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# THE TIMING AND DURATION OF ESTRADIOL ON SOCIAL BEHAVIOR AND GENE EXPRESSION IN MATURE ADULT AND AGING FEMALE RATS

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**by**

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# Dedication

I would like to dedicate this dissertation to my parents Pablo and Cynthia Garcia. Thank you for always pushing me to do my best and for never letting me give up. Your support through everything has made it possible for me to get to where I am today and for that I am eternally grateful. I love you both more than you will ever know and I am glad that I was able to accomplish this goal with you both by my side.

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# THE TIMING AND DURATION OF ESTRADIOL ON SOCIAL BEHAVIOR AND GENE EXPRESSION IN MATURE ADULT AND AGING FEMALE RATS

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Menopause causes declines in ovarian hormones such as estrogens and progesterone. During the menopausal transition, women may experience adverse symptoms such as anxiety, depression and lack of desire to interact. However, since the publication of the Women's Health Initiative (WHI) many women have been left asking questions about whether, when, and how long to take hormone treatments for menopausal symptoms. This dissertation focuses on the effects of timing and duration of estradiol ($E_2$) relative to loss of ovarian hormones (OVX) on the social brain and behavior in mature adult (MAT) and aging (AG) female rats. The results from these studies illustrated that in MAT rats, 3-months of $E_2$ given immediately following OVX decreased communications and social interactions between cagemates, as assessed in a test quantifying the number of ultrasonic vocalizations emitted, as well as time spent interacting with one another. I interpret this result to mean that the $E_2$ animals had improved social memory. In MAT rats gene expression of the neuropeptides (Oxt, Avp, Oxtr, Avpr1a) involved in regulating social behavior were also greater in the BNST, MeA, and SON of rats treated with estradiol

compared to vehicle. Because menopause typically happens at middle age, I conducted a similar experiment in middle-aged rats to assess the influence of estradiol deprivation or replacement in aging rats. Aging animals were behaviorally characterized twice (at 3 and 6-months), groups were given different timing and duration of estradiol or vehicle treatments as a model for the WHI. Similar to the MAT animals, the estradiol-treated AG rats called less than their vehicle counterparts during an ultrasonic vocalization test conducted at the 3-month testing period. However, at the 6-month testing period no differences were observed, which could be the result of aging. Oxt, Oxtr, Avp and Avpr1a showed unique gene expression patterns that were dependent upon the timing and duration of estradiol in a region-specific manner in the AG rats. Most genes were up-regulated by estradiol treatment, irrespective of timing or duration. Overall this work may benefit the field of women's health through new knowledge on consequences of age, and the timing and duration of estrogen treatment, on the social brain and behavior.

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# CHAPTER 1: GENERAL INTRODUCTION

Reproductive aging in females is highly variable in mammalian species. In humans, reproductive aging is a continuous process that begins at birth, due to a finite number of ovarian follicles, and extends through the menopausal transition when the follicular reserve is entirely depleted. The loss of follicles during this perimenopausal period causes a profound decrease in the ovarian steroid hormones, especially estradiol and progesterone. These changes lead not only to physiological symptoms but also to psychological changes.

Currently the mechanisms behind the reproductive senescence process are not entirely understood. Reproductive senescence in mammals involves changes to all three levels of the reproductive axis, which comprises the brain (especially hypothalamus), anterior pituitary gland, and ovaries. The ovarian hormonal changes during menopause are sometimes associated with an array of behavioral, psychological, and neurological symptoms. For example, a subset of perimenopausal women report increased anxiety, depression and lack of interest in social interaction (Freeman et al., 2004; Schmidtet et al., 2004; Bromberger et al., 2011). Understanding how the hormonal changes that are occurring during this transition cause behavioral changes is a growing area of research. This introduction will provide a brief background on the physiological and psychological changes that occur during aging and reproductive senescence in mammalian species. Also, part of the introduction is dedicated to describing behavioral tasks that are used to evaluate social behavior in animal models.

# MENOPAUSE

## *Menopause in women*

In women the end of reproductive aging is marked by the transition to menopause, during which neurobiological and peripheral systems begin to experience profound changes. All women will experience menopause, either naturally or surgically. The perimenopausal transition typically occurs during the 5$^{th}$ decade of life (Wise et al., 2002). A women's reproductive life can be subdivided into 3 phases. The first, premenopausal, is the period when a woman experiences regular menstrual cycles. The second phase is referred to as perimenopausal, the transition period during which women begin to have irregular menstrual cycles. Lastly there is the postmenopausal phase, defined as one year past the last menstrual cycle. It is typically during the perimenopausal period and the first few years of the postmenopausal phase that women experience menopausal neurobiological symptoms, particularly vasomotor dysfunction (hot flashes), insomnia and other sleep disturbances, mood changes, as well as affective and cognitive changes (Nelson, 2008; Benedetti et al., 2001). It is the hot flashes in particular that lead women to seek hormone replacement to improve quality of life.

Though the endocrinology of the menopausal transition is not yet completely understood, each level of the hypothalamic-pituitary-gonadal axis (HPG axis) plays a significant role in this process (Nelson et. al., 1995, Wise et. al., 1996). The ultimate marker of menopause is the depletion of ovarian follicles that leads to low levels of circulating estradiol (Saal et al., 1994). However, the transition to menopause is a gradual process. Gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus regulate

both follicle stimulating hormone (FSH) and luteinizing hormone (LH) concentrations; FSH is also regulated by ovarian inhibins and activins (Maffucci and Gore, 2006; Yin and Gore, 2006; Padmanabhan & Sharma, 2001). Elevated levels of FSH as well as decreased levels of inhibin B and anti-Mullerian hormone are thought to be indicators for the start of the transition from premenopausal to perimenopausal (Reame et al., 1998; Sowers et al., 2008). As women age pituitary gonadotropin secretion changes lead to an increase in serum FSH and eventually elevated levels of basal LH (Fitzgerald et al., 1998; Morrison et al., 2006). These elevations are due to a loss of negative feedback from declining ovarian estrogens and progesterone (Kermath & Gore, 2012). As the transition progresses, ovarian follicular depletion takes place, which cause fluctuations in ovarian hormones such as estrogens and progesterone. Early in the perimenopausal transition women can have elevated estradiol levels (Santoro, 2005). However, as the ovarian follicles decline through the transition women begin to experience periods of severe fluctuations of estrogen and progesterone, leading to the low levels associated with the postmenopausal phase (Hall, 2004; Morrison et al., 2006). Levels of other hormones such as androgens and growth factors produced by the ovary also decline (Burger et al., 2000; Davison et al., 2005). Although this description applies to women who experience menopause naturally, approximity one-quarter of a million women in the U.S. undergo surgical menopause by hysterectomy and/or oophorectomy (Henderson & Sherwin, 2007). In these women the neuroendocrine changes due to hormone deprivation occur much more precipitously, which can lead to more severe menopausal symptoms (Finch et

al., 2011). Table 1.1 shows a break down of the stages of the natural menopausal transition.

Table 1.1

| Phases | **Premenopausal** | | **Perimenopausal** | | **Postmenopausal** | |
|---|---|---|---|---|---|---|
| | *Early* | *Late* | *Early* | *Late* | *Early* | *Late* |
| Duration | Variable | Variable | Variable | ~1 - 3 years | ~2 - 6 years | Until death |
| Menstrual Cycle | Regular | Small Changes in Flow & Length | Irregular Longer length between cycles Greater fluctions in flow | Periods of amenorrhea Transition after 1 year of amernorrhea | None | None |
| Levels of E & P | Regular | Fluctuations from High to low | Fluctuations from High to low | Dramatic Fluctuations Low | Very Low | Very Low |
| Menopausal Symptoms | No | No | No | Yes | Yes | Variable |

Table 1: Phases of reproductive aging in women

This table demonstrates some of the changes that women experience during the menopausal transition. Parts of this table were adapted from the 2012 STRAW report on the stages of reproductive aging by Harlow and colleagues.

### *Rodent models of menopause*

The use of animal models to study the effects of menopause and to identify potential therapeutic treatments of adverse symptoms is vital for improving women's health. Studying reproductive aging in women is particularly difficult because it typically requires longitudinal work often over months or years. In addition it is not possible to examine the molecular and cellular changes that occur in the brain during menopause in anything other than postmortem tissue. The use of animals is also beneficial because

researchers are able to control experimental variables that cannot be controlled in humans. The two most commonly used animals to study this topic are non-human primates and rodents. Research using both of these species has contributed to a better understanding of how the endocrine system changes with age and loss of sex steroid hormones as well as mechanisms involved in behavioral and cognitive changes. Unfortunately primate studies have many of the same problems as work in humans. Menopause in the most common monkey models, e.g. rhesus macaques, does not happen until animals are nearly 30 years of age. Not only are these animals difficult to obtain; the work can be prohibitively expensive.

Therefore, rat models, while differing in some aspects of reproductive aging from humans, provide several advantages. The HPG axis in rats is similar to that of humans in many ways and is quite comparable in neuroendocrine changes that occur during the reproductive aging process (Gore, 2002). One major difference is that rats do not experience ovarian failure; rather, they undergo neuroendocrine changes at middle age that lead to a loss of drive from hypothalamus to pituitary to ovary. More specifically, at around 11-12 months of age rats begin to have irregular cycles, with estrous cycles transitioning from the typical 4-5 day pattern to a pattern of irregular cycling. Eventually, rats stop having estrous cycles altogether and either present as persistent estrus or diestrus (Kermath & Gore, 2012). Rats do not see a drastic loss of steroid hormones like women do during their transition, which can limit the utility of rodents as a model of natural menopause. Therefore, investigators have turned to a model of surgical menopause, using ovariectomy (OVX). The OVX model mimics the dramatic loss of ovarian follicles and

ovarian hormones that are seen in women who experience menopause by hysterectomy and/or oophorectomy. This model has proven valuable in understanding the role of steroid hormone loss on vascular function, osteoporosis/osteopenia, cognitive decline, affective disorders and social behavior (Castillo et al., 2005; Perez-Martin et al., 2005; Namkung-Matthai et al., 2001; Stoffel & Craft, 2004; Garcia et al., 2016).

## THE WOMEN'S HEALTH INITIATIVE (WHI)

In 1991 the National Institutes of Health started a research program called the Women's Health Initiative (WHI). The WHI lasted 15 years and was created in order to address the major causes of death, disability and poor quality of life in postmenopausal women. It is considered one of the largest randomized clinical studies to date and also one of the most controversial (Harman et al., 2006; Bestul et al., 2004; Brown, 2012). The WHI's clinical trial had three components that focused on hormone replacement therapy (HRT), dietary modification, or calcium/vitamin D supplementation. I will limit this section to the clinical trial, designed to study the effects of HRT on cardiovascular disease, osteoporosis, breast cancer, and cognition. Women with a uterus were put into the estrogen plus progesterone study in which they were given a daily estrogen [conjugated equine estrogen (Premarin)] plus progesterone [medroxyprogesterone acetate (Prempro)] tablet, or a placebo (Rossouw et al., 2002). Women without a uterus were put into the estrogen-alone study and were given either daily estrogen [conjugated equine estrogen (Premarin)] or placebo tablet (Anderson et al., 2004). Both the estrogen alone and the estrogen plus progesterone arms of the study were terminated early because the

studies found small but significantly increased risk of stroke, breast cancer, coronary heart disease, and blood clots (Alving, 2004).

The termination of the WHI left women and physicians with questions about whether, when, and for how long to take hormone treatments for menopausal symptoms. Though the WHI initially reported that there was a small but significant increase in adverse incidents in women taking hormone therapy, reanalysis suggested that there might be a critical window post-menopause during which hormone treatment is beneficial to neurobiological and other health-related endpoints. This is also supported by a limited amount of animal research showing that the timing of hormone treatment relative to OVX has significant effects on neurobiological endpoints, shown to date for basal forebrain cholinergic functions, spatial memory, hippocampal spine maintenance, and gene expression of NMDA receptors in the hypothalamus and hippocampus (Neal-Perry et al., 2005, Ottem et al., 2004, Chakraborty et al., 2003, Maffucci et. al., 2009). Therefore, it is critical to understand the risks and benefits of estrogen treatments in the context of timing and duration for women to be able to make informed decisions and to improve quality of life.

## SOCIAL BEHAVIOR

Social behavior is an area of research that is understudied in the context of menopause and HRT despite evidence that perimenopausal women experience increased anxiety, depression, and decreased desire to interact socially (Uguz et al., 2011; Deeks and McCabe, 2004; Lanza di Scalea et al., 2012; Schmidt et al., 2000). We know from

previous literature that estrogens, especially estradiol (E2), regulate these behaviors in animals as well as women (de Kloet et al., 2005; Klenerova et al., 2009; Meyer-Lindenberg et al., 2011; Rubinow, Schmidt & Roca, 1998). The use of animal models of menopause can provide a deeper insight into how sex steroid hormones regulate social behavior, and help determine underlying mechanisms involved in behavioral regulation. This section will cover the literature on the neurobiology of social behavior and current behavioral test used to examine social behavior in animals. Later in this chapter, I will review the limited research that has been conducted examining the effects of age on social behavior as well as estrogen.

***Neurobiology of social behavior: Oxytocin, vasopressin, and their receptors***

The literature examining the neurobiology of social behavior is primarily based on animal models, although there are a growing number of human studies. This section will focus on the neurobiology of social memory/recognition and social interaction based on the literature from mice, rats and humans.

Oxytocin (Oxt) and vasopressin (Avp) are two neuropeptides that have been extensively studied for their roles in social behaviors in animals and humans. In most mammalian species Oxt and Avp are synthesized in the magnocellular neurons of the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) of the hypothalamus. These neurons project to the posterior pituitary where they are released into the bloodstream to regulate water and electrolyte balance, milk letdown during lactation, and uterine contractions during parturition. In addition to these peripheral actions, Oxt and

Avp neural fibers project widely within the central nervous system (CNS). In rats and humans, Oxt is also produced in the parvocellular neurons of the PVN, which project to the olfactory bulb, dorsal and ventral hippocampus, the amygdala, the substania nigra, and nucleus of solitary tract (Buijs, 1978; Sofroniew, 1980; Swanson & Kuypers, 1980; Rinaman, 1998). In mice Oxt production has also been found in the bed nucleus of the stria terminals (BNST), medial preoptic area (mPOA), and the amygdala (Castel & Morris, 1988; Jirikowski et al., 1990; Wang et al., 1996). There are also Avp producing neurons in the POA, anterior hypothalamus, BNST, and medial amygdala (MeA) (Sofroniew, 1983; De Vries & al-Shamma, 1990; Moore & Lowry, 1998).

Actions of Oxt and Avp are mediated by specific receptors. Oxytocin receptor (Oxtr) mRNA and binding sites are abundant in the CNS, including in the PVN, MeA, BNST, ventromedial hypothalamus (VMH), central amygdala (CeA), shell of the nucleus accumbens, and the ventral subiculum of the hippocampus (Brinton et al., 1984; De Kloet et al., 1986; Tribollet et al., 1988; Tribollet et al., 1990; Yoshimura et al., 1993; Clipperton-Allen et al., 2012). Unlike Oxt, which has only one receptor, three receptors have been identified for Avp. The two Avp receptors involved in social behavior are vasopressin receptor 1a (Avpr1a) and vasopressin receptor 1b (Avpr1b); the third receptor vasopressin receptor 2 (Avpr2) is not abundant in the CNS. Avpr1a, the most abundant of the Avp receptors in the CNS, is detected in the olfactory bulb, hippocampus, suprachiasmatic nucleus (SCN), PVN, SON, amygdala, arcuate nucleus (ARC), substantia nigra, and nucleus of the solitary tract (Ostrowski et al., 1994; Szot et al., 1994; Arakawa & Deak, 2010). Avpr1b is predominately found in the anterior

pituitary, hippocampus (CA2 region), and the anterior amygdala (Young et al., 2006). These different populations and projections likely represent diverse actions of these neuropeptides on behaviors and neurobiological functions.

Studies in rats, mice and humans have demonstrated that Oxt, Avp, and their receptors play a significant role in regulating aspects of social behaviors such as social recognition and social interaction. If a male rat is exposed to a juvenile conspecific, and then given a low dose intracranial administration of Oxt 5 minutes following the first encounter, this leads to the experimental rat spending less time investigating the same juvenile conspecific during the second exposure 120 minutes later (Benelli et al., 1995). Both male and females also show impaired social recognition if given an Oxt antagonist (Benelli et al., 1995; Engelmann et al., 1998; Popik & Vetulani, 1991). Male and female rats also show increased social recognition when given peripheral injections of Avp (Bluthe et al., 1990; Bluthe and Dnatzer, 1990). However, research looking at the effects of Avp antagonist found that it impaired social recognition in female rats but not male rats (Englemann et al., 1998; Bluthe et al., 1990; Bluthe and Dnatzer, 1990). Studies have found that Avp is mainly responsible for male-typical social behaviors such as aggression and reproduction (Donaldson and Young, 2008). Research done using Oxt (OxtKO), Oxtr (OxtrKO), and Avpr1a (Avpr1aKO) knockout mice has illustrated how important these peptides are for social recognition. OxtKO, OxtrKO, or Avpr1aKO mice do not habituate to the repeated presentation of a familiar mouse unlike their wild-type counterparts (Choleris et al., 2003; Ferguson et al., 2000; Lee et al., 2008; Takayanagi et al., 2005; Bielsky et al., 2004). OxtKO and OxtrKO mice cannot distinguish between a familiar

mouse and a novel mouse, which illustrates a lack of social recognition (Takayanagi et al., 2005; Choleris et al., 2006). All three types of knockout mice have impaired social interaction (Egashira et al., 2007; Choleris et al., 2006; Pobbe et al., 2012).

Though the literature is more limited when it comes to research on Oxt and Avp in human social behavior, there are some studies demonstrating that these peptides do play such a role. In male and female adults Oxt has been found to increase approach behavior and positive affect, which leads to higher levels of trust (Heinrichs & Domes, 2008; Kosfeld et al., 2005). The authors speculated that this result was attributable to a reduced neuroendocrine stress response as well as through blockade of anxiety (Heinrichs & Domes, 2008). Trust is an important aspect of social affiliation and social approach in humans. A landmark study by Kosfeld and colleagues (2005) demonstrated that a single dose of intranasal Oxt in adult human subjects significantly increased trust in a trust game that required social interaction. 45% of the subjects who received Oxt reached the maximum trust level compared to the 21% in the placebo group. This study supports research that has been conducted in animals, that Oxt is necessary for prosocial approach behavior (Heinrichs & Domes, 2008). Additionally, Heinrichs and colleagues (2003) found that the combination of intranasal Oxt plus social support reduced cortisol levels as well as decreased anxiety and increased calmness in healthy men. In a double-blinded fMRI study in humans researchers found that intranasal Oxt led to increased tolerance to betrayal of trust as well as reduced activation in the amygdala, midbrain regions, and the dorsal striatum (Baumgartner et al., 2008). As in rodents, the effects of Avp on social behavior in humans appears to be sex specific. Intranasal Avp administration led women

to have increased perceived friendliness of a same-sex conspecific while in men it decreased this perception (Thompson et al., 2006). A study using fMRI by Zink and colleagues (2010) found that intranasal Avp had modulatory effect on the amygdala-medial prefrontal cortex circuit, with changes between the subgenual and supragenual cingulate cortices. It has been postulated that these changes reflect disinhibition of the amygdala because research has demonstrated that the medial prefrontal cortex regulates amygdala activity via negative feedback (Quirk et al., 2003).

***Behavioral tests to study social memory and social interaction in rodents***

The emergence of standardized behavioral testing in rodents has improved our understanding of how drugs, genetics, and various aspects of the environment affect specific behaviors, including learning, memory, depression, anxiety, mating, aggression, and social behavior. This section will focus on common behavioral paradigms and apparati used to study social memory and social interaction. In addition, I will discuss a novel paradigm designed to look at familiar female-to-female social interaction as well as how ultrasonic vocalizations can be used to measure social memory.

Tests of social memory and social interaction typically use either the habituation-dishabituation paradigm or a social discrimination paradigm. In the habituation-dishabituation, the experimental animal is presented with a novel conspecific either for a single trial (Thor & Holloway, 1982; Thor, 1980) or over multiple trials (Sánchez-Andrade & Kendrick, 2011; Choleris et al., 2003; Choleris et al., 2007; Spiteri & Ågmo, 2009). The conspecific is then removed for a period of time (minutes, hours, or days) and

then reintroduced to the experimental animal after the allotted time. If the animal has habituated to the conspecific, meaning it no longer views it as a novel, it should show decreased social interaction compared to its initial interaction with the conspecific. Then, the first conspecific is removed and a novel one is put in its place; this typically results in increased social interaction, termed dishabituation.

The social discrimination paradigm has two steps. In the first step the experimental animal is either presented with one conspecific or two conspecifics at the same time for a specified duration (single trial or over multiple trials). The conspecific or conspecifics are then removed for a period of time (minutes, hours, or days). For step 2 the familiar conspecific is reintroduced to the experimental rat along with a novel conspecific after having been separated for a specific duration of time (Reilly et al., 2015; Choleris et al., 2006; Engelmann et al., 1995). If the experimental animal spends more time with the novel conspecific it is demonstrating social memory because rats are inclined to investigate novel animals or objects over familiar ones.

A novel paradigm for studying social memory is proposed in this dissertation. The first step is to pair-house rats of the same sex, age, and hormone status for an allotted amount of time (for this dissertation, either 3 or 6 months). In step 2 the cagemates are separated from each other for a set amount of time, in my case, 1 week. Then, similar to the habituation-dishabituation paradigm, the cagemates are reintroduced to each other and social interaction is observed using a setup similar to the social discrimination paradigm. After the 1-week separation period, one of the cagemates is chosen to be the experimental rat and given a choice between her cagemate and a novel conspecific of the

same sex, age, and hormone status. Both of these paradigms allow for evaluation of social memory and social interaction.

Social memory tests typically take place in either the experimental animal's home cage (Bielsky et al., 2004; Ferguson et al., 2000), a neutral open field box (Gregus et al., 2005; Tõnissaar et al., 2004), or a sociability 3-chamber apparatus (Reilly et al., 2015; Crews et al., 2012; Felix-Ortiz & Tye, 2014; Riedel et al., 2009). When using the experimental animal's home cage or the open field box, the experimental and the conspecific(s) are allowed to freely interact with each other, which is not the case with the sociability apparatus. The difference between using the experimental animal's home cage and the open field box is that the experimental animal has established the home cage as its territory and is therefore more likely to show aggression, especially with male rodents, or in the resident intruder test (Rammal et al., 2010; Takayanagi et al., 2005). Using the open field box the researcher takes away the element of resident territory. The sociability test also removes the aspect of resident territory since both the experimental animal and the stimulus animal(s) are only habituated to the chambers for a short period of time (Reilly et al., 2015; Crews et al., 2012; Felix-Ortiz & Tye, 2014; Riedel et al., 2009). In this setup the experimental animal is allowed to freely move throughout the 3-chambers but the stimulus rats are placed into holding cages that only allow for interactions such as nose touching and anogenital investigation. The interactions that take place using this apparatus ensures that it is being done exclusively by the experimental rat.

Ultrasonic vocalizations (USVs) are a natural form of communication in rats that

have been shown to be effective in measuring aspects of social behavior and in assessing affective state. Ultrasonic vocalizations in adults can be broken down into two distinct types of calls 22-kHz or 50-kHz. When a rat is exposed to a predator (Blanchard et al., 1991), pain (Antoniadis and McDonald, 1999; Borta et al., 2006; Wöhr et al., 2005) or drug withdrawal (Miczek & Barros et al., 1996;) they emit long 22-kHz USVs, which are though to be a reflection of a negative affective state (Antoniadis and McDonald, 1999; Borta et al., 2006; Wöhr et al., 2005). Whereas 50-kHz calls are thought to be reflective of a positive affective state (Panksepp & Burgdorf, 2003) and are seen during juvenile play (Knutson et al., 1998; Lukas & Wöhr, 2015), social exploratory activity (Brudzynski & Pniak., 2002), and mating behavior (McGinnis et al., 2003; White et al., 1990). Previous research has shown that 50-kHz USVs can be used to study social motivation and social interest (Wöhr & Schwarting, 2007; Wöhr & Schwarting, 2009). Resident intruder studies have also shown that if a female rat is exposed to the same female intruder after separation intervals the resident will emit fewer USVs each time, indicative of social memory (D'Amato & Moles, 2001; Moles et al., 2007). In my dissertation, I will illustrate how USVs can be used to measure social memory in the context of familiar female-to-female interaction.

***Social behavior and estrogen***

Though the majority of research in mice and rats exclusively used males, research on females has shown that sex steroid hormones such as estrogens and progesterone are important for normal social behavior. Estrogen treatment promotes the synthesis of Avp,

Oxt and Oxtr mRNA, which decrease significantly after OVX (Patchev et al., 1995; Ho & Lee, 1992; Sarkar et al., 1992). Oxytocin mRNA is increased by $E_2$ treatment in the POA, and its receptor is increased in the MeA (Patisaul et al., 2003; Quiñones-Jenab et al., 1997; Caldwell et al., 1989). Estrogen regulates Oxt and Avp in the SON and PVN predominantly through the estrogen receptor beta [(ERβ, *Esr2*), Winslow and Insel, 2004; Hrabovszky et al, 1998] and in the BNST, mPOA, and MeA through both ERβ and estrogen receptor alpha [(ERα, *Esr1*), Axelson and van Leeuwen, 1990; Wang & De Vries, 1995; Quinones-Jenab et al., 1997; Bale, Pederson & Dorsa, 1995; Ferguson et al., 2002]. The fact that Oxt and Avp are modulated by estrogens leads to the hypothesis that with age the lack of estrogen may affect Oxt and Avp and in turn affect social behavior (Choleris et al., 2004, Ferguson et al., 2002, Winslow and Insel, 2004).

Previous research using female ERα knockout (ERαKO) and ERβ (ERβKO) knockout mice found that each of these lines have impaired social memory and social interaction (Choleris et al., 2003; Hlinak, 1993; Egashira et. al, 2007). However, other studies found that only the ERαKO mice showed impaired social memory (Sanchez-Andrade & Kendrick, 2011; Choleris et al., 2006). It has been postulated that this discrepancy is due to different experimental protocols such as the type of stimuli used, accessibility of social stimuli, testing environment as well as number of trials (Sanchez-Andrade & Kendrick, 2011). Similar deficits are seen in rats after OVX (Hlinak, 1993; Tang et al., 2005), and this is improved by estradiol replacement therapy (Hlinak, 1993; Tang et al., 2005) or estrogen plus progesterone (Spiteri & Agmo, 2009) treatments. As additional evidence for estradiol's roles, female mice have enhanced social recognition

during the proestrous phase of their estrous cycle, the stage when estrogens and progesterone are high (Walmer et al., 1992; Sanchez-Andrade & Kendrick, 2009). Also, when estrogen levels are high during the late phase of pregnancy or during lactation, the rats show decreased neophobia to approach pups or lactating nesting material (Numan & Sheehan, 1997; Kinsley & Bridges, 1990), and this is seen if OVX rats are given hormones to mimic the hormone levels seen during late pregnancy (Fleming et al., 1989).

***Social behavior and aging***

As people age social networks begin to narrow, social roles change, negative emotions become less frequent and there is an increase in meaningful relationships (Charles & Carstensen, 2010; Fung et al., 2001; Birditt & Fingerman, 2003). With older age people report lower levels of negative affect (Carstensen et al., 2000; Deiner & Suh, 1997; Mroczek & Kolarz, 1998) and lower incidents of anxiety and major depressive disorder (Piazza & Charles, 2006). After age 60 people often report upturns in depressive symptoms (Deiner & Suh, 1997; Haynie et al., 2001; Davey et al., 2004). However, during the menopausal transition and during the first few years of menopause women experience increased anxiety, depression, and decreased desire to interact socially even if no they have no prior history of affective disorders (Uguz et al., 2011; Deeks and McCabe, 2004; Lanza di Scalea et al., 2012; Schmidt et al., 2000). Studies examining post-mortem human brain tissue found that with normal aging there is an increase in numbers of Avp (although not Oxt) expressing neurons, as well as increased nucleolar size of these neurons in the SON and PVN of the hypothalamus. These changes are

suggestive of increased peptide production and enhanced neurosecretory activity during senescence (Swaab et al., 1985; Fliers et al., 1985a; Hoogendijk et al., 1985; Lucassen et al., 1993). It is important to note that the changes seen in these studies were only observed in tissue from subjects that were 80 years or older. Also, since sample sizes are small in these studies they did not separate the subjects by sex. Though a study conducted by Ishunina and Swaab (1999) examined the effects of age and sex on vasopressin and oxytocin neurons in the SON and PVN in post-mortem human brain tissue. They found that older women (>50 years old) had significantly larger Avp cell size than younger women (≤50 years old), age was also found to correlate with cell size in women. Though they did not take into account the women's hormone status at the time of death or whether or not they were taking hormone replacement therapy.

Animal studies have also demonstrated brain and behavior changes associated with increased age. A study conducted by Salchner and colleagues (2004) found that aged male rats (30 months old) spent 75% less time partaking in active social interaction compared to young rats (3 months of age). In a study using a rat habituation-dishabituation paradigm researchers found that during the dishabituation phase there was a progressive decrease in time spent investigating a novel rat from 3 to 15 to 22 months of age (Guan & Dluzen, 1994). In contrast, Boguszewski and Zagrodzka (2002) did not see age related changes in social interaction when they compared young (~4 months) and old (~24 months) male rats. To my knowledge there have been no studies to data looking at age related changes on social memory or social interaction in female rats. One of the aims in this dissertation was to fill this gap in the literature. In addition, the effects of age

on the neurobiology involved in regulation social behavior is mixed with some studies demonstrating that vasopressin protein and mRNa decrease with age (Dorsa & Bottemiller, 1982; Fliers et al., 1985b; Terwel et al., 1992; Balmagiya & Rozovski, 1983) while others have found that it increases (Rodeck et al., 1960; Watkins & Choy, 1980; Sladek et al., 1981). Though similar to the human literature rodent's studies do not see any effects of age on oxytocin protein or mRNA (Fliers et al., 1985b; Keck et al., 2000). Both humans and rodents studies have reported elevated basal AVP plasma levels with age (Miller, 1987; Terwel et al., 1992; Johnson et al., 1994).

## SUMMARY AND CONCLUSION

Overall, it is apparent that age and the loss of ovarian hormones have significant impacts on the brain and behavior in both humans and rodents. Unfortunately, animal models looking at the role of estradiol on the social brain and behavior have focused on short-term treatment and used relatively young animals. Since the loss of estrogen happens later in life and is influenced by the aging process it is vital that research be done using middle-aged or aged animals. In addition, it is important for clinical and basic research to address the findings and limitations of the WHI so that women and physicians can be better informed about the risk and benefits of HRT. A rigorous test of the "critical window" hypothesis is badly needed and is one of the main focuses of my dissertation.

## SUMMARY OF DISSERTATION EXPERIMENTS

This dissertation focuses on the effects of estrogen therapy on social behaviors,

and on molecular properties of brain regions involved in these behaviors such as the MeA, PVN, SON, BNST, and the PFC. These interconnected regions express estrogen receptors (ERα and ERβ), and hormones that are critical to social behaviors such as oxytocin and vasopressin, along with their receptors. However, little is known about the relationship between estrogen replacement therapy, aging, and social behaviors in women. Clinical studies have shown that menopause can negatively affect a woman's social interaction and personal relationships. However women who have a strong support system (close friendships and supportive families) do not report having increased depression and/or anxiety during the different stages of menopause. Insights into the mechanistic underpinnings of the relationship between estrogen replacement therapy and social behavior are lacking, underscoring the need for animal models. Therefore, the goal of this dissertation is to help fill this gap and to gain a better understanding of how timing and duration of hormone therapy can affect social interactions, together with underlying mechanistic effects on neurobiological systems.

Proposed experiments will utilize female rats at different life stages [young adult (mature) or aging (AG)], OVX to remove ovarian hormones, and administered physiological $E_2$ or vehicle with different timing and durations as a model for informing hormone treatments post-WHI.

***Overarching Hypothesis***

Test the hypothesis that the timing and duration of estrogen therapy affects social behaviors through biological and mechanistic changes that occur in selected brain regions

as a function of aging, estradiol treatment, and their interactions.

***Chapter 2 tested the hypothesis that timing and duration of estradiol treatment relative to deprivation (OVX) affects the expression of genes in hypothalamus (PVN & SON) of mature-adult (MAT) and aging (AG) rats.***

I examined the neural circuits that underlie social behaviors in order to gain mechanistic insight into the molecular changes that occur during aging and in response to timing/duration of $E_2$ treatment. This chapter is focused on gene expression work, looking at specific brain regions (SON & PVN) that are important in social behavior. My selected targets genes were OT and AVP, their receptors (Oxtr, Avpr1a), and ERα.

***Chapter 3 tested the hypothesis that estradiol treatment relative to deprivation has significant effects on social behavior and gene expression of mature-adult female rats.***

In this chapter I specifically used mature-adult rats, to develop and refine my novel social behavioral tests. Rats were behaviorally characterized using an ultrasonic vocalizations (USVs) test and a test of sociability. In all cases, the social partners were the experimental rat's cage-mate (same age/treatment group). After behavioral testing, rats were euthanized, brains were dissected, and used for gene expression with targets chosen based on their role in social behavior, estrogen regulation, and aging.

***Chapter 3 tested the hypothesis that estradiol treatment relative to deprivation has significant effects on social behavior and gene expression of aging female rats.***

In this chapter aging female rats were used in order to examine the effects of timing and duration of estradiol treatment. Rats were behaviorally characterized using an ultrasonic vocalizations (USVs) test and a test of sociability. Behavioral testing took place at two separate time points the first was after 3-months of treatment and the second after 6-months of treatment. In all cases, the social partners were the experimental rat's cage-mate (same age/treatment group). After behavioral testing, rats were euthanized, brains were dissected, and used for gene expression with targets chosen based on their role in social behavior, estrogen regulation, and aging.

# CHAPTER 2: TESTING THE CRITICAL WINDOW OF ESTRADIOL REPLACEMENT ON GENE EXPRESSION OF VASOPRESSIN, OXYTOCIN, AND THEIR RECEPTORS, IN THE HYPOTHALAMUS OF AGING FEMALE RATS.

The Text in this section is excerpted from Garcia, A. N., Depena, C. K., Yin, W., & Gore A.C., *Molecular and Cellular Endocrinology* 419: 102-112 (2016), with permission from the journal.

**ABSTRACT**

The current study tested the "critical window" hypothesis of menopause that postulates that the timing and duration of hormone treatment determine their potential outcomes. Our focus was genes in the rat hypothalamus involved in social and affiliative behaviors that change with aging and/or estradiol ($E_2$): *Avp, Avpr1a, Oxt, Oxtr*, and *Esr2* in the paraventricular nucleus (PVN) and supraoptic nucleus (SON). Rats were reproductively mature or aging adults, ovariectomized, given $E_2$ or vehicle treatment of different durations, with or without a post-OVX delay. Our hypothesis was that age-related changes in gene expression are mitigated by $E_2$ treatments. Contrary to this, PVN *Oxtr* increased with $E_2$, and *Avpr1a* increased with age. In the SON, *Avpr1a* increased with age, Oxtr with age and timing, and *Avp* was by duration. Thus, chronological age and $E_2$ have independent actions on gene expression, with the "critical window" hypothesis supported by the observed timing and duration effects.

## INTRODUCTION

Menopause is the physiological cessation of ovarian function during which menstrual cyclicity ceases and concentrations of ovarian hormones such as estrogens and progesterone decline. Hormone replacement therapy (HRT) is currently the most commonly used therapy to treat menopausal symptoms. However, since the termination of the Women's Health Initiative (WHI) study of conjugated equine estrogens and medroxyprogesterone acetate treatment, many women have been left asking questions about whether, when, and for how long to take hormone treatments for menopausal symptoms. The WHI initially reported that there was a small but significant increase in adverse incidents in women taking hormone therapy (Rossouw et al., 2002; Manson et al., 2013). Reanalysis suggested that there might be a critical window post-menopause during which hormone treatment is beneficial to neurobiological and other health-related endpoints in women (Klaiber et al., 2005; Bhupathiraju & Manson, 2014). Therefore, it is necessary to understand the risks and benefits of estrogen treatments in the context of timing and duration for women to be able to make informed decisions and to improve quality of life.

In addition to hot flashes, neurobehavioral changes such as anxiety and depression increase at menopause in humans, which can lead to social isolation and impact the quality of life (Uguz et al., 2011; Deeks & McCabe, 2004; Lanza di Scalea et al., 2012). Oxytocin (*Oxt*) and vasopressin (*Avp*) are modulators of these behaviors in mammalian species [de Kloet et al., 2005 (mice); Klenerova et al., 2009 (rat); Meyer-Lindenberg et al., 2011 (human); Lim & Young, 2006 (voles)]. Both of these neurohypophyseal

peptides are synthesized in the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) of the hypothalamus.

Vasopressin and oxytocin neurons are regulated by estrogens, predominantly through the estrogen receptor beta (ERβ) that is the dominant ER in these regions, as demonstrated in knockout mice (Winslow & Insel, 2004) and by gene and protein expression in rats (Hrabovszky et al, 1998). ERβ protein and mRNA is also colocalized with oxytocin- and to a lesser extent with vasopressin-expressing neurons of rats and mice (Hrabovszky et al., 1998; Alves et al., 1998; Patisaul et al., 2003). However, the effects of estradiol on the regulation of vasopressin and oxytocin are mixed, and most studies utilized short-term treatment regimes. Some studies showed up-regulation [Roy et al., 1999 (mRNA, monkey); Patisaul et al., 2003 (mRNA & protein, mouse)], others down-regulation [Shughrue et al., 2002 (protein, rat); Van Tol et al., 1988 (mRNA, rat); Nomura et al., 2002 (mRNA & protein, mouse)], and still others no effect [Peter et al., 1990 (mRNA, rat); Rhodes et al., 1981 (protein, rat); Akaishi & Sakuma, 1985 (protein, rat)] of estradiol treatment. This inconsistency in results was part of our motivation for conducting the work, and to hypothesize that the effects of estrogen would be influenced by different timings and durations of treatment.

Along with oxytocin and vasopressin, we also selected *Oxtr* and *Avpr1a* gene expression as endpoints because of their role in mediating effects of their respective nonapeptides on social behaviors such as anxiety and depression (Young, 1999; Bielsky et al., 2004; Sala et al., 2011). Previous studies using RT-PCR, *in situ* hybridization, immunohistochemistry and electrophysiology have shown that mRNA and protein of the

vasopressin receptor 1a and the oxytocin receptor are expressed in the SON and PVN of rats (Hurbin et al., 1998, 2002; Gouzènes et al, 1999; Yoshimura et al., 1993). This study was designed to help fill the gap in research and to gain mechanistic insight into the molecular changes that occur during aging and in response to differential modes of treatment to assess the effects of timing and duration of $E_2$. By studying outcomes of *Avp*, *Avpr1a*, *Oxt*, *Oxtr* and *Esr2* in the PVN and SON of the hypothalamus, we can have a better understanding of the neural substrates involved in social behavior as a basis for future work testing the "critical window" hypothesis on the behaviors themselves.

## MATERIALS AND METHODS

### *Animals and Husbandry*

All animal procedures were conducted in accordance with The Guide for the Care and Use of Experimental Animals following protocols approved by The University of Austin IACUC committee and NIH standards. Female Sprague Dawley rats (Harlan) were purchased as reproductively mature adults (MAT, ~3 months, sexually naïve) or aging adults (AG, ~11 months, retired breeders). These animals are the same as those used in a separate study (Yin et al., 2015) for analyses of different brain regions. Upon arrival, rats were pair housed on a 12-hour light, 12-hour dark cycle (lights on at 0700) and received water and food ad libitum. They were allowed to acclimate to the room for two weeks prior to surgery during which time estrous cyclicity was monitored daily by vaginal lavage of sterile saline. Only females with regular 4-5 day cycles were used. All rats received ovariectomy surgery under isofluorane inhalation anesthesia. A single

injection of Rimadyl (5 mg/kg) was given at the start of surgery. Bilateral dorsolateral incisions were made through the skin, muscle, and peritoneum, and the ovaries were ligated and removed. Muscles were sutured and wound clips used to close the skin.

Animals were randomly assigned to one of eight treatment groups as illustrated in Figure 1 to test different timings and durations of hormone treatment based on those used in the WHI, in which the women experienced an average 12-year delay in treatment relative to the last menstrual period. We calculated based upon the rats' life cycle compared to humans, that 3 months to an adult rat is equal to about 5 years in a woman (Sengupta, 2013; Quinn, 2005). Capsules containing either 100% cholesterol (Veh) or 5% 17β-estradiol / 95% cholesterol ($E_2$) were implanted subcutaneously between the shoulder blades at the time of surgery. Because the aim of this study was to address and reevaluate limitations of the WHI, we chose to use continuous exposure to $E_2$ at a physiologically relevant dosage to mimic the type of treatment given in the Estrogen-alone WHI studies (The Women's Health Initiative Study Group, 1998). This treatment regime is clinically relevant, as continuous E2 treatments are still in common use for subsets of postmenopausal women.

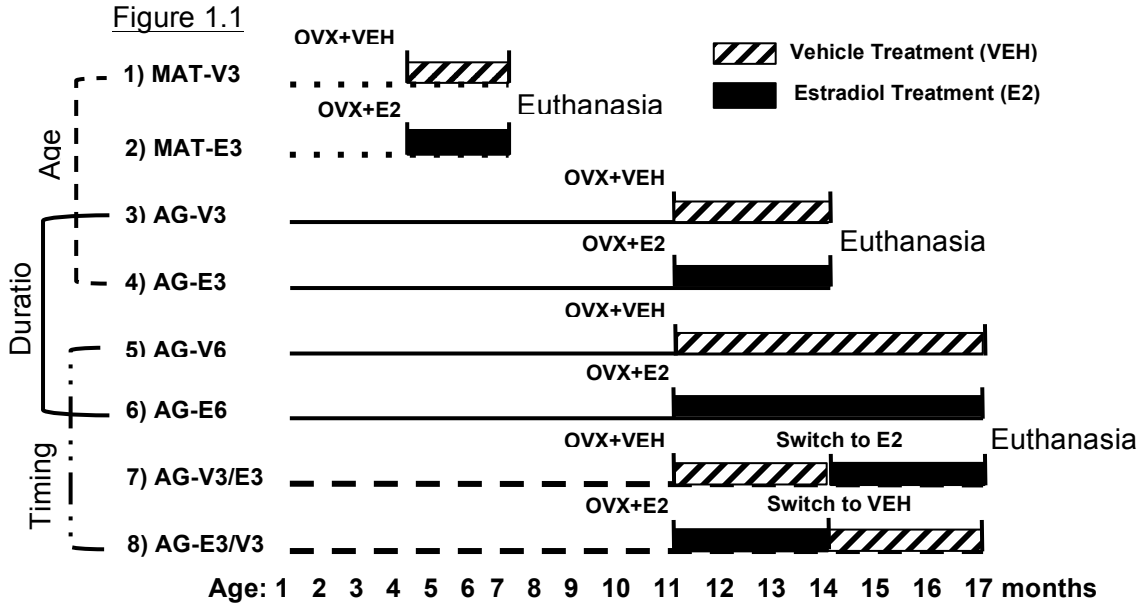


Figure 1.1: Treatment groups

The eight treatment groups are shown that were used to test age, and duration and timing of $E_2$ treatment. Reproductively mature adult (MAT) were 4 months old at the time of ovariectomy (OVX) surgery and aging rats (AG) were 11 months old at the time of OVX, and received either vehicle (cholesterol; VEH) or 17β-estradiol capsule implantation at the time of surgery. Both groups of MAT rats and two groups of AG rats received treatment for a duration of 3 months (Groups 1-4). Two groups of AG rats received treatment for duration of 6 months (Group 5 & 6). There were two AG "switch" groups that received either VEH or E2 initially at the time of OVX and were switched to the opposite treatment after 3 months, and continued on the new treatment for a 3-month duration (Group 7 & 8). This model enabled us to differentiate effects of **age** (Groups 1-4), **duration** (Groups 3-6) and **timing** (Groups 5-8).

***Tissue processing***

Rats were euthanized 3 or 6 months after OVX and hormone treatment, when mature adults (MAT) were ~7 months, and aging adults (AG) were ~14 or ~17 months of age. All animals were weighed and euthanized by rapid decapitation starting at 1330 hours with the last animal killed not later than 1600 hours; therefore they were all killed during the lights on period. Although there is a diurnal rhythm of vasopressin gene expression in the SCN, previous studies have shown that within in SON and PVN, the areas used in the present study, there is no diurnal change in vasopressin or oxytocin gene expression in rats (Uhl & Reppert, 1986, Burbach et al., 1988). In addition, when we used time of euthanasia as a covariate in statistical analyses, no time of day effect was found. Brains were removed and sectioned in 1-mm coronal sections using an ice-cold stainless steel brain matrix. Sections were placed into cryogenic storage vials that contained RNAlater (Life technologies, Grand Island, NY). Tubes were stored at 4°C overnight, then mounted onto chilled slides and placed in a -20°C freezer for storage. Using Palkovits punches and the Paxinos and Watson (2009) rat brain atlas (all coordinates are based on that atlas), bilateral micropunches were taken under a dissecting microscope. The PVN punch (1.22 mm diameter) began rostrally at ~Bregma = -0.84 mm, and extended caudally 1 mm. The SON punches (0.96 mm diameter) started rostrally at ~Bregma = -0.60 mm and extended caudally for 1 mm. The choice of sampling areas was based on previous studies showing large concentrations of magnocellular neurons containing both vasopressin and oxytocin protein in rats at this range of coordinates (Rhodes et al., 1981; Swanson & Sawchenko, 1983). The PVN has several subdivisions

(Swanson & Sawchenko, 1983), and the location of our punches includes the part that lies ventromedial to the descending column of the fornix between Bregma = -1.44 to -1.56), and the lateral magnocellar subdivision (Krieg, 1932; Swanson & Sawchenko, 1983) from Bregma = -1.72 to -1.92. Punches for each region were placed in a frozen Eppendorf tube for storage at -80°C. At the time of decapitation trunk blood was collected and allowed to clot, serum was separated and centrifuged (2300 X g for 5 minutes) then stored in Eppendorf tubes at -80°C.

***Real-time PCR assays***

RNA was extracted from frozen PVN and SON punches using an Allprep RNeasy mini kit (Qiagen, Valencia, California), according to the manufacturer's protocol. Although we started with n=10 rats per group, loss of some tissues during freezer storage caused some attrition (a subset of samples were frozen with too much liquid RNAlater that made it difficult to visualize brain landmarks; only samples were included with which we were confident in the landmarks), resulting in n=7 for most gene assays. After extraction the quality of RNA was checked on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) and all RIN values fell in the range of 8.6 to 9.9. The quantity of RNA was measured using a GloMax-Multi Detection System (Promega, Madison, WI), and ranged from 71 to 741 ng/ul in SON, and from 207 to 1550 ng/ul in the PVN. Using a high-capacity cDNA reverse transcription kit (Life Technologies, Grand Island, NY), mRNA (PVN 200 ng, SON 150 ng) was converted to single-stranded cDNA. Samples were stored at -20°C until use. Quantitative real-time PCR was used to analyze 5 genes in

the PVN and 4 genes in the SON: *Avp, Oxt, Avpr1a, Oxtr, Esr2* (FAM) in tandem with *Gapdh* (VIC). *Esr2* could not be run in SON due to the smaller amounts of RNA extracted from this smaller dissection limiting us to 4 genes maximum for analysis. All samples were run in triplicates with probe and primer predesigned gene expression assays (Life Technologies, Grand Island, NY). Target genes were amplified using Taqman universal mastermix (Life Technologies, Grand Island, NY) and detected on a ViiA7 Real time PCR machine (Applied Biosystems, Life Technologies, Grand Island, NY) with the following run parameters: 0°C for 2 minutes, 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Relative expression was determined for each sample using the comparative cycle threshold method (Pfaffl, 2001; Schmittgen & Livak, 2008). All samples were normalized to the housekeeping gene *Gapdh* and then calibrated to the median δ-cycle threshold of the young 3-month vehicle treated group. Any of the triplicates with a value of 2 SD above the mean of an individual animal was removed. Samples that amplified at or above 35ct were excluded from analysis.

***Gene expression Analysis***

Expression of each gene was analyzed using SPSS (version 22). For age (groups 1-4, Figure 1.1), two-way ANOVA was used to analyze the effects of age (MAT vs. AG) and hormone (VEH vs. $E_2$). For duration (groups 3-6, Figure 1.1), two-way ANOVA was used to analyze effects of treatment duration (3 mo vs. 6 mo) and hormone (VEH vs. E2). Interactions among variables were also analyzed. A one-way ANOVA with a Bonferroni post hoc was performed to examine the effect of timing (groups 5-8, Figure 1.1). For all

of these analyses, alpha was set at 0.05 and significant main or interaction effects were followed by two-tailed independent sample t-tests. Those data that did not pass the assumptions of normality and/or variance were transformed using either a square root or log transformation. *Avpr1a* in the PVN and *Oxtr* in the SON data did not meet assumptions even after transformation; therefore a non-parametric analysis was performed followed up with Mann-Whitney t-test.

### *Hormone assays and correlations*

Twelve serum hormones were measured, for which detailed methods and results were previously reported on these same rats in a separate study evaluating other endpoints (Yin et al., 2015). For the current study, values for serum estradiol ($E_2$), progesterone (P4), corticosterone (Cort), brain-derived neurotrophic factor (BDNF) were used for correlations with each of the target genes using Pearson's correlation on raw or square-root transformed data when a dataset did not meet assumptions of normality. For those data sets that did not meet the assumptions even after transformation, a Spearman's nonparametric correlation was used.

## RESULTS

### *Gene expression*

Paraventricular nucleus

Two genes, *Oxtr* and *Avpr1a*, were significantly affected only in comparisons of the 3-month treatment groups 1-4 (Figure 1.2). In these animals, there was a main effect

of treatment on *Oxtr* expression. Expression was increased by estradiol compared to vehicle in both the 3-month MAT and AG groups, an effect that was not seen in any of the 6-month duration groups (Figure 1.2C; $p < 0.05$). For *Avpr1a*, there was a significant main effect of age. In the comparison of the 3-month post-OVX groups, the AG animals had significantly higher gene expression than the corresponding MAT rats, but no hormone effects (Figure 1.2D; $p < 0.05$). There were no effects of age, timing, or duration on *Oxt, Avp, and Esr2* expression in the PVN (Figure 1.2A, B, E).

Supraoptic Nucleus

Three of the four genes measured, *Avp, Oxtr,* and *Avpr1a,* were significantly affected, each with a unique expression pattern (Figure 1.3). For *Avp,* two-way ANOVA of Groups 3-6 found no main effects of duration or treatment; however there was a significant interaction ($p < 0.05$). Specifically, *Avp* was significantly lower in the AG-E6 than the AG-E3 and AG-V6 (Figure 1.3B; $p < 0.05$). For *Oxtr*, a main effect of age was found in the 3-month treatment groups (1-4), with levels higher in the AG than the MAT rats (Figure 1.3C; $p < 0.05$). In addition, a timing effect was found for groups 5-8, with *Oxtr* higher in the AG-V3/E3 rats than the AG-V6 or AG-E3/V3 rats. *Avpr1a* expression also had a main effect of age in groups 1-4, with levels higher in the AG than the MAT rats (Figure 1.3D; $p<0.05$). *Oxt* gene expression was unaffected (Figure 1.3A).

Figure 1.2

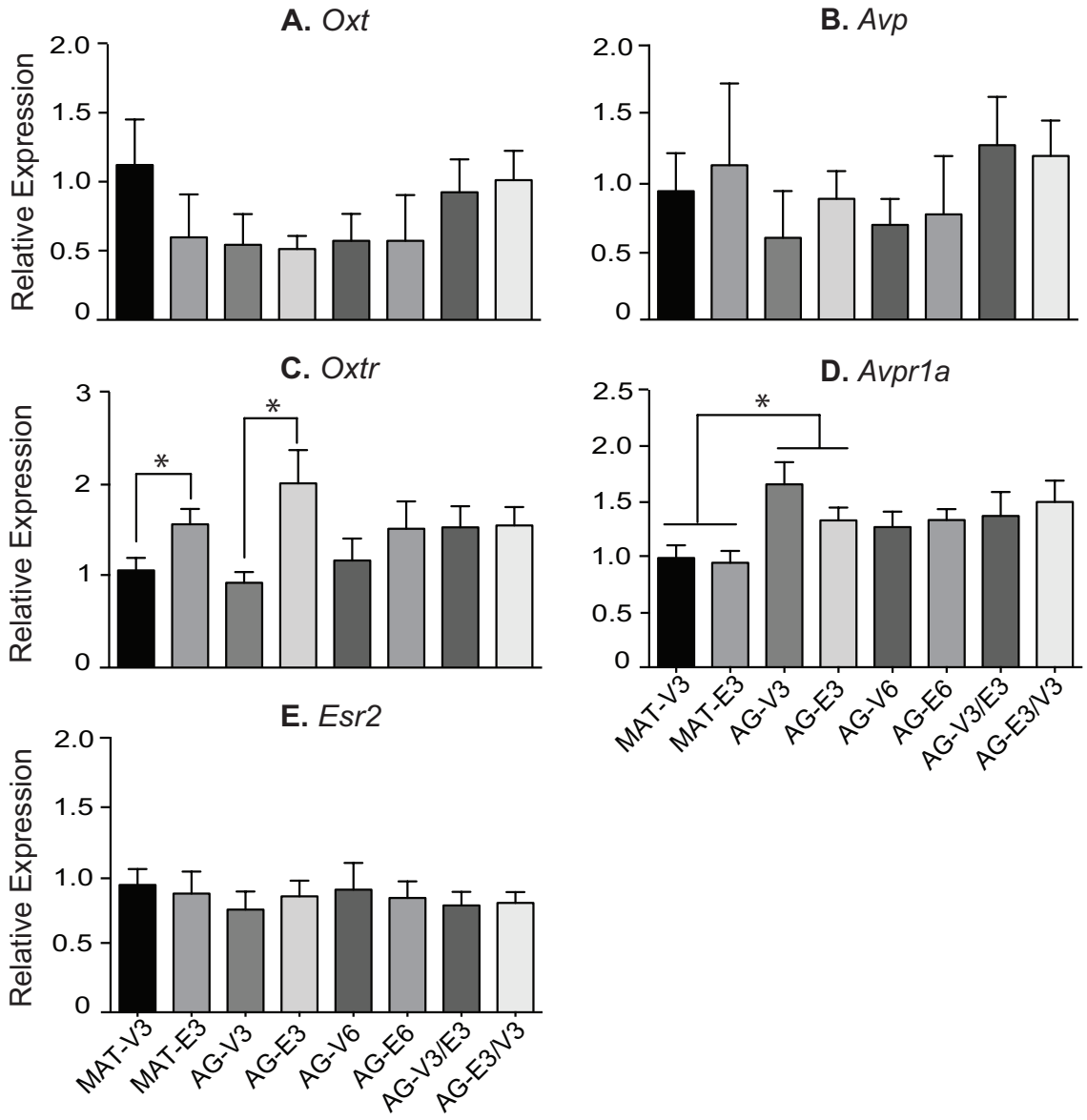


Figure 1.2: PVN relative expression data

Relative gene expression data are shown in the PVN. Note that the scale of the y-axis varies depending on the gene. Comparisons of age were made in groups 1-4, duration of hormone in groups 3-6, and timing of hormone in groups 5-8. *Oxt*, *Avp* and *Esr2* were unaffected by treatment, age, duration, or timing. For *Oxtr* there were significant hormone effects in the 3-mo treated groups. For *Avpr1a*, there was a significant age effect in those same groups. Data shown are mean + SEM. *, $p < 0.05$.

Figure 1.3

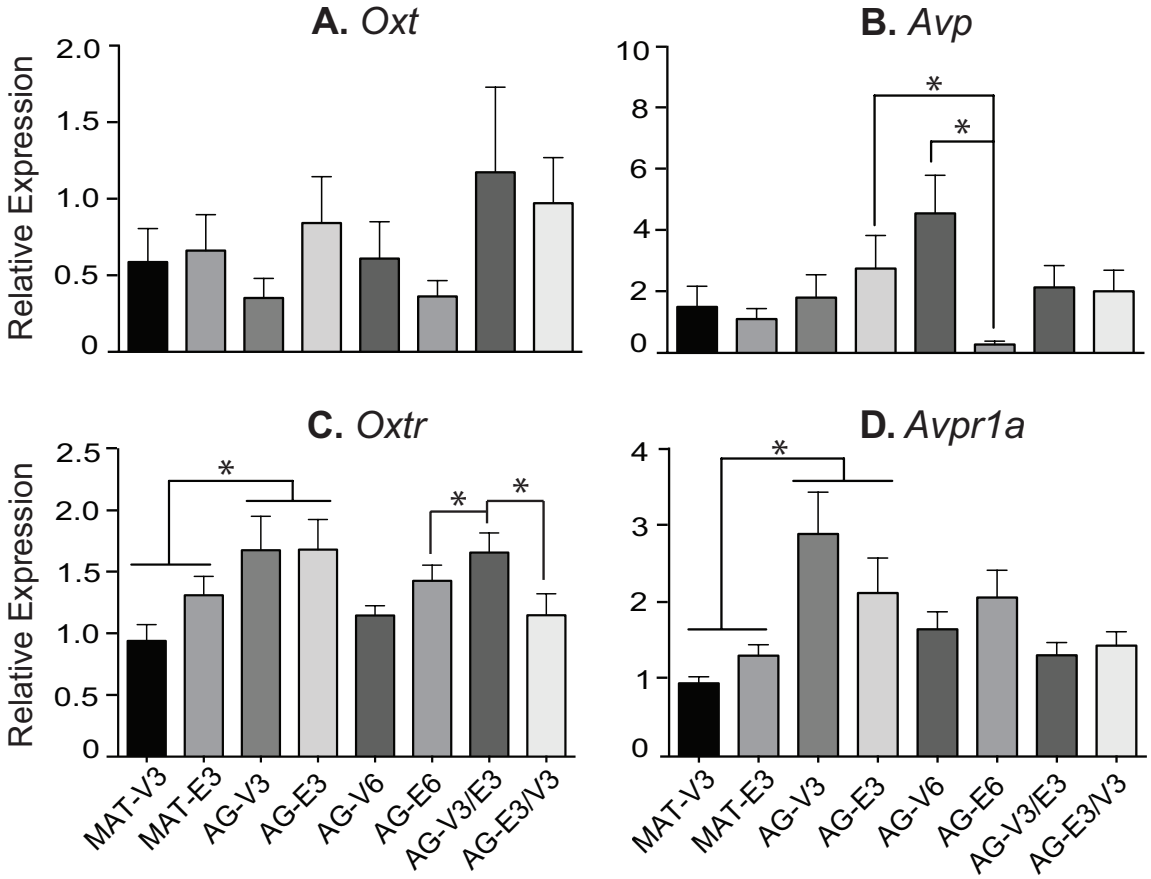


Figure 1.3: SON relative expression data

Relative gene expression data are shown in the SON. Note that the scale of the y-axis varies depending on the gene. Comparisons of age were made in groups 1-4, duration of hormone in groups 3-6, and timing of hormone in groups 5-8. *Oxt* was unaffected by treatment, age, duration, or timing. For *Avp* the E6 group had significantly higher expression than the V6 and E3 group. *Oxtr* had a significant age effect and a significant timing effect; the V3/E3 group had higher expression than the E3/V3 and E6 groups. For *Avpr1a*, there was also a significant age effect in the 3-month treatment groups. Data shown are mean + SEM. *, $p < 0.05$.

***Correlation results***

Correlation analysis was used as a hypothesis-generating method to identify relationships among genes, and between genes and hormones, as well as a way of validating the PCR results.

Gene correlations within the PVN and SON

No genes in the PVN correlated significantly with any of the genes in the SON, but within each region there were positive correlations. Within the PVN, *Oxt* was significantly correlated with *Avp* (Figure 1.4A), and *Oxtr* was significantly correlated with *Esr2* (Figure 1.4B). Within the SON, *Oxt* was significantly correlated with *Avp* (Figure 1.4C). In addition, *Oxtr* was significantly correlated with *Avpr1a* (Figure 1.4D). Detailed statistics for all of the gene correlations are provided in Supplemental Table S1.1.

Hormone and gene expression correlations

Serum hormone concentrations and assay characteristics were reported in a companion paper (Yin et al., 2015) and we refer readers to that study for graphic presentation. As published, concentrations (means $\pm$ SEM) used for the correlations were as follows for groups 1-8. Estradiol (pg/ml): 1) 24 $\pm$ 3; 2) 105 $\pm$ 11; 3) 20 $\pm$ 1; 4) 63 $\pm$ 7; 5) 17 $\pm$ 2; 6) 63 $\pm$ 5; 7) 64 $\pm$ 6; 8) 20 $\pm$ 2. The estradiol treated groups all had significantly higher E2 concentrations than their matched vehicle groups. BDNF (ng/ml): 2) 4.8 $\pm$ 0.4; 2) 3.2 $\pm$ 0.3; 3) 3.4 $\pm$ 0.3; 4) 3.2 $\pm$ 0.4; 5) 4.4 $\pm$ 0.7; 6) 2.7 $\pm$ 0.3; 7) 2.6 $\pm$ 0.3;

8) 4.0 ± 0.6. The estradiol groups had lower BDNF concentrations than their matched vehicle groups, and there was a trend for an age-related decrease in BDNF. Corticosterone (ng/ml): 1) 36 ± 8; 2) 105 ± 21; 3) 37 ± 9; 4) 47 ± 11; 5) 53 ± 9; 6) 65 ± 8; 7) 59 ± 7; 8) 39 ± 8. Reproductively mature rats had significantly higher corticosterone concentrations in the E2 group compared to vehicle, but there were no such effects in the aging rats. Progesterone (ng/ml): 1) 0.7 ± 0.1; 2) 3.8 ± 1.2; 3) 1.1 ± 0.2; 4) 1.5 ± 0.2; 5) 1.4 ± 0.2; 6) 3.5 ± 0.5; 7) 3.3 ± 0.7; 8) 1.4 ± 0.4. The estradiol-treated groups had higher $P_4$ than their matched vehicle groups.

Two genes in the PVN were significantly correlated with serum hormones. *Oxtr* was positively correlated with serum estradiol concentrations (Figure 1.5A), and *Avpr1a* was negatively correlated with corticosterone (Figure 1.5B). In the SON, *Oxtr* was positively correlated with estradiol and progesterone, and negatively correlated with brain-derived neurotrophic factor (Figure 1.5 C, D, E). Detailed statistics for these correlations can be found in Supplemental Table S1.2.

Figure 1.4

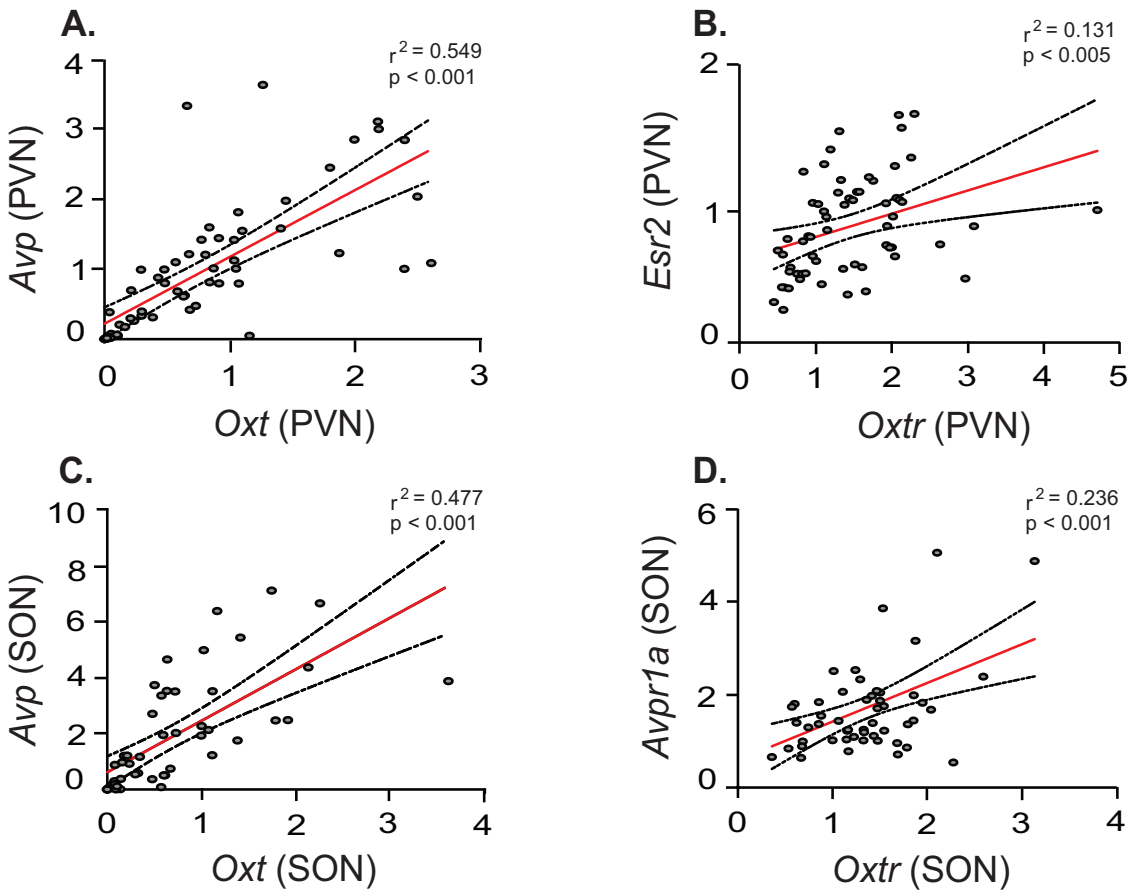


Figure 1.4: Gene correlations within the PVN and SON

Correlation are shown between expression of genes. In the PVN, significant positive correlations were found for (A) *Oxt* and *Avp*, and for (B) *Oxtr* and *Esr2*. In the SON, positive correlation were found for (C) *Oxt* and *Avp*, and for (D) *Oxtr* and *Avpr1a*.

Figure 1.5

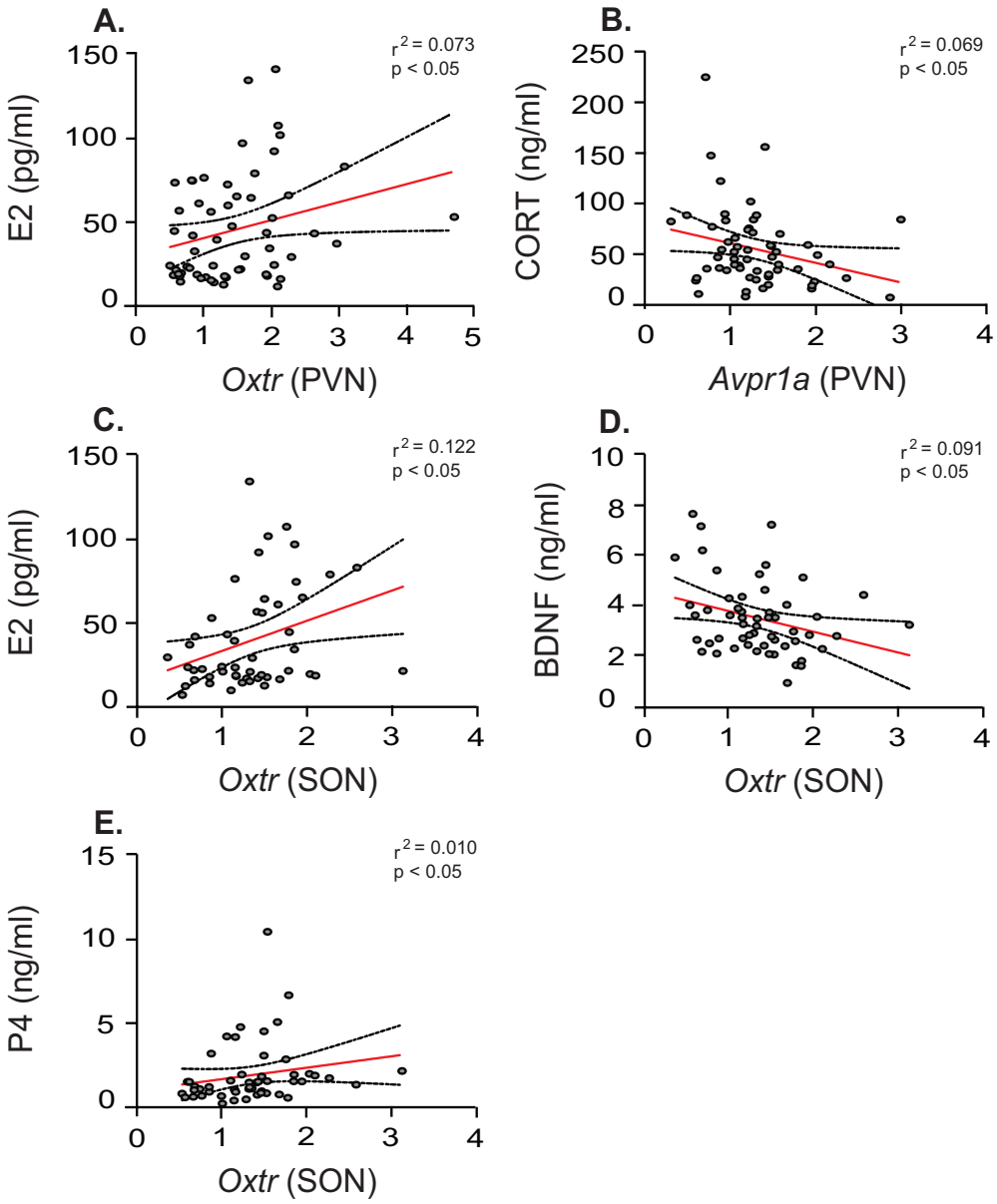


Figure 1.5: Hormone and gene expression correlations

Correlations are shown for genes and hormones. In the PVN a significant positive correlation was found for Oxtr and serum E2 (A), and a negative correlation for Avpr1a and corticosterone (B). In the SON, Oxtr positively correlated with serum E2 (C) and P4 (E), and negatively correlated with BDNF (D).
CORT = corticosterone; P4 = Progesterone

## DISCUSSION

Since the publication of the results from the WHI, which suggested an increase in adverse health outcomes in women taking hormone therapy, the question of whether there is a critical window post-menopause during which the body and brain may respond positively to hormone replacement has become paramount (Rossouw et al., 2002; Anderson et al., 2004). This study utilized a pre-clinical rat model to determine how chronological age, and timing and duration of estrogen therapy, affected genes in the PVN and SON that, to our knowledge, have not systematically been studied in this context. Oxytocin and vasopressin are regulators of social behavior in a variety of animal species and humans [reviewed in (Young & Flanagan-Cato, 2012; Winslow et al., 1993; Caldwell al., 2008; Ebstein et al., 2012)] so understanding how they change, and estrogen regulation, are translationally relevant.

Our major finding was that it was the receptors for oxytocin and vasopressin, rather than the nonapeptides themselves, which were most sensitive to hormones and age. The presence and functional importance of these receptors in PVN and SON is far less well-studied than that of the peptides themselves, but the literature in general suggests that the proteins for these receptors are indeed expressed in PVN and SON of rats (Bealer et al., 2006; Ophir et al., 2013), and that there is co-expression of the vasopressin receptor 1a protein in vasopressinergic neurons in both of these regions (Hurbin et al., 2002). Of the nonapeptides oxytocin and vasopressin, the only effect we found was an interaction between treatment and duration for *Avp* in the SON. In rats oxytocin and vasopressin affect sexual (Arletti & Bertolini, 1985; Pedersen & Boccia, 2006), aggressive (Bosch &

Neumann, 2012; Elkabir et al., 1990) and affiliative (Bosch & Neumann, 2012) behaviors, and also influence social interaction and memory (Dantzer et al., 1987; Lukas et al., 2011). Therefore, changes in expression of their receptors with age and estradiol treatment have potential functional implications and may relate to affective and social changes that can occur in some perimenopausal women (Schmidt & Rubinow, 2009).

***Gene expression in the PVN – effects of estradiol and age***

The current findings show age and hormone effects on gene expression in a region-specific manner. In the PVN, of the five genes measured, only the two-peptide receptors, *Oxtr* and *Avpr1a,* differed across the groups, each with a unique expression pattern. We were surprised not to see effects on of estradiol on *Oxt, Avp* and *Esr2* based on the literature on such effects on mRNA and protein in rats (Patisaul et al., 1999; Suzuki & Handa, 2004; Akaishi & Sakuma, 1985; Patchev et al., 1995), and age-related effects of $E_2$ on vasopressin protein in the human PVN (Ishunina & Swaab, 1999; Fliers et al., 1985a). Our lack of age-related changes in *Oxt* or *Esr2* is in line with previous research showing no E2 effect on mRNA levels in rats (Wilson et al., 2002; Yamaguchi-Shima & Yuri, 2007) or protein levels in humans (Ishunina & Swaab, 1999; Wierda et al., 1991). Differences are probably attributable to species as well as experimental design details, especially the relatively long-term $E_2$ treatments and the specific ages of our animals in the current study.

Oxytocin receptor in PVN is upregulated by estradiol in 3-month treated MAT and AG rats

For *Oxtr*, mature and aging OVX rats given estradiol for 3 months had higher expression than their vehicle treated counterparts, when euthanized at ~7 or ~14 months, respectively. No differences were found in any of the 6-month treated groups, whether given constant hormone or vehicle, or switched after 3 months. There are several interpretations of these results, including the fact that the chronological age differences between the aging rats at 14 (groups 3 and 4) and 17 months (groups 5-8) may reflect a functional transition when *Oxtr* neurons no longer respond to estradiol. Alternatively or in addition, the longer hormone treatment paradigm in the 6-month treatment group (E6), the removal of estradiol at the 3-month mark (E3/V3 group), or the delay of estradiol treatment for 3 months (V3/E3 group) may represent some adaptation to the longer treatment period post-ovariectomy.

The interactions of estradiol and aging on *Oxtr* in the PVN have not been widely studied, and there are limited studies looking at these factors independently. Young and colleagues (1997) reported that short-term (4 day) $E_2$ treatment after given one week after ovariectomy did not have a significant effect on *Oxtr* expression in the PVN. Another study found similar results in protein expression of oxytocin receptor in OVX female rats given $E_2$ or $E_2$+P starting at the time of OVX and continuing for a 16-day duration (Bealer et al., 2006). It is important to note that our treatment duration (3 or 6 months) is considerably longer than that of these published studies. Also, the current study demonstrated that estradiol treatment was only effective in increasing *Oxtr* when animals

were earlier in the aging process (~14 mo) compared to older (~17 mo) rats. However, studies have found that estradiol increases oxytocin receptor protein expression in other areas of the hypothalamus such as the supraoptic nucleus, ventromedial nucleus and medial preoptic area of rats (Bealer et al., 2006; Tribollet et al., 1990; Patchev et al., 1993) as well as the medial amygdala and bed nucleus of the stria terminalis (Patchev et al., 1993; de Kloet et al., 1986). In a study previously published study utilizing the same rats as the current study we found the *Oxtr* is upregulated by $E_2$ in the medial preoptic area (mPOA) (Yin et al., 2015).

The literature examining *Oxtr* in the context of aging is even more limited. There are no studies, to our knowledge, which have looked at the effects of aging on *Oxtr* in the PVN. However, there is one study that reported age-related increase in *Oxtr* mRNA in in the POA of aging female rats (Mobbs, 1994).

Vasopressin receptor in PVN is upregulated by age in 3-month treated MAT and AG rats

For *Avpr1a* expression in the PVN, we found an increase with age in the 3-month treated groups, but no further differences in any of the 6-month aging rats, suggesting that a plateau may be reached at 14 months of age. One interpretation of this is that the amount of *Avpr1a* is increasing as the animal ages, much as the *AVP* gene does in the human PVN (Ishunina & Swaab, 1999; Fliers et al., 1985a; Swaab et al., 1985). The fact that we do not see a response to $E_2$ in both MAT and AG rats could be due to this elevated baseline and not necessarily an impaired response to estrogen.

To our knowledge this is the first study to look at the effects of estradiol and

aging on *Avpr1a* in the PVN, although it was studied during pregnancy in prairie voles (Ophir et al., 2013). In other areas of the hypothalamus *Avpr1a* is influenced by both age and/or $E_2$ treatment. In a previous study published by our lab using the same animals as the current study we found that $E_2$ down-regulated *Avpr1a* expression in all groups despite age, timing and duration (Yin et al., 2015). This same study also found an interaction between age and $E_2$ treatment in the mPOA of MAT and AG 3-month groups (Yin et al., 2015). In addition, Kalamatianos and colleagues (2004) found that 4 days of estradiol treatment led to an increase in *Avpr1a* expression in the anteroventral periventricular nucleus of female rats. There is one study that has looked at the interaction of estradiol and aging in the preoptic area of the hypothalamus. Their results revealed that 4 days of $E_2$ injections in young (2 months) and middle-aged (8 months) rats led to young estrogen-treated OVX rats having significantly higher *Avpr1a* mRNA than their untreated counterparts, but estrogen treatment did not increase expression in the middle-aged rats (Funabashi et al., 2000). However, they did find that the middle-aged OVX animals had significantly higher *Avpr1a* expression than young OVX rats (Funabashi et al., 2000).

***Gene expression in the SON – effects of estradiol and age***

In the SON, three out of the four genes measured, *Avp, Oxtr* and *Avpr1a*, but not *Oxt*, differed across the groups. The literature on whether or not estradiol has an effect on *Oxt* in the SON is mixed, with some finding an increase or decrease, as reported for mRNA levels in rats (Peter et al., 1990; Van Tol et al., 1988), and others finding no

effects on protein levels in rats (Shughrue et al., 2002; Rhodes et al., 1981) and mice (Alves et al., 1998). One possible explanation for why we see changes in *Avp* but not in *Oxt* with $E_2$ treatment is that co-expression of ERβ protein and mRNA is higher in vasopressin than oxytocin neurons of rats (Shughrue et al., 2002; Isgor et al., 2003) and mice (Alves et al., 1998). Double-labeling studies would be needed to test this hypothesis in our model.

In addition, *Oxt* expression was not affected by age, which in part is supported by past studies. In post-menopausal women oxytocin neuronal volume and cell numbers are not altered by age (Wierda et al., 1991; Ishunina & Swaab, 1999; Hoogendijk et al., 1985), which is in line with the current study, and suggesting that there is not higher activity of oxytocin neurons when steroid hormones are lost or replaced. However, we were surprised that age did not affect *Avp*, due to the findings in humans of an increased somatic size of vasopressin neurons, as well as increased numbers of these neurons in the SON, although these studies did not taken into account hormone replacement therapy (Ishunina & Swaab, 1999; Fliers et al., 1985a; Hoogendijk et al., 1985). Yet, we did see age-related changes in both *Oxtr* and *Avpr1a* expression. It is possible that this is due to species differences, with age playing a larger role in regulating the receptors in rodents and the genes themselves in humans.

Oxytocin receptor in SON is regulated by age in 3-month treated MAT and AG rats and by timing of estradiol treatment in AG rats.

*Oxtr* expression in the SON was affected by age, with mature animals having

significantly less than their aged counterparts. In addition, the aged animals that had delayed estradiol treatment had significantly more *Oxtr* than the aged animals with 6 months of vehicle treatment and those who were deprived of treatment for 3 months prior to euthanasia. This demonstrates that even after being deprived of steroid hormones for 3 months, estradiol replacement is still effective in increasing *Oxtr*. The delayed treatment brought the aged animals back to the level similar to those who were euthanized at ~14 months. This is an indication that there is not a critical window for the effectiveness of estradiol on regulation of this gene in the SON.

The few studies published on the effects of estradiol on oxytocin receptor in the SON have reported mixed results. Young and colleagues (1997) found that there were no changes of *Oxtr* in the SON of rats during the different stages of the estrous cycle or during pregnancy. Yet, a study looking at the effects of $E_2$ and $E_2$+P treatment after OVX in female rats found that both treatments increased protein expression of oxytocin receptor in the SON compared to the control group (Bealer et al., 2006). The same study also showed that levels of the oxytocin receptor are increased during late gestation compared to early gestation and OVX control animals (Bealer et al., 2006). Similar results have been seen in pregnant prairie voles, with Oxtr being unregulated during late pregnancy, a time when steroid hormones such as estradiol are high (Ophir et al., 2013). Our results support the latter findings that estradiol does indeed play a role in the regulation of *Oxtr* in the SON. In regard to aging, *Oxtr* in the SON has not been studied. However, findings from other nuclei do indicate that aging does play a role in regulating oxytocin receptor mRNA and protein expression in the hypothalamus of the rat (Mobbs,

1994; Arsenijevic et al., 1995).

Vasopressin receptor in SON is regulated by age in 3-month treated MAT and AG rats.

In addition to seeing age effects of *Avpr1a* in the PVN we also saw age-related changes in the SON. We found an increase with age in the 3-month treated groups, but no differences in any of the 6-month aging rats. As previously mentioned, this could be due to species difference, with age affecting *Avpr1a* expression in rats and *Avp* in humans. To our knowledge, this is the first study to examine the effects of age and estradiol on *Avpr1a* expression in the SON. Previous studies indicate both age and estradiol treatment affects other parts of the hypothalamus (Kalamatianos et al., 2004; Funabashi et al., 2000).

Vasopressin in SON is down regulated by estradiol in 6-month treated AG rats.

The SON was the only region in which *Avp* changed, decreased by $E_2$ in the 6-month treatment group. As the SON ages from 14 to 17 months the responsiveness to $E_2$ treatment appears to change. Literature on the effects of estradiol on *Avp* is mixed, with some showing no effects and other showing increases. $E_2$ did not alter vasopressin protein or gene expression in the SON of mice (Nomura et al., 2002); nor did it have an effect on mRNA in the monkey (Roy et al., 1999). However, a study looking at the effects of $E_2$ post OVX in female mice found that 3 days of consecutive $E_2$ treatment led to an increase in vasopressin protein in the SON (Grassi et al., 2010). $E_2$ has also been shown to increase expression of *Avp* in the bed nucleus of the stria terminalis of rats (Han & De

Vries, 2003), and decrease mRNA and protein in the medial amygdala and lateral septum of mice (Nomura et al., 2002).

Again, we were surprised to not see any age effects in *Avp* expression. As stated previously this may be due to species differences, because previous changes in expression have been found in humans but not rodents. In addition, the findings in humans of an increased somatic size of vasopressin neurons, as well as increased numbers of these neurons did not take into account hormone replacement therapy (Ishunina & Swaab, 1999; Fliers et al., 1985a). The decrease in *Avp* in our aged rats but not our mature rats could be an indication that long-term estradiol treatment reverses the protective effects that accompany normal aging.

***Correlation results and new hypotheses***

We conducted the gene and hormone correlations as a way to generate future hypothesis based on the relationships among genes, and between genes and hormones. The *Avp* and *Oxt* genes were positively correlated with each other in both brain regions. More surprising was the significant positive correlation between *Avpr1a* and *Oxtr* in the SON; while relatively weak, it poses the possibility that these receptors are co-regulated by age and hormones. In fact, both *Avpr1a* and *Oxtr* were upregulated by age in the SON, consistent with this hypothesis. Correlations conducted between genes and hormones also identified positive and negative relationships. For example, there was a significant negative correlation between corticosterone and *Avpr1a*. There are studies showing that stress can increase corticosterone levels and *Avp* expression in the parvocellular neurons

in the PVN (Pinnock & Herbert, 2001; Ma et al., 1997; Makino et al., 1995), but relationships between corticosterone and *Avpr1a* are not as well known and merit future research. Serum $E_2$ correlated positively with *Oxtr* in both brain regions and suggests that the oxytocin receptor is an important target for menopause research.

## CONCLUSIONS, CLINICAL IMPLICATIONS, AND LIMITATIONS

The current study is the first to look at the affects of age, and timing and duration of estradiol on social behavior related genes in the PVN and SON of female rats. Results on *Avpr1a* and *Oxtr* show that most differences occurred in animals with shorter treatment durations and of younger chronological ages. There were exceptions though, as *Avp* and *Oxtr* in the SON showed some unexpected duration (*Avp*) and timing (*Oxtr*) effects. Estradiol's ability to increase *Oxtr* even after the animal has been deprived of steroid hormones for 3-months and that timing did not affect any other genes is an indication that the "critical window" hypothesis is supported for specific genes (*Avpr1a* and *Oxtr*) but not others.

These findings could also have clinical implications for women taking hormone replacement therapy. Both *Oxt* and *Avp* have been implicated in regulating a variety of behaviors such as anxiety and depression, in mice (de Kloet et al., 2005), rats (Klenerova et al., 2009), humans (Meyer-Lindenberg et al., 2011), and voles (Lim and Young, 2006). *Oxtr* and *Avpr1a* knockout mice show aberrant social and emotional behaviors, including increasesd aggression, stress and deficits in social memory (Bielsky et al., 2004; Sala et al., 2011; Takayanagi et al., 2005). The decrease of *Oxtr* that is experienced when

estradiol levels drop may contribute to these behavioral changes. However, whereas shorter-term treatment with E2 causes an increase in expression of some genes, long-term treatment did not have any effects on any of the genes except *Avp*, which was down-regulated. According to the "critical window" hypothesis there is a point in which hormone replacement therapy because more detrimental than it is helpful. This decrease in expression of *Avp* may be indicative of such a negative effects of long-term treatment.

There were several limitations to the current study. We were not able to include a delayed treatment group in our MAT animals due to the fact that they would have been entering reproductive senescence, which would have introduced a confound to our study. There were also technical pitfalls: we were only able to obtain enough RNA to assay 5 genes in PVN and 4 in SON. In addition, while the study was adequately powered to detect significant group differences, sample sizes for some of the genes were small. It is also notable that there is quite a bit of individual variability of gene expression within groups. This is not surprising considering the complex experimental model and the inevitable differences between outbred rats of strains such as Sprague-Dawley. This may be an advantage though from a clinical perspective, as humans are not inbred and symptoms and other characteristics of menopause vary enormously from woman to woman.

There are several areas that merit future investigation to enhance the clinical relevance of the work. Here, we only tested continuous $E_2$ and we did not replace progesterone, though women with a uterus may use cyclic regimes of hormones that include P4. Including P4 treatments in our study would have doubled the number of

animals needed, thus we decided to focus on addressing and reevaluating the limitations of the WHI estrogen-alone trials. Currently, we are determining direct relationships between the timing and duration of E2 treatment on neurobehavioral outcomes of animals that are behaviorally characterized. This will give us a better understanding of links between gene regulation and behavior, to inform treatment options for menopausal women.

## SUPPLEMENTAL TABLES

| Table S1.1 | ***Oxt*** **(PVN)** | ***Avp*** **(PVN)** | ***Oxtr*** **(PVN)** | ***Avpr1a*** **(PVN)** | ***Esr2*** **(PVN)** | ***Oxt*** **(SON)** | ***Avp*** **(SON)** | ***Oxtr*** **(SON)** | ***Avpr1a*** **(SON)** |
|---|---|---|---|---|---|---|---|---|---|
| ***Oxt*** **(PVN)** | ------- | 0.001* | 0.380 | 0.389 | 0.128 | 0.552 | 0.360 | 0.552 | 0.858 |
| ***Avp*** **(PVN)** | | ------- | 0.320 | 0.073 | 0.088 | 0.255 | 0.268 | 0.255 | 0.808 |
| ***Oxtr*** **(PVN)** | | | ------- | 0.189 | 0.001* | 0.823 | 0.433 | 0.823 | 0.272 |
| ***Avpr1a*** **(PVN)** | | | | ------- | 0.140 | 0.706 | 0.319 | 0.706 | 0.207 |
| ***Esr2*** **(PVN)** | | | | | ------- | 0.210 | 0.114 | 0.210 | 0.959 |
| ***Oxt*** **(SON)** | | | | | | ------- | 0.001* | 0.454 | 0.150 |
| ***Avp*** **(SON)** | | | | | | | ------- | 0.146 | 0.074 |
| ***Oxtr*** **(SON)** | | | | | | | | ------- | 0.003* |
| ***Avpr1a*** **(SON)** | | | | | | | | | ------- |

Table S1.1: Significant gene correlations

P-values are shown for the gene correlations, all of which were positive. In the PVN, significant correlations were found for *Oxt* and *Avp*, and for *Oxtr* and *Esr2*. In the SON, correlations were found for *Oxt* and *Avp*, and for *Oxtr* and *Avpr1*a. All significant p values are indicated with an asterisk.

| Table S1.2 | ***Avp*** **(PVN)** | ***Oxt*** **(PVN)** | ***Avpr1a*** **(PVN)** | ***Oxtr*** **(PVN)** | ***Esr2*** **(PVN)** | ***Avp*** **(SON)** | ***Oxt*** **(SON)** | ***Avpr1a*** **(SON)** | ***Oxtr*** **(SON)** |
|---|---|---|---|---|---|---|---|---|---|
| **E2** | 0.501 | 0.582 | 0.321 | 0.039* | 0.532 | 0.203 | 0.609 | 0.958 | 0.013* |
| **BDNF** | 0.496 | 0.985 | 0.299 | 0.651 | 0.145 | 0.481 | 0.994 | 0.186 | 0.029* |
| **CORT** | 0.112 | 0.679 | 0.031* | 0.287 | 0.685 | 0.195 | 0.227 | 0.918 | 0.675 |
| **P4** | 0.911 | 0.549 | 0.282 | 0.541 | 0.953 | 0.277 | 0.978 | 0.992 | 0.003* |

Table S1.2: P-values for correlations between the hormone assays and the target genes in both the PVN and SON.

In the PVN, a significant negative correlation was found for *Avpr1a* and corticosterone, and a positive correlation for *Oxtr* and serum E2. In the SON, *Oxtr* correlated positively with serum $E_2$ and $P_4$, and negatively with BDNF. All significant p values are indicated with an asterisk.

# CHAPTER 3: THE EFFECTS OF LONG-TERM ESTRADIOL TREATMENT ON SOCIAL BEHAVIOR AND GENE EXPRESSION IN MATURE ADULT FEMALE RATS.

## ABSTRACT

This study tested the effects of long-term estradiol ($E_2$) replacement on social behavior and gene expression in ovariectomized mature adult female rats. We used an ultrasonic vocalization (USV) test and a sociability test to examine communication, and social memory and preference, in the social context of female-female cagemates. Underlying neurobiological molecular changes were quantified in 5 brain regions involved in social behavior using a 48-gene qPCR array. In the USV test, $E_2$ profoundly decreased numbers of USVs and decreased interactions scored from videotapes. There were few effects of $E_2$ in the sociability test. For gene expression, the supraoptic nucleus (SON) was most affected: *Oxt, Oxtr* and *Avp* were increased in $E_2$ rats, and *Drd2, Htr1a*, *Grin2b*, and *Gabbr1* were decreased. Thus, $E_2$ changes communicative interactions between adult female rats, and genes involved in oxytocin, vasopressin, and neurotransmitter signaling in the SON were selectively affected.

## INTRODUCTION

The loss of ovarian estrogens during natural or surgical menopause results in a variety of symptoms that can impair the quality of life. Among the neurological and neurobehavioral symptoms reported by perimenopausal women are increased depression

and anxiety (Freeman et al., 2004; Schmidt et al., 2004; Bromberger et al., 2011). Estrogens, especially estradiol (E2), regulate these behaviors in animals as well as women (de Kloet et al., 2005; Klenerova et al., 2009; Meyer-Lindenberg et al., 2011; Rubinow et al., 1998). Clinical studies have shown that women given estradiol have decreased anxiety and depression symptoms compared to women given placebo (De Novaes Soares et al., 2001; Zweifel & O'Brien, 1997, Schmidt et al., 2000). However, there is controversy about the risks and benefits of hormone replacement therapy (HRT) following the publication of the results from the Women's Health Initiative, which suggested a small but significant increase in adverse cardiovascular and breast cancer incidents in women taking HRT (Rossouw et al., 2002; Manson et al., 2013). Although those findings have been partially discredited (Klaiber et al., 2005; Bhupathiraju and Manson, 2014), to this day the question of whether, when, and for how long HRT should be used is still debated.

Along with its effects on affective behavior are estradiol's effects on social behavior. In rodents, ovariectomy causes deficits in social interaction and social memory that are improved with administration of estradiol replacement (Hlinák, 1993; Tang et al., 2005). Mice with knockouts for the estrogen receptor α (ERα) or ERβ genes also exhibit social behavioral deficits (Choleris et al., 2006; Imwalle, Scorkakales & Rissman, 2002). This appears to translate to humans, as women with menopausal depression or anxiety report problems with interpersonal relationships and a decreased desire to engage in social interactions (Uguz et al., 2011; Deeks and McCabe, 2004; Lanza di Scalea et al., 2012; Schmidt et al., 2000). Schmidt and colleagues (2000 & 2015) have reported that

estradiol decreased social isolation during perimenopause compared to placebo, whereas another study found that hormone replacement therapy was associated with an increase in social isolation (Achat et al., 1998). Therefore, the effect of HRT on social behavior requires further investigation.

The neurobiology of social behavior involves a complex network of brain regions that signal via diverse cellular and molecular pathways. The prefrontal cortex (PFC), medial amygdala (MeA), bed nucleus of the stria terminalis (BNST) and the hypothalamus are among the most estrogen-sensitive nodes of this neural circuit (Arimatsu & Hatanaka, 1986; Kugaya et al., 2003; Han & De Vries, 2003; Garcia et al., 2016). Considering their key roles and abundant expression of ERs (Kugaya et al., 2003; Walker et al., 2003; Saper et al., 2005), they are important targets of analysis for understanding potential neuromolecular substrates involved in the estrogen-regulation of social behavior.

This study was designed in order to gain insight into how long-term estradiol treatment affects social memory and social interaction in female rats, using novel behavioral tasks designed to assess sociality in cagemates. In addition, to gain insight into the molecular changes that occur with estradiol deprivation and replacement, we systematically profiled the expression of genes that are implicated in the regulation of social behavior, and that are estrogen-sensitive.

# MATERIAL AND METHODS

### *Animals and Husbandry*

Animal procedures were conducted in accordance with The Guide for the Care and Use of Experimental Animals following protocols approved by The University of Austin IACUC committee and NIH standards. Reproductively mature adult female (MAT, 3-4 months, sexually naïve, n = 40) Sprague Dawley rats (Harlan) were purchased. Upon arrival, rats were placed on a standard 12:12 light: dark cycle (lights on at 0700) and paired housed, and given water and rat chow ad libitum. Prior to surgery they were allowed to acclimate to the room for two weeks. During this period estrous cyclicity was monitored daily by vaginal lavage of sterile saline. Only females with regular 4-5 day cycles were used. Ovariectomy (OVX) surgery was performed on all rats under isofluorane inhalation anesthesia. A single injection of Rimadyl (5 mg/kg) was given at the start of surgery. Bilateral dorsolateral incisions were made through the skin, muscle, and peritoneum, and the ovaries were ligated and removed. Muscles were sutured and wound clips used to close the skin. At the time of surgery animals were implanted subcutaneously between the shoulder blades with capsules containing either 100% cholesterol (Veh) or 5% 17β-estradiol / 95% cholesterol ($E_2$). Animals were randomly assigned to treatment groups, and pair-housed with a rat that received the same treatment for 3 months. The final number of experimental rats was n = 20 (Veh) and n= 20 ($E_2$).

### *Behavioral paradigms*

Behavioral testing began 3 months after OVX and hormone treatment, when rats

were ~7 months of age. At that time, all animals were separated from their cagemates for one week and individually housed. Testing began with an ultrasonic vocalization test (2 consecutive days) followed the next day by sociability testing (2 consecutive days). Rats were euthanized one week after the completion of testing.

Ultrasonic vocalization test (USV)

USV testing took place over 2 consecutive days in a Plexiglas tank (23L x 29W x 40H cm, Fig. 2.1a) placed inside of a sound-attenuating chamber equipped with a microphone. On Day 1, animals were allowed to habituate (Trial 1) for 5 minutes, during which USVs were recorded on the individuals. Day 2 consisted of a sequence of three 5-minute trials, referred to as Trials 2, 3, and 4, in which the cagemates were re-introduced, allowed to interact, and subsequently separated. In Trial 2 prior to placement of the rats, a removable perforated plastic grid was placed across the center of the apparatus to bisect it; it allowed for nose touching, auditory and visual contact but not gross physical contact. The cagemates were re-introduced during this trial but were separated by the grid. USVs were recorded from the pair. For Trial 3, the grid was removed and the rats were able to freely interact with one another. Videotaping was performed during this trial in order to quantify activity, time interacting, and anogenital investigation. Before the start of Trial 4 one of the cagemates was (randomly) removed from the testing chamber and placed into an identical chamber in a separate sound-attenuated chamber. Both animals were then immediately recorded for a final 5-minute trial before being individually rehoused.

USVs were recorded using UltraSoundGate hardware and software and analyzed

with Saslab Pro (all Avisoft, Germany). We were able to use the Saslab Pro software to automatically detect and quantify calls, between 45 and 70kHz, and to differentiate them as frequency modulated (FM) or non-frequency modulated (NFM). Frequency modulated calls were defined as having more than a 9kHz change in frequency. Each pair of cagemates was analyzed as a unit, because during tests when both animals were present in the same chamber, it was impossible to distinguish calls from individuals. Therefore, for Trials 1 and 4, when the animals were recorded separately, their calls were summed for statistical analysis.

During Trial 3, while rats were allowed to physically interact, the behavioral test was recorded using a digital videocamera. Recordings were subsequently analyzed by a blind observer for overall activity, interaction and anogenital investigation.

Statistical analyses were conducted using SPSS. Due to the non-homogeneity of these data sets all data were transformed using either a square root or log transformation. One pair of animals from the vehicle treated group was removed as an outlier, due to calls being more than 2 SD above the mean of the group. The final number of pairs used for USV analysis was 9 and 10 for $E_2$ vs. Veh, respectively. A repeated measures test was used to analyze the effects of treatment ($E_2$ vs. Veh) and trial (Trials 1 through 4) for total calls, non-frequency modulated calls, and frequency-modulated calls. For all of these analyses, alpha was set at 0.05 and significant main or interaction effects were followed by two-tailed independent sample t-tests. Independent sample t-test was also used to analyze the difference between treatments on overall activity, interaction and anogenital investigation. Correlations between USV behavior and calls made during trial 2, while

the animals were freely interacting, were analyzed using a Spearman's rank correlation.

Sociability test

On day 1 one of the cagemates was randomly chosen to be the experimental rat and was habituated to a Stoelting three-chamber apparatus (100L x 100W x 34.5H cm total) containing two holding cages in each corner of the side chambers, for 5 minutes prior to testing. After the habituation trial the experimental animal was placed back in its home cage, during which time its cagemate was placed in one of the two holding cages [small cage in one of the far corners (lower-left and lower-right, Fig 2.1b)]. Holding cages have bars that allow for nose touching and anogenital investigation between the bars. The second holding cages was used to house a novel (unfamiliar) rat of the same sex, age, and treatment. The experimental rat was then placed back into the center chamber and was allowed to roam freely for a 5-minute sociability test, during which her behaviors were tracked by Any-Maze software (Stoelting Co., Wood Dale, IL). Rats were re-housed separately overnight. On day 2 the cagemate that was previously used as a stimulus rat was used as the experimental rat. That rat's partner was now placed into a holding cage, and the other holding cage contained a new unfamiliar rat of the same sex, age, and treatment. Again, the experimental rat's movements were tracked by Any-Maze. The software was subsequently used to quantify time immobile, time spent in each chamber, and time spent in a smaller 19 x 19 cm zone (approximately one body length distance) designated around the stimulus rats (Fig 2.1b). After the end of day 2 of sociability testing animals were rehoused with their cagemate for one week until time of

euthanasia.

Statistics were conducted using SPSS. Due to the violation of assumptions total time freezing and time spent near stimulus rat were transformed using a log and square root transformation, respectively. Data for time immobilized near stimulus rat, time spent in chambers, and time immobilized in chambers did not meet assumptions even after transformation. Therefore, a Friedman's test was performed to analyze the effect of chambers while Wilcoxon signed-rant test was performed to analyze the effect of treatment. A false discovery rate correction was used to correct for the multiple comparisons.

Figure 2.1a

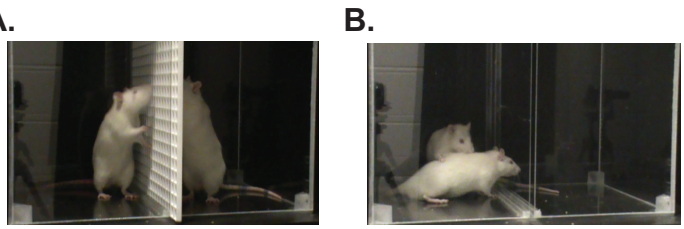


Figure 2.1a: Ultrasonic vocalization apparatus

The ultrasonic vocalization apparatus and set-up are shown for Trials 2 and 3. A) In Trial 2 the cagemates were reintroduced across a perforated plastic grid that allowed sniffing and nose-touching but no other physical interactions. USVs were recorded for 5 minutes. B) In Trial 3 the grid was removed from the apparatus and the cagemates were allowed to freely interact, and USVs recorded for 5 minutes. Trial 3 was also videotaped and scored for activity, time interacting, and time engaged in anogenital investigation. During Trials 1 and 4 the animals were alone in the apparati and recorded separately (image not shown).

Figure 2.1b

Sociability Test

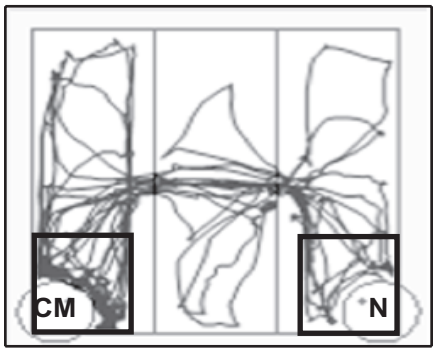


Figure 2.1b: 3-chamber sociability test apparatus

The 3-chamber sociability test apparatus is shown, with a representative behavioral path of an individual experimental rat shown as tracked by ANY-maze. The black square around each of the two corner holding cages represents the area in proximity to the stimulus rats. For the sociability test the experimental rat's cagemate (CM) was placed in one of the two corner holding cages while a novel (N) rat of the same age, sex and treatment was placed in the other. The experimental rat was placed in the center chamber and allowed to explore the apparatus for 5 minutes.

***Brain tissue processing***

Rats were euthanized at ~7 months of age. All animals were weighed and euthanized during the lights-on period at 1330 hours, with the last animal killed not later than 1600 hours, by rapid decapitation. After brains were removed they were sectioned using an ice-cold stainless steel brain matrix, 1-mm coronal sections were taken. Cryogenic storage vials that contained RNAlater (Life technologies, Grand Island, NY) were used to store sections overnight at 4°C. Sections were then mounted onto chilled slides and placed in a -80°C freezer until brain punches were taken. Bilateral punches of brain tissue were taken using Palkovits punches and the Paxinos and Watson (2009) rat brain atlas (all coordinates are based on that atlas) under a dissecting microscope. The paraventricular nucleus of the hypothalamus (PVN) punch (1.22 mm diameter) began rostrally at ~Bregma = -0.84 mm, and extended caudally 1 mm. The supraoptic nucleus (SON) punches (0.96 mm diameter) started rostrally at ~Bregma = -0.60 mm and extended caudally for 1 mm. MeA punches (1.22 mm diameter) began rostrally at ~Bregma = -1.56 mm, and extended causdally 1mm. BNST punches (0.96 mm diameter) started rostrally at ~Bregma = 0.00 mm, and extend caudally for 1mm. PFC punches (1.22 mm diameter) began rostrally at ~Bregma = 4.20 mm, and extend caudally for 1mm. Punched tissue was placed in a frozen Eppendorf tube and stored at -80°C until time of PCR. At decapitation, trunk blood was collected, allowed to clot, centrifuged (2300 X g for 5 minutes), and serum collected. Serum was stored at -80°C in Eppendorf tubes until time of hormone assays.

***Real-time PCR assays and analysis***

Extraction of RNA from frozen PVN, SON, MeA, BNST, and PFC punches was performed using an Allprep RNeasy mini kit (Qiagen, Valencia, California), according to the manufacturer's protocol. The quality of the RNA was validated using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) and all RIN values fell in the range of 8.80 to 9.90. A GloMax-Multi Detection System (Promega, Madison, WI) was used to assess the quantity of RNA. After RNA extraction mRNA (200ng) was converted to single-stranded cDNA using a high-capacity cDNA reverse transcription kit (Life Technologies, Grand Island, NY), samples then stored at -20°C until use. Samples from all 5-brain regions (n = 11 - 18 per group) were run on a customized rat Taqman low-density array (TLDA) Microfluidic 48-gene real-time PCR cards (Applied Bio- systems). Real-time PCR was performed using Taqman universal mastermix (Life Technologies, Grand Island, NY) and detected on a ViiA7 Real time PCR machine (Applied Biosystems, Life Technologies, Grand Island, NY) with the following run parameters: 0°C for 2 minutes, 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds, and 60°C for 1 minute.

The genes for the assay were chosen based on their roles in social behavior and their regulation by steroid hormones (45 genes of interest and three normalizing genes; Table 1).

Relative expression was determined for each sample using the comparative cycle threshold method (Pfaffl, 2001; Schmittgen & Livak, 2008). All samples were normalized to the geomean of the housekeeping genes *Gapdh, Rpl13a,* and *18s* and then calibrated to the median δ-cycle threshold of the vehicle treated group. Samples that

amplified at or above 35ct were excluded from analysis and any samples 2 SD above the mean of the group were removed as outliers.

The effect of treatment ($E_2$ vs. Veh) on TLDA gene expression was analyzed using independent sample t-test using SPSS. Those data that did not pass the assumptions of normality and/or variance were transformed using either a square root or log transformation. Data that did not meet assumptions even after transformation were analyzed using a Kruskal-Wallis test. For all of these analyses, alpha was set at 0.05 and the Benjamini-Hochberg false discovery rate correction method (FDR; Benjamini & Hochberg, 1995) was used to correct for the multiple comparisons.

***Estradiol hormone assay***

Levels of serum estradiol ($E_2$) were determined by radioimmunoassay (Ultrasensitive Estradiol RIA, Cat No DSL4800, Lot # 150622C, Beckman Coulter, Pasadena, CA), according to the manufacturer's directions. A single assay was used for all the samples. Samples were run in duplicates with volumes of 100 µl of serum. Assay sensitivity was 2.2 pg/ml and intrassay C.V. was 1.30%. As expected, the estradiol treated animals had significantly higher concentrations of $E_2$ ($37 \pm 3$) than did the vehicle ($14 \pm 1$) treated animals ($p < 0.01$).

# RESULTS

## *Ultrasonic vocalizations*

### USV calls

Analysis of total calls showed a significant interaction between trial and treatment ($p < 0.05$). Vehicle treated animals called significantly more than $E_2$ treated animals on Trials 2 ($p < 0.05$) and 3 ($p < 0.01$) (Fig 2.3A). When differentiated into frequency modulated (FM) and non-frequency modulated (NFM) calls, analysis of FM calls yielded a significant main effect of trial ($p < 0.01$), with more calls being emitted by both treatments on Trial 3 than Trials 1 and 4 ($p < 0.01$, Fig 2.3B). For NFM calls, a significant interaction between trial and treatment was found ($p < 0.05$) with the vehicle rats emitting significantly more NFM calls on Trial 3 ($p < 0.01$, Fig 2.3C) than the $E_2$ treated animals.

### Behavior during USV Trial 3

Videorecordings were scored for overall activity, interaction and anogenital investigation. Independent-sample t-tests showed that there was a significant difference between the treatments on all 3 behaviors ($p < 0.01$). The vehicle rats were significantly more active (Fig 2.4A) during the test and they spent more time interacting (Fig 2.4C) with their cagemate than did the $E_2$ animals. However, $E_2$ treated animals participated in significantly more anogenital investigation with their cagemate than did the vehicle treated animals (Fig 2.4E).

Correlations between total USVs and behaviors in Trial 3

Correlations were performed to examine the relationship between total calls emitted during Trial 3 and the behaviors scored during that trial. Total calls were positively correlated with activity ($p < 0.01$, Fig 2.4B) and time interacting ($p < 0.01$, Fig 2.4D). However, anogenital investigation was negatively correlated with total calls ($p < 0.01$, Fig 2.4F).

***Sociability test***

Vehicle treated animals spent significantly more time immobile during the sociability trail than $E_2$ treated animals ($p < 0.05$, Fig 2.5A).-The $E_2$ animals also spent more time immobile in close proximity to their cagemate than they did with the novel rat ($p < 0.01$, Fig 2.5B). The vehicle treated animals spent more time immobile in proximity to the novel rat than did the $E_2$ treated animals ($p < 0.05$, Fig 2.5B) Rats in both treatments spent more time in proximity to their cagemate than the novel rat ($p < 0.05$, Fig 2.5C). The vehicle treated animals on average had longer visits to their cagemate than they did with the novel rat ($p < 0.05$, Fig 2.5D).

Figure 2.3

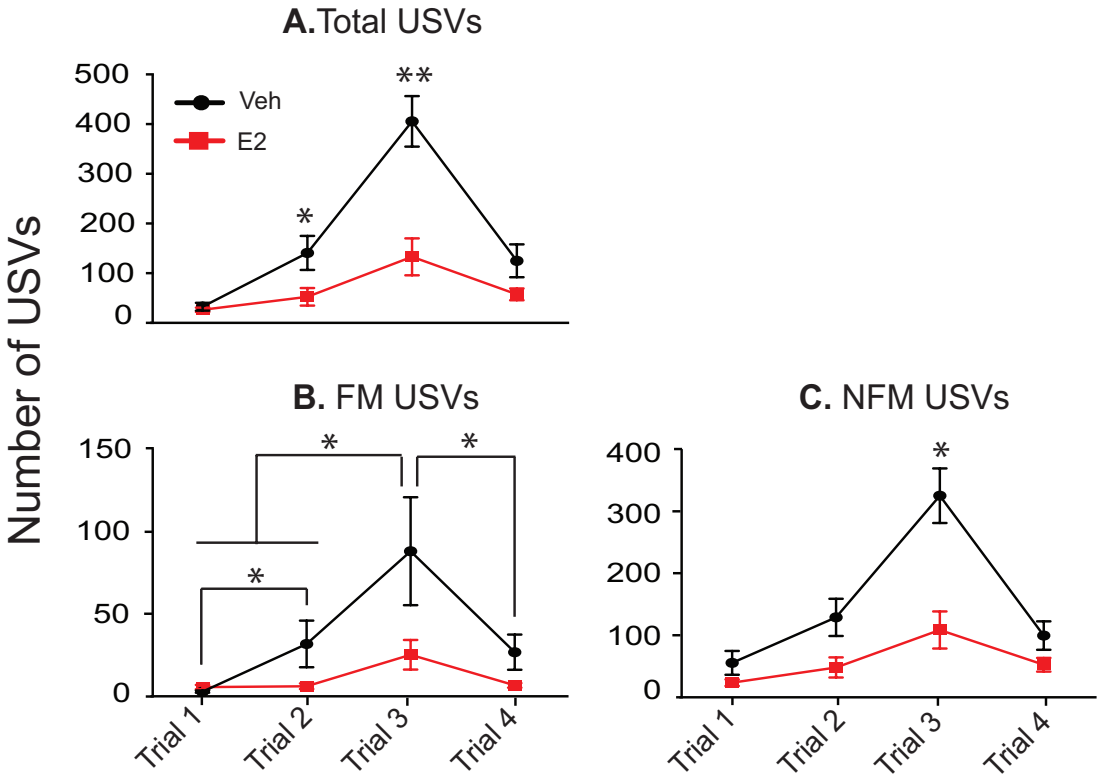


Figure 2.3: Numbers of USV calls

Note that the y-axis varies across graphs. A) The total number of USVs was significantly higher in Veh than E2 treated animals on Trials 2 and 3. B) Non-frequency modulated (NFM) calls were high in Veh than E2 rats on Trial 3. C) For FM calls both veh and E2 called significantly more on Trial 3 compared to all other trials, and more FM calls on Trial 2 than on Trial 1. However, there were no significant treatment effects for FM USVs. Data are shown $\pm$ SEM. Significant differences are shown as single asterisk for p values less than 0.05 (* = $p < 0.05$) and double asterisk for p values less than 0.01 (** = $p < 0.01$).

Figure 2.4

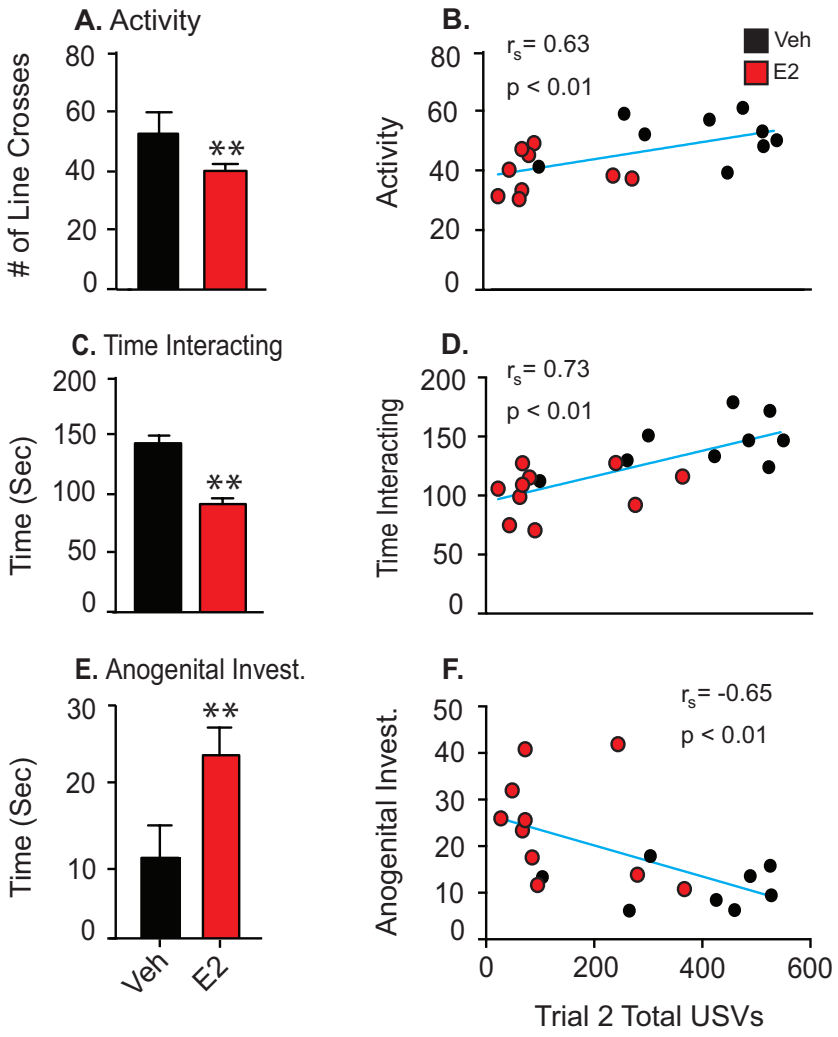


Figure 2.4: USV behavior and correlations

Behavior data are shown from videotapes taken during Trial 3 of the USV test. Note that the y-axis varies across graphs. A) Activity, measured by the number of line crosses, and C) time interacting, were significantly lower in E2 compared to Veh rats. E) By contrast, time spend participating in anogenital investigation was significantly higher in the E2 rats. Correlation analyses were performed for these 3 measures and the total number of USVs measured in Trial 3. B) Activity and D) time interacting were positively correlated with total USVs, whereas F) time spent enaged in anogenital investigation was negatively correlated with total USVs. A-C: Data are shown + SEM. Significant differences are shown as double asterisk for p values less than 0.01 (** = $p < 0.01$).

Figure 2.5

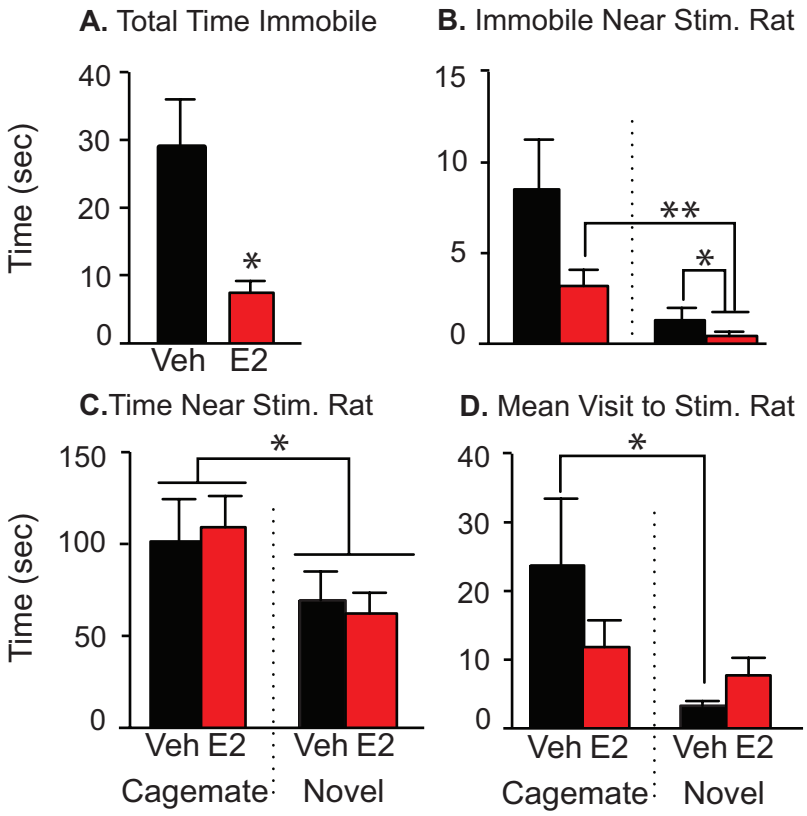


Figure 2.5: Sociability behavior data

Note that the axes vary across graphs. A) Total time spent immobile was significantly greater in Veh than E2 rats. B) When this behavior was further analyzed in proximity to the cagemate or novel stimulus rat, the E2 group spent more time immobilize near the familiar (cagemate) rat than the novel rat. There was also a treatment effect seen for time spent immobile near the novel stimulus rat, with Veh rats significantly greater than the E2 animals. C) More total time was spent near the cagemate than the novel rat, for both treatment groups. D) Veh animals had on average longer visits to the cagemate. Data are shown + SEM.

***Gene expression***

All gene expression results are summarized for PVN, BNST, MeA, SON, and PFC in Table 2.1 Statistics are reported only for genes surviving the false discovery rate test.

Paraventricular nucleus

Only one gene, progesterone receptor (*Pgr*) was significantly affected treatment in the PVN, which was upregulated by $E_2$ compared to Veh ($p < 0.01$, Fig 2.6A).

Bed nucleus of the stria terminalis

Three genes showed a significant effect of treatment in the BNST. Oxytocin receptor (*Oxtr*) was upregulated by $E_2$ ($p < 0.01$, Fig 2.6B) while estrogen receptor beta (*Esr2*) and DNA methyltransferase 3 alpha (*Dnmt3a*) were downregulated by $E_2$ ($p < 0.01$, Fig 2.6C & $p < .0.05$, Fig 2.6D, respectively).

Medial amygdala

In the MeA there were three genes that were significantly upregulated by $E_2$: *Oxtr* ($p < 0.01$, Fig 2.6E), androgen receptor [(*Ar*) $p < 0.05$, Fig 2.6F] and forkhead box P1 [(*Foxp1*) $p < 0.01$, Fig 2.6G]. Tachykinin 3 (*Tac3*) was downregulated by $E_2$ in the MeA ($p < 0.01$, Fig 2.6H).

Supraoptic Nucleus

A total of 10 genes were significantly altered by $E_2$ treatment. Of these, 3 were upregulated by $E_2$ in the SON, all related to social behavior (Fig 2.7A-C): oxytocin [(*Oxt*) $p < 0.01$], *Oxtr* ($p < 0.05$), and vasopressin [(*Avp*) $p < 0.05$]. Seven genes were significantly down-regulated by $E_2$ in the SON (Fig 2.7C–J), mainly involved in neurotransmission and epigenetic regulation: dopamine receptor $D_2$ [(*Drd2*) $p < 0.05$], serotonin receptor 1A [(*Htr1a*) $p < 0.01$], forkhead box P2 [(*Foxp2*) $p < 0.05$], histone deacetylase 2 [(*Hdac2*) $p < 0.01$], histone deacetylase 4 [(*Hdac4*) $p < 0.01$], glutamate receptor 2b [(*Grin2b*) $p < 0.05$], and GABA-B receptor 1 [(*Gabbr1*) $p < 0.05$].

Prefrontal cortex

Although two genes were upregulated by $E_2$ in the PFC, dopamine receptor D2 (*Drd2*) and *Oxtr*, they did not survive false discovery rate correction for multiple comparisons.

Figure 2.6

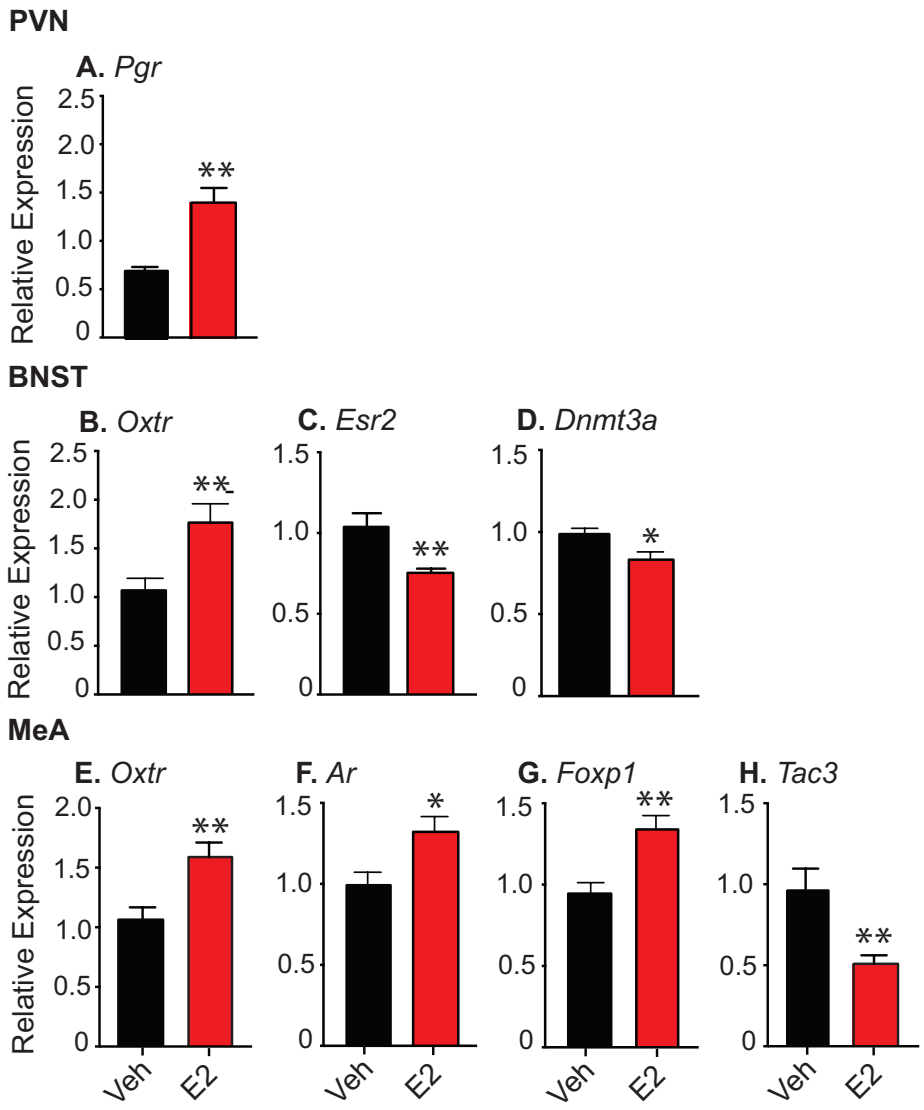


Figure 2.6: Relative gene expression data for the PVN, BNST and MeA

Note that the scale of the y-axis varies depending on the gene. A) In the PVN, *Pgr* was significantly higher in E2 than Veh rats. B-D) In the BNST, *Oxtr* was increased, and *Esr2* and *Enmt3a* decreased, by E2 compared to Veh. E-H) In the MeA, *Oxtr, Ar* and *Foxp1* were increased, and *Tac3* decreased, by E2 compared to Veh. Data are shown + SEM. Significant differences are shown as single asterisk for p values less than 0.05 (* = $p < 0.05$) and double asterisk for p values less than 0.01 (** = $p < 0.01$).

Figure 2.7

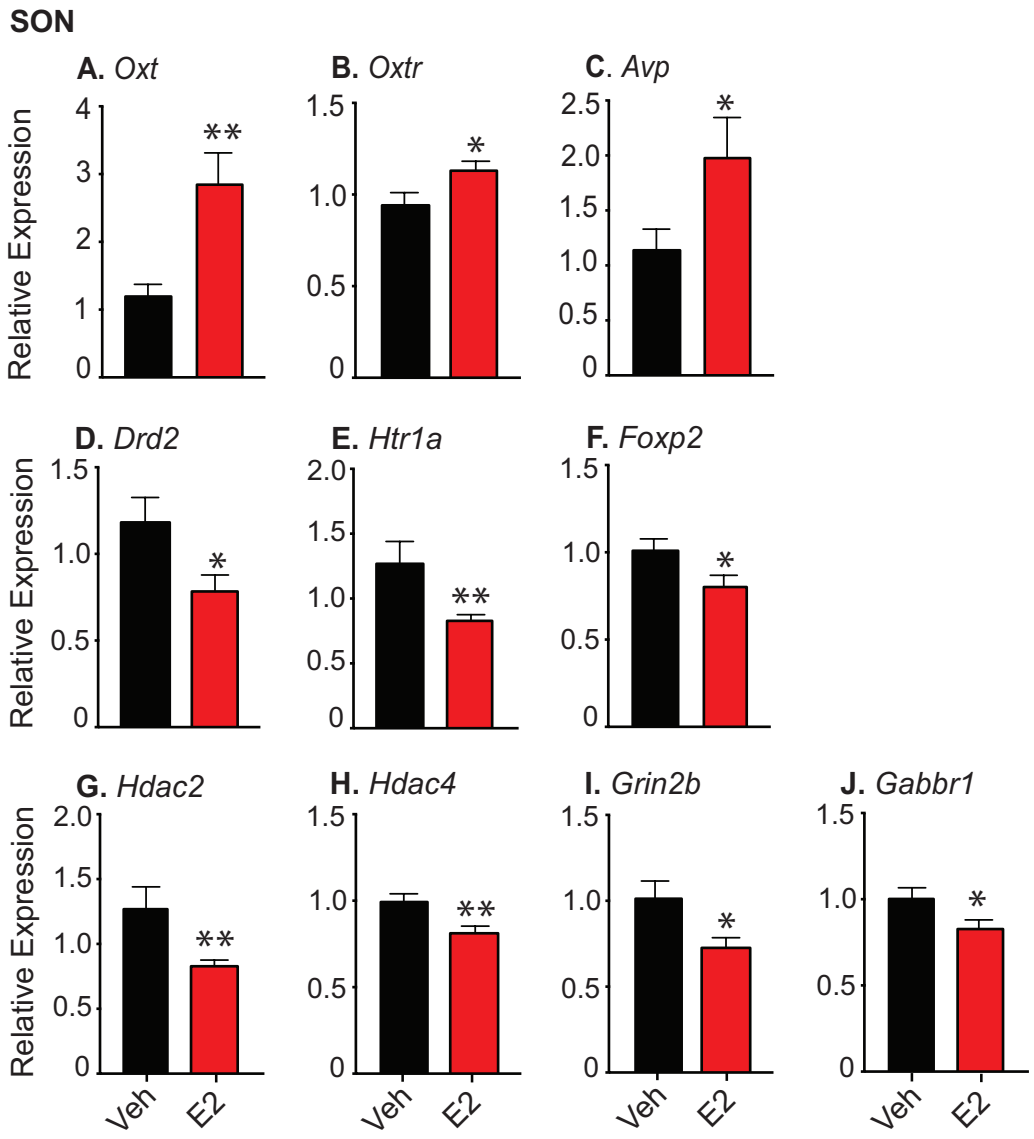


Figure 2.7: Relative gene expression data for the SON

Note that the scale of the y-axis varies depending on the gene. A-C) *Oxt*, *Oxtr*, and *Avp* were higher in estradiol than vehicle treated animals. D-J) *Drd1*, *Htr1a*, *Foxp2*, *Hdac2*, *Hdac4*, *Grin2b*, and *Gabbr1* were lower in E2 than Veh treated animals. Data are shown + SEM. Significant differences are shown as single asterisk for p values less than 0.05 (* = $p < 0.05$) and double asterisk for p values less than 0.01 (** = $p < 0.01$).

## DISCUSSION

This study assessed effects of estradiol on social behavior, and on the underlying neuromolecular circuits involved in the regulation of these behaviors. We designed and utilized novel tests of USV communications and social novelty and preference between same-sex cagemates to assess the influence of E2 on these interactions. This experimental model, focusing on social interaction and social preference between adult female rats, and USVs emitted in the context of familiar non-intruder female-to-female interactions, differs from the literature that typically focuses on opposite-sex interactions (Matochik et al., 1992b; Bialy et al., 2000; Harding & McGinnis, 2003).

Our results showed that 3 months of E2 replacement to OVX rats, roughly estimated as ~5 years in humans (Sengupta, 2013; Quinn, 2005) substantially changed these behaviors in several surprising ways. In the USV test, compared to E2 rats, vehicle rats engaged in USV calling at significantly higher levels, had greater activity and time interacting, but much lower anogenital investigation. In the sociability test, the expected preference of a rat for a novel compared to a familiar conspecific was not seen in the context of our cagemate model, where rats of both treatments spent more time near the cagemate. Assessment of gene expression in 5 brain regions revealed that the SON was most responsive to hormone treatment, and that across brain regions, the oxytocin and vasopressin signaling systems were most commonly affected, a result that likely ties back to the behavioral results.

***Effects of estradiol treatment on social behaviors***

Estradiol decreased USVs and interactions between familiar female cagemates

Studies conducted in rodents of both sexes show an increased preference for a novel over a familiar conspecific (Markham & Juraska, 2007; Carr et al., 1976; Berlyne, 1950; Bevins & Besheer, 2006). We expected to observe such a preference, and further predicted that it would be enhanced in the E2 group relative to the vehicle rats based on work conducted in other social contexts. For example, the number of USVs emitted by cycling, intact female rats is highest during proestrus, when E2 concentrations are high (Matochik et al., 1992b; Matochik et al., 1992a). Other studies in ovariectomized females rats have shown short-term treatment with E2 plus progesterone increased USVs (Matochik et al., 1992a; McGinnis & Vakulenko, 2003).

Contrary to this prediction, our E2-treated rats emitted significantly fewer total USV calls than vehicle rats on Trials 2 and 3, when the cagemates were re-introduced following a 1-week period of separation. A possible explanation for these seemingly disparate outcomes is the role of E2 social memory. Studies using a model of resident intruder have shown that if a female rat or mouse is exposed to the same female intruder several times separated by intervals the resident emitted fewer calls each time, interpreted as indication of social memory (D'Amato & Moles, 2001; Moles et al., 2007; Haney & Miczek, 1993). Thus the fewer calls being emitted by the E2 compared to vehicle rats in the current study suggests potential differences in recognition of the conspecific as their previous cagemate caused by hormone treatment. This is in line with previous studies that have found that E2 treatment after OVX led to enhanced social

recognition compared to their OVX non-treated counterparts (Hlinak, 1993; Tang et al., 2005). Beyond the resident intruder test, the context in which female USVs have been examined has been limited to mating behavior (Thomas & Barfield, 1985; Matochik et al., 1992b).

Thus, our current findings warrant further investigation of USVs as an index of social memory, and application of this type of behavioral analysis to same-sex interactions. Because our results for decreased calling in E2-treated rats was unexpected, more research is needed to better elucidate the regulatory role of estradiol on these complex behaviors.

Estradiol had little effect on social preference in a sociability test

The literature on social novelty shows that rats typically spend more time with novel than familiar animals (Markham & Juraska, 2007; Carr et al., 1976; Berlyne, 1950; Bevins & Besheer, 2006), although female rats have been shown to exhibit a lower novelty preference than males (Cyrenne & Brown, 2011). In our model using cagemates separated for one week, we found the opposite effect, namely, that the preference was for the cagemate over a novel (same-sex, same-treatment) rat. We also saw relatively modest effects of E2 treatment in the test for social preference and social interaction. This was also contrary to our original hypothesis that E2 would enhance an animal's preference for the novel rat, based on research using OVX rodents given acute injections of estradiol, and on results from studies using receptor knockout mice (ERα, ERβ) on social behaviors (Hlinak, 1993; Choleris et al., 2003; Vetter-O'Hagan & Spear, 2012). The lack of novelty

preference could be due to our paradigm that did not enable physical contact between the experimental and stimulus rats. In support of this, Vetter-O'Hagan & Spear (2012) demonstrated that in a social interaction test where the experimental animal was able to freely interact with novel animals, OVX rats spent less time interacting with a novel rat than did their intact counterparts.

***Effects of estradiol treatment on gene expression in the brain***

Five brain regions were used for qPCR profiling of a suite of genes involved in steroid hormone receptors, the vasopressin and oxytocin systems, other neuropeptides and neurotransmitters, and epigenetic modifiers. Regarding regional results, after correction for false discovery rate, no significant effects of E2 were found in the PFC. Only one gene (*Pgr*) was affected in the PVN. The BNST and MeA had 3 and 4 significant genes each. The region with greatest change was the SON. Because of the interconnectedness of the selected brain regions, we have framed the following discussion around 3 functional gene families: steroid hormone signaling, vasopressin and oxytocin signaling, and neurotransmitters involved in social behavior.

Steroid hormone receptors

Three steroid hormone receptor genes were significantly affected in our study: *Pgr* in the PVN (increased by E2), *Esr2* in the BNST (decreased by E2), and *Ar* in the MeA (increased by E2). The increase in *Pgr* in the PVN of E2 compared to vehicle rats is congruent with the literature showing this gene's estrogen responsiveness (Tetel &

Lange, 2009; Mani & Oyola, 2012). *Ar*, which was increased by E2 in the MeA, has abundant expression in this region (Simerly et al., 1990; Stanić et al., 2014). Androgen receptors are sensitive to the changes in sex steroid hormone levels during the estrous cycle in the amygdala (Feng et al., 2010). Regarding There is a high density of ERβ expressing cells throughout the BNST (Shughrue et al., 1997; Shughrue & Merchenthaler, 2001) so the down-regulation of *Esr2* by E2 was not surprising.. The literature on the regulatory effects of estradiol on ERβ in the BNST is mixed, with some studies reporting downregulation (Brown et al., 1996; Gréco et al., 2001) and others no change after $E_2$ treatment (Patisaul et al., 1999; Shima, Yamaguchi & Yun, 2003). E2 regulates expression of both estrogen receptors in a region specific manner (Patisaul et al., 1999; Gréco et al., 2001; Zhou et al., 1995); our lack of effect of E2 on *Esr1* in any region, and only one effect on *Esr2* in the BNST, may be attributable to the longer-term E2 treatment to our OVX rats relative to prior work.

Vasopressin and oxytocin signaling

Oxytocin (*Oxt*) and vasopressin (*Avp*), as well as their receptors, are involved in a variety of social behaviors (see review papers Young, 1999; Neumann & Landgraf, 2012). Mice whose genes for either *Oxt* (Choleris et al., 2003; Winslow & Insel, 2002) or *Avp1ra* receptor (Bielsky et al., 2004) have been knocked out exhibit deficits in social memory and social interaction. Interestingly, similar deficits have been seen in rats with knockouts of either estrogen receptor (Choleris et al., 2006; Imwalle et al., 2002). Similarly, removal of steroid hormones by OVX led to deficits in social interaction and

social memory that are mitigated with administration of estradiol replacement therapy (Hlinák, 1993; Tang et al., 2005).

In our gene expression work, *Oxtr* was upregulated by E2 in all 5 brain regions, but this only survived false discovery rate correction in the BNST, MeA, and SON, the latter regions which have previously been shown to express this receptor (Adan et al., 1995; Elands et al., 1988; Vaccari et al., 1998). Research on *Oxtr* has shown that elevated estrogen levels during the latter phases of pregnancy and partition were associated with increased *Oxtr* expression in the BNST and SON (Meddle et al., 2007; Young et al., 1997). Also, E2 treatment increased *Oxtr* expression in the MeA after 48 hours (Quiñones-Jenab et al., 1997) and 9 days (Patisaul et al., 2003) of treatment. In the SON, one study reported no changes in *Oxtr* across the different stages of the estrous cycle or pregnancy (Young et al., 1997), whereas *Oxtr* was increased during late gestation in rats (Bealer et al., 2006) and prairie voles (Ophir et al., 1013). Bealer and colleagues (2006) also reported that E2 treatment after OVX increased *Oxtr* expression in the SON compared to vehicle.

In the SON, along with *Oxtr,* both vasopressin (*Avp*) and *Oxt* gene expression were upregulated by E2 treatment. Estrogen regulates *Avp* and *Oxt* in the SON predominantly through the ERβ (Winslow & Insel, 2004; Hrabovszky et al, 1998), and ERβ is also colocalized with both nonapeptides in this region (Hrabovszky et al., 1998; Alves et al., 1998; Patisaul et al., 2003). However, the literature on E2 regulation of *Avp* and *Oxt* in the SON is mixed. Some studies showed that $E_2$ upregulated (Roy et al., 1999), downregulated (Shughrue et al., 2002; Van Tol et al., 1988), or had no effect on

expression (Peter et al., 1990; Rhodes et al., 1981). Our data add to this literature on the SON by showing that long-term E2 treatment up-regulates expression of genes involved in nonapeptide signaling.

Neurotransmitters involved in social and affective behavior

Depression and anxiety are twice as common in women than in men (Wong & Licinio, 2001), and menopause is associated with increases in affective dysfunctions (Freeman et al., 2004; Schmidt et al., 2004; Bromberger et al., 2011). Estrogens are believed to play a role in anxiety and depressive behavior by modulating serotoninergic and dopaminergic neural systems (Morissette & Di Paolo, 1993; van de Kar et al., 2002; Bazzett & Becker, 1994; Lammers et al., 1999). GABA and glutamate neurotransmission, also implicated in these behaviors, are also estrogen-sensitive (Petty, 1995; Brambilla et al., 2003; Hashimoto et al., 2013; Herbison, 1997; Micevych & Mermelstein, 2008).

In our study, expression of the dopamine receptor D2 (*Drd2*), serotonin receptor 1a (*Htr1a*), NMDA receptor subunit 2b (*Grin2b*), and the GABA-B receptor 1 (*Gabbr1*) were downregulated by E2, a result that was specific to the SON. Although there is a strong literature on effects of E2 on serotonin receptors and transporters, and for roles of E2 in modulating affective behaviors (McQueen, Wilson & Fink, 1997; Sumner & Fink, 1993 & 1995, Biegon & McEwen, 1982; Raap et al., 2000; Charoenphandhu et al., 2011; Mize et al., 2001, Klemenhagen et al., 2006; Parks et al., 1998; Ramboz et al., 1998, Lerer et al., 1999), little work has been conducted in the SON. It has been

postulated that E2 regulation of serotonin receptors may impact a women's responses to serotonin modulating drugs (Fischette et al., 1983; Kendall et al., 1981; Rubinow et al., 1998) and that combining both estrogens with SSRIs could lead to better therapeutic effects (Xu et al., 2009).

The dopamine receptor $D_2$ is the most abundant subtype in the central nervous system (Dailly et al., 2004), including the hypothalamus (Bouthenet et al., 1991; Mansour et al., 1990; Meador-Woodruff et al., 1991). However, most research on E2 regulation of dopaminergic signaling has focused on the mesolimbic system (Roy et al., 1990; Bédard et al., 1983; Gordon & Perry, 1983). In addition, in striatum, E2 decreased expression of the $D_2$ receptor (Lammers et al., 1999). Our result that expression of *Drd2* in the SON is decreased by long-terms E2 treatment adds to this literature.

## CONCLUSION

Our results that E2 had strong effects on genes involved in social behavioral regulation, especially in the SON, taken together with our results on USV communications, lead us to speculate that the molecular changes caused by E2 are associated with, or may even cause, the behavioral outcomes. It is interesting that the nonapeptide signaling pathways involved in social interaction and social memory, are all upregulated by E2, whereas neurotransmitter pathways are all down-regulated by E2. Clearly more work would be needed to prove a causal relationship between E2 treatment, effects on neural pathways, and behavior but our work is an important first step in identifying potential candidates.

The novel USV test developed for this study represents an emerging technique that can be used to measure social memory or behavior in other contexts (Ciucci et al., 2008; Johnson et al., 2015; Lee et al., 2015). Translation of studies in rats may help inform research in women, especially those undergoing surgical or natural menopause. While E2 has benefits in the treatment of depression and anxiety (Zweifel & O'Brien, 1997; Schmidt et al., 2000; Gambacciani et al., 2003) there is little research on the importance of the social context. In our future work, we will apply these behavioral and molecular measures to a preclinical model of menopause in rats.

| Gene | Name | PVN | BNST | MeA | SON | PFC |
|---|---|---|---|---|---|---|
| **Steroid hormone receptors** | | | | | | |
| *Esr1* | Estrogen Receptor Alpha | ------ | ------ | ------ | **0.003↓** | -- |
| *Esr2* | Estrogen Receptor Beta | ------ | **0.001↑** | ------ | ------ | -- |
| *Ar* | Androgen Receptor | ------ | ------ | **0.014↑** | ------ | -- |
| *Pgr* | Progesterone Receptor | **0.000↑** | ------ | ------ | ------ | -- |
| *Nr3c1* | Nuclear Receptor Subfamily 3, Group C, Member 1 (glucocorticoid receptor) | ------ | ------ | ------ | ------ | -- |
| **Oxytocin and vasopressin signaling** | | | | | | |
| *Avp* | Vasopressin | ------ | ------ | ------ | **0.043↑** | -- |
| *Avpr1a* | Vasopressin Receptor 1a | ------ | ------ | ------ | ------ | -- |
| *Oxt* | Oxytocin | ------ | ------ | ------ | **0.004↑** | -- |
| *Oxtr* | Oxytocin Receptor | ------ | **0.006↑** | **0.003↑** | **0.038↑** | -- |
| **Neurotransmission** | | | | | | |
| *Drd1a* | Dopamine Receptor D1A | ------ | ------ | ------ | **0.034↓** | -- |
| *Drd2* | Dopamine Receptor D2 | ------ | ------ | ------ | ------ | -- |
| *Htr1a* | 5-hydroxytryptamine (serotonin) receptor 1A, G protein-coupled | ------ | ------ | ------ | ------ | -- |
| *Htr2a* | 5-Hydroxytryptamine (serotonin) Receptor 2A, G Protein-Coupled | ------ | ------ | ------ | ------ | -- |
| *Htr2c* | 5-hydroxytryptamine (serotonin) receptor 2C, G protein-coupled | ------ | ------ | ------ | | -- |
| *Oprm1* | Opioid Receptor, Mu 1 | ------ | ------ | ------ | ------ | -- |
| *Oprk1* | Opioid Receptor, Kappa 1 | ------ | ------ | ------ | ------ | -- |
| *Oprd1* | Opioid Receptor, Delta 1 | ------ | ------ | ------ | ------ | -- |
| *Grin1* | Glutamate Receptor, Ionotropic, N-methyl D-aspartate 1 | ------ | ------ | ------ | ------ | -- |
| *Grin2a* | Glutamate Receptor, Ionotropic, N-methyl D-aspartate 2A | ------ | ------ | ------ | ------ | -- |
| *Grin2b* | Glutamate Receptor, Ionotropic, N-methyl D-aspartate 2B | ------ | ------ | ------ | **0.026↓** | -- |
| *Grin2c* | Glutamate Receptor, Ionotropic, N-methyl D-aspartate 2C | ------ | ------ | ------ | ------ | -- |
| *Grin2d* | Glutamate Receptor, Ionotropic, N-methyl D-aspartate 2D | ------ | ------ | ------ | ------ | -- |
| *Gria1* | Glutamate Receptor, Ionotropic, AMPA 1 | ------ | ------ | ------ | ------ | -- |
| *Gria2* | Glutamate Receptor, Ionotropic, AMPA 2 | ------ | ------ | ------ | ------ | -- |
| *Gabbr1* | GABA B Receptor 1 | ------ | ------ | ------ | **0.045↓** | -- |
| *Gabbr2* | GABA B Receptor 1 | ------ | ------ | ------ | ------ | -- |
| **Growth factor signaling** | | | | | | |
| *Bdnf* | Brain-Derived Neurotrophic Factor | ------ | ------ | ------ | ------ | -- |
| *Igf1* | Insulin-Like Growth Factor 1 | ------ | ------ | ------ | ------ | -- |
| *Igf1r* | Insulin-Like Growth Factor 1 Receptor | ------ | ------ | ------ | ------ | -- |
| *Egr1* | Early Growth Response 1 | ------ | ------ | ------ | ------ | -- |
| **Epigenetic signaling** | | | | | | |
| *Hdac1* | Histone Deacetylase 1 | ------ | ------ | ------ | ------ | -- |
| *Hdac2* | Histone Deacetylase 2 | ------ | ------ | ------ | **0.003↓** | -- |
| *Hdac4* | Histone Deacetylase 4 | ------ | ------ | ------ | **0.008↓** | -- |
| *Dnmt1* | DNA (cytosibe-5-)-methyltransferase 1 | ------ | ------ | ------ | ------ | -- |
| *Dnmt3a* | DNA (cytosibe-5-)-methyltransferase 3 alpha | ------ | **0.014↑** | ------ | ------ | -- |
| *Dnmt3b* | DNA (cytosibe-5-)-methyltransferase 3 alpha | ------ | ------ | ------ | ------ | -- |
| **Other** | | | | | | |
| *Crh* | Corticotropin Releasing Hormone | ------ | ------ | ------ | ------ | -- |
| *Foxp2* | Forkhead Box P2 | ------ | ------ | ------ | **0.040↓** | -- |
| *Foxp1* | Forkhead Box P1 | ------ | ------ | **0.002↑** | ------ | -- |
| *Nlgn3* | Neuroligin 3 | ------ | ------ | ------ | ------ | -- |
| *Shank1* | SH3 and Multiple Ankyrin Repeat Domains 1 | ------ | ------ | ------ | ------ | -- |
| *Tac3* | Tachykinin 3 | ------ | ------ | **0.010↓** | ------ | -- |

Table 2.1: List of 42 selected genes

List of 42 selected genes quantified using Taqman low-density arrays (does not include 3 house-keeping genes, *Gapdh, Rpl13a,* and *18s*). P-values are shown for genes that had a significant effect of treatment after false-discovery rate correction. Three genes were non-detectable and are not listed above: *Dbh, Slc6a3,* and *Slc6a4*. Estrogen effects are shown with colored arrows: up-regulation by estradiol, compared to vehicle, is shown in green up-arrows, and down-regulation in red down-arrows.

# CHAPTER 4: THE EFFECTS OF TIMING AND DURATION OF ESTRADIOL TREATMENT ON SOCIAL BEHAVIOR AND GENE EXPRESSION IN AGING FEMALE RATS

## ABSTRACT

This study focused on testing the effects of timing and duration of estradiol ($E_2$) replacement on social behavior and gene expression in ovariectomized aging female rats. Communication, social memory and preference were examined at 2 different time points (3-months & 6-months) using an ultrasonic vocalization (USV) test and a sociability test in the social context of female-female cagemates. A 48-gene qPCR array was used to look at underlying neurobiological molecular changes in 5 brain regions. At the 3-month testing period 3-months of estradiol treatment significantly decreased the number of USVs emitted and decreased the amount of time spent interacting. No differences in number of USVs emitted were observed between the 4 treatment groups at the 6-month testing period. There were few effects of timing and duration of $E_2$ in the sociability test. For gene expression, different patterns of timing and duration of estradiol were seen across the 5 brain regions. With the majority of changes seen in genes involved in regulating social behavior such as neuropeptides (Oxt, Oxtr & Avp), neurotransmitters (Drd1, Drd2, Htr2a, *Grin2d* & *Gabbr1),* and steroid hormone receptors (Esr2, Ar, Pgr). These data suggest that the timing and duration of $E_2$ treatment have specific effects on social behavior and expression of target genes involved in the regulation of these behaviors.

## INTRODUCTION

All women will experience menopause during their lifetime, be it naturally or surgically. The loss of ovarian hormones during menopause is associated with a variety of physiological changes that sometimes impair the quality of life. Hormone replacement therapy (HRT) is currently the most commonly used treatment for these menopausal symptoms. Since the early termination of the Women's Health Initiative (WHI) due to a small but significant increase in certain adverse incidents in women taking hormone therapy (Rossouw et al., 2002; Manson et al., 2013), women and physicians have been left wondering whether HRT should be taken and if so, when and for how long. Subsequent evaluations of the WHI suggested that there may be a "critical window" in which HRT is beneficial but after which it can become detrimental (Klaiber et al., 2005; Bhupathiraju & Manson, 2014). However, despite much speculation, few preclinical studies have directly tested the critical window hypothesis (Gibbs, 2000; Daniel et al., 2006).

While the most common neurobiological symptoms of perimenopausal women are hot flashes and sleep disturbances, a subset of women experience neurobehavioral changes such as anxiety and depression, sometimes leading to problems in interpersonal relationships and a lack of desire to interact socially (Uguz et al., 2011; Deeks & McCabe, 2004; Lanza di Scalea et al., 2012; Schmidt et al., 2000). Animal studies show that both the neural circuits and neurotransmitters involved in the control of social behavior, and the behaviors themselves, are subject to age- and hormone (especially estradiol) regulation. For example, with aging, rodents show increased anxiety and

decreased social interaction (Salchner et al., 2004; Guan & Dluzen, 1994; Boguszewski & Zagrodzka, 2002). Hormones are also important, as ovariectomy in female rats leads to decreased social interaction independent of age (Hlinák, 1993; Tang et al., 2005). Mice lacking either of the nuclear estrogen receptors show deficits in social interaction and social memory (Choleris et al., 2006; Egashira et. al, 2007). Short-term estradiol (E2) replacement in mature adult female rats improves social memory and increases social interaction (Hlinák, 1993; Tang et al., 2005). The neurotransmitter systems involved in regulating these behaviors, including serotoninergic and dopaminergic neurons, are highly sensitive to E2 (Morissette & Di Paolo, 1993; van de Kar et al., 2002; Bazzett & Becker, 1994). The neuropeptides oxytocin and vasopressin, and their receptors, involved in social behavioral regulation, are also E2-sensitive (Winslow & Insel, 2004; Hrabovszky et al, 1998; Quiñones-Jenab et al., 1997; Axelson & van Leeuwen, 1990; Garcia et al., 2016).

The current study sought to test the critical window hypothesis on the social behavioral phenotype, and to determine how underlying neurotransmitter systems in a defined neural network are altered by differential treatment regimes. Social behaviors were tested using a novel test of ultrasonic vocalizations (USVs) as an index of communicative and affective state, and a test of social interaction and memory (Tang et al., 2005; D'Amato & Moles, 2001; Moles et al., 2007; Hlinak, 1993). The neuromolecular phenotype of these animals was assessed using low-density qPCR arrays of relevant brain regions. An important feature of our model is that work was conducted on middle-aged female rats, as an age-appropriate model for perimenopausal women.

# MATERIAL AND METHODS

### *Animals and Husbandry*

All animal procedures were conducted in agreement with The Guide for the Care and Use of Experimental Animals following protocols approved by The University of Austin IACUC committee and NIH standards. Aged adult (AG, 11-12 months, retired breeders) female Sprague Dawley rats (Harlan) were purchased for this project. At the time of arrival animals were double housed and allowed to acclimate to their new environment for 2 weeks prior to surgery. Animals were given water and food ad libitum and kept on a 12-hour dark cycle (lights on at 0700). During the acclimation period estrous cyclicity was monitored daily by vaginal lavage of sterile saline. Only animals that had a regular 4-5 day cycles were used for the experiment. After the acclimation period all animals underwent ovariectomy (OVX) surgery under isofluorane inhalation anesthesia. At the start of the surgery each animal was given a single injection of Rimadyl (5 mg/kg) for pain. Bilateral dorsolateral incisions were made through the skin, muscle, and peritoneum, and the ovaries were ligated and removed. Muscles were sutured and wound clips used to close the skin. At the time of surgery animals were implanted subcutaneously between the shoulder blades with capsules containing either 100% cholesterol (Veh) or 5% 17β-estradiol / 95% cholesterol ($E_2$) at the time of surgery. Three months after the OVX surgery animals were anesthetized again. Half of the animals had their capsules checked to verify that they were intact. The other half of the rats had their treatments switched (E2 to vehicle, or vehicle to E2). Groups (Figure 3.1) are subsequently referred to as V6 (6 months vehicle, n = 14), E6 (6 months E2, n = 14),

V3/E3 (3 months vehicle, switched to 3 months E2, n = 14) and E3/V3 (3 months E2, switched to 3 months vehicle, n = 14). Rats were pair-housed, with both cagemates assigned to the same treatment group.

Figure 3.1

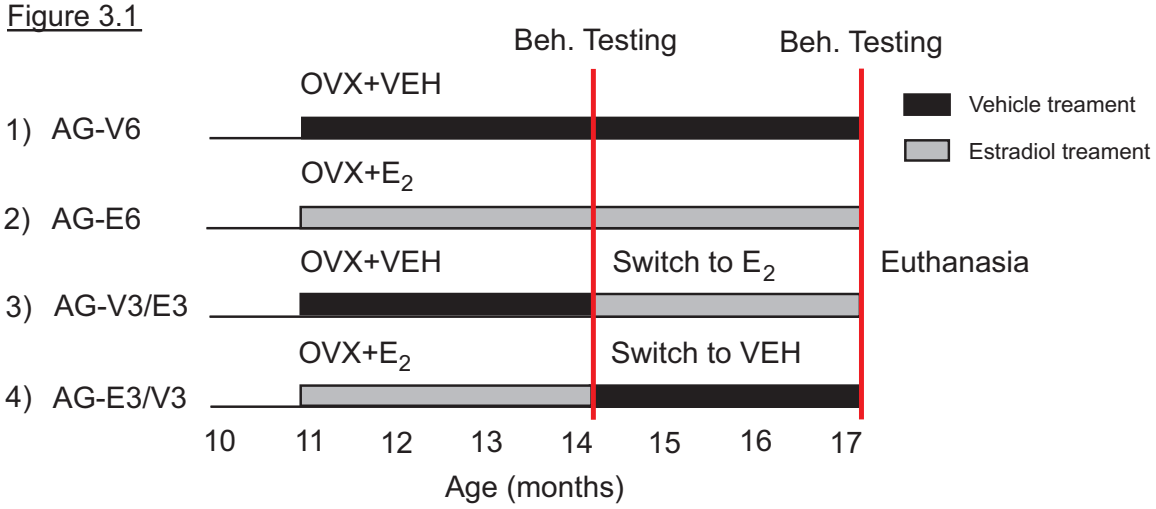


Figure 3.1: Rat model

Rat were 11 months of age at the time of OVX, and received either vehicle (cholesterol, VEH) or 17β-estradiol capsule implantation at the time of surgery. Groups 1 & 2 received treatment for a duration of 6-months. Groups 3 & 4 were the "switch" groups that received either VEH or E2 initially at the time of OVX and were switched to the opposite treatment after 3-months, and continued on the new treatment for a 3-month duration. Behavioral testing occurred twice, once at the end of the 3-month period, and again at the 6-month period.

***Behavioral paradigms***

Prior to the start of behavioral testing all animals were separated from their cagemates for one week and singly housed. Two rounds of behavioral testing were performed for this study, the first towards the end of the first 3-month period, and the second round after 6-months of treatment (3 months after switching hormone, or

verifying the capsule in rats for which the same treatment was continued. This allowed us to examine the effects of timing and duration of hormone replacement therapy on the same animals' behavior.

Ultrasonic vocalization test (USV)

Following a week of separation, USV testing was conducted on the cagemates over 2 consecutive days in a total of four 5-minute trials, during which USVs were recorded. On day 1 each rat was habituated to a rectangular Plexiglas apparatus (23L x 29W x 40H cm) for 5 minutes (Trial 1). On day 2, rats were given three sequential 5-minute trials, referred to as Trials 2, 3, and 4. In Trial 2, the apparatus was fitted with a removable plastic perforated grid that bisected the apparatus. The cagemates were placed into the apparatus separated by the grid. This was the first re-introduction of the rats after the week of isolation; they could engage in limited interactions across the grid but did not have physical contact. For Trial 3, the perforated grid was removed and the cagemates were allowed to freely interact with one another. Videorecording of this trial was performed, and recordings used to quantify activity, time interacting, and anogenital investigation of the partners. For Trial 4 the cagemates were separated into 2 identical apparati and recorded separately. Upon completion of Trial 4 on day 2, all animals were housed separate from their cagemates until they completed the sociability test.

UltraSoundGate hardware and software was used to record ultrasonic vocalizations during all four trials. Saslab Pro (Avisoft, Germany) was optimized so that we were able to use it to automatically detect and quantify calls. We found that all calls

fell in the range between 30 and 70kHz. We used these data to further differentiate calls into frequency modulated (FM) or non-frequency modulated (NFM). A call was considered to be a frequency modulated if it had more than a 9kHz change in frequency. Due to the inability to distinguish which rat was calling when the cagemates were together in the apparatus during Trials 2 and 3, we treated each set of cagemates as a unit when performing analysis. Therefore, when the animals were recorded separately during Trials 1 and 4, their calls were summed to be comparable to results in Trials 2 and 3. USV behavior that was video recorded during Trial 3 was hand scored by observer that was blind to the treatment of the animals. If the animals we participating in meaningful contact such as nose-touching or grooming it was recorded as time interacting. Time active was the number of times each animal crossed the centerline of the chamber with all four limbs.

Statistics were conducted using SPSS (Version 23). The data sets from the USV test did not meet assumptions of normality and variance even after transformations were performed. Therefore, non-parametric statistics were used. Data were tested for outliers using the Grubb's outlier test, and confirmed outliers were excluded from final analysis. Data were analyzed for the total number of calls, as well as for FM and NFM calls. Due to the non-normality both the data from the 3-month and 6-month testing period were analyzed using a Mann-Whitney tests to examine the effects of treatment during each trial. For all of these analyses, alpha was set at 0.05. In addition, a false discovery rate correction (q-value), Benjamini-Hochberg method (Benjamini & Hochberg, 1995), was used to correct for the multiple comparisons.

Sociability test

We used a 3-compartment apparatus to test discrimination and/or preference of an experimental rat for her cagemate vs. an unfamiliar same-treatment rat. There were 2 consecutive days of testing. On day 1, one of the cagemates was randomly chosen to be the experimental rat. This rat was allowed to habituate for 5 minutes to a Stoelting Plexiglas three-chamber apparatus (100L x 100W x 34.5H cm total), which contained two holding cages in each corner of the side chambers. Once habituation was completed the experimental rat was placed back into its home cage. Her cagemate was placed into one of the holding cages, and a novel rat of the same sex, age, and treatment was placed into the other holding cages. The placement of rats was randomized. Then, the experimental animal was placed into the center chamber of the apparatus and allowed to explore the entire apparatus for 5 minutes. Rats were singly housed overnight. On day 2 the original experimental rat was now used as a stimulus rat, and her cagemate used as the experimental rat. A new rat (same treatment but different from the novel rat on day 1) was used as the novel rat on day 2. The experimental rat was given 5 minutes to explore the entire apparatus. On both days, the experimental rats' movement and behavior were tracked using Any-Maze software (Stoelting Co., Wood Dale, IL). At the end of day 2 of sociability testing all animals were rehoused with their cagemates until time of euthanasia 1 week later.

Statistics were conducted using SPSS (Version 23). For data that met assumptions of normality a repeated measures test was performed to determine effects of treatment on

the behavioral measures. Data that did not meet assumptions a square root transformation was used followed by a repeated measures test. However, most of the data did not meet assumptions even after transformations; therefore a Mann-Whitney test was used to examine difference in each round and a Wilcoxon signed rank test was used to examine differences between the 3-month and 6-month testing periods. Alpha was set at 0.05 and a false discovery rate correction (q-value), Benjamini-Hochberg method (Benjamini & Hochberg, 1995), was used to correct for the multiple comparisons.

***Brain tissue processing***

Six months after OVX and a week after behavioral testing was completed, animals were euthanized between 1330-1600 hours by rapid decapitation; all animals were 17 months of age. Trunk blood was collected and the brains were removed, chilled on ice, and sectioned coronally at 1 mm increments using an ice-cold stainless steel brain matrix. Brain sections were stored overnight at 4°C in cryogenic storage vials that contained RNAlater (Life technologies, Grand Island, NY). The next day, the sections were mounted onto chilled slides. Bilateral brain punches were taken from 5 regions under a dissecting microscope using Palkovits punches and the Paxinos and Watson (2009) rat brain atlas (all coordinates are based on that atlas). The paraventricular nucleus (PVN) punch (1.22 mm diameter) began rostrally at ~Bregma = -0.84 mm, and extended caudally 1 mm. The supraoptic nucleus (SON) punches (0.96 mm diameter) started rostrally at ~Bregma = -0.60 mm and extended caudally for 1 mm. The medial amygdala (MeA) punches (1.22 mm diameter) began rostrally at ~Bregma = -1.56 mm, and

extended caudally 1mm. The bed nucleus of the stria terminalis (BNST) punches (0.96 mm diameter) started rostrally at ~Bregma = 0.00 mm, and extend caudally for 1mm. The prefrontal cortex (PFC) punches (1.22 mm diameter) began rostrally at ~Bregma = 4.20 mm, and extend caudally for 1mm. Brain punches were placed in frozen RNAse free Eppendorf tubes and stored at -80°C until time of PCR. Trunk blood that was collected at euthanasia was allowed to clot for 30 minutes, centrifuged (2300 X g) for 5 minutes, and serum collected and stored at -80°C in Eppendorf tubes until time of hormone assays.

***Real-time PCR assays and analysis***

Using an Allprep RNeasy mini kit (Qiagen, Valencia, California), according to the manufacturer's protocol, RNA was extracted from frozen PVN, SON, MeA, BNST, and PFC punches of individual rats. Using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) the quality of RNA was validated (RIN values were from 8.70 and 9.90) and quantity of RNA was assessed using a GloMax-Multi Detection System (Promega, Madison, WI). A high-capacity cDNA reverse transcription kit (Life Technologies, Grand Island, NY) was used to convert mRNA to single-stranded cDNA; all samples were then stored at -80°C until use. A customized rat Taqman low-density array (TLDA) Microfluidic 48-gene real-time PCR cards (Applied Biosystems) was used. Real-time PCR was performed using Taqman universal mastermix (Life Technologies, Grand Island, NY) and detected on a ViiA7 Real time PCR machine (Applied Biosystems, Life Technologies, Grand Island, NY) with the following run parameters: 0°C for 2 minutes, 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds, and 60°C for 1 minute.

The genes for the assay were chosen specifically for this study, based on their roles in the neurobiology of aging, social behavior, and/or regulation by steroid hormones (45 genes of interest and three normalizing genes; Supplementary Table 1). Relative expression was determined for each sample using the comparative cycle threshold method (Pfaffl, 2001; Schmittgen and Livak, 2008). All samples were normalized to the geometric mean of the housekeeping genes *Gapdh, Rpl13a,* and *18s* and then calibrated to the median δ-cycle threshold of the vehicle treated group. Samples that amplified at or above 35ct were excluded from analysis.

For gene expression data, statistics were performed using relative expression values for each sample. Data was tested for outliers using the Grubbs' outlier test and confirmed outliers were excluded from analysis. If data did not meet assumptions of normality they were transformed using either natural log or square root transformation. Data that met assumptions before or after transformation were analyzed using a one-way ANOVA with a Tukey HSD post hoc. Otherwise, a non-parametric analysis was performed using a Kruskal-Wallis test followed by a Mann-Whitney test to examine differences between each group. For all of these analyses, alpha was set at 0.05 and a false discovery rate correction (q-value), Benjamini-Hochberg method (Benjamini & Hochberg, 1995) was used to correct for multiple comparisons.

***Estradiol hormone assay***

Levels of serum estradiol ($E_2$) were determined by radioimmunoassay (Ultrasensitive Estradiol RIA, Cat No DSL4800, Lot # 150622C, Beckman Coulter,

Pasadena, CA), according to the manufacturer's directions. A single assay was used for all the samples. Samples were run in duplicates with volumes of 100µl of serum. Assay sensitivity was 2.2 pg/ml and intrassay C.V. was 1.30%. Both E6 (34 $\pm$ 5) and V3/E3 (24 $\pm$ 5) had significantly ($p < 0.01$) higher concentrations of estradiol than both the V6 (16 $\pm$ 2) and E3/V3 (14 $\pm$ 1).

## RESULTS

### *Ultrasonic vocalization test*

USV calls

In the first series of USV tests, conducted 3 months after OVX and hormone treatment, there were significant differences between E2 and vehicle treated animals in total calls on Trials 2 and 3. The $E_2$ animals emitted significantly fewer calls than the vehicle rats (Fig. 3.2A). When subdivided into FM and NFM calls, vehicle animals emitted significantly more FM calls than $E_2$ animals on Trial 2 (Fig. 3.2B) of testing. In addition, vehicle animals made significantly more NFM calls on Trials 2 and 3 (Fig. 3.2C).

There were no significant differences in the number of total (Fig. 3.2A) and NFM (Fig. 3.2C) USVs emitted between any of the treatment groups at the 6-month testing period. Analysis of FM calls showed there were significant effects of treatment during Trial 3 (Fig. 3.2B), when V6 animals emitted significantly more USVs than E6 and V3/E3 animals. E3/V3 animals made significantly more calls than E6 and V3/E3 animals.

USV behavior

Trial 3 of the USV test was videotaped, and three behaviors were scored off of the tapes; 1) time spent interacting, 2) number of anogenital investigations, and 3) activity. At the 3-month testing period, vehicle treated animals spent significantly more time interacting with their cagemate than did $E_2$ treated animals (Fig 3.3A). There were no significant differences between the two treatment groups on anogenital investigation (Fig 3.3B) or time active (Fig 3.3C). At the 6-month testing period the E3/V3 treated animals engaged in significantly less anogenital investigation than did V3/E3 and E6 treated animals (Fig 3.3B). There were no significant differences between any of the 4 treatment groups on time spent interacting (Fig 3.3A) or activity (Fig 3.3C).

Figure 3.2

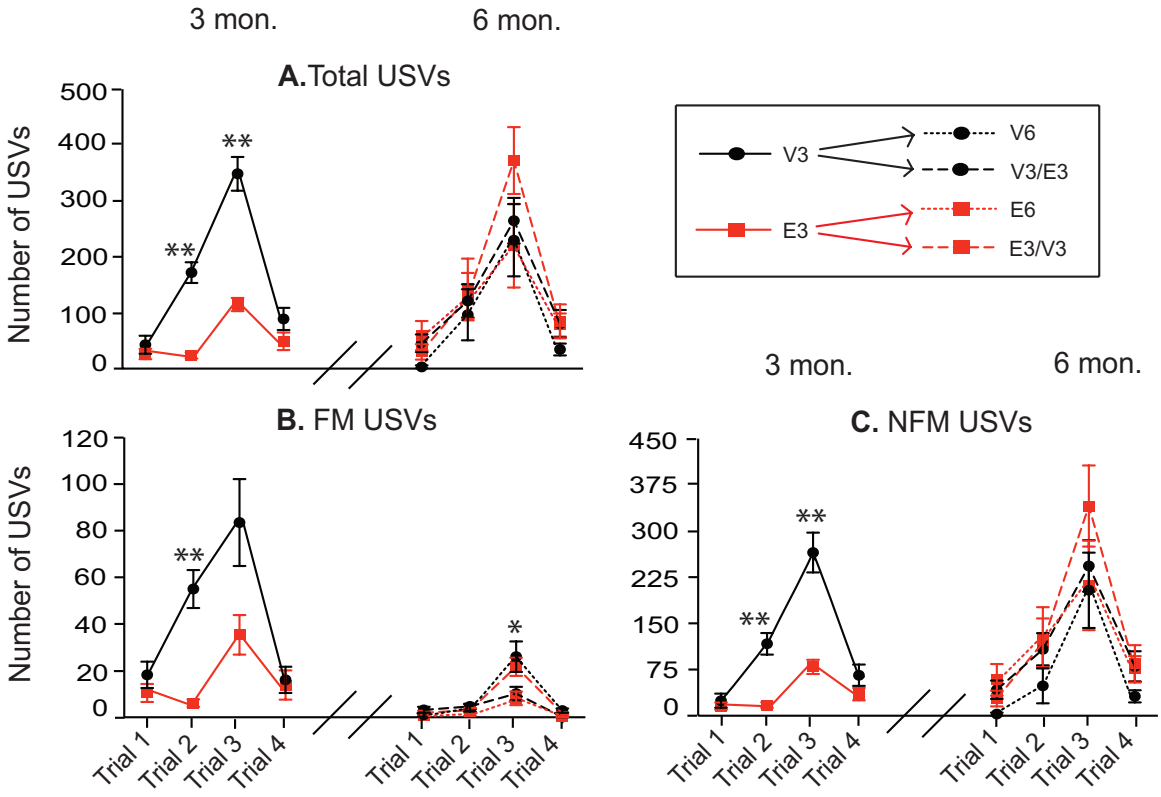


Figure 3.2: Data from ultrasonic vocalization test

Numbers of USV calls are shown the 3-month and 6-month testing periods. Hash marks on the x-axis indicate the 3-month gap between the two testing periods. The legend illustrates the transition of treatments from the 3-month to the 6-month testing period. Note that the y-axis varies across graphs. A) The total number of USVs was significantly higher in V3 than E3 animals on Trials 2 and 3 at the 3-month testing period. There were no differences between treatment groups at the 6-month testing period. B) For FM calls, the V3 group emitted significantly more calls than the E3 animals on Trial 2. At the 6-month testing period both the V6 and E3/V3 groups emitted significantly more calls than the E6 and V3/E3 groups during Trial 3. C) Non-frequency modulated (NFM) calls were higher in V3 than E3 rats on Trial 2. No differences were seen between treatment groups at the 6-month testing period. A-C: Data are shown $\pm$ SEM. Significant differences are shown as double asterisk for p values less than 0.01 (** = $p < 0.01$).

Figure 3.3

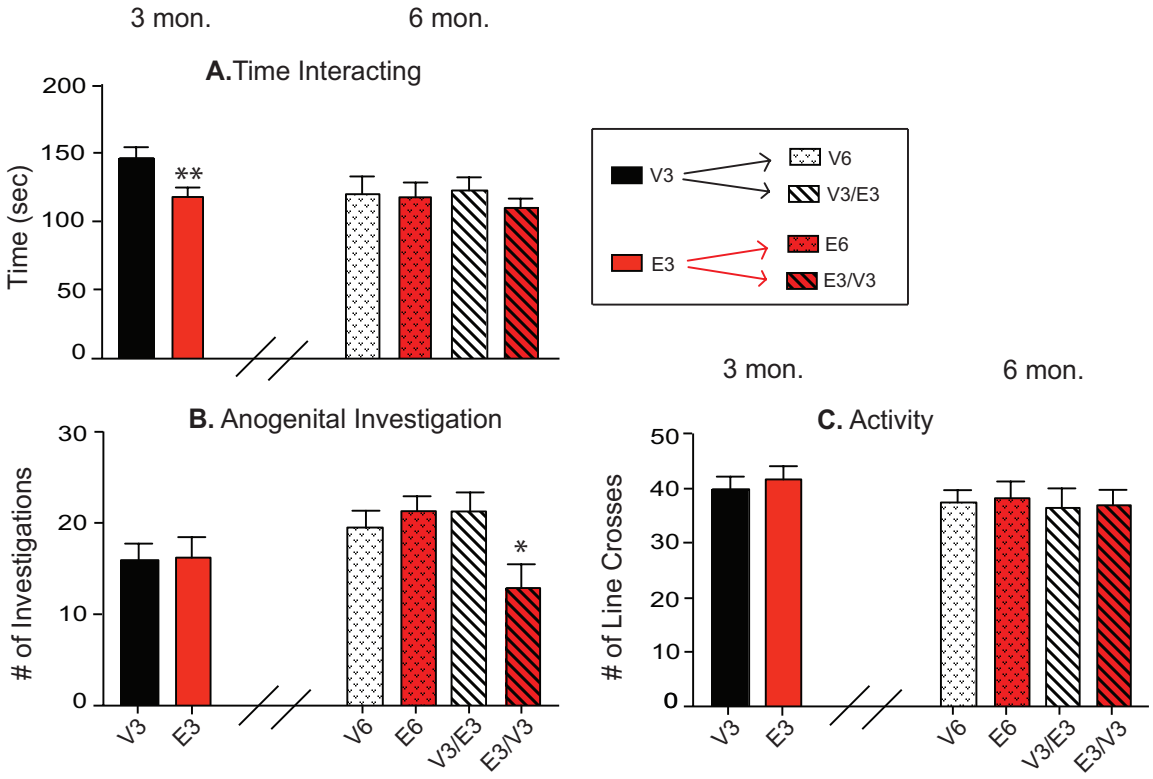


Figure 3.3: Behavior data from Trial 3 of ultrasonic vocalization test at the 3-month and 6-month testing periods

The hash marks on the x-axis indicate the 3-month gap between the two testing periods. The legend illustrates the transition of treatments from the 3-month to the 6-month testing period. Note that the y-axis varies across graphs. A) Time interacting at the 3-month testing period was significantly lower in the E3 group compared to V3. No differences were observed between groups at the 6-month testing period. B) No differences in time spent engaging in anogenital investigation were seen at the 3-month testing period. At the 6-month testing period the E3/V3 spent significantly more time engaging in anogenital investigation than the other 3 groups. C) No differences in activity, measured by the number of line crosses, were seen in either test. A-C: Data are shown + SEM. Significant differences are shown as single asterisk for p values less than 0.05 (* = $p < 0.05$) and double asterisk for p values less than 0.01 (** = $p < 0.01$).

***Sociability test***

There were few treatment effects detected in the sociability test; only significant results are presented. Estradiol treated animals at the 3-month testing period spent more time immobile (Fig 3.4A), traveled farther (Fig 3.4B), and moved significantly more quickly (Fig 3.4C) than vehicle treated animals. Percentage of the social time spent with the novel animal was calculated by dividing time spent with the novel animal by the time spent with the novel animal plus the time spent with the cagemate. When analyzing the differences between the two treatment groups on novelty preference we found that $E_2$ (64% $\pm$ 7%) animals spent a significantly larger portion of their social time with the novel rat over the cagemate than did the vehicle (40% $\pm$ 7%) treated animals. The only significant finding from the 6-month testing period was the amount of time spent with the stimulus animals. V6 treated animals spent significantly more time in proximity to their cagemate than they did with the novel animal (Fig 3.5).

Figure 3.4

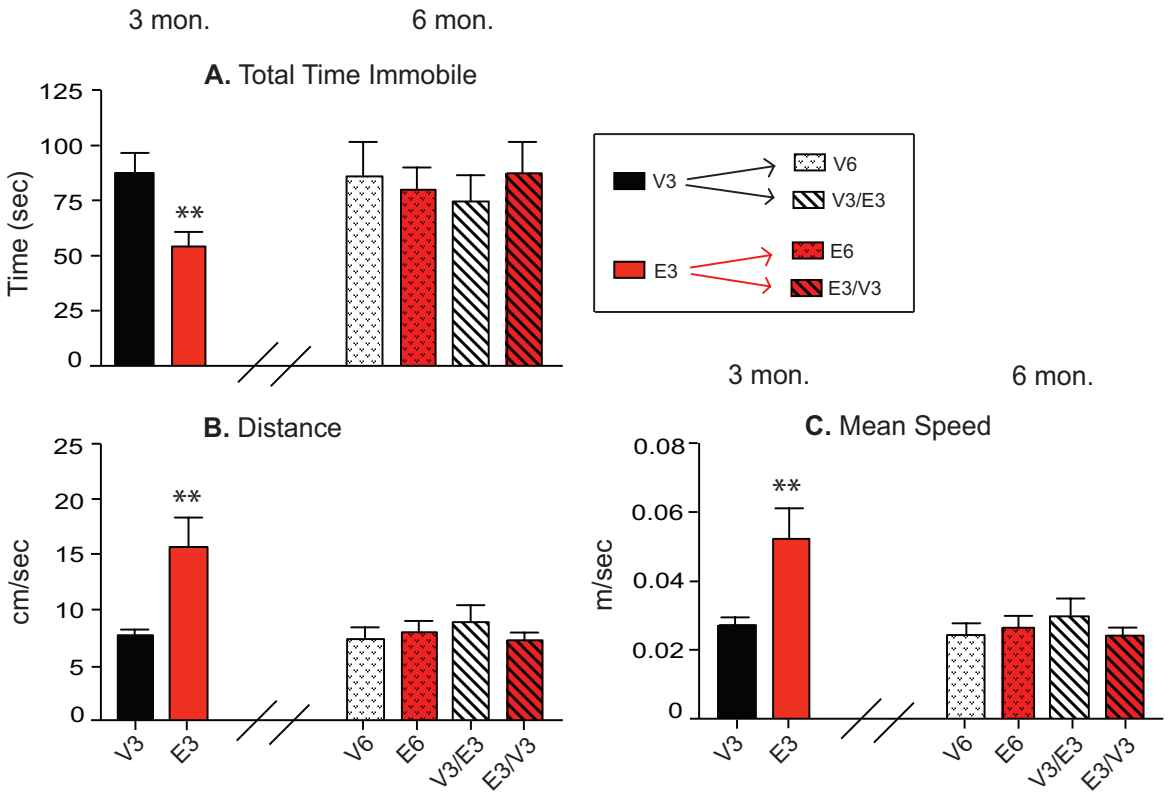


Figure 3.4: Sociability data (1/2)

The hash marks on the x-axis indicate the 3-month gap between the two testing periods. The legend illustrates the transition of treatments from the 3-month to the 6-month testing period. Note that the axes vary across graphs. A) Total time spent immobile was significantly greater in the V3 than the E3 group at the 3-month testing period. B) Total distances traveled were significantly higher in the E3 group than the V3 group at the 3-month testing period. C) The average speed of the E3 groups was significantly faster than the V3 group at the 3-month testing period. There were no treatment differences for any of these parameters at the 6-month testing period. Data are shown + SEM. Significant differences are shown as double asterisk for p values less than 0.01 (** = $p < 0.01$).

Figure 3.5

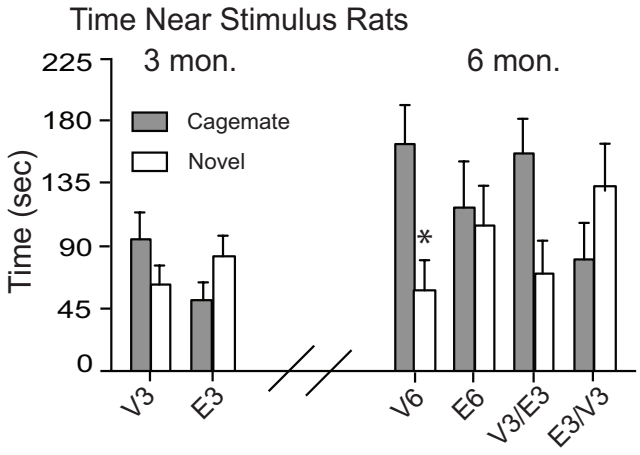


Figure 3.5: Sociability data (2/2)

Sociability results are shown for the total time spent in proximity to the stimulus rats (cagemate vs. novel). The hash marks on the x-axis indicate the 3-month gap between the two testing periods. No differences between the V3 and E3 groups were seen at the 3-month testing period. At the 6-month testing period only the V6 group showed a preference for either of the stimulus rats, spending significantly more time with their cagemate than the novel rat. Significant differences are shown as single asterisk for p values less than 0.05 (* = $p < 0.05$).

***Gene expression***

Gene expression was measured at the end of the study, from brains collected 1 week after behavioral testing was completed at the 6-month period. A summary of significant effects of treatment for the five regions, BNST, SON, PFC, PVN and MeA, is provided in Table 3.1. All genes reported as significantly affected by treatment were identified following false discovery rate correction for multiple comparisons. Supplemental Table 2.1 lists all of the genes on the customized TLDA qPCR card.

Bed nucleus of the stria terminalis

Only one gene, oxytocin receptor (*Oxtr*), was significantly affected in the BNST. There was an effect of timing and duration of treatment: *Oxtr* was lower in the E3/V3 rats compared to V6 and V3/E3 (Fig 3.6A, $p < 0.05$, $p < 0.01$, respectively).

Supraoptic Nucleus

Dopamine receptor $D_2$ (*Drd2*, Fig 3.6B, $p < 0.05$) was lower in V3/E3 rats compared to the V6 and E6 rats. *Igf1* expression was lower in the E3/V3 rats compared to V6 and E6 animals (Fig 3.6C, $p < 0.01$, $p < 0.05$, respectively). The E3/V3 group had lower *Igf1* than the V6 group ($p < 0.05$).

Prefrontal cortex

In the PFC, 4 genes had significant changes in gene expression in relation to timing and/or duration of E2 treatment. *Oxtr* expression was higher in the E6 treatment

compared to V6 rats (Fig 3.7A, $p < 0.05$). Vasopressin receptor 1a (*Avpr1a*) was higher in E6 than both V6 and E3/V3 animals (Fig 3.7B, $p < 0.01$, $p < 0.05$, respectively). Estrogen receptor beta (*Esr2*) expression was decreased in the E3/V3 treatment compared to the V6 group (Fig 3.7C, $p < 0.01$). Brain derived neurotrophic factor (*Bdnf*) was lower in both 6-month treated groups compared to the two switch groups (Fig 3.7D, $p < 0.01$).

Paraventricular nucleus

Eight genes were affected in the PVN. For most of these genes, expression was higher in one or both groups with E2 on board at the time of euthanasia, namely, the V3/E3 and/or the E6 groups. For both *Oxtr* and *Tac3* [Fig 3.8A ($p < 0.01$), 3.8H ($p < 0.05$), respectively] expression was higher in the V3/E3 than the V6 group. For *Oxt,* the E6 group had higher expression than the V6 or the V3/E3 group (Figs 3.8B, $p < 0.05$). Similar gene expression patterns were seen for *Ar, Pgr,* and *Crh*, all of which had highest expression in the V3/E3 rats (Figs 3.8C, D, and E, respectively). Specifically, *Ar* was higher in V3/E3 than in V6 or E6 rats ($p < 0.01$, $p < 0.05$, respectively). Both *Pgr* and *Crh* expression were higher in the V3/E3 than V6 and E3/V3 rats ($p < 0.01$, $p < 0.05$, respectively). For those two genes, the E6 group also had higher expression than the V6 group ($p < 0.01$). DNA methyltransferase 3a (*Dnmt3a*) expression was higher in E3/V3 compared to V3/E3 animals (Fig 3.8F, $p < 0.05$). Early growth response 1 (*Egr1*) was higher in E3/V3 than V6 animals (Fig 3.8G, $p < 0.05$).

Medial amygdala

Twelve genes were affected by timing and duration of hormone treatment in the MeA, nearly all with unique expression patterns. *Oxt* was higher in the E3/V3 rats compared to V6 and V3/E3 (Fig 3.9A, $p < 0.05$, $p < 0.01$, respectively). *Avp* was highest in the E3/V3 rats, and significantly greater than in either 6-month treatment group (Fig 3.9B, $p < 0.05$). For *Ar,* E3/V3 rats had higher expression than V6 rats (Fig 3.9C, $p < 0.01$). The dopamine receptors D1 and D2 [Fig 3.9D ($p < 0.01$), 9E ($p < 0.05$), respectively] had highest expression in the V6 rats, levels of which were significantly different from the V3/E3 animals, the latter having the lowest expression. In addition, for *Drd1*, the E6 and E3/V3 rats had higher expression than the V3/E3 rats ($p < 0.05$, $p < 0.01$, respectively). The serotonin receptor 2a (*Htr2a*, Fig 3.9F) and opioid receptor delta 1 (*Oprd1*, Fig 3.10C) had the same expression pattern, with levels higher in rats with vehicle on board at the time of euthanasia (V6, $p < 0.05$ & E3/V3, $p < 0.01$) compared to E6. GABA B receptor 1 (*Gabbr1*, Fig 3.10A) was higher in E6 than V3/E3 ($p < 0.05$). *Grin2d* had a complex expression pattern (Fig 3.10B), with expression higher in the groups with vehicle on board compared to the E2 on-board groups ($p < 0.05$). The two DNA methyltransferases, *Dnmt1a* (Fig 3.10D) and *Dnmt3a* (Fig 3.10E), as well as the glucocorticoid receptor *Nr3c1* (Fig 3.10F) had highest expression in the E3/V3 groups relative to the E6 and/or V3/E3 animals ($p < 0.01$).

Figure 3.6

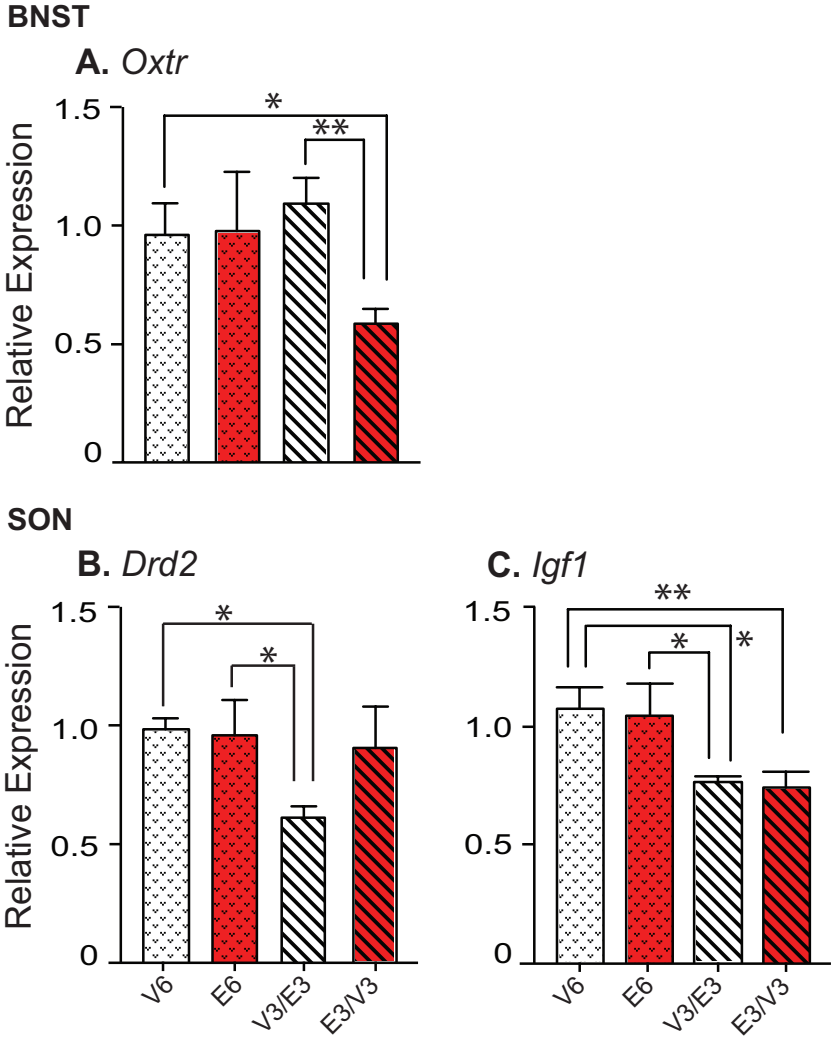


Figure 3.6: Relative gene expression data for the BNST and SON

Note that the scale of the y-axis varies depending on the gene. Data are shown + SEM. Significant differences are shown as single asterisk for p values less than 0.05 (* = $p < 0.05$) and double asterisk for p values less than 0.01 (** = $p < 0.01$).

Figure 3.7

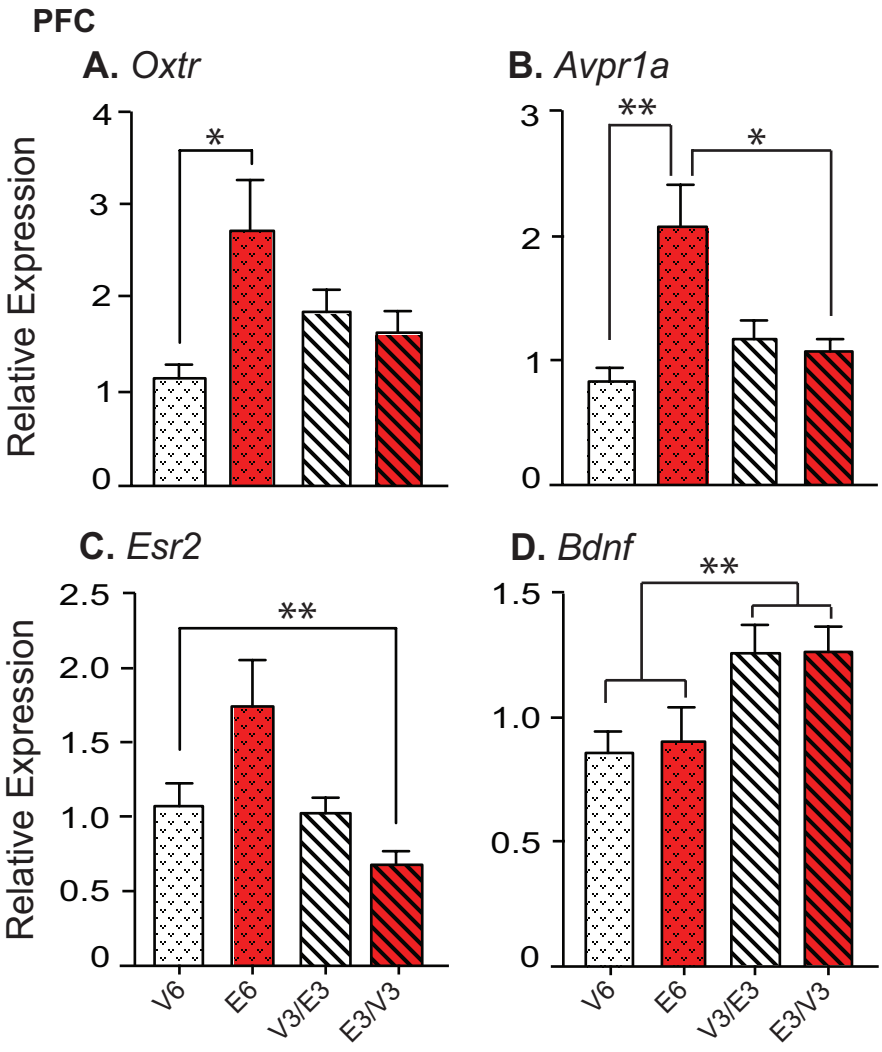


Figure 3.7: Relative gene expression data for the PFC

Note that the scale of the y-axis varies depending on the gene. Data are shown + SEM. Significant differences are shown as single asterisk for p values less than 0.05 (* = $p < 0.05$) and double asterisk for p values less than 0.01 (** = $p < 0.01$).

Figure 3.8

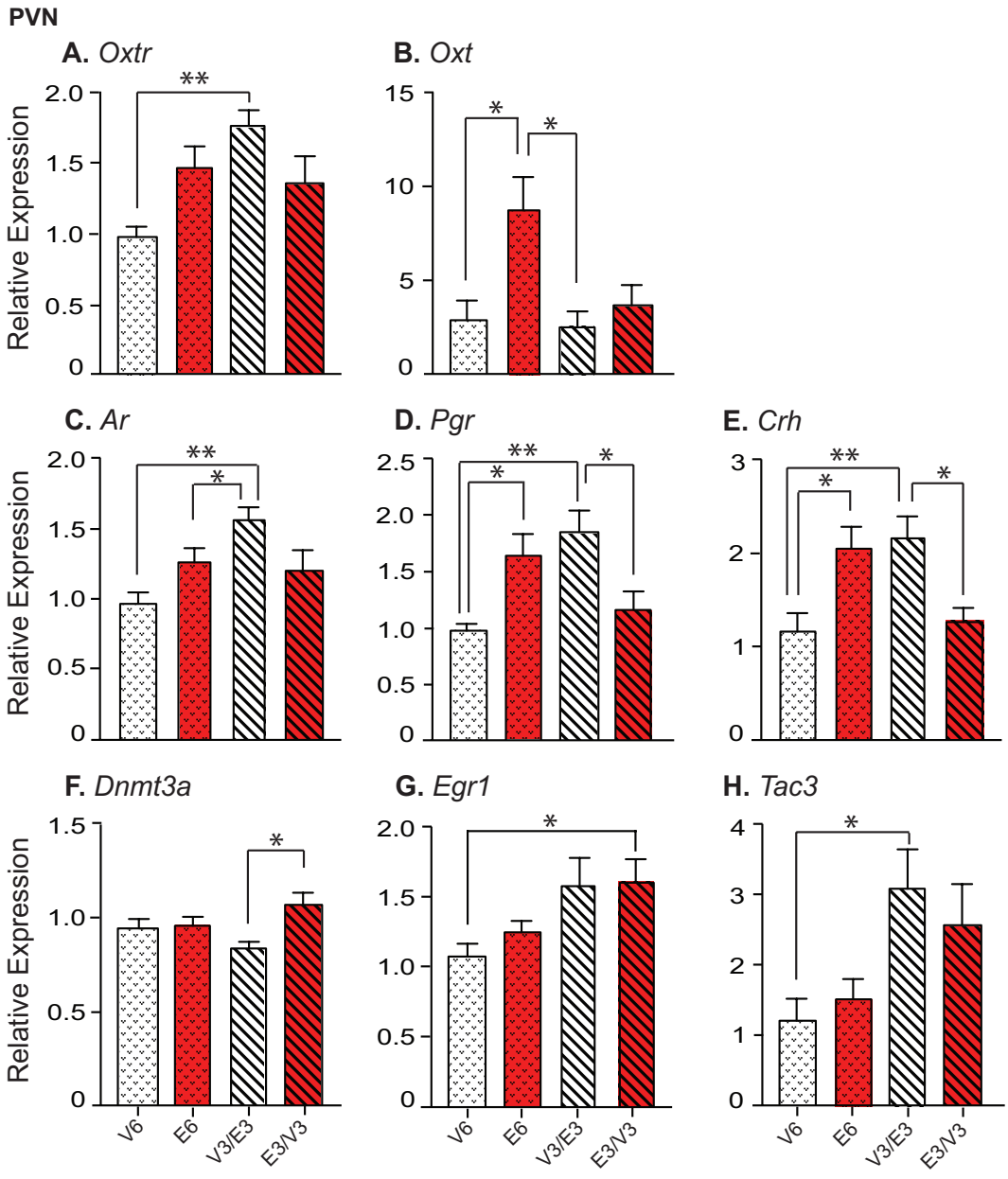


Figure 3.8: Relative gene expression data for the PVN

Note that the scale of the y-axis varies depending on the gene. Data are shown + SEM. Significant differences are shown as single asterisk for p values less than 0.05 (* = $p < 0.05$) and double asterisk for p values less than 0.01 (** = $p < 0.01$).

Figure 3.9

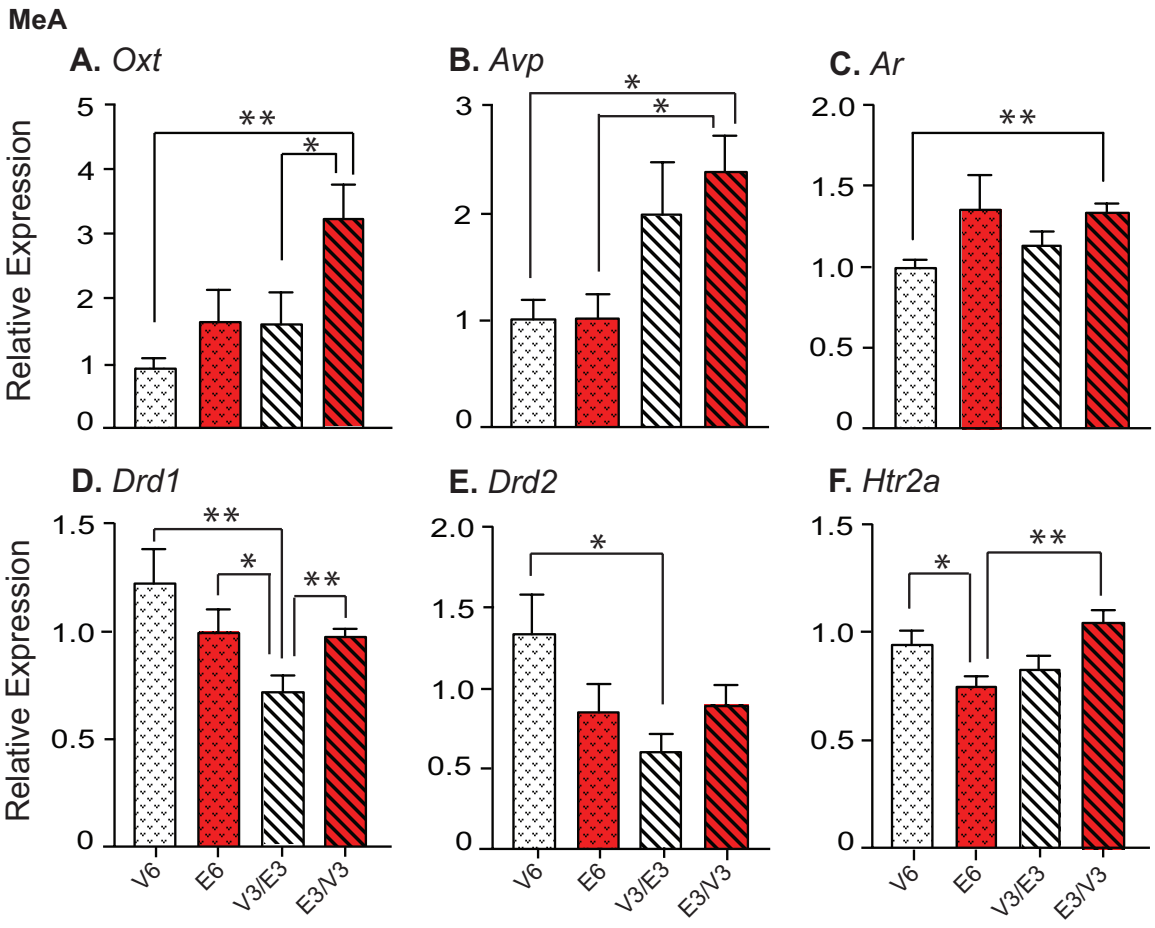


Figure 3.9: Relative gene expression data for the MeA (1/2)

Note that the scale of the y-axis varies depending on the gene. Data are shown + SEM. Significant differences are shown as single asterisk for p values less than 0.05 (* = $p < 0.05$) and double asterisk for p values less than 0.01 (** = $p < 0.01$).

Figure 3.10

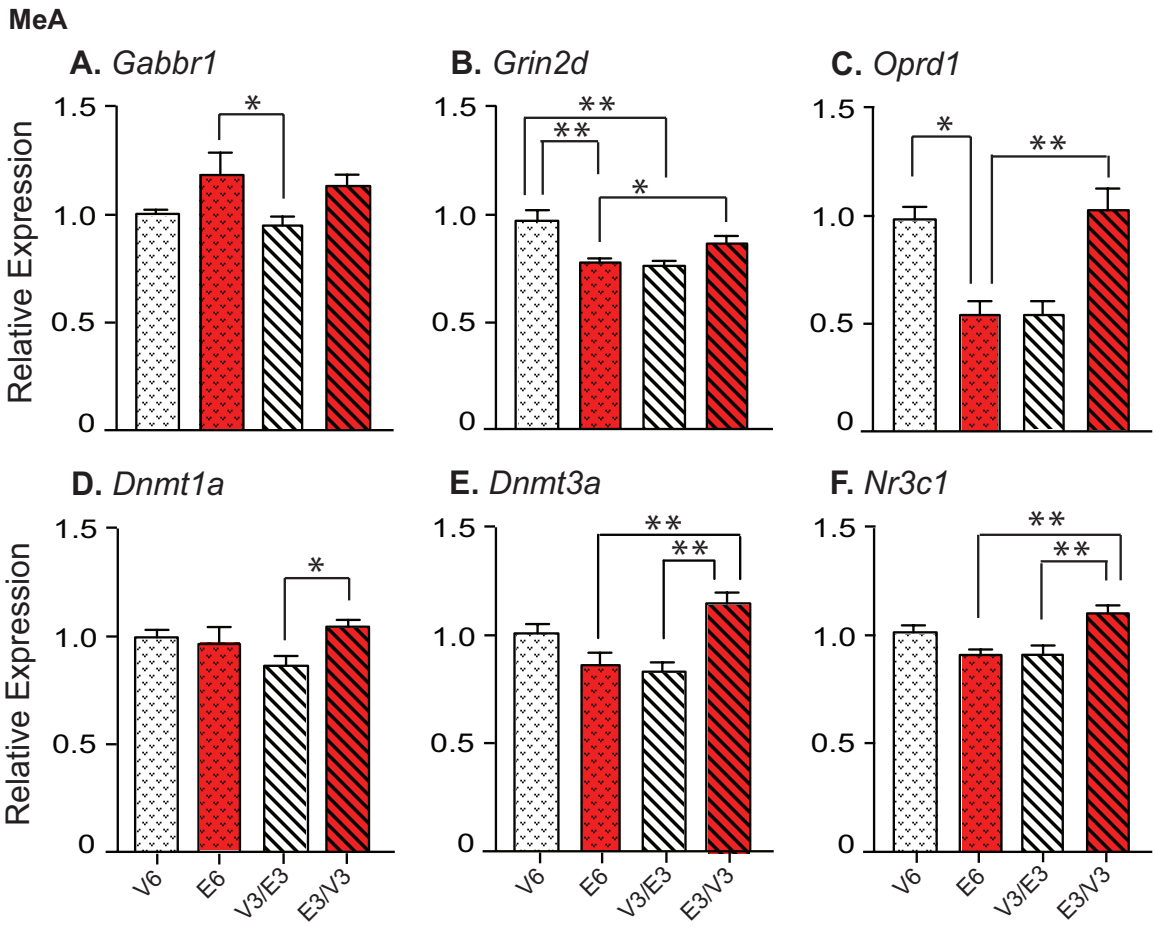


Figure 3.10: Relative gene expression data for the MeA (2/2)

Note that the scale of the y-axis varies depending on the gene. Data are shown + SEM. Significant differences are shown as single asterisk for p values less than 0.05 (* = $p < 0.05$) and double asterisk for p values less than 0.01 (** = $p < 0.01$).

# DISCUSSION

This study focused on how the timing and duration of estradiol therapy affected the brain and social behavior of aged female rats, to test the "critical window" hypothesis of hormone treatment for menopause. A specific goal of this work was to provide empirical evidence from a preclinical model that could be used to inform decisions about hormone treatments and to help address the limitations from the Women's Health Initiative. Our results showed that there are indeed specific timing and duration effects of E2 treatment on social behavior and gene expression. For our test of social communications as measured by USV emissions, E2 treated animals called much less, and showed a decrease in the amount of time spent interacting with one another, at the 3 month post-OVX testing period. This treatment effect was not observed at the 6-month testing period, suggesting that a longer duration of hormone in the context of increasing age changed this behavior. Gene expression assessment showed that there were unique patterns of timing and duration of estradiol in the 5 brain regions, with the majority of changes observed in the MeA and PVN.

### *Timing and duration of estradiol treatment on social interactions*

USV calls and behaviors were decreased by $E_2$ treatment at 3 but not 6 months of testing

At the 3-month behavioral testing period, estradiol treated animals emitted significantly fewer total, frequency-modulated, and non-frequency modulated calls in the USV test. However, this effect of treatment was not observed at the 6-month testing period. The amount of time animals spent interacting with one another was also lower in

the $E_2$ animals compared to vehicle rats at 3 but not 6 months. The differences between results in the first vs. the second set of testing periods may be attributable to the age at testing (14 vs. 17 months), the possible influence of prior experience in the behavioral test, and the nature (duration and timing) of hormone treatments. Since this was the first experiment to conduct this type of testing, future research is needed to differentiate between these possibilities, which are not mutually exclusive.

The literature examining the effects of estradiol on female USVs is limited, with studies focusing on short-term $E_2$ treatment or stages of the estrous cycle. It was reported that more USVs were emitted during the late proestrus stage, when E2 and progesterone are highest during the reproductive cycle (Matochik et al., 1992b; Matochik et al., 1992a). In addition, short-term E2 treatment after OVX in female rats increases total numbers of USVs emitted (Matochik et al., 1992b; McGinnis & Vakulenko, 2003). Though these studies show that estradiol facilitates production of USVs these studies did not look at familiar female-to-female interactions and the duration of treatment was significantly shorter compared to the long-term $E_2$ treatment used in the current study.

In parallel with the current report, we conducted a study using mature adult female rats, OVX at 4 months and given E2 or vehicle for 3 months, at which time they were tested for USV calls and interactions at 7 months of age (Garcia et al., unpublished). Those results, showing decreased USV calls in the E2 rats, were comparable to our current results from aging females tested at 14 months of age. However, we did not expect this effect to disappear when we tested the animals at the 6-month testing period, essentially irrespective of the mode (timing, duration) of hormone treatment.

It is interesting to consider the USV results at the 3-month testing period in light of behavioral outcomes scored in the same trial, when cagemates were first introduced and allowed to interact freely. The E2 animals showed less time interacting, similar to our results from the other study on mature rats (Garcia et al., unpublished). Taken in conjunction with the decrease in total number of USVs emitted, this behavioral phenotype suggests the possibility that E2 rats are demonstrating a form of social memory. Published studies in mice and rats that used a resident intruder test as well as a social recognition memory test reported that compared to vehicle, animals treated with E2 emitted significantly fewer calls and interacted less with a conspecific if it had been previously introduced to them recently; this was interpreted as the E2 animals having greater social memory (Tang et al., 2005; D'Amato & Moles, 2001; Moles et al., 2007; Hlinak, 1993). The lack of this effect at the 6-month testing period could be due a loss in sensitivity to the hormone, increased age (14 vs. 17 months), or both. With aging, there is increased anxiety and decreased social interaction compared to younger counterparts (Salchner et al., 2004; Guan & Dluzen, 1994; Boguszewski & Zagrodzka, 2002). In studies of cognitive behavior they found that delaying estradiol treatment in aged rats did not enhance spatial memory whereas young rats still show enhanced spatial memory up to 5-months post OVX (Gibbs, 2000; Daniel et al., 2006).

Furthermore, our study entailed testing rats twice, thereby introducing the potential effect of behavioral experience on the outcomes observed in the second round of testing. The fact that hormone effects appear to be blunted may represent habituation to the testing paradigm.

The typical social preference for a novel over a familiar rat is not seen in our test of social interactions among cagemates, and is not strongly influenced by E2.

Based on previous research on social novelty in rodents we hypothesized that our rats, irrespective of treatment, would spend more time with the novel rather than familiar animals (Markham & Juraska, 2007; Carr et al., 1976; Berlyne, 1950; Bevins & Besheer, 2006). Contrary to this prediction, rats tended to spend more time with their cagemate, or spent equal time with the 2 stimulus animals. Taking into account the findings from the 3-month and 6-month testing period it appears that in order for animals to display a novelty preference, as calculated by percentage of the social time spent with the novel animal, estradiol treatment must be given immediately at the time of OVX for a 3-month duration and E2 must be circulating at the time of testing. This indicates that timing and duration of estradiol therapy are important for this particular behavior because animals from the E6, V3/E3 and E3/V3 did no show a preference for the novel animal.

***Timing and duration of E2 treatment have region-specific effects on gene expression***

Several families of genes were most commonly identified in our model: the neuropeptides oxytocin and vasopressin (and receptors); dopamine receptors; other neuropeptide, neurotransmitter, and neurotrophic factor signaling systems. Several steroid hormone receptor genes were differentially affected by treatment, with different genes identified for the 5 regions. We will focus our discussion on the nonapeptide (oxytocin, vasopressin) and dopamine receptor results, followed by brief discussion of

regional differences in outcomes.

Oxytocin, vasopressin, and their receptors in the MeA, PVN and PFC, were sensitive to timing and duration of E2 in aging rats

Oxytocin (*Oxt*), vasopressin (*Avp*) and their receptors (*Oxtr*, *Avpr1a*) are among the best-studied neural systems involved in the regulation of social behaviors (see review papers Young, 1999; Neumann & Landgraf, 2012). *Oxt* and *Avp* are synthesized primarily in neurons of the SON and PVN of the hypothalamus; they, and their receptors, are also expressed in the MeA, BNST and PFC (Axelson & van Leeuwen, 1990; Bale et al., 1995; Smeltzer et al., 2006; Yoshimura et al., 1993; Young, 1999; Štefánik et al., 2015).

In the current study we found that E2 had unique timing and duration effects in the MeA, PVN and PFC. In the MeA, *Oxt* was highest in the E3/V3 switch group compared to the V3/E3 group or the V6 group. This means that immediate E2 given at the time of OVX has unique and long-lasting effects. *Avp* in the MeA was also highest in the E3/V3 group, this time relative to either 6-month treatment (V6 or E6). A different pattern and subset of genes were seen in the PVN. *Avp* expression was unaffected in this region, but *Oxt* expression was highest in the E6 group compared to either V6 or V3/E3, indicating that longer duration of estradiol has different effects from shorter-term treatment.

The receptors for oxytocin and vasopressin also had unique expression patterns, indicative of specific timing and duration effects. In the PFC, both receptors *Oxtr* and

*Avpr1a* were highest in the E6 rats. In the PVN *Oxtr* was higher in the V3/E3 compared to V6 rats, indicative of a timing (delayed E2 treatment) effect. These findings are interesting because the literature on E2 regulation of these genes is mixed, with some studies showing up-regulation (Grassi et al., 2010 [PVN]; de Vries et al., 1986 [MeA]; Patisaul et al., 2003 [MeA]), down-regulation (Nomura et al., 2002 [MeA]; Shughrue, Dellovade & Merchenthaler, 2002 [PVN]) or no changes (Nomura et al., 2002 [PVN]; Patisaul et al., 2003 [PVN]) in response to E2. Also, to our knowledge this is the first study to show effects of E2 on *Oxtr* and *Avpr1a* expression in the PFC. It is important to note that prior studies looked at relatively short-term E2 treatments and did not take into account the animals' age at euthanasia. Our findings add to the literature by showing that the age of the animal along with the timing and duration of E2 play important roles in determining its effectiveness.

Delayed E2 treatment down-regulated dopamine receptors in the MeA and SON

Dopamine receptors play roles in anxiety, depression and social behavior (Morgan et al., 2002; Leblois et al., 2010; Skuse & Gallagher, 2009; Morissette & Di Paolo, 1993; Bazzett & Becker, 1994; Lammer et al., 1999). These behaviors are sexually dimorphic in humans, with depression and anxiety higher in women than in men (Wong & Licinio, 2001). Women experiencing menopause have been shown to have increases in affective dysfunctions (Freeman et al., 2004; Schmidt et al., 2004; Bromberger et al., 2011). Dopamine receptors are also well known to be E2-sensitive (Morissette & Di Paolo, 1993; Bazzett & Becker, 1994; Lammer et al., 1999). In fact, prior work

conducted in other brain regions reported that estrogen treatment led to hyposensitivity whereas the loss of estrogen caused hypersensitivity (Roy, Buyer & Licari, 1990; Bédard et al., 1983; Gordon & Perry, 1983).

In our study, both *Drd1* and *Drd2* in the MeA were lowest in the V3/E3 rats, especially relative to the V6 group. A similar pattern was seen for *Drd2* in the SON. Our companion study conducted in mature adult rats found a similar result (Garcia et al., unpublished). Thus, the timing and duration of E2 treatment are important factors in gene regulation in this brain region.

## CONCLUSION

The current study is the first to look at the effects of timing and duration of estradiol on social behavior and gene expression in aging female rats. As a whole, our findings demonstrate that for social behavior, the "critical window" hypothesis may be age dependent, and that molecular actions caused by different modes of E2 treatment are region- and gene-specific.

The relationships between the behavioral outcomes and the neuromolecular gene profiles are not straightforward; this is not surprising considering that each gene can play multiple roles within a specific brain region due to heterogeneity within any nucleus. However, we did have some initial predictions about roles of *Oxt*, *Oxtr*, *Avp*, *Avpr1a*, *Drd1* and *Drd2* in regulating social behavior based on the literature (Choleris et al., 2006; Hlinak, 1993; Egashira et. al, 2007; Ferguson et al., 2001; Murakami et al., 2011; Paul et al., 2014; Heinrichs & Domes, 2008; Morgan et al., 2002; Leblois et al., 2010; Skuse &

Gallagher, 2009), and expected to find correlations between gene expression and social behavior. However, while many genes had altered expression at 6 months, we found no timing or duration effects of $E_2$ on social behavior at that 6-month testing period. Future work is needed to investigate gene expression in rats after 3-months of testing to relate to the time when behavioral changes are seen.

Nevertheless, several aspects of our study were novel. The USV test designed for this study appears to be a robust test for social interactions, and potentially for measuring social memory, in rodents. The application to same-sex cagemates adds a new social context to previous studies utilizing USV communications as indices of sexual or motivational status. Our findings may also have implications for human research examining the role of estradiol in a social context, which is currently very limited. Translation of this research may be able to demonstrate that the "critical window" hypothesis is indeed relevant to anxiety, depression, and a sense of social isolation in women experiencing menopause naturally or surgically.

| | | Relative to V6 | | | Relative to E6 | | Relative to V3EV |
|---|---|---|---|---|---|---|---|
| | **Gene** | **E6** | **V3E3** | **E3V3** | **V3E3** | **E3V3** | **E3V3** |
| **BNST** | *Oxtr* | --- | --- | ↓ | --- | --- | ↑ |
| **SON** | *Igf1* | --- | ↓ | ↓ | ↓ | --- | --- |
| | *Drd2* | --- | ↓ | --- | ↓ | --- | --- |
| **PFC** | *Oxtr* | ↑ | --- | --- | --- | --- | --- |
| | *Avpr1a* | ↑ | --- | ↑ | --- | --- | --- |
| | *Esr2* | --- | --- | ↓ | --- | --- | --- |
| | *Bdnf* | --- | ↑ | ↑ | ↑ | ↑ | --- |
| **PVN** | *Oxtr* | --- | ↑ | --- | --- | --- | --- |
| | *Oxt* | ↑ | --- | --- | ↓ | --- | --- |
| | *Ar* | ↑ | ↑ | --- | --- | --- | --- |
| | *Pgr* | ↑ | ↑ | --- | --- | --- | ↓ |
| | *Crh* | ↑ | ↑ | --- | --- | --- | ↓ |
| | *Dnmt3a* | --- | --- | --- | --- | --- | ↑ |
| | *Egr1* | --- | --- | ↑ | --- | --- | --- |
| | *Tac3* | --- | ↑ | --- | --- | --- | --- |
| **MeA** | *Oxt* | --- | --- | ↑ | --- | --- | ↑ |
| | *Avp* | --- | --- | ↑ | --- | ↑ | --- |
| | *Ar* | --- | --- | ↑ | --- | --- | --- |
| | *Drd1* | --- | ↓ | --- | ↓ | --- | ↑ |
| | *Drd2* | --- | ↓ | --- | --- | --- | --- |
| | *Htr2a* | ↓ | --- | --- | --- | ↑ | --- |
| | *Gabbr1* | --- | --- | --- | ↓ | --- | --- |
| | *Grin2d* | ↓ | ↓ | --- | --- | ↑ | --- |
| | *Oprd1* | ↓ | --- | --- | --- | ↑ | --- |
| | *Dnmt1a* | --- | --- | --- | --- | --- | ↑ |
| | *Dnmt3a* | --- | --- | --- | --- | ↑ | ↑ |
| | *Nr3c1* | --- | --- | --- | --- | ↑ | ↑ |

Table 2.1: Significant changes in gene expression

List of significant changes in gene expression in each of the 5 brain regions. Down-regulation is indicated by red arrows and up-regulation is indicated by green.

## SUPPLEMENTAL TABLE

| Gene | Name |
|---|---|
| **Steroid hormone receptors** | |
| *Esr1* | Estrogen Receptor Alpha |
| *Esr2* | Estrogen Receptor Beta |
| *Ar* | Androgen Receptor |
| *Pgr* | Progesterone Receptor |
| *Nr3c1* | Nuclear Receptor Subfamily 3, Group C, Member 1 (glucocorticoid receptor |
| **Oxytocin and vasopressin signaling** | |
| *Avp* | Vasopressin |
| *Avpr1a* | Vasopressin Receptor 1a |
| *Oxt* | Oxytocin |
| *Oxtr* | Oxytocin Receptor |
| **Neurotransmission** | |
| *Drd1a* | Dopamine Receptor D1A |
| *Drd2* | Dopamine Receptor D2 |
| *Htr1a* | 5-hydroxytryptamine (serotonin) receptor 1A, G protein-coupled |
| DBH | Dopamine Beta-Hydroxylase (Dopamine Beta-Monooxygenase) |
| Slc6a3 | Solute Carrier Family 6 (Neurotransmitter Transporter, dopamine), Member 3 |
| Slc6a4 | Solute Carrier Family 6 (Neurotransmitter Transporter, dopamine), Member 4 |
| *Htr2a* | 5-Hydroxytryptamine (Serotonin) Receptor 2A, G Protein-Coupled |
| *Htr2C* | 5-hydroxytryptamine (serotonin) receptor 2C, G protein-coupled |
| *Oprm1* | Opioid Receptor, Mu 1 |
| *Oprk1* | Opioid Receptor, Kappa 1 |
| *Oprd1* | Opioid Receptor, Delta 1 |
| *Grin1* | Glutamate Receptor, Ionotropic, N-methyl D-aspartate 1 |
| *Grin2a* | Glutamate Receptor, Ionotropic, N-methyl D-aspartate 2A |
| *Grin2b* | Glutamate Receptor, Ionotropic, N-methyl D-aspartate 2B |
| *Grin2c* | Glutamate Receptor, Ionotropic, N-methyl D-aspartate 2C |
| *Grin2d* | Glutamate Receptor, Ionotropic, N-methyl D-aspartate 2D |
| *Gria1* | Glutamate Receptor, Ionotropic, AMPA 1 |
| *Gria2* | Glutamate Receptor, Ionotropic, AMPA 2 |
| *Gabbr1* | GABA B Receptor 1 |
| *Gabbr2* | GABA B Receptor 1 |
| **Growth factor signaling** | |
| *Bdnf* | Brain-Derived Neurotrophic Factor |
| *Igf1r* | Insulin-Like Growth Factor 1 Receptor |
| *Igf1* | Insulin-Like Growth Factor 1 |
| *Egr1* | Early Growth Response 1 |
| **Epigenetic signaling** | |
| *HDAC1* | Histone Deacetylase 1 |
| *HDAC4* | Histone Deacetylase 4 |
| *HDAC2* | Histone Deacetylase 2 |
| *DNMT1* | DNA (cytosibe-5-)-methyltransferase 1 |
| *DNMT3a* | DNA (cytosibe-5-)-methyltransferase 3 alpha |
| *DNMT3b* | DNA (cytosibe-5-)-methyltransferase 3 alpha |
| **Other** | |
| *Crh* | Corticotropin Releasing Hormone |
| *Foxp2* | Forkhead Box P2 |
| *Foxp1* | Forkhead Box P1 |
| *Nlgn3* | Neuroligin 3 |
| *Shank1* | SH3 and Multiple Ankyrin Repeat Domains 1 |
| *Tac3* | Tachykinin 3 |

Table S2.1: List of 45 neuroendocrine genes quantified using Taqman low-density arrays (does not include 3 house-keeping genes, *Gapdh, Rpl13a,* and *18s*).

# CHAPTER 5: GENERAL DISUCSSION

My overall goal was to examine how the timing and duration of estrogen therapy affect the social brain and behavior as a function of aging, estradiol treatment, and their interactions. When taken together my 3 experiments provide evidence that estradiol plays a role in regulating social behavior and gene expression. In particular all three research chapters found that estradiol has specific effects of neuropeptides *Oxt* and *Avp* as well as their receptors, which are known to be involved in regulating social behavior. Chapter 4 demonstrates that the "critical window" hypothesis holds up for these specific neuropeptides as well as neurotransmitters such as *Drd1*, *Drd2*, *Htr1a*, and *Htr2a*. Overall, these experiments support my hypothesis that estradiol plays a role in regulating social behavior and that timing and duration of estradiol affects the underlying mechanisms involved in the regulation of social behaviors.

## ESTROGEN REGULATION OF SOCIAL BEHAVIOR

With chapters 3 and 4 I was able to examine the effects of timing and duration of estradiol treatment on social communication, memory, and preference. Both the mature adult and aging female rats that were treated with 3-months of estradiol spent significantly less time interacting with their cagemate than the vehicle animals which is another indication of increases social memory. Surprisingly, in the experiment described in chapter 4 I found that at the 6-month testing period there was no differences between groups on total amount of USVs emitted nor on time spent interacting. One of the contributing factors for this lack of treatment effect at the 6-month testing period could be

age. Research examining age related changes in rats have demonstrated that aged animals have increased anxiety, decreased social interaction and novelty preference compared to younger counterparts (Salchner, Lubec & Singewald, 2004; Guan & Dluzen, 1994; Boguszewski & Zagrodzka, 2002). It is interesting that the aging animals in chapter 4 showed a similar pattern to the mature adult rats in chapter 3 after 3-months of treatment considering the animals used in chapter 3 were approximately 7-months of age at the time of testing and the animals in chapter 4 were approximately 14-months of age at the first round of testing. It appears that there may be a critical time between 14- and 17-months of age in which there is a loss in sensitivity to the hormone in female rats. The literature looking at age related changes on social memory and social interaction are very limited. Though a previous study found that there was a significant difference in time spent interacting with a novel male rat between male rats that were 15-months old and rats that were 22-months old with the older rats spending less time exploring the novel animal (Guan & Dluzen, 1994). To my knowledge there are no studies to data that compare social memory or social interaction between young and aging female rats. These findings indicate that aging in females may affect social behavior in a similar manor as it does in males. Further investigation is warranted on this topic and future studies should examine social memory and social interaction in the context of aging in female rats.

I must also note that since our study had two identical testing periods it is plausible that the animals were habituated to the test, which had an impact on the results. However, I think this to be unlikely because of the large gap in time between testing periods and the fact that they were only exposed to the USV chamber for a total of 2 days

in the first round of testing. Past research has shown that even after several trials in the same testing apparatus rats will still demonstrate increased social interaction if a novel animal is introduced (Sánchez-Andrade & Kendrick, 2011; Choleris et al., 2003; Choleris et al., 2007; Spiteri & Ågmo, 2009).

For the sociability test, I saw few effects of estradiol on social interaction and novelty preference in the experiments in chapters 3 and 4. Though in chapter 3 I found that both vehicle and estradiol treated animals preferred to spend more time with their cagemate that they did with the novel animal. This was an unique finding because previous literature shows that rats typically spend more time with novel than familiar animals (Markham & Juraska, 2007; Carr et al., 1976; Berlyne, 1950; Bevins & Besheer, 2006), but it is important to note that these studies were conducted using male rats. One study using both male and female rats found that female rats exhibit a lower novelty preference than males (Cyrenne & Brown, 2011). Also, at the 3-month testing period estradiol decreased total time immobile and increased total distance traveled and mean speed. By contrast, at the 6-month testing period all groups were around the same for all 3 of these measures. I attribute this to the age of the animals, as age has been shown to decrease locomotor activity in male and female rats (Willig et al., 1987; Jucker et al., 1988; Altun et al., 2007). A list of significant changes that were found for the USV and sociability test in chapters 3 & 4 are provided in Supplemental Table S3.1.

## TIMGING AND DURATION OF ESTRADIOL ON GENES INVOLVED IN SOCIAL AND AFFECTIVE BEHAVIOR

One of the main goals of this dissertation was to gain a better understanding of how estrogen regulates the underlying molecular mechanisms involved in social behavior. The neuropeptides oxytocin, vasopressin and their receptors have all been shown to be regulated by estradiol. For chapters 2 and 4 there were specific regulatory patterns of timing and duration of estradiol, and in Chapter 3 3-months of estradiol treatment up-regulated all of these genes in a region specific manor. In chapters 2 and 4 similar experimental designs were used: both included 6-month treatment groups, which received either estradiol or vehicle, and they also included two switch groups that started on either estradiol or vehicle then switched treatments after 3 months. In chapter 2, I only focused on 5 genes and 2 brain regions whereas in chapter 4 I looked at 42 genes and 5 brain regions.

When I compare groups, genes and brain regions that are the same in both experiments I did not find complimentary findings. In chapter 2 I did not find any effects of timing and duration on *Oxt*, *Avp* or their receptors in the PVN, whereas in chapter 4 delaying estradiol treatment for 3-months increased *Oxtr* expression compared to the V6 group. Also, 6-months of estradiol treatment increased *Oxt* expression compared to both V6 and V3/E3 group in the PVN. Additionally when I compared the mature adult groups (3-months of vehicle or estradiol) in chapters 2 and 3 I did not find similar results. In chapter 2 I did not find any effects of treatment for these groups in the SON. However, in

chapter 3 *Oxt*, *Oxtr*, and *Avp* were all up-regulated by estradiol compared to vehicle. The discrepancy in these findings could be attributed to the small number of animals in some of the groups in chapter 2. Some of the tissue samples from those animals were damaged due to over exposure to RNAlater. Rather than risk using a brain that I could not distinguish the regions I opted to use a smaller number of tissues to guarantee that I was getting the correct brain regions. However, this could be a major contributing factor to the lack of similar results between my experiments. With a small sample size I may have missed differences that would have been significant with a larger sample size. Thus the rest of this section will focus on the findings from chapter 3 and 4. Table 3.1-3.3 compare gene expression results between chapters 2, 3, and 4. In addition, all significant gene expression findings for each of the experiments are provided in Supplemental Table S3.2.

In chapter 3, before performing a false discovery rate (FDR), *Oxtr* expression was up-regulated by estradiol in all 5 brain regions. After FDR *Oxtr* was significantly up-regulated in the BNST, MeA, and SON. This supported by previous literature showing that *Oxtr* expression is increased in the BNST and SON in the latter phases of pregnancy and partition (Meddle et al., 2007; Young et al., 1997). Also, short-term estradiol treatment after OVX increases *Oxtr* expression in the MeA (Quiñones-Jenab et al., 1997; Patisaul et al., 2003). Both *Oxt* and *Avp* expression were increased in the SON after 3-months of estradiol treatment. Previous research has demonstrated that estrogen regulates *Avp* and *Oxt* in the SON predominantly through the ERβ (Winslow and Insel, 2004; Hrabovszky et al, 1998). Yet, our findings are contrary to other studies in rodents that have examined the effects of estradiol on *Oxt* and *Avp* expression in the SON, most of

them have found that estradiol treatment decreases expression if administered after OVX (Shughrue et al., 2002; Van Tol et al., 1988) while others found not changes in expression (Peter et al., 1990; Rhodes et al., 1981). These studies used either acute injections of estradiol or very short-term treatments (>1 month). Longer term treatments are more clinically relevant since 3-months in a rats life is thought to be equivalent to 5 years in humans (Sengupta, 2013; Quinn, 2005), which is comparable to a duration of time women would be on hormone replacement therapy. The fact that we see an increase in regulation could be an indicator that estradiol must be on board for a relatively long period of time before an increase is observed.

When these results are taken into consideration with the results from the USV test it is probable that the increase we see in these neuropeptides from estradiol plays a role in the increase in social memory. This conclusion is supported by previous research that has found that OVX female rats also show impaired social memory and this deficit is restored after estradiol (Hlinak, 1993; Tang et al., 2005) or estrogen plus progesterone treatment (Spiteri & Ågmo, 2009). Also, oxytocin and oxytocin receptor knockout mice do not habituate to the repeated presentation of a familiar mouse unlike their wild-type counterparts (Choleris et al., 2003; Ferguson et al., 2000; Lee et al., 2008; Takayanagi et al., 2005; Bielsky et al., 2004). OxtKO and OxtrKO mice cannot distinguish between a familiar mouse and a novel mouse, which illustrates a lack of social recognition (Takayanagi et al., 2005; Choleris et al., 2006).

In chapter 4 there were a variety of timing and duration effects of estradiol on the neuropeptides involved in social behavior. In the MeA, immediate $E_2$ given at the time of

OVX but removed 3-months prior to euthanasia was able to induce increased *Oxt* and *Avp* expression. In contrast, in the PVN *Oxt* expression was highest in the E6 group compared to either V6 or V3/E3, indicating that longer duration of estradiol has different effects from shorter-term treatment. Interestingly we found that *Oxtr* and *Avpr1a* expression was increased by 6-month estradiol treatment in the PFC. To my knowledge this is the first study to show effects of estradiol on these receptors in the PFC. These findings are important because previous literature looking at the effects of estradiol on these genes is mixed, with some studies showing up-regulation (Grassi et al., 2010 [PVN]; de Vries et al., 1986 [MeA]; Patisaul et al., 2003 [MeA]), down-regulation (Nomura et al., 2002 [MeA]; Shughrue, Dellovade & Merchenthaler, 2002 [PVN]) or no changes (Nomura et al., 2002 [PVN]; Patisaul et al., 2003 [PVN]). These varying responses to estradiol may be responsible for the lack of differences that were observed in the social behavior tests at the 6-month testing period. Understanding the relationships between the behavioral outcomes and the neuromolecular gene profiles are not straightforward; this is not surprising considering that each gene can play multiple roles within a specific brain region due to heterogeneity within any nucleus. However, as stated previously, the lack of differences in social behavior between the groups at the 6-month testing period could have been due to the age of the animals at the time of testing. The effect of age appears to have abrogated estradiol's up-regulation of these neuropeptides from inducing behavioral changes.

Interestingly, in the MeA of the aging animals and the SON of the mature adult rats, 3 months of estradiol given immediately following OXV led to a decrease in *Drd2*

expression. In addition, *Htr2a* was decreased by 6-months of estradiol treatment in the MeA of the aging animals and *Htr1a* was decreased by 3-months in the SON of the mature adult rats. These genes have been shown to be involved in regulating affective disorders such as anxiety and depression (Dunlop & Nemeroff, 2007; Noble, 2003; Arias et al., 2001; Frisch et al., 1999). The current findings are important because it demonstrates that estradiol does not just regulate these genes in the mesolimbic system, which has been the focus of a majority of studies (Roy et al., 1990; Bédard et al., 1983; Gordon & Perry, 1983; Lammers et al., 1999). The fact that both the mature adult and aging female rats show a decrease in these genes with estradiol treatment means that estradiol could have benefits in the treatment of affective disorders such as depression despite age. Previous research has shown that estradiol does play a regulatory role in modulating affective disorders (McQueen, Wilson & Fink, 1997; Sumner & Fink, 1993 & 1995, Biegon & McEwen, 1982; Raap et al., 2000; Charoenphandhu et al., 2011; Mize, Poisner & Apler, 2001, Klemenhagen et al., 2006; Parks et al., 1998; Ramboz et al., 1998, Lerer et al., 1999).

| Gene | Brain region | Age (Ch.2) | Treatment (Ch.2, 3, & 4) | Timing & Duration (Ch. 2 & 4) |
|---|---|---|---|---|
| ***Oxt*** | SON | | E3 > V3 (Ch.3) | |
| | PVN | | | E6 > V3 & V3/E3 (Ch.4) |
| | MeA | | | E3/V3 > V6 & V3/E3 (Ch.4) |
| | | | | |
| ***Oxtr*** | SON | AG > MAT | E3 > V3 (Ch.3) | V3/E3 > E6 & E3/V3 (Ch.2) |
| | PVN | | E3 > V3 (Ch.2) | V3/E3 > V6 (Ch. 4) |
| | MeA | | E3 > V3 (Ch.3) | |
| | BNST | | E3 > V3 (Ch.3) | V6 & V3/E3 > E3/V3 (Ch. 4) |
| | PFC | | E6 > V6 (Ch.4) | |
| | | | | |
| ***Avp*** | SON | | E3 > V3 (Ch.3) | V6 > E6 & E3 (Ch.2) |
| | PVN | | | E3/V3 > V6 & E6 (Ch. 4) |
| | | | | |
| ***Avpr1a*** | SON | AG > MAT | | |
| | PVN | AG > MAT | | |
| | PFC | | | E6 > V6 & E3/V3 (Ch. 4) |

Table 3.1: Oxt, Oxtr, Avp, Avpr1a gene expression changes across chapters 2-4

This table allows for comparison of genes that were studied in parallel in chapters 2, 3, and 4. Changes in expression are broken down by age, treatment, as well as timing and duration of treatment.

| Brain Region | Gene | Mature Adult Rats (Chapter 3) | Aging Rats (Chapter 4) |
|---|---|---|---|
| **SON** | *Esr1* | E3 < V3 | |
| | *Avp* | E3 > V3 | |
| | *Oxt* | E3 > V3 | |
| | *Oxtr* | E3 > V3 | |
| | *Drd1a* | E3 < V3 | |
| | *Drd2* | | V3/E3 < V6 & E6 |
| | *Grin2b* | E3 < V3 | |
| | *Gabbr1* | E3 < V3 | |
| | | | |
| | *Hdac2* | E3 < V3 | |
| | *Hdac4* | E3 < V3 | |
| | *Foxp2* | E3 < V3 | |
| | *Igf1* | | V3/E3 > V6 & E6 |
| | | | E3/V3 > V6 |
| **PVN** | *Oxtr* | | V3/E3 > V6 |
| | *Oxt* | | E6 > V6 & V3/E3 |
| | *Ar* | | V3/E3 > V6 & E6 |
| | *Pgr* | E3 > V3 | E3/V3 > V6 & E3/V3 |
| | | E6 > V6 (Ch.4) | |
| | *Crh* | | E6 & V3/E3 > V6 |
| | | | V3/E3 > E3/V3 |
| | *Dnmt3a* | | V3/E3 < E3/V3 |
| | *Egr1* | | E3/V3 > V6 |
| | *Tac3* | | V3/E3 > V6 |
| **MeA** | *Oxt* | | E3/V3 > V6 & V3/E3 |
| | *Oxtr* | E3 > V3 | |
| | *Avp* | | E3/V3 > V6 & E6 |
| | *Ar* | | E3/V3 > V6 |
| | *Drd1* | | V3/E3 < V6, E6, & E3/V3 |
| | *Drd2* | | V3/E3 < V6 |
| | *Htr2a* | | E6 < V6 & E3/V3 |
| | *Gabbr1* | | E6 > V3/E3 |
| | *Grin2d* | | E6 & V3/E3 < V6 |
| | | | E6 < E3/V3 |
| | *Oprd1* | | E6 < V6 & E3/V3 |
| | *Dnmt1a* | | V3/E3 < E3/V3 |
| | *Dnmt3a* | | E3/V3 > E6 & V3/E3 |
| | *Nr3c1* | | E3/V3 > E6 & V3/E3 |
| | *Foxp1* | E3 > V3 | |
| | *Tac3* | E3 < V3 | |
| **BNST** | *Esr2* | E3 > V3 | |
| | *Oxtr* | E3 > V3 | E3/V3 < V6 & V3/E3 |
| | *Dnmt3a* | E3 > V3 | |
| **PFC** | *Oxtr* | | E6 > V6 (Ch. 4) |
| | *Avpr1a* | | E6 > V6 & E3/V3 |
| | *Esr2* | | E3/V3 < V6 |
| | *Bdnf* | | V6 < V3/E3 & E3/V3 |
| | | | E6 < V3/E3 & E3/V3 |

Table 3.2: Gene expression changes in chapters 3 & 4

This table allows for comparison of genes that were studied in parallel in chapters 2 and 4. Changes in expression are broken down by treatment as well as timing and duration of treatment. Important to note that chapter 3 used mature adult female rats (~7 months) while chapter 4 used aging female rats (~17 months).

# CONCLUSION, CLINICAL IMPLICATIONS, AND FUTURE DIRECTIONS

The results of this dissertation add significant insight into the effects of timing and duration of estradiol treatment on gene expression and behaviors involved in social behavior. The overall findings from this dissertation suggest that 1) estradiol treatment may only be beneficial to social memory for a specific period of time immediately following ovariectomy; 2) there are age-related changes in social memory in female rats; 3) estradiol treatment increases expression of neuropeptides involved in the regulation of social behavior in mature adult female rats; 4) there are unique patterns of timing and duration of estradiol on neuropeptides involved in the regulation of social behavior in aging female rats; 5) the increase in neuropeptide expression in aging female rats is not able to compensate for the age-related changes in social behavior; and 6) estradiol decreases expression of dopamine and serotonin receptors which may relate to affective disorders. These results may have important clinical implications, as menopause is associated with an increase in anxiety, depression, and social isolation. Previous research has postulated that estradiol's regulation of serotonin receptors could impact a woman's responses to serotonin modulating drugs, which are often used to treat anxiety and depression (Fischette, Beigon & McEwen, 1983; Kendall, Stancel & Enna, 1981; Rubinow, Schmidt & Roca, 1998). Research has focuses mostly on the role of estradiol in depression and anxiety but the literature on its role in social behavior is limited and warrants further research. The effects of timing and duration of estradiol on gene expression suggests that the "critical window" hypothesis is important for some genes but

not for others and that its effects are region specific. More studies need to be conducted on this topic in order to gain a more comprehensive understanding of timing and duration of estradiol on gene expression.

In addition, the USV test used in this dissertation has proven to be a robust way to way to measure social memory in the context of familiar female-to-female social interactions. It would be interesting for a future study to examine the differences in USVs when female rat is exposed to her cagemate and a novel female rat. In this dissertation this setup was used in the sociability test but we did not expose the cagemates to novel animals during the USV test. The sociability test did not allow for direct physical interaction and no USVs were recorder during the trials. By slightly modifying the setup of the USV test to include a novel animal it would help to further validate the use of this test for measuring social memory. This dissertation also helped to fill a gap in the USV literature examining the types of communications between same-sex cagemates, which to my knowledge has not been done previously. Though there were no timing or duration effects seen in the USV test, future research should examine these treatments in other social and affective behavioral tests. Overall this work represents a unique contribution to our understanding of the effects of estradiol treatment following the loss of steroid hormones and will consequently benefit the field of women's health.

## SUPPLEMENTAL TABLES

| Behavior | MAT | AG | |
|---|---|---|---|
| **USVs** | **3 mon.** | **3 mon.** | **6 mon.** |
| *Total* | $E_2$ < Veh (T2 & 3) | $E_2$ < Veh (T2 & 3) | ----- |
| *FM* | ----- | $E_2$ < Veh (T2) | E6 & V3/E3 < V6 & E3/V3 |
| *NFM* | $E_2$ < Veh (T3) | $E_2$ < Veh (T2 & 3) | ----- |
| **USV Behavior** | **3 mon.** | **3 mon.** | **6 mon.** |
| *Activity* | $E_2$ < Veh | ----- | ----- |
| *Time Interacting* | $E_2$ < Veh | $E_2$ < Veh | ----- |
| *Anogenital Invest.* | $E_2$ > Veh | ----- | E3/V3 < E6, V6 & V3/E3 |
| **Sociability** | **3 mon.** | **3 mon.** | **6 mon.** |
| *Total Time Immobile* | $E_2$ < Veh | $E_2$ < Veh | ----- |
| *Immobile Near Stim.* | $E_2$ CM > $E_2$ Nov<br>Veh Nov > $E_2$ Nov | ----- | ----- |
| *Time Near Stim.* | $E_2$ & Veh CM > $E_2$ & Veh Nov | ----- | V6 CM > V6 Nov |
| *Mean Distance* | ----- | $E_2$ > Veh | ----- |
| *Mean Speed* | ----- | $E_2$ > Veh | ----- |

Table S3.1: Significant behavioral changes in the USV and sociability test for chapter 3 & 4

Abbreviations: T2 = Trial 2, T3 = Trial 3, CM = Cagemate, Nov = Novel

| Brain Region | Gene | Chapter 2 (All groups) | Chapter 3 (MAT) | Chapter 4 (AG) |
|---|---|---|---|---|
| SON | *Esr1* | ----- | E3 < V3 | ----- |
| | *Oxt* | ----- | E3 > V3 | ----- |
| | *Oxtr* | MAT-V3 & E3 < AG-V3 & E3<br>AG-V3/E3 > AG-E6 & AG-E3/V3 | E3 > V3 | ----- |
| | *Avp* | AG-E6 < AG-V6 & AG-E3 | E3 > V3 | ----- |
| | *Avpr1a* | MAT -V3 & E3 < AG-V3 & E3 | ----- | ----- |
| | *Drd1a* | ----- | E3 < V3 | ----- |
| | *Drd2* | ----- | ----- | V3/E3 & E3/V3 < V6 |
| | *Grin2b* | ----- | E3 < V3 | ----- |
| | *Gabbr1* | ----- | E3 < V3 | ----- |
| | *Hdac2* | ----- | E3 < V3 | ----- |
| | *Hdac4* | ----- | E3 < V3 | ----- |
| | *Foxp2* | ----- | E3 < V3 | ----- |
| PVN | *Oxt* | ----- | ----- | E6 > V6 & V3/E3 |
| | *Oxtr* | MAT-V3 < MAT-E3<br>AG-V3 < AG-E3 | ----- | V3/E3 > V6 |
| | *Avpr1a* | MAT -V3 & E3 < AG-V3 & E3 | ----- | ----- |
| | *Pgr* | ----- | E3 > V3 | ----- |
| | *Ar* | ----- | ----- | V6 < E6 & V3/E3 |
| | | ----- | ----- | V3/E3 > E3/V3 |
| | *Crh* | ----- | ----- | V6 < E6 & V3/E3<br>V3/E3 > E3/V3 |
| | *Dnmt3a* | ----- | ----- | V3/E3 > E3/V3 |
| | *Egr1* | ----- | ----- | E3/V3 > V6 |
| | *Tac3* | ----- | ----- | V3/E3 > V6 |
| MeA | *Oxt* | ----- | ----- | E3/V3 > V6 & E3/V3 |
| | *Avp* | ----- | ----- | E3/V3 > V6 & E6 |
| | *Oxtr* | ----- | E3 > V3 | ----- |
| | *Drd1* | ----- | ----- | V3/E3 < V6, E6 & E3/V3 |
| | *Drd2* | ----- | ----- | V3/E3 < V6 |
| | *Htr2a* | ----- | ----- | E6 < V6 & E3/V3 |
| | *Ar* | ----- | E3 > V3 | E3/V3 > V6 |
| | *Grin2d* | ----- | ----- | V6 > V6 & V3/E3<br>E6 < E3/V3 |
| | *Gabbr1* | ----- | ----- | E6 > V3/E3 |
| | *Oprd1* | ----- | ----- | E6 < V6 & E3/V3 |
| | *Dnmt1a* | ----- | ----- | V3/E3 < E3/V3 |
| | *Dnmt3a* | ----- | ----- | E3/V3 > E6 & V3/E3 |
| | *Nr3c1* | ----- | ----- | E3/V3 > E6 & V3/E3 |
| | *Foxp1* | ----- | E3 > V3 | E3/V3 < V6 & V3/E3 |
| | *Tac3* | ----- | E3 < V3 | ----- |
| BNST | *Esr2* | ----- | E3 > V3 | ----- |
| | *Oxtr* | ----- | E3 > V3 | ----- |
| | *Dnmt3a* | ----- | E3 > V3 | ----- |
| PFC | *Esr2* | ----- | ----- | E3/V3 < V6 |
| | *Oxtr* | ----- | ----- | E6 > V6 |
| | *Avpr1a* | ----- | ----- | E6 > V6 & E3/V3 |
| | *Bdnf* | ----- | ----- | V6 & E6 < V3/E3 & E3/V3 |

Table S3.2: Significant changes in gene expression for chapters 2, 3 & 4

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