is however the other possibility that alteration in the positive feedback itself could be a function of decreased NE release in the MPA.

CHAPTER-4

CHRONIC ESTROGEN EXPOSURE-INDUCED SUPPRESSION OF HYPOTHALAMIC CATECHOLAMINERGIC ACTIVITY IN FEMALE RATS: POSSIBLE INVOLVEMENT OF NITRIC OXIDE

A. Introduction

Chronic estrogen exposure (CEE) has been shown to disrupt estrus cycles in rats leading to a constant estrus-like condition [Chapter-1]. It has also been shown to alter the steroid-induced positive feedback on the LH surge leading to complete abolishment of the LH surge with a corresponding suppression of norepinephrine release in the MPA [Chapter-3]. A Similar suppression of catecholamine activity was also observed in different parts of the preoptico suprachiasmatic tuberoinfundibular system [Chapter-2]. The mechanism involved in the suppression of NE activity in the MPA is not known in this CEE model.

Although the number of studies demonstrating the pathological effects of chronic estrogen is limited, a few acute studies have shown that estrogen can indeed instigate an inflammation like reaction. Administration of estradiol valerate has been shown to cause gliosis [88, 119], an inflammatory reaction of astroglial cells, in the arcuate nucleus of the hypothalamus.

Gliosis is the inflammatory reaction of astroglial cells in the nervous system. One of the hallmarks of an inflammatory process is the increased production of nitric oxide [130]. Nitric oxide is a free radical which is known to be produced at higher levels in many pathological conditions [131, 132]. Increased activity of NOS is associated with many pathological conditions [133]. One of the many

reactions of nitric oxide is the nitration of proteins [134, 135]. Nitration of proteins might include the nitration of thiol residues or of the tyrosine residues [129]. One of the effects of NO action on proteins is the formation of 3-Nitrotyrosine, which is formed by reaction of peroxynitrite with tyrosine residues [127]. Peroxynitrite is the immediate product of the reaction between nitric oxide and superoxide radicals. Measurement of 3-Nitrotyrosine residues on proteins has been used as the marker of nitric oxide activity in those cells/tissues [136]. Hence in our study, measurement of 3-nitrotyrosine residues would serve as an index of the nitric oxide induced damage in specific regions of the hypothalamus in our CEE model.

The reason for the decrease in the activity of the MPA-NE in our model could be one or more of the following, namely, decreased production of the enzymes involved in NE biosynthesis, namely TH and Dopamine-beta Hydroxylase (DBH), decreased activity of the same, increased degradation of the catecholamines so produced either in the presynaptic or synaptic regions by Monoamine Oxidase (MOA) and/or Catechol-O-Methyl Transferase (COMT), or decreased availability of the catecholamine precursor, L-Tyrosine. Ara., et.al., [134] have shown that, nitration of tyrosine hydroxylase, would inactivate [127] the enzyme in vitro and in vivo. Nitric Oxide could in turn be produced from nitric oxide synthase present in the neurons or in the astrocytes. Both inducible and neuronal NOS isoforms are known to be present in the central nervous system. Inducible NOS is known to be present in both the astrocytes and in neuronal cells.

In this study, we tested the hypothesis that CEE-induced decrease in MPA-NE could be due to increase in NO production, thereby leading to nitration of TH

resulting in its inactivation. Thus, we measured the nitrate/nitrite levels in catecholaminergic nucleus of the brain stem region (A1, A2 & A6) as a marker of NO activity, and the direct measurement of nitrotyrosine residues on the TH molecule in the MPA, as a marker of, both, NO activity in that region and NO-induced inactivation of the enzyme [134] and hence, indirectly the catecholamine content.

B. Materials and Methods

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 groups of 3 per cage in temperature (23±2°C) and light-controlled (lights on from 0500 to 1900 h) animal rooms. They were given food and water *ad libitum*. The animals were used for the experiment 2 weeks after arrival. All the protocols followed in this study were approved by the University Committee for Animal Care and Use at Michigan State University.

Treatment

Vaginal smears were obtained in the morning hours between 0800-1000 h. Those animals showing regular 4-day cycles were chosen for the experiment. The animals were randomly divided into different treatment groups. Animals in the control group were sham implanted and those in the treatment groups were implanted subcutaneously with slow-release estrogen pellets under mild halothane anesthesia. The pellets were capable of releasing 17-β estradiol at the rate of 20 ng per day for a period of 30, 60 or 90 days (Innovative Research America, Sarasota, FL). The animals receiving estrogen pellets were divided into 3 groups based on the duration of estrogen exposure which was for 30, 60 or 90 days (E-30, E-60 and E-90 respectively). Vaginal cytology was recorded daily as described in the previous chapter for all the animals. At the end of the treatment period (30, 60 or 90 days), animals were sacrificed by rapid decapitation (under

protocols approved by the AUCAUC) at 1200 hrs, along with their respective controls. The brain along with brain stem was removed and quickly frozen on dry ice. These tissues were stored at -70°C freezer until the time of sectioning.

Brain Sectioning

At the time of sectioning, the brains (or brain stems) were mounted on a chuck and kept inside the cryostat (Slee, London, UK) for 1 hour to attain that temperature (-10°C), before sectioning. Serial brain (or brain stem) sections of 300μm thickness were obtained using a cryostat maintained at -10°C. The sections were transferred to cover slips which were placed on a cold stage set at -10°C. The medial preoptic area (MPA) in the case of hypothalamus and the A1, A2 & A6 nuclei in the case of brain stem, were located with the help of a rat brain stereotaxic atlas [94] and microdissected using the Palkovits' microdissection technique [28]. Tissue samples were obtained bilaterally and included all subdivisions of individual nuclei. They were stored at -70°C until they were used for assay procedures.

Measurement of Nitrates in brain homogenates:

A commercially available kit (Total nitric oxide assay kit, Assay Designs Inc., Ann Arbor, MI) using the Griess reaction procedure to measure nitrate level was used to measure nitrate concentrations in the brain stem nuclei. This is a complete kit for the quantitative determination of total nitric oxide (NO). It involves the enzymatic conversion of nitrate to nitrite, by the enzyme nitrate reductase, followed by colorimetric detection of nitrate as a colored azo dye product of the Griess reaction that absorbs visible light at 540 nm. This kit allows

for the total determination of both NO products, i.e., nitrates and nitrites in the sample by conversion of all sample nitrates into nitrite, followed by the determination of total concentration of nitrite in the sample. The sensitivity of the kit is 1.35 µmoles/liter. Only samples from the Control, E-60, E-90 and old constant estrus groups were used in the nitrate assay.

Immunoprecipitation of Tyrosine Hydroxylase

Microdissected tissue samples was incubated in a total volume of 50 µL containing 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 ortho vanadate, pH 7.4) and 0.5 µg of Anti-Tyrosine Hydroxylase antibody (Chemicon Intl., Temecula, CA.) for 12 hours at 4°C followed by the addition of 100 µL of Protein-A Agarose slurry. This was then mixed well and incubated at 4°C for 1.5 hours. The mixture containing TH and TH 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. This was then centrifuged at high speed for 10 minutes. The resultant supernatant was carefully transferred and stored in a sterile vial. This supernantant, which contains the TH, would be used for western blotting procedure.

Detection of Nitrotyrosine residues by Western Blotting:

Ten microliters of the supernatant containing TH alone was loaded onto two separate 12% SDS- polyacrylamide gel and electrophoresed at 70 volts 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 (Either anti-rat tyrosine hydroxylase, 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 horse radish peroxidase(SIGMA, Saint Louis, MO), for 4 hours. Bands were visualised using 4-Chloro-1-Naphthol (Biorad, Hercules, CA). Using densitometric screening pixel intensities of bands was determined using a Kodak Digital Science Image analysis system.

Statistical Analysis

Linear regression was used to calculate the nitrate levels in different treatment groups. Non-linear regression was used to calculate the IL-1 levels in different groups. One-way ANOVA was used to analyze the nitrate, IL-1 levels and the ratio of the intensities of nitrotyrosine residues and tyrosine hydroxylase (obtained through western blotting). Post hoc analyzes performed using Fischer's LSD test.

C. Results

Effects of chronic estrogen exposure on nitrate levels in discrete areas of the brain stem

Exposure of intact rats to estrogen for 60 and 90 days was not found to affect nitrate (Mean±S.E.; μM/μg protein) in the A1 region (7.1±0.8 and 8.7±0.7 for E-60 and E-90 respectively). The nitrate level of old constant estrus rats (104±0.6) was significantly higher as compared to estrus-controls (6.5±1.5) at $p<0.05$. ($P = 0.0316$, $F = 3.678$) [Figure 39].

In the A2 nucleus, there was a non-significant rise in nitrate levels upon 60 (12.4 ± 1.05) and 90 days (13.6±0.95) of E2 treatment and a significant increase in its level in OCE groups (16.9 ± 1.15) as compared to estrus controls(11.5 ± 0.45; $p<0.05$) ($P=0.0053$, $F=5.843$) [Figure 40].

In the A6 nucleus, a significant increase in nitrate levels was apparent only upon 90 days of estrogen exposure (16.7 ± 0.98) as compared to estrus-controls (12.3 ± 1.3). We also found significantly higher nitrate levels in the OCE group (17.1 ± 1.3) ($P = 0.0187$, $F=4.246$) [Figure 41].

Chronic estrogen exposure increases nitration of tyrosine hydroxylase in medial preoptic area [Figure 45 & 46]

In the MPA, exposure to estrogen for 90 days caused a significant increase in the nitration index (1.274 ± 0.38 units) compared to those of controls (0.323 ± 0.026 units) as measured by the ratio of the intensities of bands for nitrotyrosine residues and those of tyrosine hydroxylase in the same samples. Its levels were

also higher in old constant estrus rats (OCE) (1.024 ± 0.14 units; P=0.0127, F=4.874).

Chronic E2 exposure causes significant elevation in the levels of IL-1 levels in the brain stem noradrenergic regions.

The concentration (mean ± SEM pg/mg protein) of IL-1 in the A1 region of the brain stem increased significantly after 90 days of estrogen exposure (88.2 ± 3.4) as compared to controls (75.7 ± 4.6) in estrus. These levels were similar to what was observed in aging constant estrous rats (89.6 ± 2.5) at $p<0.05$ level (P= 0.0206, F=4.035). Sixty days of estrogen treatment did not have any effect [Figure 42].

A2 region showed significant elevation of IL-1 levels upon exposure to E2 for 60 (101.7±12.7) and 90 (106.2±4.9) days compared to controls in estrus (79.1±8.202), similar to what was observed in aging constant estrous rats (99.5±4.3) (P=0.0194, F=4.06) [Figure 43].

Chronic effects of estrogen on the induction of IL-1 were very evident in the A6 region of the brain stem (one-way ANOVA $P<0.0001$; F=23.79). Significant elevation in the IL-1 levels were found in this region upon 60 (73.6±5.5) and 90 (88.2±3.4) days of treatment and was found to be highly significant ($P<0.0001$) when compared to the controls in estrus (43.4±5.1). IL-1 levels in old constant estrous animals had significantly higher IL-1 levels (89.6±2.5; $P<0.01$) compared to controls in estrus [Figure 44].

Nitrate Levels in the A1 brain stem region

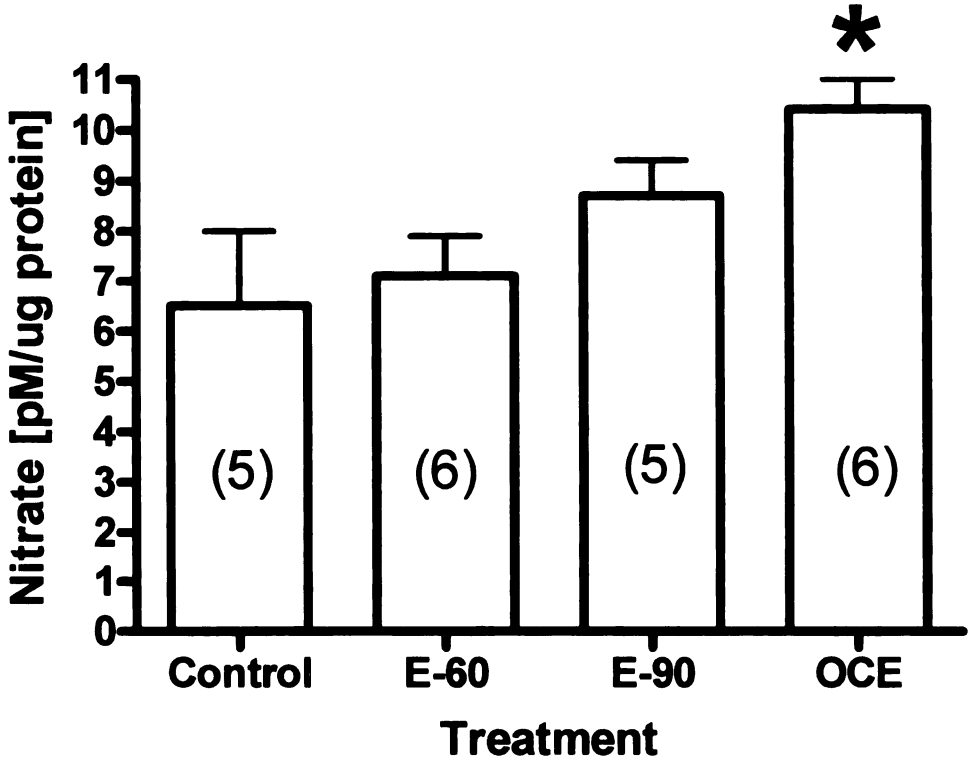

Figure 39: There is a non-significant rise in nitrate levels upon E2 treatment, direcly proportional to the duration of treatment. Aged animals in the state of constant estrous (OCE) had significantly (*) higher nitrate levels ($p<0.05$) compared to controls. Number of animals per group is indicated in parenthesis. The data shown as mean ± SEM.

Nitrate Levels in the A2 brain stem region

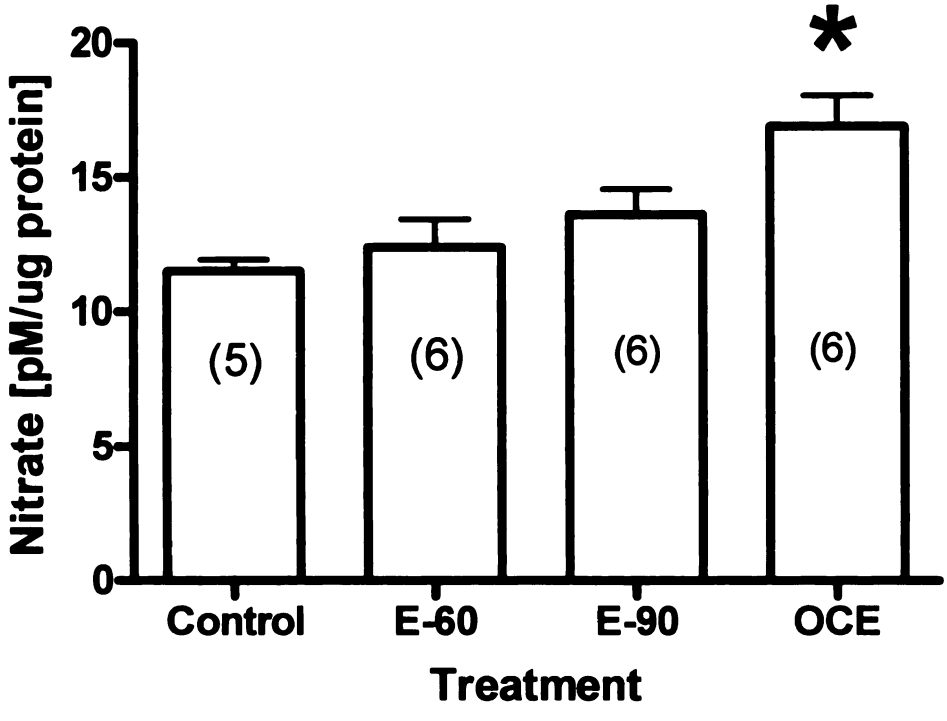

Figure 40: Increase in Nitrate levels in the A2 noradrenergic cell body region of the brain stem. Though there is an increasing trend in nitrate levels upon 60 and 90 days of E2 treatment compared to control animals, only old constant estrous animals had significantly (*) higher nitrate levels compared to both controls ($p<0.01$) and E-60 ($p<0.05$). Number of animals per group is indicated in parenthesis. The data shown as mean ± SEM.

Nitrate Levels in the A6 brain stem region

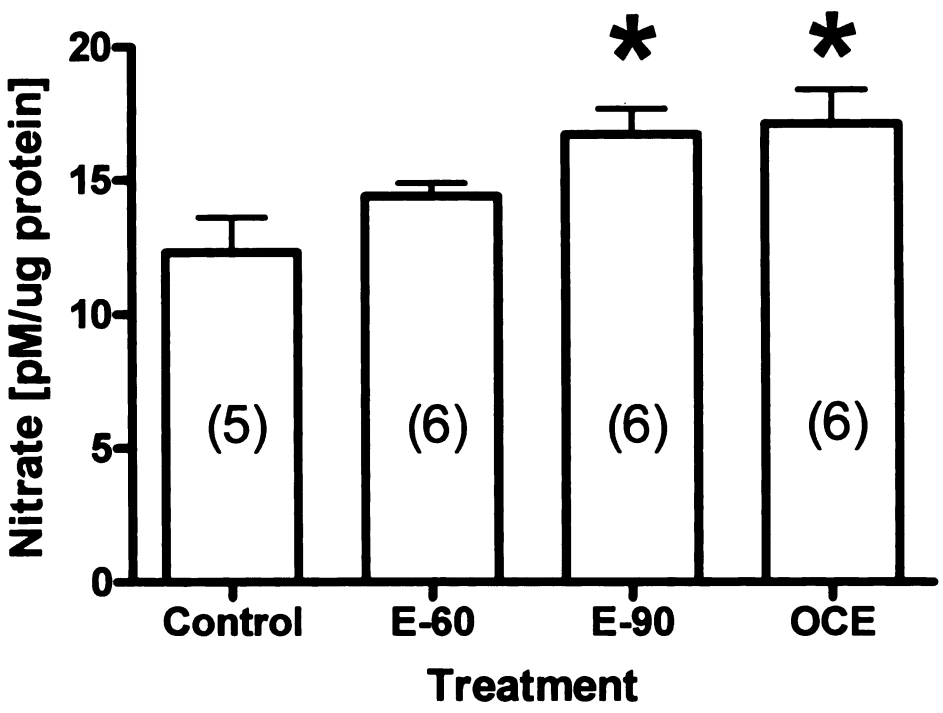

Figure 41: Chronic E2 treatment (90 days) significantly (*) increases the nitrate levels in the A6 region of the brain stem compared to controls at $p<0.05$. This is similar to what is seen in aged animals in the state of constant estrous (OCE) which had significanly (*) higher levels compared to controls at $p<0.05$. Number of animals per group is indicated in parenthesis. The data shown as mean ± SEM.

IL-1β Levels in the A1 brain stem region

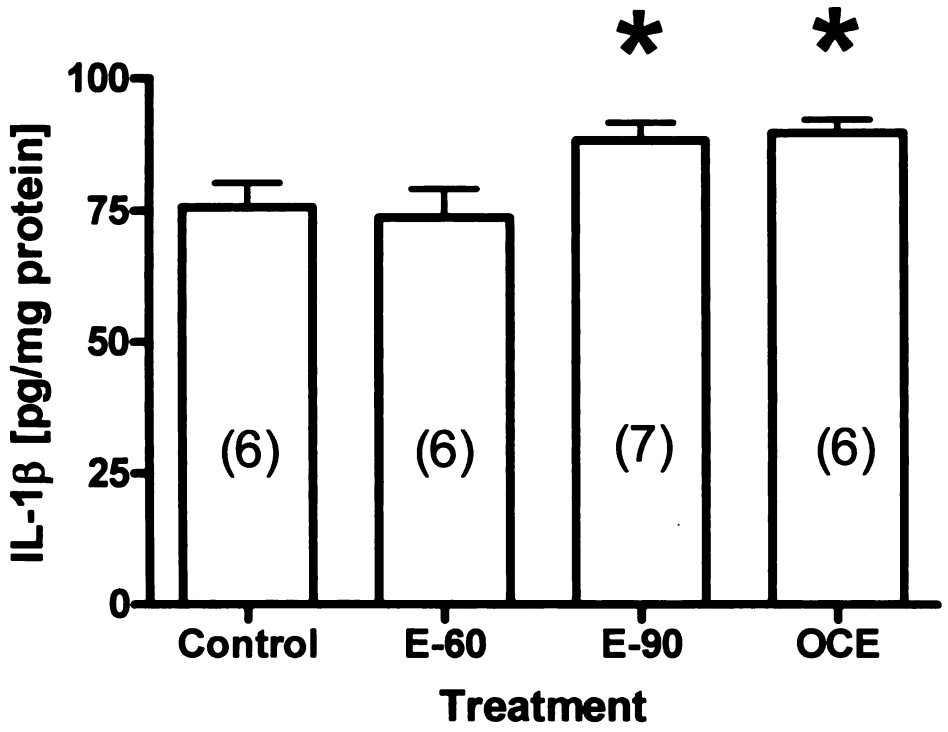

Figure 42: There is a significant rise in IL-1 levels upon E2 treatment for 90 days. Aged animals in the state of constant estrous (OCE) had significantly (*) higher IL-1β levels ($p<0.05$) compared to controls. Number of animals per group is indicated in parenthesis. The data shown as mean ± SEM.

IL-1β Levels in the A2 brain stem region

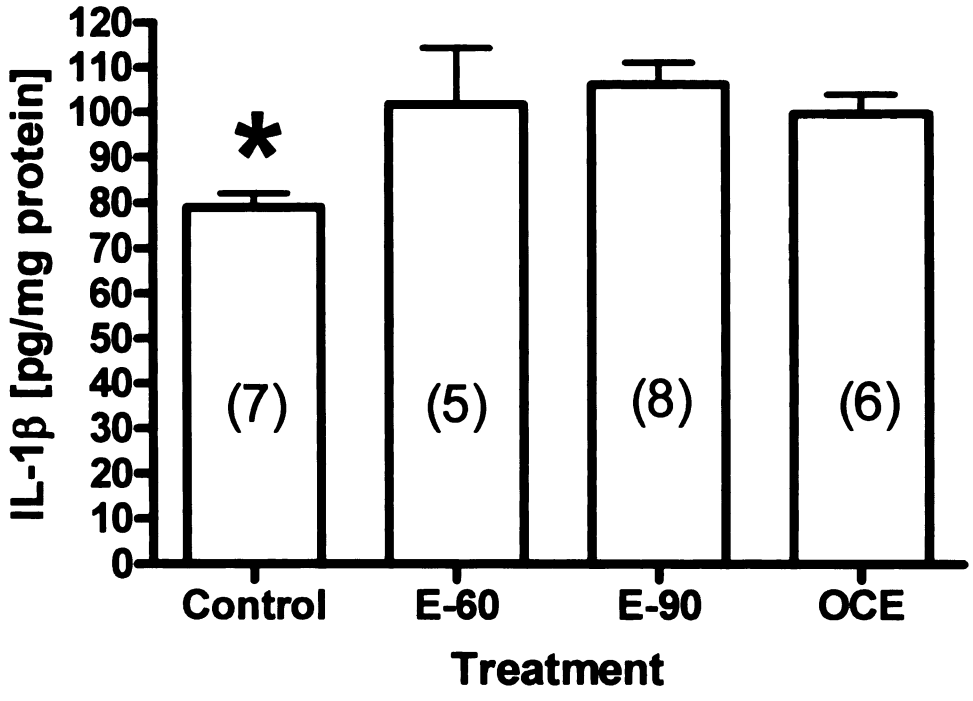

Figure 43: Significant elevation in IL-1 levels in E2-treated persistent estrous rats was seen. This was similar to those of aging persistent estrous animals (OCE) which also had significantly higher levels of IL-1 compared to controls. The controls were young animals age-matched to E2-90 group. Asterics '*' indicates significant difference from rest of the groups at $p<0.05$ level. Number of animals per group is indicated in parenthesis. The data shown as mean ± SEM.

IL-1β levels in the A6 brain stem region

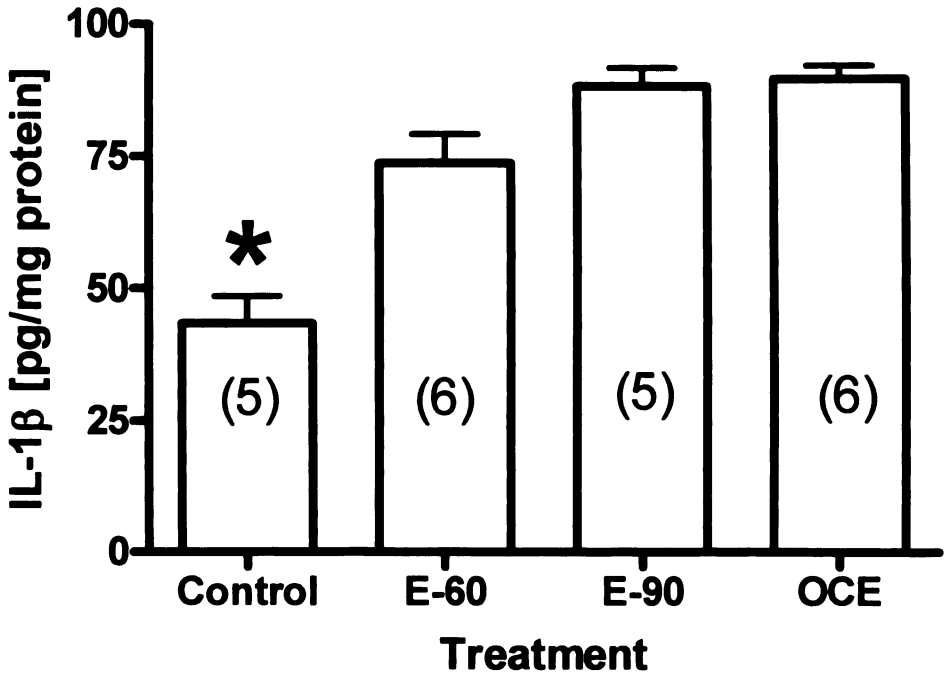

Figure 44: Chronic E2 treated persistent estrous animals and old persistent estrous animals had significantly higher IL-1β compared with controls in estrus, age-matched to E2-90 group. Comparison made at $p<0.05$ level. Number of animals per group is indicated in parenthesis. The data shown as mean ± SEM.

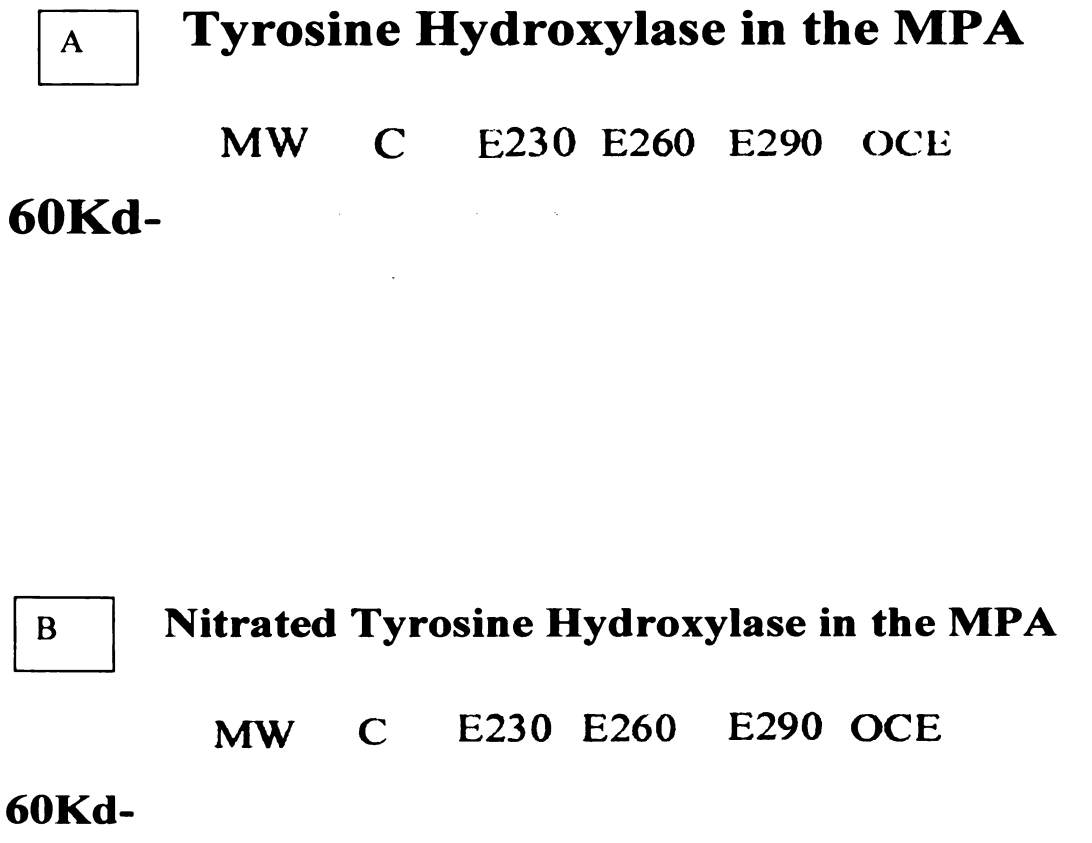

Fig. 45. Figure showing sample western blots probed for Tyrosine hydroxylase (A) and nitrotyrosine (B). The ratio of the intensities of B to A was used to find out the degree of nitration of TH.

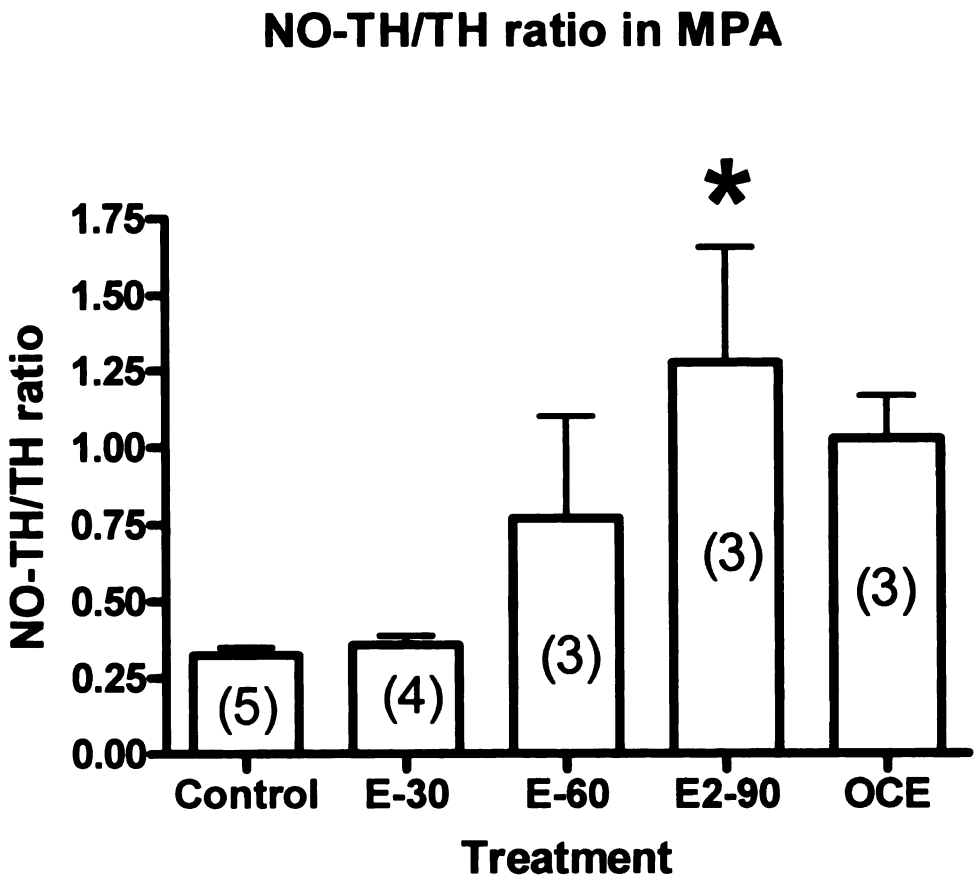

Figure 46: The graph indicates the increase in the nitration of TH residues upon chronic E2 exposure and in aging persistent estrous animals as compared with controls in estrus, age-matched to E2-90 group. Comparison was made at $p<0.05$ level. Number of animals per group is indicated in parenthesis. The data shown as mean ± SEM.

D. Discussion

In summary, chronic estrogen exposure for 60 or 90 days produced non-significant elevations in the levels of nitrate in the brain stem catecholaminergic A1 and A2 nuclei and a significant increase in its levels in A6 region. Nitrate levels were significantly higher in all the nuclei tested in old constant estrus rats. We also found a significant increase in the nitration of TH in the MPA of E2-90 and OCE animals.

Though the result of this study strongly supports the hypothesis that estrogen exposure can cause a reduction in catecholaminergic activity through the increase in nitrate production and nitration of TH, it is yet to be confirmed whether the nitration of TH in these animals is accompanied by loss of TH activity. It is well known that nitration of proteins by NO is mediated through formation of peroxynitrite [134, 135], which causes the nitration of aminoacid residues. Though the reaction of peroxynitrite with proteins always results in nitration of tyrosine residues, it need not be accompanied by loss of TH activity. In fact, it has been shown that the loss of activity of TH is not because of the nitrotyrosine residues, but is because of the oxidation of sulfhydryl groups in the protein by peroxynitrite [137]. Whether the enzyme gets inactivated or not depends on the pH of the environment in which peroxynitrite reacts with the protein [137]. Hence, though presence of nitrotyrosine groups may serve as an index of NO production in those nuclei, it does not indicate for sure whether or not the enzyme is active. Therefore, future studies should look at the activity of TH in these animal models. This can be accomplished by in vitro or in vivo

methods. Results from Chapters 2 and 3 indicate that this is a good possibility since we observed a decrease in NE concentrations and release after estrogen exposure.

Since nitration of proteins is mediated through peroxynitrite intermediate and since peroxynitrite is produced by the reaction between nitric oxide and superoxide radicals, it is possible that increased nitration might also reflect the increased production or availability of superoxide radicals in the medial preoptic area of these animals. Hence some knowledge of the activity of the superoxide dismutase (SOD), the enzyme involved in scavenging the superoxide radical, and the content of the SOD, might throw some light on the mechanisms involved in the CEE-induced decline in NE activity.

A similar increase in the nitration of TH has been shown in the median eminence of these rats before [unpublished report]. Hence, it appears that there is a general tendency in these animals to exhibit an increased NO activity in the hypothalamic and brain stem nuclei relevant for reproduction. Whether this NO is produced by the neuronal nitric oxide synthase (nNOS) or by the inducible nitric oxide synthase (iNOS) is not known. Again, whether this NO is derived from the neuron or from astroglial cells is yet to be clarified.

An increase in the activity of NO in chronically E2 treated animals (E-90) is akin to the old constant estrus rats which naturally exhibit higher E2 levels. It is possible that these animals might have increased activity of NADPH oxidase leading to more production of superoxide radical, thereby leading to more peroxynitrite production, which has been implicated in LPS-induced killing of

oligodendrocyte cells [138]. There is evidence to show that estrogen may induce NO production by iNOS through a receptor mediated process [139]. Hence the increased production of NO in these animals could be attributed to the same reason. Since there is evidence to support the action of estrogen in causing gliosis in the hypothalamic regions [88], it may be presumed that chronic estrogen exposure might cause an overall inflammation-like reaction at the level of hypothalamus leading to more production of nitric oxide through iNOS enzyme. This needs further investigation.

The fact that the nitrate levels in the A1 and A2 regions were not significantly higher might suggest the fact that either these nuclei are resistant to induction, by chronic E2 exposure, of NO production or that they may have some mechanism of taking care of the excess NO produced. Also, the fact that there is a significant rise in nitrate levels in the A6 nucleus of only E-90 rats might suggest that probably the suppression of catecholamines in the hypothalamic areas or that of estrus cycles might not be a direct result of increase in NO production, since changes in NE, LH and estrus cycles happen way before by 60 days of E2 treatment. Also, it suggests that decline in the NE activity in MPA is probably not mediated by increased activity of NO in the brain stem nuclei. But it is possible that this result might explain the permanent alterations in estrus cycles upon withdrawal after 90 days of E2 treatment as compared with 60 days of treatment. This is yet to be investigated.

Though the results of this study may suggest the lack of cause and effect relationship between NO activity and catecholamine activity or estrus cycle, it

must be tested mechanistically by the use of specific inhibitors of NOS activity. Also, we do not know whether the nitration of TH is a means of some kind of regulatory mechanism or just a random reaction of NO with TH.

In summary, chronic E2 exposure causes significant increases in NO activity in the brain stem and hypothalamic nuclei implicated in the governance of LH surge. It must be clarified whether this increase in NO is responsible for any alterations in the PST system with respect to catecholamines or LH secretion. Also, the possibility of suppression of catecholamine activity by estrogens acting through estrogen receptors of the brain stem must be investigated.

SUMMARY AND CONCLUSIONS

This study attempted to explore the possibility of one of the ovarian humors, estrogen, playing an important role in the reproductive aging process in rats, specifically through the involvement of the hypothalamo-pituitary system, via changes in the catecholaminergic activity at least in specific, if not all, areas of the hypothalamus that are concerned with reproduction.

In this exploration, interesting observations were made with respect to the estrous cycle upon chronic estrogen exposure, and the effects of its withdrawal. The phenomenon of 'plasticity' in the estrous cycle physiology was indeed observed. This study is the first of its kind to report the same, during the withdrawal phase of estrogen exposure, after 60 days of estrogen treatment-induced persistent estrous state. The nature of estrous cycle, with respect to its duration, upon withdrawal after 90 days of E2 exposure resembled what would be observed in a middle aged rat that is undergoing a transition phase from regular cycler to the persistent estrous state. Hence, in a way, it was possible to create both a persistent estrous rat model (60 and 90 days of E2 treatment) and a model that would mimic the middle aged irregular cyclers (E2-90 withdrawal groups). These rat models could be used to study the nature of changes that would be observed in aging animals with respect to the neuroendocrine system governing reproduction.

Similar aging-like changes were observed with respect to the serum levels of estrogen and progesterone, specifically, that of their ratio (E2/P4) which was

found to be elevated upon estrogen exposure for 60 and 90 days, and this state of elevated E2/P4 ratio resembled that found in aging persistent estrous rats. The finding that ovaries of chronic E2 treated rats had multiple follicles indicate ovaries being "a possible source" of serum E2, though it does not prove the same. However, similarities could be drawn between the chronic E2 exposed (60 and 90 days exposure) rat model and that of aging persistent estrous rats (OCE) with respect to the above mentioned factors: Both had increased serum E2 levels, decreased serum P4 levels and hence, increased E2/P4 ratio, along with the presence of multiple follicles and absence of fresh corpora lutea. Thus with respect to estrous cycles, serum hormonal profiles and ovarian histology, we were able to develop a rat model which mimics aging persistent estrous rats, and which could be used to study the phenomenon of reproductive aging. This forms the subject of chapter-1.

The other interesting observation made with respect to the effect of estrogen withdrawal is the significant decrease in the weight of anterior pituitary of these animals. This was accompanied by a significant decrease in the body weight of these animals compared to the E-90 group, but was similar to the E-30 controls. However, since both the withdrawal groups are not accompanied by their respective controls, whether this decrease in the body weight of these animals during withdrawal phase is a result of estrogen withdrawal or other factor(s) in the colony could not be ascertained. Since estrogen is known to suppress the body weight (at levels higher than what we used), a decrease in the body weight after estrogen withdrawal needs further investigations with proper controls. Also,

the levels of estrogen in their blood need to be measured (on the day of estrus, for comparison with rest of the groups).

Profound alteration in the level of LH was seen upon chronic exposure to estrogen where significantly low levels were seen compared to controls in estrus. Similar changes in the Norepinephrine content were noted in the medial preoptic area. The idea that NE might play a major role in the induction of estrogen-induced constant estrus is well supported through the results of this study. We have shown that chronic E2 treatment not only decreases the concentration of NE in the MPA, but also decreases the NE release in the same region upon steroid priming in ovariectomized

Does Beneficial Effects Of Estrogen Mediated Through Progesterone? [A Digression]

Special attention to the elevation of serum P4 levels upon 30 days of E2 exposure is warranted. It is well known that estrogen treatment can have beneficial effects with respect to the prevention of inflammatory changes following traumatic brain injury and in stroke, in rat models. This is especially true with reference to cortex and hippocampus regions of the brain. Similar beneficial effect has also been shown in such models using progesterone and its metabolite [140-143]. The results from this current study indicate the induction of progesterone secretion by chronic estrogen treatment (30 days). This leads one to ponder whether the beneficial effects of estrogen treatment in the brain injured

model are actually mediated through increase in the serum progesterone level. If so, what would be source of this progesterone, ovaries or adrenals (or both)?

Since most of the studies showing the beneficial effects of estrogen are with reference to the immediate aftermath of the injury to the brain, it is possible that this is mediated through increased P4 secretion immediately after E2 treatment. It is possible that this beneficial effect may not be seen in these animals if they were pretreated with estrogen for chronic periods (60 or 90 days as in this study) of time before subjecting them to such traumatic injury, since chronic E2 treatment for more than 30 days (like 60 and 90 days of treatment) brings back the P4 levels to that of controls (chapter-1). Hence I propose that "estrogen-induced increase in the serum progesterone level is probably responsible for the beneficial effects seen with respect to estrogen treatment in traumatic injury and stroke models, in rats. Also, this estrogen-induced progesterone secretion, and hence the beneficial effects of estrogen treatment (through P4), could be abolished upon chronic E2 treatment". If true, this would raise concern regarding the use of estrogen as a possible therapeutic or prophylactic agent, specifically against the chronic estrogen usage. Future studies towards this must be undertaken (Does people on chronic estrogen treatment are at a disadvantage in any ways?). Also, whether the outcome would be the same in both sexes needs to be taken into consideration, since the source of progesterone has not been clarified by this study.

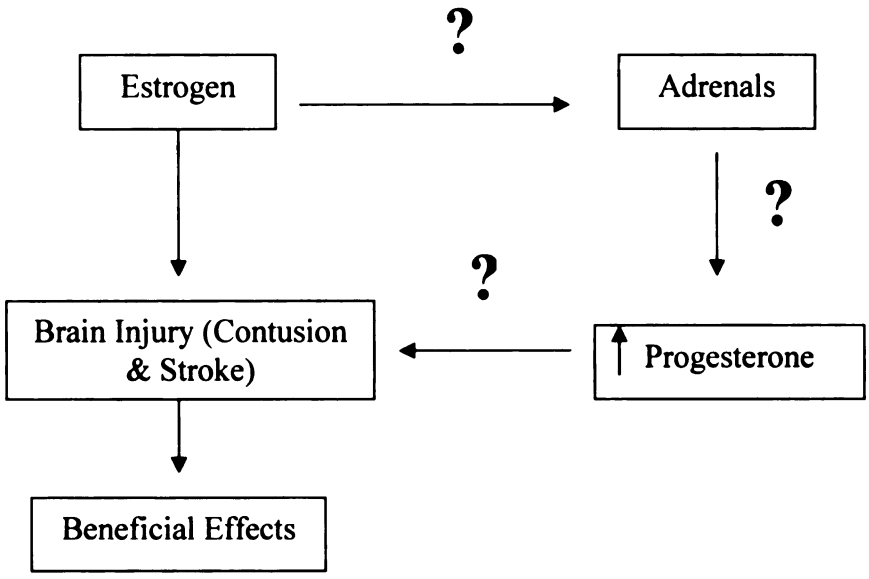

Figure 47: Schematic of a hypothesis explaining the possible mechanism by which estrogen treatment imparts beneficial effects in traumatic brain injury and stroke models. The involvement of Adrenals and Progesterone is indicated.

Estrogen-Induced Progesterone Secretion: What Could It Mean From Biological Standpoint?

One of the interesting observations made in our study is that, chronic E2 treatment for 60 days raised serum corticosterone levels, which was normalized upon 90 days of treatment [not published]. Similarly, 30 days of E2 treatment increased the serum P4 levels significantly higher than controls. Treatment for 60 and 90 days with estrogen brought back the P4 levels back to normal [chapter-1]. Both these findings taken together, along with the fact that both the hormones are produced from the adrenals, and that progesterone serves as the precursor for corticosterone, might mean an operation of some kind of homeostatic mechanism, involving adrenals, to a sudden disturbance in the hormonal (estrogen) levels, where progesterone production is stepped up to counteract the effects of estrogen (The fact that E2 and P4 have antagonistic functions is well

known). Also, the fact that both progesterone and corticosterone levels were brought back to normal despite continued E2 treatment indicates the habituation response in operation. Thus, I propose the operation of a novel homeostatic mechanism involving adrenals which is responsive to changes in the levels of serum estrogen, and that this pathway uses progesterone as a main mediator". The validity of this hypothesis needs to be verified. Also, whether such mechanism, if present, operates through neural (neuroendocrine) system or autonomic is open to question.

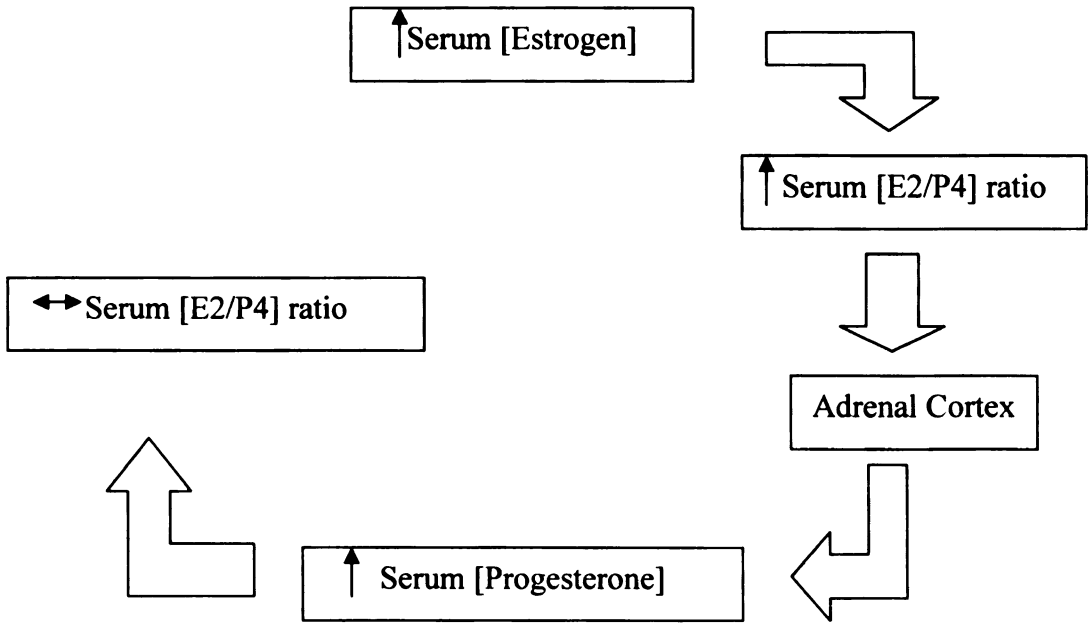

Figure 48: Schematic of the hypothesis explaining the homeostatic function of adrenal cortex during increase in the serum E2 level during 30 days of E2 treatment. The involvement of adrenal cortex and progesterone is proposed. Note: The habituation of the adrenal cortex to chronically increased E2 levels is not shown.

Thirty Days Of Estrogen Treatment Did Not Affect Estrous Cycles. Why?

The first 30 days of estrogen treatment did not really have any great impact on the estrous cycles. Most of the animals were regularly cycling during this period. This period is also characterized by high progesterone levels [chapter-1]. It is well known that estrous cycles could be reinitiated and maintained for some period of time (~6-19 cycles) in old persistent estrous rats by the administration of progesterone. All of these above facts raise the question of whether the high level of progesterone in the E2-30 group is responsible for the maintenance of regular cycles in these animals, and whether the decrease in the serum P4 levels with concomitant increase in serum E2 levels [and hence a shift in the E2/P4 ratio] upon continuous treatment precipitated the induction of constant estrus state in majority of E2-60 and E2-90 groups. In other words, will the rats exhibit regular estrous cycles during the first 30 days of E2 treatment if the rise in the serum P4 is prevented? Further investigations are needed to answer this question.

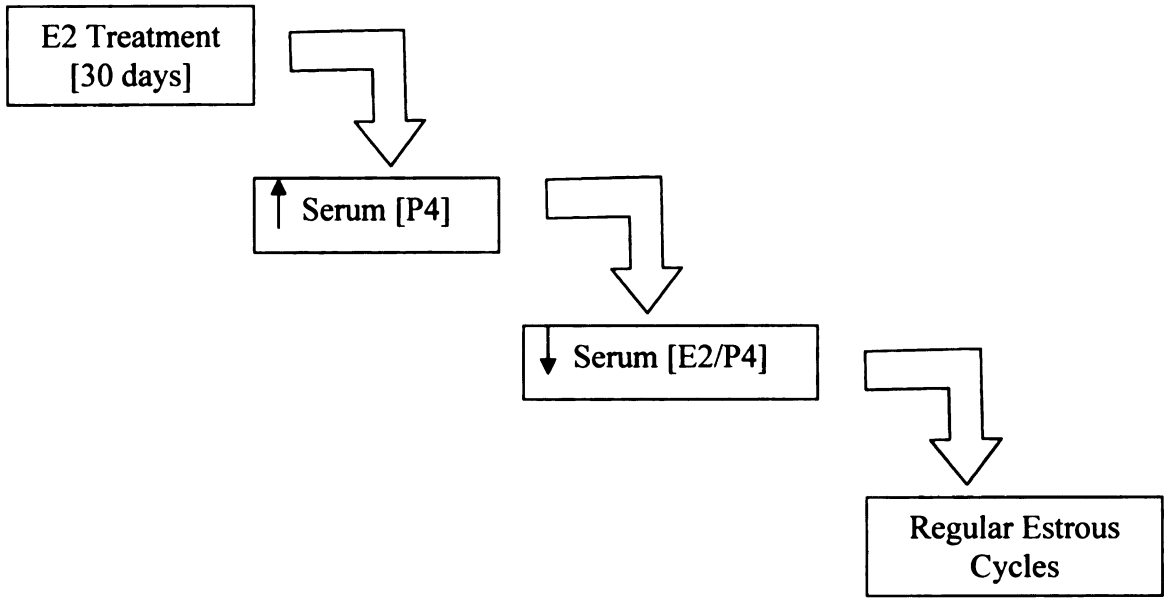

Figure 49: Schematic showing the proposed hypothesis to explain the occurrence of regular estrous cycles in 30 day E2 treated rats despite the p. The ratio of E2 and P4 is thought to be the factor which would determine whether regular estrous cycles would ensue or not.

E2-Withdrawal Group: Possible Model for Middle-Aged Irregular Cyclers?

The fact that rats treated for 60 days with estrogen became persistent estrus and that withdrawal of E2 after this treatment period allowed regular cycles in majority of these animals indicates the plastic nature of estrous cycles. This plasticity is lost upon 90 days of E2 treatment after which, during the withdrawal phase, the animals exhibited irregular cyclicity. These animals could be used as a model to mimic the middle-aged irregular cyclers where in profound alterations in the LH surge amplitude, timing and responsiveness of the HP-axis to steroid priming are reported [31, 80]. Whether they really mimic these middle-aged cyclers with respect to LH secretion (surge) and changes in the catecholamine activities in discrete areas of the brain involved in estrous cycles needs to be

verified. Also, the time of occurrence of persistent estrous state in these withdrawal groups is worth monitoring, compared to sham implanted controls.

Loss of Responsiveness to Steroid Priming Upon Chronic Estrogen Treatment: Role of Norepinephrine

From the results of chapter-3 it could be seen that chronic E2 treatment completely abolished the responsiveness to steroid priming which is manifested in the observation of dampened LH surge (serum) and NE release (MPA). Though a correlative relationship could be established between the NE and LH in these animals, whether decline in the NE is the sole cause of absence of LH surge could not be established. Also, the argument that these animals lack responsiveness to steroid priming needs more clarification with respect to NE. Does lack of responsiveness to steroid priming (possibly at the brain stem level) leads to decrease in NE release, OR decline in NE release by itself constitute the reason for the lack of responsiveness to steroids needs clarification.

The Brain Stem Connection

We observed changes in the levels of nitrates and IL-1 beta in the brain stem noradrenergic nuclei with respect to estrogen treatment and aging. Though we did not find a profound change in the nitrate levels with respect to treatment, we however did find significant difference in aging animals with respect to controls. The chronic E2 treatment did however tended to raise the nitrate levels with respect to duration of treatment. Similarly, IL-1 beta levels were found to significantly rise with respect to duration of E2 treatment. We however did not

find any dose response. The fact that there is an increase in the levels of nitrates (not significant) and IL-1 in these animals indicates the possibility of the role of inflammatory mediators in the process of estrogen mediated alterations in the neuroendocrine system. Whether this means an ongoing inflammatory process or not cannot be answered. Since this finding is a correlation, whether they play any role in the reproductive aging process needs to be investigated. Given the fact that these brain stem nuclei project to different areas of the brain [144] (cortex, hippocampus and the limbic system) and spinal cord [145], and hence are possibly involved in the control of other bodily functions including cardiovascular regulation [145], the effects of chronic E2 exposure on these functions needs to be investigated.

BIBLIOGRAPHY

1. Huang H, Meites J. Reproductive capacity of aging female rats. . Neuroendocrinology 1975; 17: 289-295.

2. Long J, Evans H. The oestrous cycle in the rat and its associated phenomena. Memoirs Univ Calif 1922; 6: 1-111.

3. Lu K, Hopper B, Vargo T, Yen S. Chronological changes in sex steroid, gonadotropin and prolactin secretion in aging female rats displaying different reproductive states. Biology of Reproduction 1979; 21: 193-203.

4. Aschheim P. [Results Provided by Heterochronic Grafts of the Ovaries in the Study of the Hypothalamo-Hypophyso-Ovarian Regulation of Senile Rats.]. Gerontologia 1964; 10: 65-75.

5. Felicio LS, Nelson JF, Gosden RG, Finch CE. Restoration of ovulatory cycles by young ovarian grafts in aging mice: potentiation by long-term ovariectomy decreases with age. Proc Natl Acad Sci U S A 1983; 80: 6076-6080.

6. Finch CE, Felicio LS, Mobbs CV, Nelson JF. Ovarian and steroidal influences on neuroendocrine aging processes in female rodents. Endocr Rev 1984; 5: 467-497.

7. Lu JK, Gilman DP, Meldrum DR, Judd HL, Sawyer CH. Relationship between circulating estrogens and the central mechanisms by which ovarian steroids stimulate luteinizing hormone secretion in aged and young female rats. Endocrinology 1981; 108: 836-841.

8. Kohama SG, Anderson CP, Osterburg HH, May PC, Finch CE. Oral administration of estradiol to young C57BL/6J mice induces age-like neuroendocrine dysfunctions in the regulation of estrous cycles. Biol Reprod 1989; 41: 227-232.

9. Tsai HW, Legan SJ. Chronic elevation of estradiol in young ovariectomized rats causes aging-like loss of steroid-induced luteinizing hormone surges. Biol Reprod 2001; 64: 684-688.

10. Tsai HW, Legan SJ. Loss of luteinizing hormone surges induced by chronic estradiol is associated with decreased activation of gonadotropin-releasing hormone neurons. Biol Reprod 2002; 66: 1104-1110.

11. Brown-Grant K, Exley D, Naftolin F. Peripheral plasma estradiol and luteinizing hormone concentrations during the estrous cycle of the rat. Journal of Endocrinology 1970; 48: 295-296.

12. Butcher R, Collins W, Fugo N. Plasma concentration of LH, FSH, prolactin progesterone and estradiol-17beta throughout the 4-day estrous cycle of the rat. Endocrinology 1974; 94.

13. Eto T, Masuda H, Suzuki Y, Hosi T. Progesterone and pregn-4-en-20a-ol-3-one in rat ovarian venous blood at different stages in reproductive cycle. Jpn J Anim Reprod 1962; 8: 34-40.

14. Hashimoto I, Hendricks D, Anderson L, Melampy R. Progesterone and pregn-4-en-20a-ol-3-one in ovarian venous blood during various reproductive states in the rat. . Endocrinology 1968; 82: 333-341.

15. Hashimoto I, Wiest W. Correlation of the secretion of ovarian steroids with functionof a single generation of corpora lutea in the immature rat. Endocrinology 1969; 84: 873-885.

16. Gay V, Midgley AJ, Niswender G. Patterns of gonadotrophin secretion associated with ovulation. Fed Proc 1970; 29: 1880-1887.

17. Smith M, Freeman M, Neill J. The control of progesterone secretion during theestrous cycle and early pseudopregnancy in the rat: Prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. Endocrinology 1975; 96: 219-226.

18. Kalra SP, Kalra PS. Neural regulation of luteinizing hormone secretion in the rat. Endocr Rev 1983; 4: 311-351.

19. Kalra SP. Observations on facilitation of the preovulatory rise of LH by estrogen. Endocrinology 1975; 96: 23-28.

20. Legan SJ, Coon GA, Karsch FJ. Role of estrogen as initiator of daily LH surges in the ovariectomized rat. Endocrinology 1975; 96: 50-56.

21. Legan SJ, Karsch FJ. A daily signal for the LH surge in the rat. Endocrinology 1975; 96: 57-62.

22. Everett J. Neurobiology of reproduction in the female rat. Monographs on Endocrinology 1989; 32: 2-7.

23. Wolfe JM BE, Wright AW. The estrous cycle and associated phenomena in a strain of rats characterized by a high incidence of mammary tumors

together with observations on the effects of advancing age on these phenomena. Amer J Cancer 1940; 38: 383-398.

24. MohanKumar PS, MohanKumar SM, Arbogast L, Quadri SK, Voogt JL. Effects of chronic hyperprolactinemia on tuberoinfundibular dopaminergic neurons. Proc Soc Exp Biol Med 1998; 217: 461-465.

25. MohanKumar PS, MohanKumar SM, Quadri SK. Deprenyl stimulates the release of luteinizing hormone from the pituitary in vitro. Life Sci 1997; 61: 1783-1788.

26. MohanKumar PS, MohanKumar SM, Quadri SK, Voogt JL. Responsiveness of tuberoinfundibular dopaminergic neurons to 5-hydroxytryptophan: effects of aging. Endocrine 1998; 9: 33-37.

27. Mohankumar PS, Thyagarajan S, Quadri SK. Tyrosine hydroxylase and DOPA decarboxylase activities in the medical preoptic area and arcuate nucleus during the estrous cycle: effects of aging. Brain Res Bull 1997; 42: 265-271.

28. ThyagaRajan S, MohanKumar PS, Quadri SK. Cyclic changes in the release of norepinephrine and dopamine in the medial basal hypothalamus: effects of aging. Brain Res 1995; 689: 122-128.

29. Mohankumar PS, Thyagarajan S, Quadri SK. Correlations of catecholamine release in the medial preoptic area with proestrous surges of luteinizing hormone and prolactin: effects of aging. Endocrinology 1994; 135: 119-126.

30. MohanKumar SM, MohanKumar PS. Aging alters norepinephrine release in the medial preoptic area in response to steroid priming in ovariectomized rats. Brain Res 2004; 1023: 24-30.

31. Wise PM, Dubal DB, Wilson ME, Rau SW, Bottner M. Minireview: neuroprotective effects of estrogen-new insights into mechanisms of action. Endocrinology 2001; 142: 969-973.

32. Thompson DL, Pomerantz DK, Weick RF. Characteristics of pulsatile luteinizing hormone secretion in middle-aged ovariectomized rats. Biol Reprod 1985; 33: 827-834.

33. Browman L. Light in its relation to activity and oestrous rhythms in the albino rat. J Exp Zool 1937; 75: 375-388.

34. Holloszy JO, Smith EK, Vining M, Adams S. Effect of voluntary exercise on longevity of rats. J Appl Physiol 1985; 59: 826-831.

35. Barraclough CA. Production of anovulatory, sterile rats by single injections of testosterone propionate. Endocrinology 1961; 68: 62-67.

36. Mobbs CV, Kannegieter LS, Finch CE. Delayed anovulatory syndrome induced by estradiol in female C57BL/6J mice: age-like neuroendocrine, but not ovarian, impairments. Biol Reprod 1985; 32: 1010-1017.

37. Harris G. Neural control of the pituitary gland. Physiol Rev 1948; 28: 139.

38. Everett JW, Sawyer CH. A neural timing factor in the mechanism by which progesterone advances ovulation in the cyclic rat. Endocrinology 1949; 45: 581-595, illust.

39. Barraclough CA, Sawyer CH. Inhibition of the release of pituitary ovulatory hormone in the rat by morphine. Endocrinology 1955; 57: 329-337.

40. Barraclough CA, Sawyer CH. Blockade of the release of pituitary ovulating hormone in the rat by chlorpromazine and reserpine: possible mechanisms of action. Endocrinology 1957; 61: 341-351.

41. Cordova T, Ayalon D, Lander N, Mechoulam R, Nir I, Puder M, Lindner HR. The ovulation blocking effect of cannabinoids: structure-activity relationships. Psychoneuroendocrinology 1980; 5: 53-62.

42. Hillarp N-A. Studies on the localzation of hypothalamic centers controlling the gonadotrophic functions of the hypophysis. Acta Endocrinol 1949; 2: 11-23.

43. Terasawa E, Wiegand SJ, Bridson WE. A role for medial preoptic nucleus on afternoon of proestrus in female rats. Am J Physiol 1980; 238: E533-539.

44. Clemens JA, Meites J. Neuroendocrine status of old constant-estrous rats. Neuroendocrinology 1971; 7: 249-256.

45. Halasz B, Gorski RA. Gonadotrophic hormone secretion in female rats after partial or total interruption of neural afferents to the medial basal hypothalamus. Endocrinology 1967; 80: 608-622.

46. Tejasen T, Everett JW. Surgical analysis of the preoptico-tuberal pathway controlling ovulatory release of gonadotropins in the rat. Endocrinology 1967; 81: 1387-1396.

47. Everett JW. Ovulation in Rats from Preoptic Stimulation through Platinum Electrodes. Importance of Duration and Spread of Stimulus. Endocrinology 1965; 76: 1195-1201.

48. Terasawa E, Kawakami M, Sawyer CH. Induction of ovulation by electrochemical stimulation in androgenized and spontaneously constant-estrous rats. Proc Soc Exp Biol Med 1969; 132: 497-501.

49. Wuttke W, Meites J. Effects of electrochemical stimulation of medial preoptic area on prolactin and luteinizing hormone release in old female rats. Pflugers Arch 1973; 341: 1-6.

50. Kalra SP. Mandatory neuropeptide-steroid signaling for the preovulatory luteinizing hormone-releasing hormone discharge. Endocr Rev 1993; 14: 507-538.

51. Baker BL, Dermody WC, Reel JR. Distribution of gonadotropin-releasing hormone in the rat brain as observed with immunocytochemistry. Endocrinology 1975; 97: 125-135.

52. King JC, Parsons JA, Erlandsen SL, Williams TH. Luteinizing hormone--releasing hormone (LH-RH) pathway of the rat hypothalamus revealed by the unlabeled antibody peroxidase-antiperoxidase method. Cell Tissue Res 1974; 153: 211-217.

53. Setalo G, Vigh S, Schally AV, Arimura A, Flerko B. LH-RH-containing neural elements in the rat hypothalamus. Endocrinology 1975; 96: 135-142.

54. Shivers BD, Harlan RE, Morrell JI, Pfaff DW. Immunocytochemical localization of luteinizing hormone-releasing hormone in male and female rat brains. Quantitative studies on the effect of gonadal steroids. Neuroendocrinology 1983; 36: 1-12.

55. Wiegand SJ, Terasawa E. Discrete lesions reveal functional heterogeneity of suprachiasmatic structures in regulation of gonadotropin secretion in the female rat. Neuroendocrinology 1982; 34: 395-404.

56. Wiegand SJ, Terasawa E, Bridson WE. Persistent estrus and blockade of progesterone-induced LH release follows lesions which do not damage the suprachiasmatic nucleus. Endocrinology 1978; 102: 1645-1648.

57. Wiegand SJ, Terasawa E, Bridson WE, Goy RW. Effects of discrete lesions of preoptic and suprachiasmatic structures in the female rat. Alterations in the feedback regulation of gonadotropin secretion. Neuroendocrinology 1980; 31: 147-157.

58. Pfaff D, Keiner M. Atlas of estradiol-concentrating cells in the central nervous system of the female rat. J Comp Neurol 1973; 151: 121-158.

59. Pettus EH, Wright DW, Stein DG, Hoffman SW. Progesterone treatment inhibits the inflammatory agents that accompany traumatic brain injury. Brain Res 2005; 1049: 112-119.

60. Leranth C, MacLusky NJ, Shanabrough M, Naftolin F. Catecholaminergic innervation of luteinizing hormone-releasing hormone and glutamic acid decarboxylase immunopositive neurons in the rat medial preoptic area. An electron-microscopic double immunostaining and degeneration study. Neuroendocrinology 1988; 48: 591-602.

61. Leranth C, MacLusky NJ, Sakamoto H, Shanabrough M, Naftolin F. Glutamic acid decarboxylase-containing axons synapse on LHRH neurons in the rat medial preoptic area. Neuroendocrinology 1985; 40: 536-539.

62. Muller E, Nistico, G. Brain Messengers and the Pituitary. Academic Press, Inc., San Diego, California 92101. 1989.

63. Bushnik T, Bielajew C, Konkle AT. The substrate for brain-stimulation reward in the lateral preoptic area. I. Anatomical mapping of its boundaries. Brain Res 2000; 881: 103-111.

64. King JC, Tobet SA, Snavely FL, Arimura AA. LHRH immunopositive cells and their projections to the median eminence and organum vasculosum of the lamina terminalis. J Comp Neurol 1982; 209: 287-300.

65. Kalra PS, Kalra SP. Temporal changes in the hypothalamic and serum luteinizing hormone-releasing hormone (LH-RH) levels and the circulating ovarian steroids during the rat oestrous cycle. Acta Endocrinol (Copenh) 1977; 85: 449-455.

66. Kalra SP, Kalra PS. Dynamic changes in hypothalamic LH-RH levels associated with the ovarian steroid-induced gonadotrophin surge. Acta Endocrinol (Copenh) 1979; 92: 1-7.

67. Ching M. Correlative surges of LHRH, LH and FSH in pituitary stalk plasma and systemic plasma of rat during proestrus. Effect of anesthetics. Neuroendocrinology 1982; 34: 279-285.

68. Sarkar DK, Chiappa SA, Fink G, Sherwood NM. Gonadotropin-releasing hormone surge in pro-oestrous rats. Nature 1976; 264: 461-463.

69. Kalra SP, McCann SM. Effects of drugs modifying catecholamine synthesis on plasma LH and ovulation in the rat. Neuroendocrinology 1974; 15: 79-91.

70. Kalra PS, Kalra SP, Krulich L, Fawcett CP, McCann SM. Involvement of norepinephrine in transmission of the stimulatory influence of progesterone on gonadotropin release. Endocrinology 1972; 90: 1168-1176.

71. Hosny S, Jennes L. Identification of alpha1B adrenergic receptor protein in gonadotropin releasing hormone neurones of the female rat. J Neuroendocrinol 1998; 10: 687-692.

72. Drouva SV, Laplante E, Kordon C. alpha 1-adrenergic receptor involvement in the LH surge in ovariectomized estrogen-primed rats. Eur J Pharmacol 1982; 81: 341-344.

73. Crowley WR, O'Donohue TL, Wachslicht H, Jacobowitz DM. Effects of estrogen and progesterone on plasma gonadotropins and on catecholamine levels and turnover in discrete brain regions of ovariectomized rats. Brain Res 1978; 154: 345-357.

74. Rance N, Wise PM, Selmanoff MK, Barraclough CA. Catecholamine turnover rates in discrete hypothalamic areas and associated changes in median eminence luteinizing hormone-releasing hormone and serum gonadotropins on proestrus and diestrous day 1. Endocrinology 1981; 108: 1795-1802.

75. Simpkins JW, Huang HH, Advis JP, Meites J. Changes in hypothalamic NE an DA turnover resulting from steroid-induced LH and prolactin surges in ovariectomized rats. Biol Reprod 1979; 20: 625-632.

76. Kalra SP, Kalra PS, Chen CL, Clemens JA. Effect of norepinephrine synthesis inhibitors and a dopamine agonist on hypothalamic LH-RH serum gonadotrophin and prolactin levels in gonadal steroid treated rats. Acta Endocrinol (Copenh) 1978; 89: 1-9.

77. Simpkins JW, Kalra PS, Kalra SP. Temporal alterations in luteinizing hormone-releasing hormone concentrations in several discrete brain regions: effects of estrogen-progesterone and norepinephrine synthesis inhibition. Endocrinology 1980; 107: 573-577.

78. Quadri SK, Kledzik GS, Meites J. Reinitiation of estrous cycles in old constant-estrous rats by central-acting drugs. Neuroendocrinology 1973; 11: 248-255.

79. Thyagarajan S, Meites J, Quadri SK. Deprenyl reinitiates estrous cycles, reduces serum prolactin, and decreases the incidence of mammary and pituitary tumors in old acyclic rats. Endocrinology 1995; 136: 1103-1110.

80. Cooper RL, Conn PM, Walker RF. Characterization of the LH surge in middle-aged female rats. Biol Reprod 1980; 23: 611-615.

81. Steger RW, Huang HH, Chamberlain DS, Meites J. Changes in control of gonadotropin secretion in the transition period between regular cycles and constant estrus in aging female rats. Biol Reprod 1980; 22: 595-603.

82. Simpkins JW, Mueller GP, Huang HH, Meites J. Evidence for depressed catecholamine and enhanced serotonin metabolism in aging male rats: posssible relation to gondotropin secretion. Endocrinology 1977; 100: 1672-1678.

83. Steger RW, Huang HH, Meites J. Relation of aging to hypothalamic LHRH content and serum gonadal steroids in female rats. Proc Soc Exp Biol Med 1979; 161: 251-254.

84. Watkins BE, Meites J, Riegle GD. Age-related changes in pituitary responsiveness to LHRH in the female rat. Endocrinology 1975; 97: 543-548.

85. Finch CE, Felicio LS, Flurkey K, Gee D, Mobbs CV, Nelson JF, Osterburg HH. Studies on ovarian-hypothalamic-pituitary interactions during reproductive aging in C57BL/6J mice. . Peptides 1980; 1(suppl.1): 163-175.

86. Everett J. The restoration of ovulatory cycles and corpus luteum formation in persistent estrous rats by progesterone. Endocrinology 1940; 27: 681-686.

87. Aschheim P. Relation of Neuroendocrine system to Reproductive Decline in Female Rats. in Neuroendocrinology of Aging by Meites, J 1983: 83-97.

88. Brawer JR, Naftolin F, Martin J, Sonnenschein C. Effects of a single injection of estradiol valerate on the hypothalamic arcuate nucleus and on reproductive function in the female rat. Endocrinology 1978; 103: 501-512.

89. Brawer JR, Schipper H, Naftolin F. Ovary-dependent degeneration in the hypothalamic arcuate nucleus. Endocrinology 1980; 107: 274-279.

90. Long JA EH. The oestrous cycle in the rat and its associated phenomena. Memoirs Univ Calif 1922; 6: 1-111.

91. Sieck G, Nance D, Gorski R. Estrogen modification of feeding behaviour in the female rat: influence of metabolic state. Physiol Behav 1978; 21: 893-897.

92. Tarttelin MF, Gorski RA. The effects of ovarian steroids on food and water intake and body weight in the female rat. Acta Endocrinol (Copenh) 1973; 72: 551-568.

93. Braestrup C, Andersen H, Randrup A. The monoamine oxidase B inhibitor deprenyl potentiates phenylethylamine behaviour in rats without inhibition of catecholamine metabolite formation. Eur J Pharmacol 1975; 34: 181-187.

94. Paxinos GaW, C. The rat brain in stereotaxic coordinates. 4th ed. San Diego: Academic Press. xxvi [237] p of plates. 1998.

95. Witkin JW, Paden CM, Silverman AJ. The luteinizing hormone-releasing hormone (LHRH) systems in the rat brain. Neuroendocrinology 1982; 35: 429-438.

96. Sawchenko PE, Swanson LW. The organization of noradrenergic pathways from the brainstem to the paraventricular and supraoptic nuclei in the rat. Brain Res 1982; 257: 275-325.

97. Chan-Palay V, Zaborszky L, Kohler C, Goldstein M, Palay SL. Distribution of tyrosine-hydroxylase-immunoreactive neurons in the hypothalamus of rats. J Comp Neurol 1984; 227: 467-496.

98. Barraclough CA, Wise PM. The role of catecholamines in the regulation of pituitary luteinizing hormone and follicle-stimulating hormone secretion. Endocr Rev 1982; 3: 91-119.

99. Negro-Vilar A, Dickerman E, Meites J. PSH-releasing factor activity in plasma of rats after hypophysectomy and continuous light. Endocrinology 1968; 82: 939-944.

100. Kopin IJ, Gordon EK. Metabolism of norepinephrine-H3 released by tyramine and reserpine. J Pharmacol Exp Ther 1962; 138: 351-359.

101. Glowinski J, Axelrod J. Effect of Drugs on the Uptake, Release, and Metabolism of H3-Norepinephrine in the Rat Brain. J Pharmacol Exp Ther 1965; 149: 43-49.

102. Corrodi H, Hanson LC. Central effects of an inhibitor of tyrosine hydroxylation. Psychopharmacologia 1966; 10: 116-125.

103. Weiner RI, Shryne JE, Gorski RA, Sawyer CH. Changes in the catecholamine content of the rat hypothalamus following deafferentation. Endocrinology 1972; 90: 867-873.

104. Clifton DK, Sawyer CH. LH release and ovulation in the rat following depletion of hypothalamic norepinephrine: chronic vs. acute effects. Neuroendocrinology 1979; 28: 442-449.

105. Clifton DK, Sawyer CH. Positive and negative feedback effects of ovarian steroids on luteinizing hormone release in ovariectomized rats following chronic depletion of hypothalamic norepinephrine. Endocrinology 1980; 106: 1099-1102.

106. Simpkins JW, Kalra SP. Blockade of progesterone-induced increase in hypothalamic luteinizing hormone-releasing hormone levels and serum gonadotropins by intrahypothalamic implantation of 6-hydroxydopamine. Brain Res 1979; 170: 475-484.

107. Simpkins JW, Advis JP, Hodson CA, Meites J. Blockade of steroid-induced leuteinizing hormone release by selective depletion of anterior hypothalamic norepinephrine activity. Endocrinology 1979; 104: 506-509.

108. Skynner MJ, Sim JA, Herbison AE. Detection of estrogen receptor alpha and beta messenger ribonucleic acids in adult gonadotropin-releasing hormone neurons. Endocrinology 1999; 140: 5195-5201.

109. Hrabovszky E, Steinhauser A, Barabas K, Shughrue PJ, Petersen SL, Merchenthaler I, Liposits Z. Estrogen receptor-beta immunoreactivity in luteinizing hormone-releasing hormone neurons of the rat brain. Endocrinology 2001; 142: 3261-3264.

110. Kallo I, Butler JA, Barkovics-Kallo M, Goubillon ML, Coen CW. Oestrogen receptor beta-immunoreactivity in gonadotropin releasing hormone-expressing neurones: regulation by oestrogen. J Neuroendocrinol 2001; 13: 741-748.

111. Laflamme N, Feuvrier E, Richard D, Rivest S. Involvement of serotonergic pathways in mediating the neuronal activity and genetic transcription of neuroendocrine corticotropin-releasing factor in the brain of systemically endotoxin-challenged rats. Neuroscience 1999; 88: 223-240.

112. Legan SJ, Tsai HW. Oestrogen receptor-alpha and -beta immunoreactivity in gonadotropin-releasing hormone neurones after ovariectomy and chronic exposure to oestradiol. J Neuroendocrinol 2003; 15: 1164-1170.

113. Butler AA, Le Roith D. Control of growth by the somatropic axis: growth hormone and the insulin-like growth factors have related and independent roles. Annu Rev Physiol 2001; 63: 141-164.

114. Simerly RB, Carr AM, Zee MC, Lorang D. Ovarian steroid regulation of estrogen and progesterone receptor messenger ribonucleic acid in the anteroventral periventricular nucleus of the rat. J Neuroendocrinol 1996; 8: 45-56.

115. Zhou Y, Shughrue PJ, Dorsa DM. Estrogen receptor protein is differentially regulated in the preoptic area of the brain and in the uterus during the rat estrous cycle. Neuroendocrinology 1995; 61: 276-283.

116. Arteaga-Lopez PR, Dominguez R, Cerbon MA, Mendoza-Rodriguez CA, Cruz ME. Differential mRNA expression of alpha and beta estrogen receptor isoforms and GnRH in the left and right side of the preoptic and anterior hypothalamic area during the estrous cycle of the rat. Endocrine 2003; 21: 251-260.

117. Childs GV, Unabia G, Komak S. Differential expression of estradiol receptors alpha and beta by gonadotropes during the estrous cycle. J Histochem Cytochem 2001; 49: 665-666.

118. Plata-Salaman CR, Ilyin SE, Turrin NP, Gayle D, Flynn MC, Bedard T, Merali Z, Anisman H. Neither acute nor chronic exposure to a naturalistic (predator) stressor influences the interleukin-1beta system, tumor necrosis factor-alpha, transforming growth factor-beta1, and neuropeptide mRNAs in specific brain regions. Brain Res Bull 2000; 51: 187-193.

119. Brawer JR, Beaudet A, Desjardins GC, Schipper HM. Pathologic effect of estradiol on the hypothalamus. Biol Reprod 1993; 49: 647-652.

120. Knobil E, Neill. The Physiology of Reproduction. 1994; 2 (2nd Edition): 633-639.

121. Herbison AE. Noradrenergic regulation of cyclic GnRH secretion. Rev Reprod 1997; 2: 1-6.

122. Hancke JL, Wuttke W. Effects of chemical lesion of the ventral noradrenergic bundle or the medial preoptic area on preovulatory LH release in rats. Exp Brain Res 1979; 35: 127-134.

123. Jennes L, Jennes ME, Purvis C, Nees M. c-fos expression in noradrenergic A2 neurons of the rat during the estrous cycle and after steroid hormone treatments. Brain Res 1992; 586: 171-175.

124. Okazaki K, Okazaki S, Nakamura H, Kitamura Y, Hatayama K, Wakabayashi S, Tsuda T, Katsumata T, Nishikawa A, Hirose M. A repeated 28-day oral dose toxicity study of genistein in rats, based on the

'Enhanced OECD Test Guideline 407' for screening endocrine-disrupting chemicals. Arch Toxicol 2002; 76: 553-559.

125. Liaw JJ, He JR, Hartman RD, Barraclough CA. Changes in tyrosine hydroxylase mRNA levels in medullary A1 and A2 neurons and locus coeruleus following castration and estrogen replacement in rats. Brain Res Mol Brain Res 1992; 13: 231-238.

126. Montemayor ME, Clark AS, Lynn DM, Roy EJ. Modulation by norepinephrine of neural responses to estradiol. Neuroendocrinology 1990; 52: 473-480.

127. Blanchard-Fillion B, Souza JM, Friel T, Jiang GC, Vrana K, Sharov V, Barron L, Schoneich C, Quijano C, Alvarez B, Radi R, Przedborski S, Fernando GS, Horwitz J, Ischiropoulos H. Nitration and inactivation of tyrosine hydroxylase by peroxynitrite. J Biol Chem 2001; 276: 46017-46023.

128. Xu Y, Stokes AH, Roskoski R, Jr., Vrana KE. Dopamine, in the presence of tyrosinase, covalently modifies and inactivates tyrosine hydroxylase. J Neurosci Res 1998; 54: 691-697.

129. Ignarro L. Nitric oxide-Biology and Pathobiology. New York: San Diego Acdemic Press; 2000.

130. Iravani MM, Kashefi K, Mander P, Rose S, Jenner P. Involvement of inducible nitric oxide synthase in inflammation-induced dopaminergic neurodegeneration. Neuroscience 2002; 110: 49-58.

131. Iravani MM, Liu L, Rose S, Jenner P. Role of inducible nitric oxide synthase in N-methyl-d-aspartic acid-induced strio-nigral degeneration. Brain Res 2004; 1029: 103-113.

132. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol 1996; 271: C1424-1437.

133. Bagasra O, Michaels FH, Zheng YM, Bobroski LE, Spitsin SV, Fu ZF, Tawadros R, Koprowski H. Activation of the inducible form of nitric oxide synthase in the brains of patients with multiple sclerosis. Proc Natl Acad Sci U S A 1995; 92: 12041-12045.

134. Ara J, Przedborski S, Naini AB, Jackson-Lewis V, Trifiletti RR, Horwitz J, Ischiropoulos H. Inactivation of tyrosine hydroxylase by nitration following exposure to peroxynitrite and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Proc Natl Acad Sci U S A 1998; 95: 7659-7663.

135. Beckman JS. Protein tyrosine nitration and peroxynitrite. Faseb J 2002; 16: 1144.

136. Haddad IY, Pataki G, Hu P, Galliani C, Beckman JS, Matalon S. Quantitation of nitrotyrosine levels in lung sections of patients and animals with acute lung injury. J Clin Invest 1994; 94: 2407-2413.

137. Kuhn DM, Aretha CW, Geddes TJ. Peroxynitrite inactivation of tyrosine hydroxylase: mediation by sulfhydryl oxidation, not tyrosine nitration. J Neurosci 1999; 19: 10289-10294.

138. Djebaili M, Guo Q, Pettus EH, Hoffman SW, Stein DG. The neurosteroids progesterone and allopregnanolone reduce cell death, gliosis, and functional deficits after traumatic brain injury in rats. J Neurotrauma 2005; 22: 106-118.

139. Binko J, Murphy TV, Majewski H. 17Beta-oestradiol enhances nitric oxide synthase activity in endothelium-denuded rat aorta. Clin Exp Pharmacol Physiol 1998; 25: 120-127.

140. Galani R, Hoffman SW, Stein DG. Effects of the duration of progesterone treatment on the resolution of cerebral edema induced by cortical contusions in rats. Restor Neurol Neurosci 2001; 18: 161-166.

141. Roof RL, Hoffman SW, Stein DG. Progesterone protects against lipid peroxidation following traumatic brain injury in rats. Mol Chem Neuropathol 1997; 31: 1-11.

142. Roof RL, Duvdevani R, Heyburn JW, Stein DG. Progesterone rapidly decreases brain edema: treatment delayed up to 24 hours is still effective. Exp Neurol 1996; 138: 246-251.

143. Roof RL, Duvdevani R, Stein DG. Gender influences outcome of brain injury: progesterone plays a protective role. Brain Res 1993; 607: 333-336.

144. Newman DB, Liu RP. Nuclear origins of brainstem reticulocortical systems in the rat. Am J Anat 1987; 178: 279-299.

145. Maisky VA, Doroshenko NZ. Catecholamine projections to the spinal cord in the rat and their relationship to central cardiovascular neurons. J Auton Nerv Syst 1991; 34: 119-128.