Chronic Estradiol exposure-harmful effects on behavior, cardiovascular and reproductive functions
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Running title: Estradiol, hormones, anxiety, hypertension

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Abstract:

Estradiol (E2) is a female hormone that is produced largely by the ovaries, but also by the adrenal glands, fat and liver. It is present in the circulation of both males and females. Many studies in the literature have described how E2 is beneficial to the body in terms of preventing bone loss, affording protection in ischemia reperfusion injury, relieving symptoms of menopause, maintaining vaginal health, and helping with ovarian failure or hypogonadism. Beneficial effects on the brain have been reported to include protection against memory loss, neuronal degeneration, changes in cognition, mood and behavior. However, the effects of E2 exposure on the neuroendocrine system have not been understood completely. This is because differences in doses, preparation and duration of exposure have produced variable results ranging from beneficial, to no change, or to detrimental. Studies in our lab over the last few years have shown that chronic exposures to low levels of E2 in young rats can produce specific effects on the neuroendocrine system. We have observed that these exposures can induce reproductive senescence, hypertension, anxiety-like behavior, and cause degenerative changes in specific neuronal populations leading to hyperprolactinemia. The purpose of the review is to present evidence from the literature for these effects and to discuss the underlying molecular mechanisms.
Introduction:

Estrogens are female hormones that are responsible for the onset and progression of female reproductive cycles and the development of secondary sexual characteristics typical of females. There are three kinds of estrogens in circulation: estrone (E1), estradiol (E2) and estriol (E3). Of these, E2 is present at higher levels, fluctuates throughout the life of females, and much of the endogenous estrogenic activity has been attributed to this hormone. E2 plays important roles in the regulation of reproduction and energy metabolism, and influences a number of other hormones such as thyroid hormones (Santin & Furlanetto 2011). It is also reported to produce a variety of beneficial effects especially when it is used to restore estrogens in older women or ovariectomized animal models. These effects include preventing bone loss in early postmenopausal women (Cooper et al. 1999), preventing age-related changes in the skin (Patriarca et al. 2013), improving insulin sensitivity in ovariectomized animals (Shen et al. 2014) and in postmenopausal women (Raudaskoski et al. 1999), preventing the incidence of type 2 diabetes early in menopause (Pereira et al. 2015), and improving cognitive functions after focal ischemia in experimental animals (Soderstrom et al. 2009) and in postmenopausal women with Alzheimer’s disease (Wharton et al. 2011).

There are numerous studies describing the beneficial effects of E2 exposure on the brain and many of these beneficial effects have been observed after acute E2 exposures (Bagger et al. 2005), (Whitmer et al. 2011), (Zhang et al. 2018). However, the effects of chronic E2 exposures have not been explored in great detail. These exposures are more relevant since humans are exposed to a variety of estrogens.
compounds from endogenous estrogens to phytoestrogens and estrogenic endocrine disruptors in the environment (Adeel et al. 2017). Most of these exposures occur at low, persistent levels and their effects on neuroendocrine functions are not understood, recognized or are frequently dismissed. More recently, these effects have received considerable attention from the scientific community and regulatory bodies (Vandenberg et al. 2012). This is because low dose exposures may contribute to many non-communicable diseases such as hypertension, reproductive and metabolic disorders that are widely prevalent today (Gore et al. 2015). Therefore, it is important to understand the effects of chronic exposure to low levels of E2 on neuroendocrine functions and its relationship to the development of these disorders. E2 is capable of influencing a variety of neurotransmitter systems (Barth et al. 2015) that in turn, could mediate its effects on neuroendocrine function. For the purpose of this review, we will focus on norepinephrine (NE). This is because the catecholamines, NE and dopamine (DA) have been implicated in the neuroendocrine regulation of reproductive functions for a long time. There are several early studies in the 1970s and 1980s that have used agonists, inhibitors, catecholamine depletors and neurotoxins that suggest that catecholamines play an important role in GnRH and prolactin regulation and alterations in behavior (Mueller & Nistico 1989). NE is believed to play a prominent role especially when ovarian steroids are administered (Mueller & Nistico 1989). Moreover, there is strong evidence to suggest that catecholamines are important for the regulation of cardiovascular function (Blessing & Reis 1982, Blessing et al. 1982, Madden & Sved 2003).
**Chronic E2 exposure and reproductive functions:**

**E2 in reproductive physiology:** Reproduction is a complex process involving coordinated regulation of the brain stem, the hypothalamus, the anterior pituitary and the gonads. When follicles in the ovaries mature, they produce E2. Increase in circulating E2 levels stimulates brainstem noradrenergic neurons (Barraclough 1994) to release NE, which in turn stimulates gonadotropin releasing hormone (GnRH) neurons in the hypothalamus (Mohankumar *et al.* 1994). E2 can also act directly on GnRH neurons to stimulate them (Herbison & Pape 2001). Upon stimulation, these neurons release GnRH via their terminals located in the median eminence and GnRH enters the portal circulation to reach the anterior pituitary and acts on gonadotrophs (Schally *et al.* 1971). Here, it stimulates the release of luteinizing hormone (LH), which, in turn acts on the ovaries to cause ovulation. This process is common to mammals, including humans (Barraclough & Wise 1982).

Female rats attain puberty when they are about 2-months old and begin to have regular estrous cycles that are 4-5 days long. Each day is a specific stage of the cycle and the rat goes through proestrous, estrous, metestrous and diestrous in one cycle (Long & Evans 1922). Proestrous is marked by a gradual rise in circulating E2 in the afternoon that stimulates noradrenergic neurons in the brain stem (McEwen *et al.* 2012) and increases NE release in the medial preoptic area (MPA) of the hypothalamus (Mohankumar *et al.* 1994), an area intimately involved in GnRH regulation (Herbison 1997). This activates GnRH neurons leading to the preovulatory surge in LH that causes ovulation (Wise *et al.* 1981, Wise 1984, Mohankumar *et al.* 1994). Age-related reduction in NE activity in the MPA plays a
role in subduing the LH surge during middle-age (MohanKumar & MohanKumar 2004). This causes irregular cyclicity as the animals reach 11-12 months of age. Soon after that, rats enter a state of acyclicity called ‘constant estrus or persistent estrus’ because there is not enough LH to induce ovulation and they are stuck in the “estrus” state (Steger et al. 1980). It is believed that repeated exposure to endogenous E2 peaks during each estrous cycle is the primary cause for loss of LH surges and cessation of estrous cyclicity in rodents (Lu et al. 1981, Nelson et al. 1981).

In our studies, we mimicked this phenomenon by exposing intact young (2-3 month old) cycling rats to low levels of exogenous E2 (slow release pellets implanted subcutaneously, capable of releasing 20 ng Estradiol-17β/day) for 30, 60 or 90 days and evaluated NE activity in the MPA, serum LH, serum estradiol and estrous cyclicity. A majority (about 80%) of the animals that were exposed to E2 for 30 days exhibited regular estrous cycles and had circulating E2 levels of about 35pg/ml. When they were exposed to E2 for 60 days, their E2 levels increased to 70pg/ml and 70-80% of them were in persistent estrus, however, a majority of them returned to normal cyclicity at the end of E2 exposure (about 70%). More than 80% of the animals that were exposed to E2 for 90 days were in persistent estrus and did not regain normal cycles at the end of E2 exposure. These animals had circulating E2 levels of 95pg/ml. In these animals, there was a significant reduction in NE in the MPA and serum LH correlating well with the loss of cyclicity (Kasturi et al. 2009). The serum levels of E2 in these animals were in the range of 45-80 pg/ml which corresponds to the levels observed during the afternoon of proestrus in
regularly cycling animals (Lu et al. 1979). In other words, rats implanted with E2 pellets were continuously exposed to proestrus levels of E2. If we consider the normal aging process in rats, they experience roughly 7 estrous cycles (and therefore 7 days of proestrus) a month. They enter the constant estrous state after 8 months of regular cycling (or after 56 days of proestrus). In our studies, the dose of E2 used made animals enter constant estrous after 60 days of exposure (Kasturi et al. 2009, Gilbreath et al. 2014). These results suggest that chronic exposure to 20ng of E2/day accelerates reproductive aging.

Other studies in ovariectomized rats have found that E2 implants can suppress GnRH activation and block the steroid-induced LH surge (Tsai & Legan 2001, Tsai & Legan 2002). These implants were capable of generating circulating E2 levels of 75pg/ml and were kept in place for a maximum of 4 weeks. The steroid-induced LH surges began to decrease in amplitude by 2 weeks and were completely absent after 4 weeks of E2 exposure (Tsai & Legan 2002). These investigators also reported that the reduction in LH levels after E2 exposure did not involve changes in estrogen receptor (ER) expression on GnRH neurons (Legan & Tsai 2003). Further, using the ovariectomized rat model, they demonstrated that acute E2 exposure was capable of suppressing NE release in the hypothalamus (Legan & Callahan 1999).

In an effort to understand the underlying mechanism by which chronic E2 exposure decreases NE levels, we have focused on the biosynthetic pathway for NE. Tyrosine Hydroxylase (TH) is the rate-limiting enzyme in the synthesis of NE and dopamine. One means by which noradrenergic activity can be reduced is by nitration of tyrosine residues on TH (Huie & Padmaja 1993, Ischiropoulos et al.
Peroxynitrite, an oxidant that arises from the reaction of nitric oxide and superoxide anion, gives rise to nitrating species that can nitrate tyrosine moieties on proteins (Radi 2013). This can lead to inactivation of protein function, particularly of proteins like TH which contain several tyrosine residues in their structure (Ara et al. 1998). The tyrosine moieties in TH are clustered around the active center of the enzyme (Imam et al. 2001) and nitration of the tyrosine residues causes steric hindrance, which, in turn inhibits the activity of this enzyme (Imam et al. 2001). Thus, measurement of nitration of TH can be used as an indicator of degeneration of noradrenergic neurons. In fact, we have shown that there is a gradual increase in nitration of TH in the MPA with increasing duration of E2 exposure that parallels the reduction in NE levels (Kasturi et al. 2013). Therefore, it is possible that E2-induced increase in nitric oxide metabolism in the hypothalamus could cause nitration of TH and lead to a loss of noradrenergic neuronal function.

Brain derived neurotrophic factor (BDNF) is another likely candidate to be involved in the neuroendocrine effects of chronic E2 exposure. BDNF is widely distributed throughout the brain including cerebral and cerebellar cortex, hypothalamus, midbrain, hindbrain and the brain stem and is considered to be a protective factor (Hofer et al. 1990, Wetmore et al. 1990, Merlio et al. 1992, Masana et al. 1993, Viant et al. 2000). E2 is a potent regulator of BDNF (Singh et al. 1995, Sohrabji et al. 1995, Wang & Zheng 1998, Kenny et al. 2000, West et al. 2001, Wu et al. 2004, Yan et al. 2004) since the BDNF gene promoter region has an estrogen responsive element (Amantea et al. 2005). Moreover, ERs have been identified in BDNF-synthesizing neurons (Miranda et al. 1993). E2 and BDNF have overlapping
actions in the forebrain, and act through common second messengers such as ERK1 and ERK 2 (Singh et al. 1999). BDNF and NE can be co-localized in neurons (Hwang et al. 2013) and can influence each others’ expression (Juric et al. 2008, Sakata & Duke 2014). Therefore, there is a possibility that changes in BDNF expression can affect NE levels and vice versa. In the chronic E2 exposure model, we have found that BDNF levels decrease in specific brain regions such as the amygdala (Balasubramanian et al. 2014). However, there are no studies exploring the involvement of BDNF in the neuroendocrine regulation of reproductive functions. Further studies are needed to explore the role of BDNF in the regulation of noradrenergic activity in the context of GnRH regulation after chronic E2 exposure.

Other possible mechanisms of decreasing NE levels in the MPA involve increasing GABA release locally (Demling et al. 1985, Sirivelu et al. 2009a), or by increasing cytokine levels which can in turn act on brainstem NE neurons (Sirivelu et al. 2012) and inhibit NE synthesis. E2 can directly stimulate cytokine production from a variety of cells including peritoneal macrophages (Calippe et al. 2008). In our model, E2 exposure elevated IL-1β levels in specific brain regions such as the MPA (unpublished) and the arcuate nucleus. (MohanKumar et al. 2011). The source of proinflammatory cytokines in our model is not clear. It is likely that E2 acts through its receptors to increase cytokines. These receptors have been identified in specific cell types such as ependymal, endothelial cells, neurons and glial cells in several areas of the hypothalamus and the brain (Langub & Watson 1992, Paech et al. 1997, Azcoitia et al. 1999, Cardona-Gomez et al. 2000, Donahue et al. 2000, Gundlah et al. 2000, Hosli et al. 2000, Su et al. 2001, Garcia-Ovejero et al. 2002).
Cytokines such as IL-1β are known to cross the blood brain barrier and inhibit NE synthesis by acting on brain stem noradrenergic neurons (Sirivelu et al. 2009b) or suppress NE release by increasing GABA levels locally in the hypothalamus (Sirivelu et al. 2009a). Therefore, the cytokines generated locally in response to chronic E2 exposure could act similarly.

Kisspeptin, neurokinin B and dynorphin could also be potential mediators in chronic E2-induced suppression of reproductive functions. KNDy neurons that secrete these mediators are believed to relay the negative feedback effects of E2 on GnRH secretion (Rance 2009), (Mittelman-Smith et al. 2012). Chronic exposure to E2 was found to decrease KNDy neuron size and spine density and reduce the expression of kisspeptin and neurokinin B in these neurons. These changes could potentially contribute to the reduction in GnRH levels after chronic E2 exposure (Cholanian et al. 2015). It should be noted that these KNDy neurons are distinct from subpopulations of kisspeptin positive neurons in the hypothalamus that are involved in stimulating GnRH secretion (Herbison 2008).

**Chronic E2 exposure effects on TIDA neurons:**

Tuberoinfundibular dopaminergic (TIDA) neurons are responsible for secreting dopamine that enters the portal circulation to inhibit prolactin secretion by the anterior pituitary (Fuxe 1964). The number of dopamine receptors in the anterior pituitary increases sharply during the afternoon of proestrous, coinciding with the occurrence of the surge in plasma prolactin (Heiman & Ben-Jonathan 1982a). During this time frame, dopamine concentrations in the portal circulation and dopamine turnover in the median eminence drop (Ben-Jonathan et al. 1977,
Rance et al. 1981), suggesting that the reduction in dopamine levels contribute to
the increase in prolactin secretion. E2 is believed to be one of the contributing
factors for the increase in prolactin, either by decreasing dopaminergic activity in
TIDA neurons or possibly through a direct effect on the anterior pituitary (Raymond
E2 treatment for up to 10 days produced a dramatic increase in prolactin mRNA and
serum prolactin levels (Lawson et al. 1993). E2 also decreases the number of
dopamine binding sites in the anterior pituitary (Heiman & Ben-Jonathan 1982b).
These effects are probably mediated through ERs located on lactotrophs (Keefer et
al. 1976). Although these studies were acute in nature, they provided clues to what
might happen during the natural aging process in rats.

Female rats enter the constant estrous state when they are 11-12 months of
age and they remain in this state for about 6 months. Prolactin levels in the early
stages of constant estrous are about 40 ng/ml and increase to around 200 ng/ml
towards the end of constant estrous (Goya et al. 1991). The reason for this several
fold increase has been attributed to a reduction in dopamine levels in the median
eminence (Sarkar et al. 1984). Initially, it was believed that the persistent increase
in E2 levels in the constant estrous state continuously stimulated prolactin
secretion. This, in turn increased dopamine production in TIDA neurons to turn
down prolactin production (Simpkins 1983, Sarkar et al. 1984). However, with time,
the continuous stimulation by prolactin causes TIDA neurons to become exhausted
ultimately leading to lower dopamine production (Simpkins 1983, Sarkar et al.)
There are other physiological changes that occur with aging in the hypothalamus leading to the notion that other mechanisms could also be in play. Gliosis is a phenomenon that is commonly observed with aging. E2 exposure is known to increase astrocyte activity specifically in the arcuate nucleus of the hypothalamus where TIDA neuronal cell bodies are located (Kohama et al. 1995, Stone et al. 1998). Moreover, glial fibrillary acidic protein (GFAP) that is produced by activated astrocytes has an estrogen responsive element in its promoter region (Stone et al. 1998). We have found an increase in GFAP expression in the chronic E2 exposure model after 60 and 90 days of exposure that was comparable to what is seen in constant estrous rats (MohanKumar et al. 2011). Glial cells are highly dynamic cells that are responsible for maintaining homeostasis in the brain (Araque 2006). Although astrocytes are the predominant glial cells in the CNS, microglia also contribute significantly to the pathophysiology of the CNS. Microglia when activated, are capable of releasing a variety of proinflammatory cytokines, including IL-1β (Lynch et al. 2010). In the chronic E2 exposure model, we found significant elevations in IL-1β levels in the arcuate nucleus after 60 and 90 days of exposure and paralleled IL-1β levels in old constant estrous rats (MohanKumar et al. 2011). E2 stimulates microglia most probably through a toll-like receptor (Young et al. 2014). Besides cytokines, microglia have been implicated in the generation of a variety of other molecules: superoxide and other oxidants through the NADPH oxidase pathway and nitric oxide and related molecules through the inducible nitric oxide synthase (iNOS) pathway (Block & Hong 2007). They can also decrease the supply of protective factors, such as BDNF and insulin-like growth factor-1 (IGF-1).
In fact, chronic E2 exposure increases nitric oxide levels in the arcuate nucleus and the levels are similar to what is seen in constant estrous rats (MohanKumar et al. 2011). Elevations in nitric oxide-related free radicals can cause nitration of TH as explained previously. Peroxynitrite, a powerful oxidant that is formed by the reaction between nitric oxide and superoxide anion, reacts with the metal center of TH and nitrates tyrosine residue 423. This results in a loss of enzyme activity (Blanchard-Fillion et al. 2001). Loss of TH function, not only impairs the synthesis of NE, but also drastically affects dopamine production since it is a synthesizing enzyme required for both NE and dopamine (Nakashima et al. 2009). We have been able to block this effect in iNOS knockout mice, but not IL-1 receptor knockout mice (our unpublished observations), suggesting that iNOS plays a key role in this phenomenon. Besides nitration of TH, dopamine itself is capable of being nitrated. In fact, there is experimental evidence that indicates that significant amounts of dopamine can get nitrated at pH 7 and nitrated dopamine fails to bind to D1 dopamine receptor(Daveu et al. 1997). These studies indicate that nitric oxide-related free radical generation can have a significant impact on aging processes and processes that mimic aging.

**Chronic E2 exposure and cardiovascular function:**

It is well established that the prevalence of hypertension increases with age. In the US, roughly 1 in 3 people are affected by high blood pressure and only half of them maintain good control over their blood pressures using medication or lifestyle changes(Booth et al. 2017). While roughly 25% of men and 19% of women develop hypertension when they are 35 years of age, the prevalence is 66% in men
and 78% in women when they are 75 years or older (Hayward & Kelly 1997). The reason for the dramatic change in older women is attributed to loss of estrogen due to menopause and related changes in arterial walls (Lima et al. 2012). This led to the increasing use of hormonal replacement therapy (HRT) in postmenopausal women as a way to replace the loss of endogenous estrogen. Use of HRT in postmenopausal women became controversial after the results from 2 large randomized clinical trials, namely the Women’s Health Initiative (WHI) (Manson et al. 2003) and the Heart and Estrogen/Progestin Replacement Study (HERS I and II) (Hulley et al. 2002) (Hulley et al. 1998), showed increased incidence of cardiovascular diseases like stroke and myocardial infarction in women on HRT.

Table 2 provides a list of studies using various estrogenic preparations and their effects on cardiovascular function.

In light of these findings, it was important to understand the effects of chronic E2 exposure on the cardiovascular system. In fact, the dose of E2 used in the chronic exposure model mimicked oral contraceptive use in young women and HRT in older women. Chronic E2 exposure increased systolic and diastolic blood pressure (BP) without affecting heart rate in female rats (Subramanian et al. 2011). In fact, systolic BP begins to rise 3 weeks after the beginning of E2 exposure (Subramanian et al. 2017). A number of studies (both basic and clinical) have reported increases in blood pressure in experimental animals and women after varying doses and duration of E2 exposure (Lim et al. 1987, Woods 1988, Byrne et al. 1994, Chasan-Taber et al. 1996, Olatunji & Soladoye 2006, Olatunji & Soladoye 2008). In contrast to these findings, shorter durations of exposure (3 or 8 weeks)
to E2 (1.5mg, s.c.) have shown beneficial effects on the cardiovascular system both in ovariectomized and experimental hypertensive animals (Brosnihan et al. 1997, Gimenez et al. 2006). In these animals, there was a reduction in mean arterial pressure due to reduced synthesis of and response to Angiotensin II (Brosnihan et al. 1997) and an improvement in microvascular density (Gimenez et al. 2006).

Collectively, these findings support the idea that whether E2 exposure is detrimental or beneficial to the cardiovascular system depends largely on the dose, duration of E2 exposure and the age of the animal.

There are several central mechanisms by which chronic E2 exposure could increase BP. Some of the central regions involved in BP regulation include the rostral ventrolateral medulla (RVLM), the nucleus tractus solitarius (NTS) and area postrema of the brainstem, periventricular regions, such as the Subfornical organ (SFO) and the organum vasculosum lamina terminalis (OVLT), and hypothalamic nuclei, such as the paraventricular nucleus (PVN), Supraoptic nucleus (SON), the lateral hypothalamus, and medial septal area (Menani et al. 2014). Chronic E2 exposure could affect one or several of these areas to produce a concerted effect on BP. Activation of neurons in the PVN and RVLM could also result in activation of the sympathetic nervous system through the intermediolateral cell column of the spinal cord (Westerhaus & Loewy 1999, Guyenet 2006).

Since chronic low dose E2 exposure is capable of inducing oxidative stress in specific hypothalamic areas, it is likely that this could be a possible molecular mechanism that contributes to increased BP in this model. In fact, oxidative stress has been implicated in the pathogenesis of hypertension in other experimental
animal models of obesity, angiotensin II- or salt-induced hypertension (Kishi et al. 2004, Chan et al. 2005, Stocker et al. 2007, Hirooka 2008, Oliveira-Sales et al. 2009, Hilzendeger et al. 2012, Konno et al. 2012). In the chronic low dose E2 exposure model, the levels of superoxide, a reactive oxygen species, were elevated after 90 days of exposure and both superoxide and BP parameters were markedly reduced when rats were treated with resveratrol, a natural phenol and antioxidant (Subramanian et al. 2011). The source of the superoxide anion is not clear. As explained previously, microglia could be a potential source of this molecule. Microglia express ER alpha (Sierra et al. 2008) and they also contain NADPH oxidase, that gives them the ability to generate superoxide and produce neurodegenerative changes in chronic conditions (Surace & Block 2012). In fact, NADPH oxidase gene expression is increased in the RVLM after chronic E2 exposure (Subramanian et al. 2015). This agrees well with published evidence that NADPH oxidase is activated in the RVLM of neurogenic hypertensive rats contributing to oxidative stress in these models (Hirooka 2008, Bai et al. 2009, Hirooka 2011, Kishi & Hirooka 2012). There is some evidence to suggest that oxidative stress in the RVLM could activate the sympathetic nervous system leading to hypertension (Agarwal et al. 2011, Winklewski et al. 2015).

Angiotensin II (Ang II), a vasoconstrictor peptide, has also been reported to increase oxidative stress leading to hypertension (Young & Davisson 2015). To our surprise, both Ang II levels and gene expression in the RVLM were unaffected in the chronic E2 exposure model. However, a vasoconstrictor peptide, endothelin-1 (ET-1), was dramatically up-regulated in the RVLM of E2-treated animals after 90 days.
of exposure (Subramanian et al. 2017). ET-1 has been shown to activate the sympathetic nervous system (Bruno et al. 2011). It is also capable of increasing superoxide production through activation of NADPH oxidase to cause vasoconstriction in the aorta of rats (Loomis et al. 2005, Dammanahalli & Sun 2008). Therefore, it is very likely that ET-1 increases NADPH oxidase in the RVLM as well. This effect is most probably produced through the ETA receptor, since the gene expression of this receptor was upregulated in the chronic E2 exposure model, both in the RVLM and the PVN (Subramanian et al. 2017).

Nitration of proteins is present at a basal level in normal tissues and is important for certain signaling pathways. However, when there is serious imbalance in the generation of nitrating species, there is abnormal level of nitration that leads to altered cardiovascular function (Turko & Murad 2002). Nitration of free radical scavenging enzymes such as manganese Superoxide dismutase (MnSOD) could also result in accumulation of free radicals. MnSOD is capable of undergoing peroxynitrite-dependent nitration of the tyrosine residue (Tyr34) that is located very close to the active site of the enzyme. This results in loss of enzyme activity leading to a gradual change in mitochondrial redox homeostasis that further aggravates the oxidative insult (Radi 2013). Besides nitration of specific enzymes, myofibrillar proteins in the heart and aorta can be nitrated especially in hypertensive animals (Cabassi et al. 2001). Further studies are needed to determine if similar changes are obvious in the chronic E2 exposure model.

Unlike other neurogenic hypertensive models such as spontaneously hypertensive rats or Ang II/salt-induced hypertension, chronic E2 exposure only
results in a moderate increase in mean arterial pressure. This represents the current scenario in society where women are exposed to a very small dose of estrogenic compounds on a day-to-day basis in the form of oral contraceptives, cosmetics, or hormone replacement therapy. It is possible that this constant exposure could move a woman from the pre-hypertensive state to a hypertensive state over a period of time. Hence, it is of utmost clinical importance to understand the mechanisms behind chronic E2-related hypertension.

Chronic E2 exposure and behavior:

In the previous sections, we described the effects of low dose chronic E2 exposures on neuroendocrine functions related to reproduction and prolactin secretion and its impact on the development of moderate hypertension. Besides these effects, E2 is also capable of affecting behavior. The most common association is between E2 and mood swings in women. Although mood disorders are common in women of all reproductive ages, the incidence is higher during the luteal phase of menstrual cycle (premenstrual syndrome (PMS) and premenstrual dysphoric disorder (PMDD) and during the perimenopausal period (anxiety and depression). Sex hormones, particularly E2, are strongly implicated in the pathogenesis of these behavioral disorders as their incidence coincides with periods of drastic fluctuations in circulating levels of E2 in women. A recent study has found a significant association between oral contraceptive use in adolescents and subsequent use of anti-depressants, suggesting chronic exposure to E2 may impact mental health (Skovlund et al. 2016). Further, clinical studies in post-menopausal women who are exposed to E2 in the form of hormonal replacement therapy (HRT) [conjugated
equine estrogen (CEE) alone/plus medroxyprogesterone acetate] have also reported minimal or modest adverse effects on cognition and mood associated with its long-term use (Espeland et al. 2004), (Espeland et al. 2017). Some of the early studies, such as Women’s Health Initiative Memory Study (Shumaker et al. 2004), (Espeland et al. 2004) and Heart and Estrogen/progestin Replacement Study (HERS) (Grady et al. 2002), found an increased risk for dementia and decreased cognitive profile in older postmenopausal women treated with E2 alone or E2 plus progestin pills. As these studies involved women who initiated HRT later in their life, concerns were raised as to the timing of HRT initiation in postmenopausal women. This led to the proposal of ‘critical window hypothesis’ which states that the outcomes of HRT depend on the timing of initiation of treatment with respect to age and/or the onset of menopause, with benefits limited to early initiation of treatment (Resnick & Henderson 2002). According to this theory, there is a critical window around perimenopause when hormonal withdrawal increases the susceptibility of the brain to pathologic processes and initiation of HRT around that time might provide the maximal benefits (Marder & Sano 2000). Supporting this idea, clinical studies like Research into Memory, Brain function and Estrogen Replacement (REMEMBER) pilot study, Cache county study, and Cognitive and Affective Study (KEEPS-Cog) of the Kronos Early Estrogen Prevention Study (KEEPS) showed that early initiation of HRT around the perimenopausal period was associated with better cognitive outcomes, reduced risk of Alzheimer’s disease, anxiety and depression (Zandi et al. 2002, MacLennan et al. 2006, Gleason et al. 2015). Neuroimaging studies also found that women who started using HRT around
perimenopause had larger hippocampal volume and better memory performance than the never-users (Erickson et al. 2010) (Maki et al. 2011). One study that investigated the effects of a combination of estrogen and progestogen on verbal memory in women younger than 65 years of age and did not find a significant association with short and long-delay free recall (Maki et al. 2007). On the other hand, another study found improvements in verbal memory (Linzmayer et al. 2001).

Based on these studies, a clear consensus does not exist on whether timing of HRT affects its outcomes. Also, some of these studies involve estrogen plus progestin pills, hence the extent to which progesterone contributes to these outcomes needs to be clarified. While the concept of hormonal therapy for menopause is centered around the fact that stabilizing fluctuating estrogen levels will attenuate the adverse outcomes of menopausal transition, perimenopause is also characterized by periods of increased E2 levels. Whether this period of exposure to elevated E2 levels programs the brain for increased susceptibility to anxiety and depression as observed in postmenopausal women needs to be addressed.

Other studies have observed variable effects of estrogen on behavior. Factors such as dose and duration of estrogen exposure, presence or absence of ovaries, and age of the animals at the time of exposure might be responsible for the differences observed in terms of anxiogenic versus anxiolytic effects of estrogen treatment (Table 3). For example, in other rodent studies, the beneficial effects of chronic estrogen treatment (3 weeks) in improving depression was observed only at low doses (0.3-3μg/day) and not at high doses (10μg/day) which produces supra-physiological estradiol levels (Okada et al. 1997). Further, ovariectomized rats
displayed increased activity in the open field test only when treated with estrogen for 5 days and not 35 days (Luine et al. 1998). In some cases, even acute estrogen treatment (0.75μg for 2 days) has been reported to induce depression-like behavior (Galea et al. 2002). In comparison with all these studies, the dose of estrogen used in our studies was much lower (20 ng of E2/day) and the duration of exposure was much longer (12 wks) (Kasturi et al. 2009). This resulted in lower BDNF mRNA and protein levels in the amygdala, but not in the hippocampus (Balasubramanian et al. 2014). In fact, there was downregulation of one specific BDNF exon (exon VI) (Balasubramanian et al. 2014) which has an estrogen responsive element in its structure (Sohrabji et al. 1995). This was associated with a reduction in dopamine (DA) levels in the amygdala that has been linked to anxiety in humans (Kienast et al. 2008) and rodents (Perez de la Mora et al. 2012). Further studies are needed to determine if chronic E2-induced reduction in BDNF directly influences DA levels, or if DA levels are decreased independent of BDNF action as a result of TH nitration in the amygdala.

**Conclusions:**

We need to consider that E2 is a pleiotropic hormone. It could have a number of beneficial effects as numerous studies in the literature indicate. However, it needs to be used in moderation and with caution. Many of these reported beneficial effects have been observed with higher doses of E2 and for varying durations. According to the studies described here, it appears that chronic exposures to low levels of E2 have widespread effects that are deleterious (Fig 1).

These are important to consider since most of the environmental exposures to...
estrogenic compounds occur at low levels and for prolonged periods of time. The deleterious effects on the nervous, cardiovascular and reproductive systems described here are based on the generation of oxidative stress and other mechanisms. If the underlying mechanism (oxidative stress) appears as a constant feature for all these effects, it should be possible to correct all of them using antioxidants.

Acknowledgement:

Work presented here from the MohanKumar laboratory was supported by NIH AG 027697 to PSM. We would like to thank Ms. Stephanie Pfeiffer, Educational resource center, UGA for preparing the figure and Ms. Elyssa Jacob for her editorial assistance.

Declaration of Interests:

The authors declare that they have no conflicts of interest.
References:
Blessing WW, Sved AF & Reis DJ 1982 Hypertension with elevated plasma vasopressin after lesions of noradrenergic neurons in the rabbit medulla oblongata. Trans Assoc Am Physicians 95 79-85.

Block ML & Hong JS 2007 Chronic microglial activation and progressive dopaminergic neurotoxicity. Biochem Soc Trans 35 1127-1132.


Cholanian M, Krajewski-Hall SJ, McMullen NT & Rance NE 2015 Chronic oestradiol reduces the dendritic spine density of KNDy (kisspeptin/neurokinin B/dynorphin) neurones in the arcuate nucleus of ovariectomised Tac2-enhanced green fluorescent protein transgenic mice. J Neuroendocrinol 27 253-263.

Cooper C, Stakkestad JA, Radowicki S, Hardy P, Pilate C, Dain MP & Delmas PD 1999 Matrix delivery transdermal 17beta-estradiol for the prevention of bone loss in


Gilbreath ET, MohanKumar SM, Balasubramanian P, Agnew DW & MohanKumar PS 2014 Chronic exposures to low levels of estradiol and their effects on the ovaries and reproductive hormones: Comparison with aging. *Endocr Disruptors (Austin)* **2**.


Kasturi BS, MohanKumar SM, Sirivelu MP & MohanKumar PS 2009 Chronic exposure to low levels of oestradiol-17beta affects oestrous cyclicity, hypothalamic norepinephrine and serum luteinising hormone in young intact rats. *J Neuroendocrinol* 21 568-577.


Kohama SG, Brown SA, Finch CE & McNeill TH 1992 Chronic estradiol administration did not cause loss of hypothalamic LHRH or TIDA neurons in young or middle-aged C57BL/6J mice. Brain Res 574 341-344.


Long JA & Evans HM 1922. The oestrous cycle in the rat and its associated phenomena. In Memoirs of the University of California, pp. 5-137.

Loomis ED, Sullivan JC, Osmond DA, Pollock DM & Pollock JS 2005 Endothelin mediates superoxide production and vasoconstriction through activation of NADPH

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


Luine VN, Richards ST, Wu VY & Beck KD 1998 Estradiol enhances learning and memory in a spatial memory task and effects levels of monoaminergic neurotransmitters. *Horm Behav* 34 149-162.


Rance N, Wise PM, Selmanoff MK & Barraclough CA 1981 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 108 1795-1802.


Stocker SD, Meador R & Adams JM 2007 Neurons of the rostral ventrolateral medulla contribute to obesity-induced hypertension in rats. *Hypertension* 49 640-646.


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


Wise PM 1984 Estradiol-induced daily luteinizing hormone and prolactin surges in young and middle-aged rats: correlations with age-related changes in pituitary responsiveness and catecholamine turnover rates in microdissected brain areas. *Endocrinology* 115 801-809.


Young CN & Davisson RL 2015 Angiotensin-II, the Brain, and Hypertension: An Update. *Hypertension* 66 920-926.


Table 1:
Effects of chronic administration of different estrogenic preparations on TIDA activity in animal models.

Table 2:
Chronic administration of various estrogenic preparations and their impact on cardiovascular functions.

Table 3:
Effects of various estrogenic preparations administered on a chronic basis on behavioral indices.

Figure 1 Legend:
Schematic above depicts the effects of chronic low dose E2 exposure on three distinct brain regions: The hypothalamus, brain stem and the limbic system (hippocampus and amygdala). E2-induced glial activation may play a role in both the hypothalamus and the brainstem. Effects on the hypothalamus involve activation of cytokines and oxidative stress-related pathways leading to nitration of tyrosine hydroxylase that results in reduction in NE and DA levels leading to ovulatory failure and mammary/pituitary tumors respectively. Effects on the brain stem involve increasing ET-1 levels and generation of free radicals that may promote endothelial dysfunction or increased sympathetic nerve activity leading to hypertension. Effects on the hippocampus and amygdala may include reduction in BDNF levels and monoamines leading to anxiety.
Schematic above depicts the effects of chronic low dose E2 exposure on three distinct brain regions: The hypothalamus, brain stem, and the limbic system (hippocampus and amygdala). E2-induced glial activation may play a role in both the hypothalamus and the brainstem. Effects on the hypothalamus involve activation of cytokines and oxidative stress-related pathways leading to nitrination of tyrosine hydroxylase that results in reduction in NE and DA levels leading to ovulatory failure and mammary/pituitary tumors respectively. Effects on the brain stem involve increasing ET-1 levels and generation of free radicals that may promote endothelial dysfunction or increased sympathetic nerve activity leading to hypertension. Effects on the hippocampus and amygdala may include reduction in BDNF levels and monoamines leading to anxiety.
<table>
<thead>
<tr>
<th>Species</th>
<th>Type of E2</th>
<th>Gonadectomy</th>
<th>Route</th>
<th>Dose</th>
<th>Duration</th>
<th>Age at exposure</th>
<th>Effects on TIDA neurons and Prolactin</th>
<th>Citation/PMID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female Holtzman rats</td>
<td>17β-estradiol (E2) or diethylstilbestrol</td>
<td>Yes</td>
<td>Subcutaneous infusion via silastic capsules</td>
<td>4 hours to 10 days</td>
<td>Adult hood</td>
<td>Prolactin mRNA in the anterior pituitary doubled on the 10th day of treatment. Pituitary prolactin levels paralleled mRNA levels until day 7 then decreased significantly at day 10. Serum prolactin levels also significantly increased at day 10 with fluctuations during earlier timepoints.</td>
<td>Lawson et al., 1993 8412486</td>
<td></td>
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<tr>
<td>Female rats</td>
<td>Estradiol benzoate</td>
<td>Yes</td>
<td>Subcutaneous infusion via silastic capsules</td>
<td>6, 12 and 18 days</td>
<td>Adult hood</td>
<td>Serum prolactin concentrations were markedly increased at all timepoints whereas the rate of DOPA accumulation was increased at 6 days but not at 12 days, and was decreased at 18 days. The concentration of DA in the median eminence was reduced at all timepoints.</td>
<td>Demarest et al., 1984 6504266</td>
<td></td>
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<tr>
<td>Female Sprague-Dawley rats</td>
<td>17β-estradiol</td>
<td>Yes</td>
<td>Subcutaneous infusion via silastic capsules and Intramuscular injections</td>
<td>40 µg - Once a week</td>
<td>7 weeks</td>
<td>Adult hood</td>
<td>Subcutaneous or i.m. administration of estrogen for 7 weeks induced a marked hyperprolactinemia with no detectable changes in THmRNA in the arcuate nucleus. However, 30 weeks after estrogen withdrawal, TH neuron numbers were back to normal and serum prolactin returned to basal levels.</td>
<td>Morel et al., 2009 19744546</td>
</tr>
<tr>
<td>Female Wistar rats</td>
<td>17β-estradiol</td>
<td>Yes</td>
<td>ICV infusion</td>
<td>1 µm using Alzet osmotic minipumps</td>
<td>1, 3 and 7 days</td>
<td>Adult hood</td>
<td>While serum prolactin and prolactin mRNA increased initially at 3 days, there was no change at 7 days of E2 treatment. Similarly, DOPAC/DA ratio was significantly higher at day 1, but no longer different at 3 and 7 days of treatment.</td>
<td>Maeda et al., 1996 8957741</td>
</tr>
<tr>
<td>Study</td>
<td>Treatment</td>
<td>Route</td>
<td>Vehicle</td>
<td>Dose</td>
<td>Duration</td>
<td>Timing</td>
<td>Outcome</td>
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<tr>
<td>Mohankumar et al., 2011</td>
<td>Female Sprague-Dawley rats</td>
<td>17β-estradiol</td>
<td>No</td>
<td>Subcutaneous slow release pellets</td>
<td>20ng/day</td>
<td>30, 60 or 90 days</td>
<td>4-5 months old</td>
<td>Chronic estrogen treatment increased GFAP, IL-1β and nitrate levels in the arcuate nucleus and increased nitration of TH in the median eminence. This was associated with increase in serum prolactin levels.</td>
</tr>
<tr>
<td>Kohama et al., 1992</td>
<td>Female C57BL/6J mice</td>
<td>17β-estradiol</td>
<td>No</td>
<td>Through drinking water</td>
<td>850 µg/kg/day</td>
<td>3 months</td>
<td>4 and 9 months old</td>
<td>Loss of cyclicity due to chronic E2 treatment was not associated with changes in the number of TIDA neurons in the arcuate nucleus.</td>
</tr>
<tr>
<td>Csakvari et al., 2008</td>
<td>Male and female CFY albino rats</td>
<td>17β-estradiol</td>
<td>Yes</td>
<td>Single dose by subcutaneous injection</td>
<td>45 µg/kg/day</td>
<td>24 hrs</td>
<td>3 months</td>
<td>In females, E2 treatment decreased the numerical density of GABAergic synapses on TH immunoreactive neurons, but not on nondopaminergic neurons, whereas in males, E2 treatment increased inhibitory synapses onto nondopaminergic neurons but did not affect the number of inhibitory terminals onto TH-IR neurons.</td>
</tr>
<tr>
<td>Species</td>
<td>Type of E2</td>
<td>Gonadectomy</td>
<td>Route</td>
<td>Dose</td>
<td>Duration</td>
<td>Age at exposure</td>
<td>Effects on cardiovascular function</td>
<td>Citation/PMID</td>
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<tr>
<td>Female SD rats</td>
<td>Ethinyl estradiol plus norgestrel</td>
<td>Oral gavage</td>
<td>Oral gavage</td>
<td>0.1 µg/day and 1.0 µg/day and 1.0 µg/day (Low EEN) and 10.0 µg/day respectively (High EEN)</td>
<td>6 weeks</td>
<td>12 weeks</td>
<td>Elevates SBP and endothelial dysfunction</td>
<td>Olatunji et al., 2016 27447455</td>
</tr>
<tr>
<td>ethynylestradiol</td>
<td>s.c. injection</td>
<td>0.2 µg/day</td>
<td>14 weeks</td>
<td>10-12 weeks</td>
<td>Elevates SBP</td>
<td>Geraghty BP et al., 1990 2170069</td>
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<tr>
<td>17b-estradiol</td>
<td>s.c. implant</td>
<td>0.05 mg and 0.1 mg</td>
<td>21 days</td>
<td>180 and 250</td>
<td>E2 replacement prevents cardiac hypertrophy and interstitial fibrosis induced by DOCA-salt administration.</td>
<td>Shenoy V et al., 2009 19747516</td>
<td></td>
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<tr>
<td>Chimeric mice</td>
<td>17b-estradiol</td>
<td>Yes</td>
<td>s.c. implant</td>
<td>5.6 µg/day</td>
<td>10 weeks</td>
<td>12 weeks</td>
<td>17β-estradiol is atheroprotective</td>
<td>Shelton KA et al., 2013 23395521</td>
</tr>
<tr>
<td>ApoE(-/-) mice</td>
<td>17b-estradiol</td>
<td>Yes</td>
<td>Oral</td>
<td>6 µg/day</td>
<td>12 weeks</td>
<td>11 weeks of age and E2 exposure at 12 weeks</td>
<td>E2 treatment reduces ROS levels and atherosclerosis progression in apoE(-/-) mice</td>
<td>Wing LY et al., 2009 19546345</td>
</tr>
<tr>
<td>ApoE(-/-) mice</td>
<td>17b-estradiol</td>
<td>Yes</td>
<td>On western diet</td>
<td>1.1 and 6µg/day</td>
<td>90 days</td>
<td>Ovariectomized at 28 days and E2 exposure at 42 days of age</td>
<td>Low dose increased and high dose decreased atherosclerotic plaque</td>
<td>Freudenberder T et al., 20177692</td>
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<tr>
<td>Model</td>
<td>Estradiol Type</td>
<td>Receptor Availability</td>
<td>Route</td>
<td>Initial Concentration</td>
<td>Duration</td>
<td>Treatment Duration</td>
<td>Notes</td>
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<tr>
<td>Cynomolgus macaques</td>
<td>estradiol</td>
<td>Yes</td>
<td>oral</td>
<td>1 mg/d micronized E2</td>
<td>8 months</td>
<td>1 month or 54 months</td>
<td>E2 treatment 1 month after ovx inhibits macrophage accumulation in carotid artery but not in the 54 month group</td>
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<tr>
<td>Female mice</td>
<td>17b-estradiol</td>
<td>Yes</td>
<td>s.c. implant</td>
<td>150-200 pg/ml</td>
<td>5,14, 28,49 days</td>
<td>9-11 weeks</td>
<td>E2 is able to suppress reactive gliosis</td>
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<tr>
<td>C57BL/6 mice</td>
<td>17b-estradiol</td>
<td>No</td>
<td>s.c. implant</td>
<td>0.21 mg/day</td>
<td>3 months and 1 month</td>
<td>17 months - every month until 20 months of age; 17 months - implanted once at 20 months of age</td>
<td>Chronic E2 improved infarct outcomes in female mice but acute treatment did not.</td>
<td></td>
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</table>

Sophonsritsuk A et al., 2013
23615645

McAsey et al., 2006
16226751

Liu F et al., 2012
22053957
<table>
<thead>
<tr>
<th>Species</th>
<th>Type of E2</th>
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<th>Effects on cardiovascular function</th>
<th>Citation/PMID</th>
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<tr>
<td>Sprague-Dawley female rats</td>
<td>17β-estradiol (E2)</td>
<td>No</td>
<td>Subcutaneous implantation of slow release pellets</td>
<td>1.8 µg pellets which release 20 ng per day</td>
<td>90 days</td>
<td>4-5 months</td>
<td>Increases anxiety like behavior in open field test (OFT), No change in elevated plus maze test (EPM)</td>
<td>Balasubramian et al., 2014 24361909</td>
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<tr>
<td>Sprague-Dawley male and female rats</td>
<td>17β-estradiol, ERα agonist propyl pyrazole triol (PPT), ERβ agonist diarylpropionitrile (DPT)</td>
<td>Yes</td>
<td>Daily subcutaneous injections</td>
<td>17β-estradiol-0.25mg/kg, PPT and DPT-1mg/kg</td>
<td>4 days</td>
<td>60-90 days</td>
<td>DPN and E2-treated females reduced anxiety behavior in EPM and OFT test. PPT increased, anxiogenic behaviors such as the number of fecal boli and time spent grooming in females. DPN produced similar anxiolytic effects in male rats too.</td>
<td>Lund et al., 2005 15514081</td>
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<tr>
<td>Sprague-Dawley female rats</td>
<td>Estradiol benzoate</td>
<td>Yes</td>
<td>Subcutaneous injection</td>
<td>10 µg/kg</td>
<td>3 hours</td>
<td>Adult</td>
<td>Anxiogenic effects in EPM</td>
<td>Mora et al., 1996 90444444</td>
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<tr>
<td>C57Bl/6 N female mice</td>
<td>17β-estradiol, ERα agonist propyl pyrazole triol (PPT), ERβ agonist diarylpropionitrile</td>
<td>Yes</td>
<td>Subcutaneous injection</td>
<td>E2- 0.025 and 0.25 mg/kg, other drugs 1mg/kg BW</td>
<td>2 hours</td>
<td>3 months and 3 weeks</td>
<td>E2 produced dose dependent changes Low dose- Anxiogenic effects in OFT High dose-Anxiolytic effects in EPM GPR30 agonist-anxiogenic in EPM and OFT, No changes with PPT or DPT treatment</td>
<td>Kastenberger et al., 2012 22143579</td>
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<tr>
<td>Study</td>
<td>Treatment</td>
<td>Route</td>
<td>Dose</td>
<td>Duration</td>
<td>Effect</td>
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<td>Bekku et al., 2005</td>
<td>17β-estradiol</td>
<td>Subcutaneous injection</td>
<td>0.3, 1.5, 3, 15 and 30 µg/kg/day</td>
<td>14 days</td>
<td>Anti-depressive effect at 15 and 30µg dose-Shortened the duration of immobility in forced swimming test. Increase in uterine weight</td>
<td>ICR albino female mice</td>
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<td>Walf et al., 2010</td>
<td>17β-estradiol</td>
<td>Subcutaneous injection</td>
<td>10µg</td>
<td>1 hour</td>
<td>Decreased anxiety and depression behavior in open field, mirror chamber, light-dark transition and forced swim test. No changes in motor activity and coordination</td>
<td>Congenic C57BL/6 background female mice</td>
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<td>Walf et al., 2009</td>
<td>17β-estradiol</td>
<td>Subcutaneous injection</td>
<td>0.09 mg/kg once weekly</td>
<td>14 weeks</td>
<td>Anti-anxiety and depressive effect-More time on open arms in elevated maze test and less immobile time in forced swim test</td>
<td>Long-Evans female rats (not clearly mentioned, but this group used LE rats in their previous studies)</td>
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<tr>
<td>Walf et al., 2009</td>
<td>17β-estradiol</td>
<td>Chronic subcutaneous infusion via silastic capsules</td>
<td>0.03 and 0.09 mg/kg</td>
<td>Exp 1: 2 and 6 months Exp 2: 3.5 months</td>
<td>Experiment 1: Increase in incidence of mammary tumors and uterine weight Experiment 2: Increased sexual behavior and enhanced sexual receptivity (i.e., lordosis in response to mounting by a male rat)</td>
<td>Long-Evans female rats</td>
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<tr>
<td>Strain</td>
<td>Treatment</td>
<td>Route</td>
<td>Dose</td>
<td>Duration</td>
<td>Age</td>
<td>Outcome</td>
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<tr>
<td>C57BL/6 female mice</td>
<td>E2, ERα agonist propyl pyrazole triol (PPT); or ERβ agonist diarylpropionitrile (DPT)</td>
<td>Subcutaneous injection</td>
<td>10 µg/kg or 30 µg/kg/day</td>
<td>4 weeks</td>
<td>Adult</td>
<td>E2 (both doses) and ERβ agonist (high dose only) reversed depression like behavior in sucrose preference test. Similarly, E2 and ERβ agonist at high dose reduced anxiety like behavior in EPM and OFT. ERα agonist had no effect on behavior.</td>
<td>Xu et al., 2016 26928197</td>
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<tr>
<td>C57BL/6 female mice</td>
<td>Estradiol benzoate</td>
<td>Chronic subcutaneous infusion via silastic capsules</td>
<td>25,50 or 75 µg in 0.03ml sesame oil</td>
<td>10 days</td>
<td>8-9 weeks</td>
<td>Increase in fear and anxiety in EPM, OFT, Dark and light transition test and conditioned fear learning test. Dose dependent increase in uterine weight</td>
<td>Morgan et al., 2001 11716576</td>
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<tr>
<td>Sprague-Dawley female rats</td>
<td>17β-estradiol</td>
<td>Subcutaneous injection (4 weeks after OVX)</td>
<td>30 µg/day</td>
<td>7 days</td>
<td>Adult</td>
<td>Reversed OVX-induced depressive behavior in forced swimming test. Increased number of crossings and rearings in OFT.</td>
<td>Li et al., 2014 24710472</td>
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<tr>
<td>Female Wistar rats</td>
<td>17β-estradiol</td>
<td>Chronic subcutaneous infusion via silastic capsules</td>
<td>2 capsules with 400 µg/8 µl in each capsule</td>
<td>30 days</td>
<td>Young (Y)—3 months old; adult (AD)—7–8 months old; and middle-aged (MA)—12–13 months</td>
<td>Improved spatial reference memory of adult and middle-aged rats and anti-depressive behavior in young and adult rats in forced swimming test</td>
<td>Kiss et al., 2012 22085882</td>
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<tr>
<td>Study</td>
<td>Treatment</td>
<td>ND</td>
<td>Route</td>
<td>Dosage</td>
<td>Follow-up</td>
<td>Age Range</td>
<td>Cognitive Function Test</td>
<td>Outcome</td>
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<tr>
<td>Heart and Estrogen/progestin Replacement Study (HERS) involving 2763 women with coronary disease</td>
<td>Conjugated estrogen (0.625 mg) plus medroxyprogesterone acetate (2.5 mg)</td>
<td>ND</td>
<td>Oral</td>
<td>One tablet daily</td>
<td>Mean age of 71 +/- 6 years</td>
<td>Cognitive function test: Women assigned to hormone replacement scored worse on the Verbal Fluency test compared to placebo group</td>
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<tr>
<td>Women's Health Initiative Memory Study</td>
<td>Conjugated equine estrogen (CEE)</td>
<td>Yes-Prior Hysterectomy</td>
<td>Oral</td>
<td>One tablet daily (0.625 mg of CEE)</td>
<td>Mean follow-up of 5.4 years</td>
<td>65 to 79 years</td>
<td>Global cognitive function test: Women assigned to CEE scored less in Modified Mini-Mental State Examination (3MMSE) than the placebo group. The effect was more pronounced among women with lower cognitive function at baseline</td>
<td></td>
</tr>
<tr>
<td>Women's Health Initiative Memory Study</td>
<td>Conjugated equine estrogen alone or Conjugated estrogen (0.625 mg) plus medroxyprogesterone acetate (2.5 mg)</td>
<td>ND</td>
<td>Oral</td>
<td>One tablet daily</td>
<td>Mean follow-up of 5.21 years</td>
<td>65 to 79 years</td>
<td>Estrogen alone and estrogen plus progestin resulted in increased risks for dementia</td>
<td></td>
</tr>
</tbody>
</table>

Grady et al., 2002 12459399
Espeland et al., 2004 15213207
Shumaker et al., 2004 15213206
<p>| Early versus Late Intervention Trial (ELITE) | 17β-estradiol | Both intact and women with oophorectomy were involved | Oral | Once daily | 2-5 years + additional 2.5 years | Early initiators scored significantly higher than late initiators on the MMSE, Early initiators were significantly faster than never users on the TMTA (Attention and Concentration Tasks) | No difference between early and late groups on verbal memory, executive functions, or global cognition | Henderson et al., 2013 24277815 |</p>
<table>
<thead>
<tr>
<th>Study Description</th>
<th>Hormone Treatment</th>
<th>Treatment Duration</th>
<th>Research Outcomes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cognitive follow-up study of women enrolled in the Melbourne Women’s Midlife Health Project</td>
<td>Hormone therapy</td>
<td>~11 years</td>
<td>fMRI-perimenopausal HT users showed increased activation in the left hippocampus and decreased activation in the parahippocampal gyrus bilaterally compared with never users in the verbal recognition task. Each of these patterns of activation was associated with better memory performance on the imaging memory task.</td>
<td>Maki et al., 2011 21078303</td>
</tr>
<tr>
<td>Cognitive and Affective Study (KEEPS-Cog) of the Kronos Early Estrogen Prevention Study (KEEPS)</td>
<td>17β estradiol in the transdermal product and estrone in oral conjugated equine estrogens (CEE)</td>
<td>Upto 4 years</td>
<td>No effect on cognitive outcomes, women treated with o-CEE showed improvements in depression and anxiety symptoms. No effect of transdermal E2 on mood</td>
<td>Gleason et al., 2015 26035291</td>
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<td></td>
<td>Oral and transdermal</td>
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**ND** = Not defined.
| Women's Health Initiative Memory Study of Younger Women (WHIMSY) | CEE with or without medroxyprogesterone acetate | ND | Oral | 0.625 mg CEE with or without 2.5 mg medroxyprogesterone acetate | Mean of 7 years | Postmenopausal women aged 50 to 55 years | No overall sustained benefit or risk to cognitive function in women on CEE based therapies | Espeland et al., 2013 23797469 |