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Stress and Anxiety Reactivity as a Function of Ovarian Hormones in the Female Rat

by

Wendy J. Smith, M.A.

A Thesis

In

The Department

of

Psychology

Presented in partial fulfillment of the Requirements
for the degree of Doctor of Philosophy at
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ABSTRACT

Stress and anxiety reactivity as a function of ovarian hormones in the female rat.

Wendy Smith, Ph.D.
Concordia University, 2006

Female rodents and primates have an ovulatory cycle, such that they are able to reproduce when in a particular hormonal state. Many behavioral differences are observed in many species as a function of hormonal state, including, for example, sexual receptivity, aggression, and anxiety. Interestingly, sexual behavior and stress reactivity appear to be mutually inhibitory processes, whereby sexually non-receptive rats will attend more to stress stimuli, and sexually receptive rats will attend primarily to the sexual stimuli. In the current set of experiments, the differences in expectancy of anxiety and stress reactivity as a function of ovarian hormone was explored in the female rat. The results indicate that contextual conditioned fear is more robust in non-sexually receptive females, lending more support to the notion of mutually inhibitory processes. The current findings also indicate that benzodiazepines were effective in reducing stress reactivity in hormone-treated rats, suggesting that GABA may interact with estrogen and/or progesterone to reduce conditioned fear and stress reactivity. Perhaps in primates there is a similar underlying mechanism, where a dysfunction in the interaction between ovarian hormones and GABA modulates the increased anxiety and stress seen in some women and primates premenstrually.

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CONTRIBUTION OF AUTHORS

For the co-authored paper included in Chapters 2, 3, and 4, the candidate, Wendy Smith, made a substantial contribution in designing and implementing the experiments; and in analysing the data from the experiments under the supervision of Dr. Pfaus.

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CHAPTER 1

GENERAL INTRODUCTION

Differences exist in a variety of behaviors and cognitive functions across the ovulatory cycle in different species. These differences are the result, in part, of differences in estrogen (E) and progesterone (P) levels across the cycle that set up the capacity of females to ovulate and come into behavioral estrus. There also appears to be a difference in how organisms process stimuli as a function of hormonal state, such that during times where hormone levels are high there is decreased reactivity to stress, whereas when hormone levels are low there is increased reactivity to stressful situations. From an evolutionary perspective, a reduction in the relative importance of stressful stimuli during times when a female is sexually receptive allows her to attend primarily to the goal of reproduction. What happens to this decreased attentiveness to threat when the female is in an environment previously associated with stress? If environmental stimuli are given a different valence depending on hormonal state during prior experience with those stimuli, this could shed some light on how hormonal variations in women are tempered by women's expectancies, and in particular, how expectancies of stress alter – and are altered by – the hormonal milieu that exists at the moment.

The goal of this thesis was to investigate whether the differences in anxiety responding observed in female rats are solely the function of hormonal state, or additionally are dependent on the rat's prior experience with stress in a particular context, both internally (hormonal state) and externally (environment). Chapters 2 and 3 explore this question by varying factors such as experience with stress, and hormonal state. Chapter 4 explored the behavioral effects of treating rats with diazepam, a

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benzodiazepine, and fluoxetine, a selective serotonin reuptake inhibitor. In this chapter, previous research investigating hormonal differences in behaviors, as well as possible mechanisms of action are reviewed, and finally, in Chapter 5 the results of the current studies are explored and presented in light of preexisting literature.

Ovarian hormone fluctuations across the ovulatory cycle

Female mammals all have an ovulatory cycle, which, broadly stated, consists of the growth of an ovarian follicle, the release of this follicle from the ovary, and the development of the corpus luteum. These events are regulated by cyclic changes in levels of circulating hormones, in particular, gonadotrophin releasing hormone (GnRH), luteinizing hormone (LH), follicle stimulating hormone (FSH), estradiol (E) and progesterone (P). The ovulatory cycle is defined as the menstrual cycle in women, and the estrous cycle in rodents (Freeman, 1994).

The menstrual cycle is classified into phases based primarily on the developmental stage of the follicle: the follicular phase, the ovulatory phase, and the luteal phase. The follicular phase begins with the first day of menstruation (Maxson & Hargrove, 1988). At this time, secretion of LH, E, and P are at their lowest rates, while FSH concentations are higher in response to feedback from decreased rates of E during the premenstrual period (Asso, 1983). FSH stimulates the growth of several ovarian follicles, and by about the sixth day of the cycle, one of the antral follicles is designated the Graafian (dominant) follicle, and begins to grow rapidly (Barbieri, 1993). The Graafian follicle secretes increasing levels of E from theca interna and graulosa cells. E peaks at approximately 300 pg/ml at about 12 days after the onset of menstruation, and

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remains at this level for about 48 hours (Asso, 1983). Together with FSH, E stimulates the production of LH receptors on the Graafian follicle. Further, E causes increased release of GnRH by the hypothalamus, and, in turn, an LH surge in the late follicular phase, and a smaller increase in FSH (Barbieri, 1993). A small increase in P is also observed prior to the LH surge (Asso, 1983).

The ovulatory phase lasts for 36 hours, beginning with the LH surge, and ending with ovulation. At ovulation, the Graafian follicle ruptures, releasing its ovum. The ovum is transported to the uterus by fallopian tube contractions and the actions of prostaglandins, and if fertilization does not occur, the ovum will eventually travel out through the vagina (Barbieri, 1993). The ruptured Graafian follicle fills with blood, the granulosa and theca cells of the follicle proliferate, and the blood is replaced with luteal cells, becoming a corpus luteum (Asso, 1983). This marks the beginning of the luteal phase of the cycle.

The corpus luteum secretes high levels of P and E, which inhibit secretion of LH and FSH. If pregnancy occurs, the corpus luteum persists, and levels of P and E remain elevated. If there is no fertilization, levels of these hormones drop at approximately Day 24 of the cycle, causing degeneration of the corpus luteum (Barbieri, 1993). FSH begins to rise in order to initiate follicular growth at the beginning of the next cycle. The epithelium of the endometrium is disrupted, and menstruation occurs. The whole cycle lasts between 14 and 35 days (Brown, 1994).

The mammalian reproductive cycle, called the estrous cycle, differs from that of the primate, in that menstruation does not occur. The term estrus along with its various suffixes (pro-, di-, and met-) was first coined in 1900 by Heape (cited in Freeman, 1994).

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The rat's estrous cycle lasts an average of 4 to 5 days. Although much shorter than the menstrual cycle, the hormonal changes occurring during the estrous cycle are similar in timing to those occurring during the menstrual cycle. Diestrus begins with ovarian steroid hormone levels being at their lowest, and parallels the follicular phase. As the egg matures, E secretion increases along with a rise in FSH that causes the maturation of ovarian follicles. E binds to E receptors in the hypothalamus (Barbacka-Surowiak, Surowiak & Stolosowa, 2003), producing a GnRH surge from the hypothalamus, which in turn causes the release of a surge of LH from the pituitary. LH causes the follicle to rupture, and ovulation to occur (Carter, 1992). An increase in P is also seen during this time. These increases in GnRH, LH, and P occur during proestrus, and mark the beginning of behavioral estrus, a time of sexual receptivity (Freeman, 1994). Sexual receptivity continues through estrus, and levels of E and P are significantly lowered, beginning to rise again towards the end of diestrus.

Figure 1 illustrates levels of E and P during the estrus and menstrual cycles. As can be seen, the hormonal milieu during ovulation is similar in both of these cycles, and in both species, denotes the time where copulation could result in fertilization.

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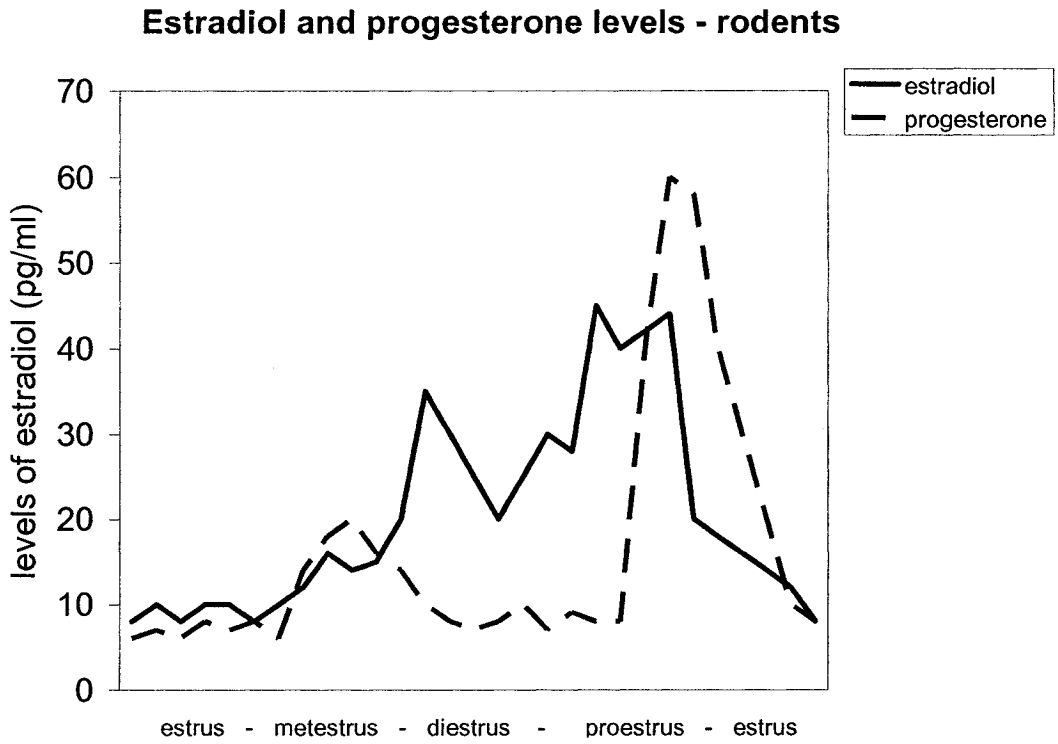

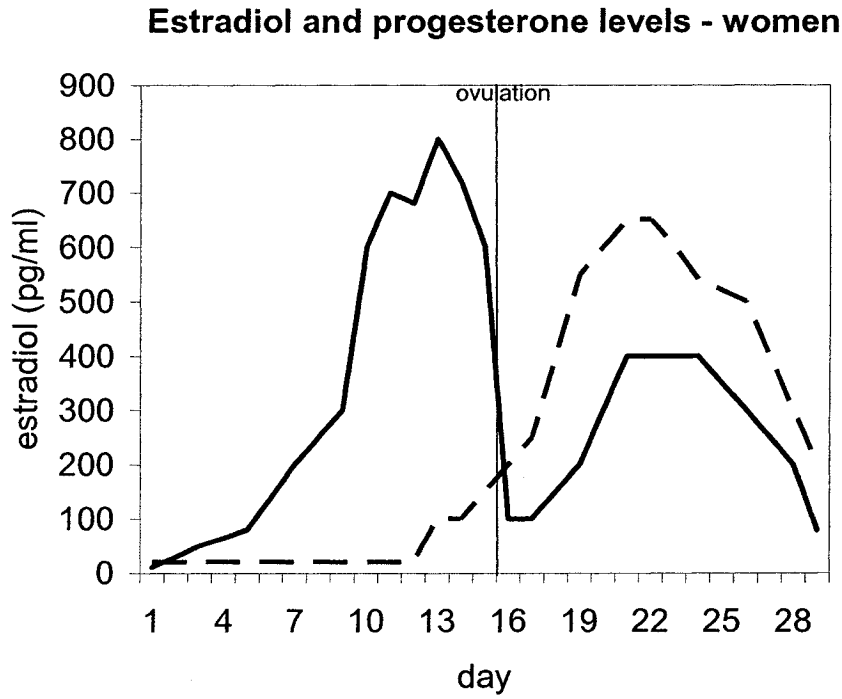

Figure 1. Levels of estrogen and progesterone across the ovulatory cycle in female rats and in women.

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When female rats are OVX, although there is no production of E and P from the ovaries, there is a small production from the adrenals, but no fluctuation of these hormones across days (Pfaff, Schwartz-Giblin, McCaarthy & Kow, 1994). As discussed in more detail below, the significant decrease in circulating levels of these hormones results in a decrease in sexual receptivity.

Differences in sexual behavior across ovulatory cycles

In mammals with an estrous cycle, such as rats, females are sexually receptive only during behavioral estrus. In primates, although sexual receptivity is not limited to any particular phase of the menstrual cycle, there are strong indications that sexual arousal and desire are enhanced during ovulation, a time when sexual activity could result in fertilization.

Humans. During ovulation, women have been found to have enhanced sexual desire, as measured by increased female-initiated sexual behaviors, masturbation, sexual fantasies, and increased arousability and attention toward sexual stimuli (e.g. Bancroft, 1984; Matteo & Rissman, 1984; Meuwissen & Over, 1992; Slob, Bax, Hop, Rowland & van der Werff ten Bosch, 1996; Wallen, 1995). During ovulation, women also report feeling more attractive, and having a greater interest in attending social situations where they may meet potential partners than during any other period in the cycle (Halselton & Gangestad, 2006). Krug et al. (2000) have demonstrated a corresponding increase in the amplitude of the late positive component of an event-related brain potential, the P300, during the ovulatory phase when women were shown sexual stimuli, suggesting a stronger valence for these stimuli during this phase. An earlier study by Krug et al,

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(1994) indicates a higher rating by women of stimuli being sexual during the ovulatory phase. Together, these findings indicate that women have higher sexual interest during ovulation that at other phases of the menstrual cycle.

Primates. Female rhesus monkeys living in dyads with males can engage in sexual behavior throughout the cycle, however, when living in large natal groups, they approach, solicit, and mate primarily mid-cycle, or during the ovulatory period characterized by the peak in estradiol levels (Wallen, 1990). Female rhesus monkeys will bar-press at a higher rate for access to a male during ovulation than during other phases of the cycle (Kevern, 1976). Female rhesus monkeys also look longer at faces of male rhesus monkeys during times with high levels of E (Lacreuse, Martin-Malivel, Lange & Herndon, 2006), suggesting that they have higher levels of sexual interest during times when they are fertile.

Rodents. Lordosis, the measure of sexual receptivity in the female rat, is a reflexive behavior characterized by arching of the back, elevation of the rump, dorsoflexion of the tail, and extension of the neck, displayed when a receptive female is stimulated by a male (Pfaff & Modianos, 1985). Lordosis is dependent on estrogen and progesterone. A single intromission or manual vaginocervical stimulation (VCS) produces lordosis in the female rat (Rodriguez-Sierra, Crowley & Komisaruk, 1975). As well, it produces increased activation of Fos in the medial preoptic area, the lateral septum, the bed nucleus of the stria terminalis, the paraventricular hypothalamic nucleus, the ventromedial hypothalamus, the medial amygdala, the arcuate, the lateral septum, the lateral habenula, the ventral premammillary nuclei, midbrain regions, the cortex, and the straitum (e.g. Erskine, 1993; Pfaus, Kleopoulos, Mobbs, Gibbs & Pfaff, 1993; Pfaus,

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Marcangione, Smith, Manitt & Abillamaa, 1996; Tetel, Getzinger & Blaustein, 1993), many of which are areas that contain E receptors. In naturally cycling females, lordosis is shown during behavioral estrus. In OVX, EB-treated rats, at least 16 hours is required for rats to begin to show lordosis (Green et al, 1970). EB alone is sufficient to produce lordosis (Soderton, 1981), as well as many other proceptive behaviors, including pacing in the form of level changing in the bilevel chambers, and hopping and darting (Pfaus, Smith & Coopersmith, 1999). The addition of P in EB primed rats reduced the amount of EB required for lordosis to occur (Sodersten, 1981), and produced higher levels of appetitive level changing (i.e. level changing in the bilevel chamber before the male was introduced), active solicitation of the male, and high lordosis quotients and lordosis reflex magnitudes (Pfaus et al., 1999). When rats were given lower levels of EB and P, there was a reduction in the number of appetitive level changes, as well as lower lordosis quotients and lordosis reflex magnitudes (Pfaus et al., 1999). When OVX females, and gonadally-intact females in Diestrus, are presented with a male, contrary to showing lordosis, they will attempt to box the male, or escape from him. Further support that hormonal state is an essential determinant of sexual receptivity is provided by a study showing that female rats in Proestrus and Estrus chose a male over a female rat more frequently during a runway-choice task than rats in Diestrus (Eliasson & Meyerson, 1975). The incentive value for cues associated with sexual activity is also heightened when animals have higher levels if EB and P. OVX rats, treated with EB alone or EB+P, were mated in one of 2 compartments, and later showed a preference for this compartment (Oldenburger, Everitt & de Jonge, 1992).

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Differences in stress behaviors across ovulatory cycles

Differences in many other behaviors besides sexual have been seen in primates and rodents as a function of hormonal variation. For example, variations in feeding have been seen across the ovarian cycle where food intake is highest during the luteal phase in women (e.g. Lissner, Stevens, Levistsky, Rasmussen & Strupp, 1988; Pelkman, Chow, Heinbach & Rolls, 2001) and primates (Czaja, 1978; Rosenblatt, Dyrenfurth, Ferin & Vande Wiele, 1980), and lowest during the estrus phase in rodents (Tartellin & Gorski, 1971; Eckel, Houpt & Geary, 2000). Differences have also been recorded in alcohol use as a function of ovarian hormones. Although findings have been contradictory, it appears that women ingest more alcohol during the luteal phase of the cycle (e.g. Gill, 1997; Lindman, Koskelainen & Eriksson, 1999), and rats lever pressed at a higher rate for ethanol during the diestrus phase of the cycle than during estrus or proestrus phases (Roberts, Smith, Weiss, Rivier & Koob, 1998). This suggests that when a female is sexually receptive, non-sexual, pleasurable stimuli decrease in valence, and sexual stimuli increase in valence.

Interestingly, it seems that when a female is sexually receptive, she is able to withstand higher levels of stress and pain relative to when she is not, suggesting that procreation takes precedence over avoidance of potential danger. There is more direct evidence of this in rodents (see below), but in humans and primates, research findings support the idea of different stress reactivity and susceptibility across the ovulatory cycle.

Humans. There have been reports of a difference in stress responding in women across the menstrual cycle. In a review of 44 studies of attempted and completed suicide across the menstrual cycle, Saunders and Hawton (2006) determined a correlation

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between the early follicular and late luteal phases, times when E levels are lower, and increased suicide attempts. A recent review of the literature of physiological response to stress in postmenopausal and pregnant women, women using oral contraceptives, and naturally cycling women reported many changes in the autonomic nervous system (e.g. cardiovascular response, catecholamines), and in the HPA axis (e.g. cortisol response) that differed with differing levels of E: higher E generally correlated with lower responsiveness to stress (Kajantie & Phillips, 2006).

Anxiety differences as a function of ovarian hormones have been reported. The incidence of generalized anxiety disorder among post-menopausal women, a time with lower levels of E, is about 10%, which is double the incidence in the overall population (Weissman & Klerman, 1977; Wittchen & Hoyer, 2001). Self-reports of anxiety are higher at times when E levels are lower, that is during the postpartum and premenstrual periods (Arpels, 1996), and after menopause (Torizuka, Mizowaki & Hanawa, 200, cited in Walf & Frye, 2005). Thus, physiological response to stress and self-reports of anxiety appear to be modulated by E. Differences in women's mood across the menstrual cycle, in particular, increased depression and anxiety during the late luteal phase, have been recognized for the past 70 years (Rubinow & Roy-Byrne, 1984). Endocrine dysfunction, particularly during the luteal phase, has long been assumed to underlie premenstrual symptoms (e.g. Frank, 1931; Greene & Dalton, 1953; Rubinow et al., 1988), owing in part to the symptomatology being reported only during the late luteal phase (Backstrom et al., 1983), and the lack of symptoms before puberty, during pregnancy, after menopause (Reid, 1988), or during anovulatory cycles (DeVane, 1991). However, although some researchers have found evidence for a role of ovarian hormones in

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premenstrual symptoms, such as decreased levels of E (Klaiber, Broverman, Vogel & Kobayshi, 1979), others have not (e.g. Backstrom et al., 1983; Schmidt, Purdy, Moore, Paul & Rubinow, 1994; Taylor, 1979). At this time, it is still not known to what extent hormonal differences contribute to premenstrual symptoms.

Primates. Studies of primates report increased aggression in rhesus monkeys during the days before menstruation (Sassenrath, Rowell & Hendrickx, 1973). There have also been reports of increased feeding and social withdrawal in baboons during the premenstrual period (Hausfater & Skoblick, 1985). However, findings of another study indicates that high levels of E in rhesus monkeys are associated with agonistic interactions with other females, as well as increased affiliative interactions with males (Wallen & Tannenbaum, 1997). These results along with others discussed above, suggest that female primates that are sexually receptive focus on stimuli that will lead them to copulate, whereas they are more reactive to other stimuli, leading to changes in social and interactive behaviors when they are not sexually receptive.

Rodents. Similar differences observed in women's responses to stress, and their experience of anxiety across the menstrual cycle, can be seen in rats across the estrous cycle. For example, placing a rat in a shuttle box where the floor on one side is electrified and produces footshock, allows the rat to either escape the shock by running to a non-electrified component, or to avoid it by running to the other compartment when a conditioned stimulus, such as a tone, that was paired with the footshock, is present, or to freeze in place, a behavior coined as learned helplessness. Diestrus rats show more learned helplessness behavior than estrus rats (Jenkins, Williams, Kramer, Davis & Petty, 2001). Relative to rats in proestrus and estrus, rats in diestrus have longer latencies to

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enter both open and closed arms of the elevated plus maze (Reddy & Kulkarni, 1999), and spend less time exploring the open arms (Marcondes, Miguel, Melo & Spadari-Bratfisch, 2001; Mora, Dussaubat & Diaz-Veliz, 1996), suggesting higher levels of anxiety during diestrus. Similarly, lower levels of anxiolytic-like behavior have been reported for rats in proestrus relative to diestrus or estrus rats in the social interaction and defensive burying tasks (Frye, Petralia & Phodes, 2000). These differences suggest that, as do women, female rats have an increased threshold for anxiety when circulating hormone levels reflect sexual receptivity. In fact, female rats have been shown to cross an electrified grid to reach a male, but only when they are in behavioral estrus (Jenkins, 1928; Nissen, 1929; Warner, 1927; cited in Matthews et al., 1997), suggesting that ovulating rats are willing to withstand higher levels of anxiety and pain in order to achieve contact with a male.

The differences across the estrous cycle in anxiety response are also seen in OVX, hormone-treated rats. OVX rats treated with E show less anxiety in the defensive burying paradigm (Luo, Kiff, Makara, Lolait & Aguilera, 1994), increased exploration of the open arms in a plus-maze (Mora, Dussaubat & Diaz-Velliz, 1996; Nomikos & Spyraki, 1988), a higher response rate in a conflict situation (Roderiguez-Sierra, Howard, Pollard & Hendricks, 1984), more central crossings in the open field (Bowman, Ferguson & Luine, 2002), and spend more time in the central area of the open field (Hiroi & Neumaier, 2006) than untreated rats, suggesting lower levels of anxiety with administration of E. A subchronic dose of EB administered to OVX rats caused a decrease in anxiety behaviors as measured by the elevated plus maze, but not the social interaction test (Koss, Gehlert & Shekhar, 2004).

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Sexually motivated rats may take more risks, and be more willing to expose themselves to danger in order to attain the goal of reproduction. This may be the result of decreased perception of the danger, or perhaps increased tolerance to pain and/or anxiety. Bangasset and Shors (2004) conducted a series of studies using eyeblink conditioning and demonstrated that there is no decrease in perception of US strength. There have been some reports of slight differences in pain thresholds as a function of estrus cycle phase (e.g. Martinez-Gomez, Cruy, Salas, Hudson & Pacheco, 1994; Vinogradova, Zhukov & Batuev, 2003), however, these differences are small and attributable to other factors, including time of day and type of test used. Together, these findings suggest that increased risk-taking is likely because of increased motivation and focus on the goal of copulation. Together with the literature reviewed above, this suggests that when rats are sexually receptive, they attend less to anxiety-inducing or stressful stimuli, because they are driven by sexual motivation. Conversely, non-sexually receptive rats are more focused on dangers in the environment, thus they are more reactive to stressful and anxiogenic stimuli. Interestingly, although research has investigated the effects of stress on sexual behavior in the male rat (e.g. Almeida, Kempinas & Lamano Carvalho, 2000; Menendez-Patterson, Florez-Lozano, Fernandez & Marin, 1978), there has been little research investigating this same question in female rats. A recent study showed that 10 days chronic stress consisting of starvation, low temperature, immobilization, and changes in diurnal rhythm, led to a decrease in sexual receptivity (lower frequencies of lordosis during a 30 minutes period) in estrus rats, as well as a decrease in E levels (Yoo, Chung, Park & Cho, 2005).

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Proposed mechanisms for ovarian hormone effects on anxiety

There is a higher incidence of depression (e.g. Angst et al, 2002; Kornstein et al., 2000) and anxiety (e.g. Cloitre et al., 2004; Pigott, 2003) in women than in men, and, as discussed above, women experience differences in mood across the menstrual cycle. Because decreased levels of 5-HT have been implicated in depression (e.g. Asberg, Thoren & Traskman, 1976; Westernberg & Verhoeven, 1988), anxiety (Van Pragg, 1988), and irritability (Steinberg, Annable, Young & Liyanage, 1999a), it may be that 5-HT dysfunction might make women with PMS more vulnerable to altered mood during normal menstrual fluctuations in ovarian and/or adrenal hormones (Odber, Cawood & Bancroft, 1998; Su, Schmidt, Danaceau, Murphy & Rubinow, 1997). Whole-blood 5-HT levels are lower in women with PMS than in controls during the last 10 days of the menstrual cycle, suggesting that abnormal fluctuations of 5-HT may be involved in the etiology of premenstrual symptoms (Rapkin et al., 1987). 5-HT agonists, including fluoxetine (Pearlstein & Stone, 1994; Su, Schmidt, Danaceau, Tobin et al., 1997; see review by Dimmock, Wyatt, Jones & O'Brien, 2000), L-tryptophan (Steinberg, Annable, Young & Belanger, 1994; Steinberg, Annable, Young & Liyanage, 1999b), clomipramine (Sundblad, Modigh, Anderson & Eriksson, 1992), and citalopram (Freeman, Jabara, Sondheimer, & Auletto, 2001) have had some success in relieving symptoms such as irritability, dysphoria, sadness, anxiety, and physical discomfort premenstrually, suggesting that 5-HT dysfunction may underlie symptoms. However, other researchers have failed to find support for the hypothesized role of 5-HT in PMS. Veeninga and Westenberg (1992) found no difference between women with and without a DSM-III-R diagnosis of late luteal phase dysphoric disorder (LLPDD) in platelet 5-HT uptake, and

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suggest that previous research findings of 5-HT differences between women with and without a LLPDD diagnosis are the result of underlying mood disorders, such as depression, in the diagnosed women. Contrary to this hypothesis is the finding that the SSRI sertraline was effective in relieving symptoms of PMS, whereas placebo and desipramine, a tricyclic antidepressant, were not (Freeman, Rickels, Sondheimer & Polansky, 1999).

Steiner and Pearlstein (2000) suggest that SSRIs may effectively alleviate premenstrual symptoms not because of their actions on 5-HT, but because they cause increased synthesis of allopregnenolone which may act on the $GABA_A$ receptor to relieve symptoms of dysphoria and irritability. In fact, plasma GABA levels are lower in women with PMDD than in asymptomatic controls (Halbreich et al., 1996; Sundstrom et al., 1998). Further, Monteleone et al. (2000) reported that allopregnenolone levels are lower in women with PMS than asymptomatic women during the luteal phase, and that the variation between luteal and follicular phase levels of this hormone are 3 times lower in PMS women. The authors suggest that lower levels of allopregnanolone in women with more severe premenstrual symptoms may result in lower levels of GABA, thereby increasing symptoms such as irritability and anxiety.

E has effects on 5-HT synthesis, metabolism, and receptor distributions. Bigeon and McEwen (1982) showed that a single dose of EB in OVX rats caused an immediate decrease in 5-HT1 receptors, only to be followed 48-72 hours later with an increase in these receptors in the amygdala, hypothalamus, and preoptic area. Acute administration of EB downregulates $5\text{-}HT_{1A}$ receptor mRNA in the limbic system of female rats (Osterlund & Hurd, 1998), and a reduction in $5\text{-}HT_{1A}$ receptor binding in medial

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amygdala and hippocampus was found with 2 weeks of chronic EB (Osterlund, Halldin & Hurd, 2000).

Although changes in both pre- and post-synaptic 5-HT tone have been found as a function of estrus cycle phase (Bethea, 1993; Farmer, Isakson, Coy & Renner, 1996; Gundlah, Simon & Auerbach, 1998), there have been no reductions in anxiety behaviors on open field or elevated plus maze tests as a function of E and fluoxetine treatment (Taylor, Farr, Klinga & Weiss, 2004). There is some evidence for a differing anxiolytic effect of benzodiazepines as a function of hormonal state. A reduction in anxiety behaviors in the elevated plus maze was observed in OVX rats treated with EB+P and diazepam relative to those given diazepam but not EB+P (Bitran, Hilvers & Kellogg, 1991). Another benzodiazepine, alprazolam, was effective in reducing anxiety on the social interaction test only when administered in conjunction with EB in OVX rats (Koss et al., 2004). Finally, in a conflict-operant paradigm, female rats tested under the influence of diazepam during estrus and proestrus showed an increase in immediate punished reinforcement, suggesting lower levels of anxiety, than female rats treated with diazepam and tested during diestrus (Molina-Hernandez, Contreras & Tellez-Alcantara, 2001).

At least some of E's anxiolytic effects appear to be the result of E actions in the hippocampus. For example, administration of EB subcutaneously or infused directly into the dorsal hippocampus resulted in an increase in number of central crossings in the open field, an increase in time spent on the open arms of the elevated plus maze, and a decrease in time immobile in the forced swim test (Walf & Frye, 2006; Walf & Frye, 2005). Daily subcutaneous injections of 10 μg EB for 2 days resulted in improved spatial

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memory retention in a water maze task (Sandstrom & Williams, 2004), as did direct infusion of EB into the hippocampus (Packard & Teather, 1997). These findings suggest that E in the hippocampus has anxiolytic effects, as well as effects on learning and memory.

The mechanisms underlying the effects of E in the hippocampus have been explored. The hippocampus goes through many changes in morphology and physiology during the estrous cycle, including increased density and number of dendritic spines and synapses in the hippocampal CA1 region (Woolley, Gould, Frankfurt & McEwen, 1990; Woolley & McEwen, 1992), as well as increased synaptic plasticity (Good, Day & Nuir, 1999; Warren, Humphreys, Juraska & Greenough, 1995). In fact, the induction of LTP in the hippocampus follows the fluctuations of E: induction is highest in the afternoon of proestrus, when E levels are highest, and it is lower during estrus and diestrus, when E levels are also lower (Warren et al., 1995). There is an increase in fos expression in the CA1 and CA3 regions of the hippocampus in intact female rats administered EB (Rudick & Woolley, 2000). Furthermore, after 2 daily injections of 10 μg EB in ovariectomized rats, there is an increase in dendritic spine density of CA1 pyramidal neurons (Gould, Woolley, Frankfurt & McEwen, 1990; Woolley & McEwen, 1993), which is associated with an increase in the sensitivity of these neurons to NMDA receptor-mediates synaptic input (Weiland, 1992), and a decreased threshold for induction of long-term potentiation (Cordoba-Montoya & Carrer, 1997), all of which ultimately result in the behavioral observation of decreased response to anxiety.

When EB is administered directly to the medial amygdala, or injected subcutaneously, there is a reduction in anxiety behaviors, as evidenced by more central

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crossings in the open field, and more time spent on the open arms of the elevated plus maze (Walf & Frye, 2006; Frye & Walf, 2004). These results indicate that E's actions in the amygdala may have anxiolytic effects on behavior.

Results of some studies suggest that the decrease in anxiety behavior in rats is a function of P. Administration of P to OVX rats increases time spent in the open arms of the elevated plus maze (Bitran, Shiekh & McLeod, 1995), decreases time spent burying an electrified probe (Martinez-Mota, Estrada-Camarena, Lopez-Rubalcava, Contreras & Fernandez-Guasti, 2000), and increases the amount of time water deprived rats spend licking an electrified water spout (Rodriguez-Sierra, Hagley & Hendricks, 1986). P may have these anxiolytic effects through actions at the GABA receptor (Majewska, Harrison, Schwartz, Barker & Paul, 1986), perhaps via it's metabolites, including 5α-pregnan-3α-ol-20-one (3α,5α-THP), and 3α-hydroxy-5α-pregnan-20-one (allopregnanolone). In support of this hypothesis, systemic administration of 3α,5α-THP produces increased time spent in the open arms of the elevated plus maze (Bitran, Hilvers & Kellogg, 1991), and number of central crossings in the open field (McCarthy, Felenberg, Robbins, Pfaff & Schwartz-Giblin, 1995). Blocking the metabolism of progesterone into 3α,5α-THP with finasteride, a 5α-reductase inhibitor, attenuated anxiolytic behavior of rats in behavioral estrous in elevated plus maze, open field, and defensive burying tests (Rhodes & Frye, 2001). Blocking the metabolism of progesterone with a 3-hydroxysteroid dehyrogenase inhibitor, indomethacin, increased time spent burying the electrified probe in the defensive burying test, suggesting increased anxiety (Gallo & Smith, 1993). Furthermore, reductions in anxiety behaviors on the elevated plus maze, open field, and defensive burying tests were seen whether finasteride was administered systemically, or

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directly infused to the hippocampus, indicating that the hippocampus is a likely place where these neurosteroids may exert their anxiolytic actions (Rhodes & Frye, 2001) by increasing the time that the GABA-gated Cl^- channel is open (Majewska et al., 1986), resulting in increased GABAergic inhibition (Patenaude, Nurse & Lacaille, 2001; Smith, Waterhouse & Woodward, 1987).

The studies reviewed above illustrate that although manipulations of ovarian hormones produce clear behavioral effects on anxiety, there are many theories, but no conclusions, of how these hormones may be affecting behavior.

Expectancy and conditioning

The research presented above suggests a strong influence of hormonal levels in a differential reactivity to stress in women, other female primates, and female rats, across the ovulatory cycle. However, expectancy has been postulated to underlie some of the differences in mood and stress reactivity noted across the menstrual cycle in women. When women are led to believe that they are premenstrual, they report more symptoms than when they believe they are intermenstrual (Ruble, 1977), particularly if they are told that these symptoms are normal and to be expected (AuBuchon & Calhoun, 1985; Marvan & Escobedo, 1999; Olasov & Jackson, 1987).

Prior experiences, conditioning, and learning can have important influences on behaviors that are typically thought to be purely under the control of hormones, or other physiological variables. For example, as discussed above, sexual behavior, particularly in the rat, is thought to be a function of varying hormonal levels. Pfaus and colleagues (2001) suggest that there is a role of learning and conditioning in sexual behavior.

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Conditioned place preference is seen in both male and female rats given the choice of being in a chamber where they previously copulated versus one where they did not (Everitt, 1990; Parades & Alonso, 1997), and in fact a reduction in latency for penile erections was observed in male rats that were placed in a chamber where they have previously copulated (Sachs & Garinello, 1978), suggesting an important effect of learning. Further, rats that are provided with early sexual experiences with an almond-scented sexually receptive female show a conditioned ejaculatory preference for almond-scented females (Kippin & Pfaus, 2001). The results of these studies all provide evidence that a behavior that is thought to be hormonally mediated can also be influenced by prior experience and learning.

Fear conditioning allows the investigation of how people and animals learn to predict danger from previous experiences. Fear conditioning occurs when a previously neutral, conditioned stimulus (CS) is paired with an aversive unconditioned stimulus (US) to produce an unconditioned fear response (UR; Rescorla, 1968). Following one, or several pairings of the CS-US, the CS comes to develop an emotional significance, and is capable of eliciting the UR upon presentation of the CS alone (LeDoux, 1998; Wilensky, Schafe & LeDoux, 2000). Conditioned fear has been postulated to contribute to certain psychiatric disorders. For example, fear conditioning both to explicit and contextual cues has been postulated as a model for the intrusive memories in post traumatic stress disorder (PTSD; Grillon & Morgan, 1999; Grillon, Southwick & Charney, 1996). In panic disorder, there is an avoidance of certain places or situations because of a belief that panic attacks will occur in these places, suggesting that there is fear conditioning to these cues (Rauch, Shin & Wright, 2003). Fear conditioning can be demonstrated in the

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laboratory. In one study, participants were exposed to slides of snakes and spiders (CS) that were paired with a loud noise (US) and their heart rate (UR) was measured. Following a few CS-US pairings, presentation of the slides alone was sufficient to produce increases in heart rate (Sandin & Chorot, 1989).

In the rat, typically a CS is a tone or a light, and it is paired with a US, such as footshock. The UR can range from a variety of innate, species-specific behaviors seen in response to danger, including freezing or escape responses; autonomic nervous system responses, such as changes in blood pressure or heart rate; and changes in hormones released from the pituitary and adrenal glands (LeDoux, 2003). Sex differences in conditioned fear have been investigated, and, to date, no differences have been found between male and female rats in freezing during presentation of a tone previously paired with shock (i.e. Maren, De Oca & Fanselow, 1994; Pryce, Lehmann & Feldon, 1999). Perhaps because of the lack of sex differences found, there is a paucity of research investigating differences in fear conditioning across the estrous cycle. Avoidance conditioning, where rats have to learn to perform an avoidance response to avoid a negative consequence, such as footshock, and increased acquisition or retention is thought to indicate higher levels of fear (Banerjee, 1971; Nelson & Young, 1998), has been shown to be differentially affected by ovarian hormone levels. Avoidance response was facilitated during diestrus relative to other cycle phases, and enhanced in OVX rats not treated with EB and/or P (Diaz-Veliz, Sots, Dussaubat & Mora, 1989). This suggests that E and/or P serve to decrease the fear associated with the CS. Fear conditioned freezing can be significantly reduced with direct infusion of flesinoxan, a 5-HT_{1A} receptor agonist, into the amygdala or the hippocampus, whereas injections into the medial

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prefrontal cortex do not have the same effect (Li et al, 2006), suggesting that stimulation of postsynaptic 5-HT_{1A} receptors in the hippocampus and amygdala account for the reduction in conditioned freezing caused by flesinoxan.

Contextual fear conditioning

It has become apparent that context can serve an important moderating role in determining the salience of other cues. For example, drug studies have shown that context plays a role in habituation to many drugs of abuse (e.g. McSweeney, Murphy & Kowal, 2005), such that more drug is needed to produce the same effect when it is administered in an environment where it has previously been administered. Interestingly, findings from some studies of therapeutic effectiveness in extinguishing phobias suggest that context may also determine whether individuals show signs of anxiety when presented with their feared stimuli, and that fears may return when individuals are removed from the therapeutic environment (Mineka, Mystkowski, Hladek & Rodriguez, 1999; Rodriguez, Craske, Mineka & Hladek, 1999), indicating that extinction is greatly influenced by contextual conditioning. This effect has been supported by laboratory studies, where skin conductance (UR) was measured in a context in which participants are exposed to acquisition of the association between a particular image (CS) and white noise (US), and findings indicate that re-exposure to the context caused an increase in skin conductance response, even when the association had been extinguished (Vansteenwegen et al., 2005; 2006).

In animal studies of contextual fear conditioning, an experimental chamber (CS) is paired with a mild footshock (US), and freezing behavior (UR) results (e.g. Blanchard

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& Blanchard, 1969; Bolles, 1970; LeDoux, Iwata, Pearl & Reis, 1986). Whereas sex differences in cued conditioned fear have not been found, Maren and colleagues (1994) demonstrated that male rats froze more than female rats during re-exposure to a context associated with fear conditioning. Among female rats, differences have been found in use of spatial or contextual cues as a function of ovarian hormones. Sava and Markus (2005) note that in the water maze, proestrus females are affected by and attend to a wide range of stimuli, whereas estrus females are affected more by cues that are closer to the goal, and therefore more salient. Similarly, Korol et al. (2002; 2004) demonstrated that proestrus females are affected more by extramaze cues whereas estrus females are more reliant on egocentric cues, such as muscle response, in solving a navigational task.

The differences in use of contextual cues as a function of ovarian hormones may underlie observed differences in contextual fear conditioning as a function of ovarian hormones. Using a contextual conditioned fear paradigm, Tropp and Markus (2001) demonstrated that rats in proestrus froze less to context than those in estrus, suggesting that higher circulating E and P resulted in less conditioning. Similarly, OVX rats froze more in a contextual fear conditioning paradigm than cycling rats, but the freezing was significantly reduced if the OVX rats were treated with EB (Gupta, Sen, Diepenhorst, Rudick & Maren, 2001). Contrary to these findings, other studies report findings of higher levels of freezing in OVX mice that were implanted with an EB capsule compared to those implanted with an oil capsule (Jasnow, Schulkin & Pfaff, 2006; Morgan & Pfaff, 2001). Perhaps the discrepancy in findings is the result of constant elevated levels of EB compared to cyclical increases in EB seen in naturally cycling rats, or those treated with systemic injections rather than hormone implants.

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Hippocampus, amygdala, and anxiety

In the case of auditory conditioned fear in the rat, sensory inputs to the amygdala, such as those from the auditory thalamus and auditory cortex, terminate mainly in the basolateral amygdala (BLA; e.g. Mascagni, McDonald & Coleman, 1993; Romanski & LeDoux, 1992), which is comprised of the lateral, basal, and accessory basal nuclei (Donley, Schulkin & Rosen, 2005). Visual information necessary for conditioning to a light, for example, is processed and transmitted to the BLA via the inferotemporal cortex and the perirhinal cortex (Davis & Lee, 1998; Shi & Davis, 2001). The BLA is interconnected with the central amygdala (CeA), which sends outputs to various structures involved in fear responses, including the bed nucleus of stria terminalis, lateral hypothalamus, and the periaquedutal gray (Kim & Jung, 2006; LeDoux, 1996; McDonald, 1998).

Fear conditioning is largely dependent on the amygdala (LeDoux, 2003). Aggleton (1993) proposes that in addition to being a necessary site for the formation of the association between the US and CS, the amygdala is also responsible for assessing positive and negative reinforcement qualities of stimuli, and acquiring response contingencies. In the rat, lesions of the amygdala results in an impairment of the acquisition and the expression of fear conditioning as measured by a variety of responses, including freezing (e.g. Baker & Kim, 2004; Blanchard & Blanchard, 1972; Kim & Davis, 1993) and increases in blood pressure (Iwata, LeDoux & Reis, 1986). In primates, lesions of the amygdala produce a reduction in fear, and decreased vigilance to a snake and to unfamiliar conspecifics (e.g. Aggleton & Passingham, 1981; Kalin, Shelton, Davidson & Kelley, 2001). In humans, a positive correlation has been determined

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between the magnitude of conditioned fear responses, as measured by electrodermal fluctuations, and the amount of cerebral blood flow in the right amygdala (Furmark, Fiscer, Wik, Larsson & Fredrikson, 1997; Morris, Ohman & Dolan, 1998). Imaging studies in humans have shown that the amygdala is activated with presentation of a CS that had previously been paired with a US (e.g. Cheng, Knight, Smith, Stein & Helmstetter, 2003; LaBar, Gateby, Gore, LeDoux & Phelps, 1998), and a more recent study suggests that activation of the amygdala with presentation of the CS also correlates with skin conductance response in study participants (Knight, Nguyen & Bandettini, 2005).

Contextual fear conditioning requires both the amygdala and the hippocampus (Kim & Fanselow, 1992; Maren, Aharonov & Fanselow, 1997). There are projections from the ventral hippocampus (CA1) to the basolateral amygdala (Canteras & Swanson, 1992), and current theories suggest that the amygdala modulates the storage of a contextual fear memory in the hippocampus (Huff & Rudy, 2004; Huff, Wright-Hardesty, Higgins, Matus-Amat & Rudy, 2005). Inactivation of BLA via direct injection of muscimol either immediately prior to or immediately following exposure to the context was found to impair the rat's memory for the context (Huff & Rudy, 2004), and pre- or post-training NMDA lesions of the BLA impaired freezing when rats were re-exposed to the context (Cousens & Otto, 1998).

Pretraining lesions of the ventral hippocampus have been found to reduce freezing following contextual conditioned fear, but only when a tone was paired with the context and the shock (Bannerman et al., 2003; Richmond et al., 1999), suggesting that the ventral hippocampus is not required for the acquisition and expression of contextual

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conditioned fear. Contrary to this finding, although pretraining lesions of the dorsal hippocampus impair freezing upon context re-exposure following fear conditioning (Philips & LeDoux, 1992; Young, Bohenek & Fanselow, 1994), lesions in this area have no effect on auditory or olfactory fear conditioning (Otto & Poon, 2006; Rawlins & Tanner, 1998). The importance of the dorsal hippocampus in contextual, but not auditory, conditioned fear is supported by findings of a separate study, where microinjections of D-amphetamine into the dorsal hippocampus administered immediately after context, tone, and footshock pairings increased conditioned freezing to the context, but not to the tone, 24 hours later (White & Salinas, 2003).

The hippocampus and the amygdala are therefore necessary areas for the acquisition and expression of contextual conditioned fear. In addition, the research discussed above suggests that these areas are possible sites for the effects of ovarian hormones, 5-HT, and GABA in reducing anxiety.

Current studies

The research reviewed above suggests that sexual behavior and stress reactivity in the female animal are mutually inhibitory processes – when a female is sexually receptive, she is less reactive to stress, and vice versa. The premenstrual symptoms experienced by women in the week before they menstruate provide another example of this opponent process: Hormones are low, and stress reactivity is high. As discussed above, there is no clear link, however, between hormone levels and premenstrual symptoms, which has led many researchers to the hypothesis that it is the combination of many factors that is responsible for symptomatology (e.g. Anson, 1999; Jarvis &

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McCabe, 1991; Woods, Lentz, Mitchell, Shaver & Heitkemper, 1998). It is thought that premenstrual symptoms are the result of bio-psycho-social factors, where cyclical variations in the hormonal milieu are interpreted based on expectancies formed from past experience and learning about menstruation (Anson, 1999). One group of researchers postulate the existence of an experiential state, characterized by changed beliefs, cognition, memories, perceptions, affects, and neurobiology, occurring in some women during the few days prior to menstruation (Rubinow & Schmidt, 1989). Hormonal fluctuations occurring 5-10 days before menstruation may signal the onset of this theorized experiential state, leading to the expression of some premenstrual symptoms, in particular, anxiety, dysphoria, and irritability.

It is difficult to examine the multitude of factors, including context, expectation, and sensitivity to hormonal or neurotransmitter fluctuations, that might contribute to the induction of premenstrual symptoms in women. However, because of the cyclical hormonal changes that occur in rodents in a similar fashion to the way they occur in women, and because of the changes in anxiety, aggressive, and depressive behaviors seen in rodents, primates and women as a function of ovarian hormones, the rat may provide a model to allow us to begin to elucidate the relative contributions of ovarian hormones and pre-exposure to stress in relation to levels of anxiety. In addition to possibly shedding some light on the development and maintenance of premenstrual symptoms, understanding the relative contributions of hormones and conditioning in the female rat will allow us to understand more the propsed opponent process between stress and sexual activity. Thus, the overall goal of this thesis was to explore the behavioral differences in

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stress and anxiety reactivity in rats as a function of ovarian hormones and expectancy of stress.

The experiments described in Chapter 2 had the goal of comparing differences in anxiety reactivity in cycling and OVX, hormone-treated rats that had been pre-exposed to inescapable footshock in a contextual conditioned fear paradigm. Behavior in the open field test and on the elevated plus maze was examined as a function of hormonal state, as well as whether rats had previously been fear conditioned. Chapter 3 extends these results by adding more behavioral measures to the assessment of anxiety in OVX, hormone-treated rats. In addition, this chapter examined the importance of the rat being exposed to the contextually-cued chamber to produce increases in anxiety response, to answer the question of whether hormonal state was sufficient to signal conditioned fear in the rat. Chapter 4 examined the effects of 2 different drugs, diazepam and fluoxetine, in reducing anxiety behaviors following contextual fear conditioning.

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CHAPTER 2:

EFFECTS OF HORMONAL STATES AND CONDITIONED ANXIETY ON ELEVATED PLUS-MAZE PERFORMANCE IN GONADALLY INTACT AND OVARIECTOMIZED RATS

Levels of ovarian hormones fluctuate over the estrus cycle in the rat, with highest levels of estrogen (E) and progesterone (P) during proestrus, when the rat is becoming sexually receptive (Frye, Petralia & Rhodes, 2000), and the lowest levels in the morning of diestrus I (Cole & Jones, 1995). Ovarian hormones exert an influence on many more processes than reproduction (e.g. Barntess & Waldbillig, 1984; Leuner, Mendolia-Loffredo & Shors, 2004; Wade & Gray, 1979), including learning and memory (e.g. Diaz-Veliz, Urresta, Dussaubat & Mora, 1994; Gallo & Smith, 1993; Marcondes, Miguel, Melo & Spadari-Bratfisch, 2001; Serevino et al., 2004), and anxiety (e.g. Blizzard, Lippman & Chen, 1975; Gorzalka, Wilkie & Hanson, 1995).

Differences exist on a variety of measures of anxiety across the estrus cycle. Relative to rats in proestrus and estrus, rats in diestrus are less active in an open field (Butcher, Collins & Fugo, 1974), show more ulceration following restraint stress (Inoue, Tschiya & Koyama, 1996), exhibit decreased open-arm exploration in the elevated plus maze (Mora, Dussaubat & Diaz-Veliz, 1996), and have an impairment in the acquisition of conditioned avoidance responding (Ebner, Richardson & Riccio, 1981; Sfikakis, Spyraki, Sitaras & Varonos, 1978; Smith, Schramek & Pfaus, 2006), all of which suggest increased anxiety during diestrus. Frye, Petralia and Rhodes (2000) used a series of behavioral tests to measure anxiety across the estrus cycle, including the elevated plus maze test, social interaction, the open field, defensive burying, and the holeboard task. Findings suggested decreased anxiety in proestrus rats compared to rats in other phases of

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the estrus cycle or male rats. In another study, increased exploration in an open field was found in group-housed mice relative to individually-housed mice, but this difference as a function of housing was not seen in mice tested in proestrus relative to other estrus phases [44], suggesting that the hormonal state during proestrus creates a milieu where the impact of certain stressors is reduced. Severino et al. (2004) found that estrus phase was a more important predictor of anxiety than early handling in plus maze performance. Rats were handled during the first 10 days after delivery and exposed to the elevated plus maze. Previously-handled rats tested in diestrus were less anxious than non-handled rats tested in diestrus, but this difference in anxiety as a function of handling was not observed in rats tested in estrus, suggesting lower levels of baseline anxiety as well as anxiety reactivity during estrus. Together, the results of these studies suggest that rats are more anxious during diestrus, when plasma levels of E and P are low compared to proestrus and estrus when hormone levels are higher.

The importance of ovarian hormones in modulating anxiety reactivity has also been demonstrated in ovariectomized (OVX) rats. OVX rats treated with EB and/or P show more exploration of the open arms in a plus maze (Morgan & Pfaff, 2001), and a higher response rate in a conflict situation (Sandstrom & Williams, 2004) than OVX rats not given hormone replacement, suggesting lower levels of anxiety. OVX rats treated with estradiol and exposed to 21 days of restraint stress showed more exploration of inner sectors of an open field than OVX rats not treated with estradiol, suggesting that E has anxiolytic properties (Bowman, Ferguson & Luine, 2002). Rats tested in the absence of hormone priming showed higher levels of anxiety than those tested with EB+P in anticipatory freezing, proportion of central crossings in the open field, and number of

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contacts with the electrified probe in the defensive burying test, suggesting that female rats are more sensitive to contextual cues that predict shock in the absence of ovarian hormones (Somponpum, Holmes, Seckl & Russell, 2004). Intact rats in diestrus were found to spend less time in the open arms of an elevated plus-maze than rats in proestrus, suggesting higher levels of anxiety, but this difference was abolished if estradiol was administered to the diestrus rats in levels sufficient to mimic levels during proestrus (McCarthy, McDonald, Brooks & Goldman, 1996).

E serves not only to decrease anxiety in female rats, but may also increase learning and memory. EB has been shown to improve memory of platform location in a water maze task in ovariectomized rats, whether it is administered acutely (Palanza, Gioiosa & Parmigiani, 2001), or chronically (Rodriguez-Sierra, Howard, Pollard & Hendricks, 1984). Acute or chronic administration has also been shown to improve performance during acquisition of a radial arm maze (Diaz-Veliz et al., 1994), and during associative memory formation using trace eyeblink conditioning (Leuner, Mendolia-Loffredo & Shors, 2004). A recent study suggests that rats have preferred learning strategies across the estrus cycle, preferring to use place strategies (i.e. returning to the same spatial location where reward was in a previously learned maze) during proestrus, and response strategies (i.e. making the same directional turn on the maze that was previously rewarded) during estrus (Korol, Malin, Borden, Busby & Couper-Leo, 2004), suggesting that gonadally-intact rats are able to discriminate hormonal state. In other studies, discrimination of hormonal state in OVX rats required high doses of EB and/or P (Feldon & Weiner, 1989; Gorzalka, Wilkie & Hanson, 1995; Stewart, Krebs & Kaczender, 1967). However, using a more sensitive task, conditioned flavor aversion,

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Costanzo, Riccio and Kissinger (1995) demonstrated that OVX females injected with lower levels of EB and P (6 μg EB and .5 mg P) showed state dependent retention. These results suggest that female rats may discriminate hormonal state, whether it is naturally occurring, in gonadally intact rats, or induced in OVX rats.

The purpose of the present study was to examine whether a hormone state paired with a stressor can come to elicit higher levels of anxiety in animals simply as a result of being in that same hormonal state. Two experiments were conducted. In the first, gonadally-intact rats were trained and tested always in the same phase of the estrus cycle (proestrus, estrus, or diestrus). In the second experiment, rats were ovariectomized and injected with estradiol benzoate (EB) or the sesame oil vehicle (O) followed by either P or the sesame oil vehicle sesame oil to produce 4 groups: EB+P, EB+O, O+P, and O+O. In both studies, rats were subjected to inescapable foot-shock while in the particular hormonal state for 5 cycles of 4 to 5 days. On the 6^{th} cycle, their behavior on the elevated plus-maze was recorded. The elevated plus maze (Pellow & File, 1986) was selected because it is the most commonly used measure of anxiety (Ramos, Burton, Mormede & Chaouloff; 1997). The paradigm provides measures of two specific aspects of anxiety; approach avoidance towards aversive stimuli (number of open arm entries and time spent in the open arms), and activity in novel environments (number of closed arm entries; Ramos & Mormede, 1998).

It was hypothesized that there would be a main effect of stress expectancy, such that rats that were trained with shock would have higher anxiety responses on the elevated plus maze. Given the known anxiolytic effects of ovarian hormones, it was also hypothesized that OVX rats trained and tested in the presence of EB+P would exhibit less

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anxiety than those trained and tested without these hormones, and that diestrus rats would show less anxiety than proestrus rats. Finally, an interaction between these 2 factors was expected, such that the non hormone-treated, or diestrus rats that were trained with shock were expected to have the highest levels of anxiety.

Methods

Animals and surgery

Female Long-Evans hooded rats (Charles River, St. Constant, QC; 200g-250g) were housed in groups of two in plastic cages (36 X 26 X 19 cm) in a colony room maintained on a reverse 12:12 hour light/dark cycle (lights off at 08:00), and at a constant temperature of 21°C, and food and water were continuously available.

Beginning one week after arrival, vaginal smears were taken daily from gonadally-intact females for at least three weeks (approximately five 4-day cycles) before behavioral testing began. Smears were examined under the microscope, and a predominance of nucleated epithelial cells determined that the rat was in proestrus, a predominance of cornified epithelial cells determined that she was in estrus, and a predominance of leukocytes determined that she was in diestrus I or II [16]. For the purposes of this study, no differentiation was made between diestrus I and II. Smears were verified every day, and only rats that had a regular 4-5 day cycle over 4 consecutive cycles were used in this study.

Ovariectomies were performed on a second group of rats via bilateral lumbar incisions under ketamine hydrochloride (50 mg/ml) and xylazine hydrochloride (4 mg/ml), mixed at a ratio of 4:3 respectively, and injected intraperitoneally in a volume of

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1 ml/kg of body weight. Animals were given a week to recover prior to beginning experimental training sessions.

Hormone condition and training sessions

Gonadally-intact females were randomly assigned to be trained in one of 3 phases of the estrous cycle: proestrus (N=17), estrus (N=22), or diestrus (N=25). Throughout the course of the experiment, smears were obtained daily to ensure rats were tested in the appropriate phase of the estrus cycle.

Ovariectomized rats were assigned randomly to one of 4 hormone groups: EB+P (N = 17), EB+O (N = 18), O+P (N = 19), or O+O (N = 21). Subcutaneous injections of EB (10 μg/0.1 cc of sesame oil) or 0.1 cc of sesame oil were administered 48 h before each behavioral training or testing session, and progesterone (P; 500 μg/0.1 cc of sesame oil) or 0.1 cc of sesame oil 4 h before a session.

In both experiments, rats were further randomly assigned to one of two stress conditions, shock or no shock. For each experiment, at least one rat from each condition was tested on each test day. All sessions took place during the middle third of the dark phase of the light/dark cycle. During each training session, rats were placed in a 30 X 30 cm Plexiglas box with horizontal metal rods placed 5 cm above the floor. Rats in the shock condition were placed in the box for a 5-minute period, after which a series of 11 0.8 mA foot shocks were delivered at 30 s intervals through the metal rods. Rats in the no shock condition were placed in the box for 15 min. Each rat was subjected to 5 training trials, at approximately 4-day intervals, always under the same hormone and shock condition.

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Behavioral testing

Four days after the fifth training trial, rats completed behavioral testing. At least one rat from each condition was tested on each day. Rats were placed in the Plexiglas box for 5 min where they had previously received shock or no shock, and then removed and placed into an arena (60 x 60 x 60 cm) for 5-minutes. Rats were then were tested on the elevated-plus maze. The elevated-plus maze was a dark Plexiglas T-maze, elevated 70 cm off the ground, and comprised of two open arms (10 X 50 X 1cm) facing each other, two closed arms opposite from each other (10 X 50 X 20 cm), and a central platform (10 X 10 cm) (Pellow & File, 1986). Rats were placed on the central platform of the maze facing the point where the open and closed arms meet. The number of closed and open arm entries was recorded, and a ratio was calculated as proportion of entries that were on the open arms. Latencies to enter the closed and open arms, and time spent on the open and closed arms, were also recorded over a 5 minute period.

Statistical analyses

Raw data were rank-ordered and ANOVAs were performed on the ranks for the elevated plus maze variables. A 4x2 (estrus phase x shock) ANOVA was conducted on each variable. There were between 8 and 13 rats in each phase x stress condition group. Raw data from Experiment II were also ranked, and a 4x2 (hormone condition x shock or no shock) ANOVA was conducted on each of the plus maze variables. There were between 8 and 13 rats in each hormone x stress condition. For all significant main effects or interactions, posthoc analyses of individual mean differences were done using the Tukey HSD method, $P<0.05$.

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Results

Effect of phase of estrous cycle

Figure 1 shows the number of open arm entries made on the elevated plus maze as a function of estrus cycle phase and shock. The ANOVA detected a main effect of shock ($F(1,58) = 8,228$, $P < 0.01$). Rats that were shocked made fewer open arm entries than those that were not shocked.

Figure 2 shows the number of closed arm entries made on the elevated plus maze as a function of estrus cycle phase and shock. The ANOVA detected a main effect of shock ($F(1,58) = 5.762$, $P<0.05$). Rats that were shocked made fewer closed arm entries than rats that were not shocked. The ANOVA also detected a main effect of phase in the number of closed arm entries made ($F(2,58) = 3.341$, $P<0.05$). Posthoc analyses revealed that rats in diestrus made significantly less closed arm entries than did rats in proestrus.

The ratio of open to total arm entries was calculated. There were no significant differences as a function of estrus cycle phase or shock, and no interaction was found between the 2 variables.

Figure 3 shows the latency to enter the open arms of the elevated plus maze as a function of estrus cycle phase and shock. The ANOVA detected a main effect of shock ($F(1,58) = 5.467$, $P<0.05$). Shocked rats had a longer latency to enter the open arms than did rats that were not shocked.

No significant differences were found in the latency to enter the closed arm, or in the amount of time spent in the closed or open arms.

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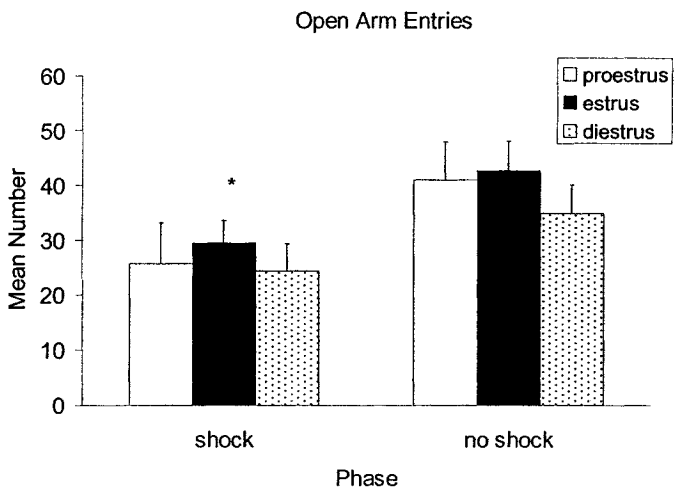

Figure 1. Number of open arm entries on the elevated plus maze as a function of estrus cycle phase and shock. * shock < no shock, $P<0.05$.

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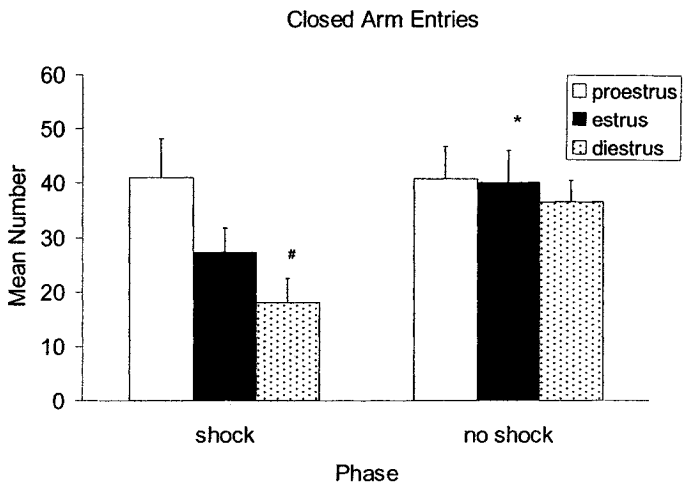

Figure 2. Number of closed arm entries on the elevated plus maze as a function of estrus cycle phase and shock. * shock > no shock, $P<0.05$; # diestrus < proestrus, $P<0.05$.

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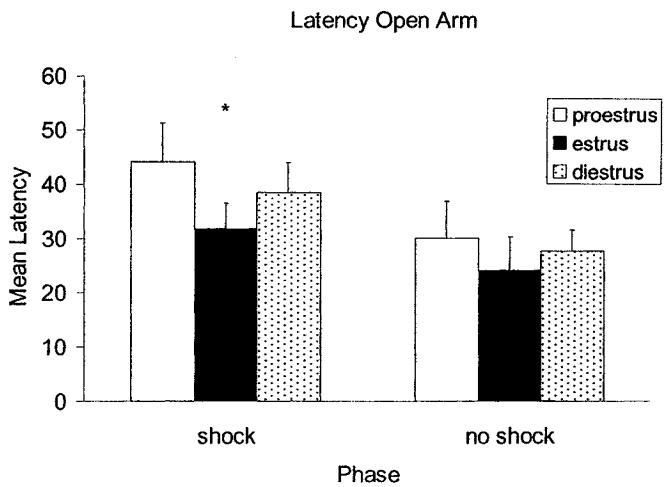

Figure 3. Latency to enter the open arm of the elevated plus maze as a function of estrus cycle phase and shock. * shock > no shock, $P<0.05$.

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3.2. Effect of hormone replacement in OVX rats

Figure 4 shows the number of open arm entries made on the elevated plus maze as a function of hormone condition, and shock in OVX rats. The ANOVA detected a main effect of hormone ($F(3,67) = 5.451$, $P<0.01$). Post-hoc tests revealed that rats treated with EB+P made more open arm entries than those treated with O+P, or O+O.

Figure 5 shows the number of closed arm entries made on the elevated plus maze as a function of hormone condition, and shock. The ANOVA detected a significant interaction between hormone and shock in the number of closed arm entries made ($F(3,67) = 2.819$, $P<0.05$). Post-hoc analyses revealed that rats treated with EB+P and shocked made significantly more closed arm entries than the O+P shocked and the O+O shocked rats.

The ratio of open arm entries was calculated, and no significant differences were found as a function of hormone, shock, or the interaction of these variables.

Figure 6 shows the time spent on the open arms as a function of hormone condition and shock. The ANOVA detected a main effect of hormone ($F(3,67) = 3.941$, $P<0.05$). Post hoc analyses revealed that rats treated with EB+P spent more time on the open arms than rats treated with O+O.

Figure 7 shows the time spent on the closed arms as a function of hormone condition and shock. The ANOVA detected a main effect of hormone ($F(3,67) = 3.464$, $P<0.05$). Rats treated with EB+P spent less time on the closed arms that rats treated with O+O.

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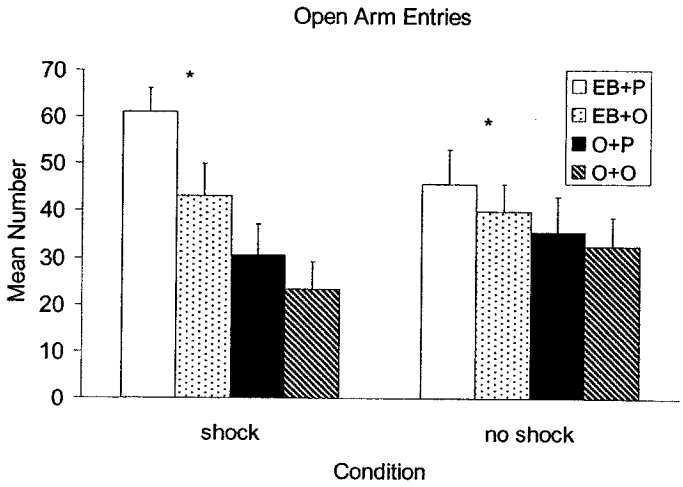

Figure 4. Number of open arm entries on the elevated plus maze as a function of EB, P, and shock. * EB > O, $P<0.05$.

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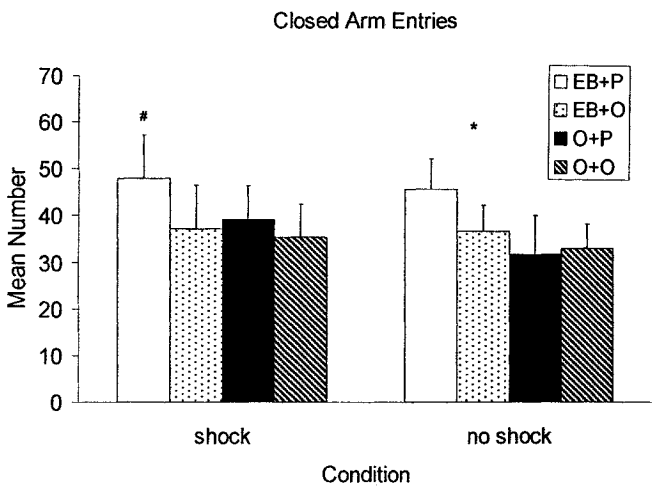

Figure 5. Number of closed arm entries on the elevated plus maze as a function of EB, P, and shock. * EB, shock < O, shock, $P<0.05$, # EB+P, shock > O+P, shock, O+O shock, $P<0.05$.

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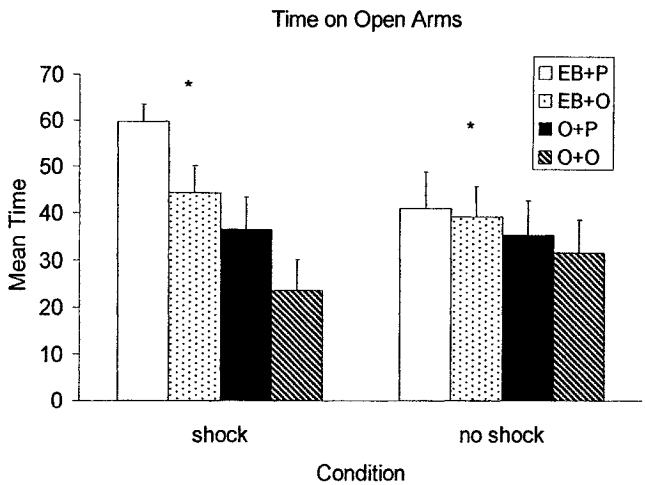

Figure 6. Time spent on the open arms of the elevated plus maze as a function of EB, P, and shock. * EB > O, $P<0.05$.

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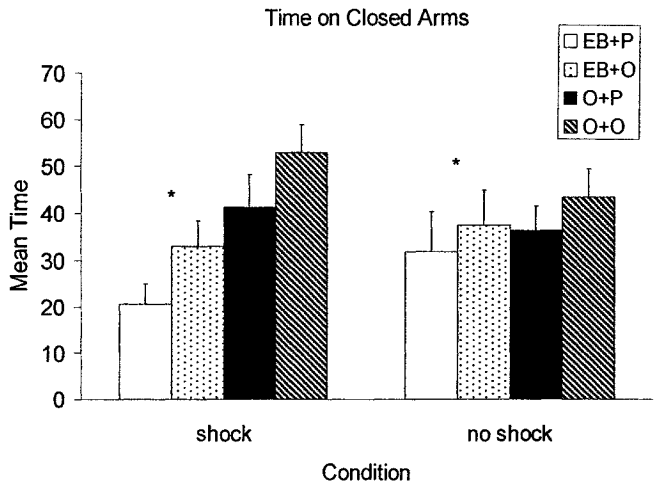

Figure 7. Time spent on the closed arms of the elevated plus maze as a function of EB, P, and shock. * EB < O, $P<0.05$.

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There was no siginicant difference in the latency to enter the open arm of the elevated plus maze as a function of hormone condition and shock, cand no significant differences were found in the latency to enter the closed arms of the elevated plus maze as a function of hormone condition or stress.

Discussion

In the current study, a reduction in anxiety was observed in OVX rats given hormone replacement. Those rats made more open arm and less closed arm entries than O-treated rats, and spent more time on open and less time on closed arms. They also had a shorter latency to enter the open arm than O-treated rats. Among intact animals, pre-treatment with shock was a more important predictor of response, in that rats that were shocked made more closed arm and less open arm entries, and had a longer latency to enter the open arm. The only significant estrus phase difference was that diestrus rats made fewer closed arm entries than proestrus rats. This suggests that actions of circulating E and/or P in the brain reduce anxiety behaviors in response to stress in both intact and OVX, hormone-treated rats. Rats in proestrus and estrus, or those treated with EB+P, are known to locomote more than those with lower circulating levels of these hormones. It is unlikely that this increase in locomotion accounts for the differences in elevated plus maze activity seen in the current study, because the EB+P treated rats made more open but not closed arm entries. Increases in locomotion alone would have been expected to increase entries into both open and closed arms.

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Evidence for a reduction in anxiety as a function of E has previously been shown in rats (Harris & Westbrook, 2001); mice (Osterlund & Hurd, 1998); and post-menopausal women treated with E replacement therapy (e.g. Shors, Lewczyk, Pacynski, Mathew & Pickett, 1998). More than one mechanism has been postulated to underlie E's anxiolytic properties. Hypothalamic-pituitary-adrenal (HPA) axis activity has been linked to anxiety behaviors in rats (e.g. Costanzo, Riccio & Kissinger, 1995; Walf & Frye, 2005), and there is some evidence to suggest that E interacts with the HPA axis to modulate this decrease in anxiety (reviewed in Bodo & Rissman, 2006). In support of this hypothesis, a sex difference in adrenocorticotrophin and glucocorticoid levels has been found, with higher levels in females, which correlates with higher circulating levels of E (Handa, Burgess, Kerr & O-Keefe, 1994). Walf & Frye (2005) demonstrated that EB had anxiolytic effects on behaviors of female rats in the elevated plus maze and forced swim test, and these effects correlated with lower corticosterone levels. It has been suggested that E receptor β in the PVN may modulate the HPA-axis response to stress (Isgor, Cecchi, Kabbaj, Akil & Watson, 2003; Somponpun, Holmes, Seckl & Russell, 2004).

Another possible mechanism for E's anxiolytic effects is that E modifies the serotonergic system (e.g. Bowman, Ferguson & Luine, 1974; Williams & Uphouse, 1989). 5-HT has been shown to modulate anxiety (e.g. Bodo & Rissman, 2006; Graeff, 2002). Osterlund et al. (1998; 2000) demonstrated that EB downregulates 5-HT_{1A} mRNA in the limbic system of female rats, and E has been shown to increase 5-HT receptors in the hypothalamus, preoptic area, and amygdala (Bigeon & McEwan, 1982). A recent study showed an increase in time spent in the open arms of the elevated plus maze, suggesting lower levels of anxiety, in OVX EB-treated rats, and this decrease in anxiety

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also correlated with decreased 5-HT concentrations in the frontal cortex and hippocampus, among other areas (Pandaranandaka, Poonyachoti & Kalandakanond-Thongsong, 2006).

E has been found to increase P receptors (Pfaff & McEwan, 1983), and to increase oxytocin binding sites in the ventromedial hypothalamus (VMH) and central amygdala (CEA; McCarthy, McDonald, Brooks & Goldman, 1996). Oxytocin in the VMH is involved in female sexual behavior but not in anxiety behaviors, whereas the reverse is true for oxytocin in the CEA (Bale, Davis, Auger, Dorsa & McCarthy, 2001). Thus, the anxiolytic effects of E and P may be occurring in the amygdala, a site which is also involved in conditioned fear (Phillips & LeDoux, 1992). EB+P administered subcutaneously or infused directly into the amygdala of OVX rats decreased time spent freezing in a defensive freezing task (Frye & Walf, 2004), suggesting a role for ovarian hormones in the amygdala in anxiety behaviors. E and P have effects on other systems, including GABA. Both of these hormones have been shown to increase GABA syntheis (Weiland, 1992), and increase GABA binding in the basolateral amygdala and dorsolateral septal nucleus (Canonaco, Tavolaro & Maggi, 1993). These hormones also have different effects on GABA, for example, P has been found to regulate GABAA receptor subunits in the hippocampus (Weiland & Orchinik, 1995), and E has been found to increase GABAergic inhibition in the hippocampus (Murphy, Cole, Greenberger & Segal, 1998). Given that the anxiolytic effect of benzodiazepines is thought to involve an augmentation of GABAA receptor binding (Cole & Jones, 1995; Fanselow & Helmstetter, 1988; Harris & Westbrook, 2001; Waddington & Olley, 1977; Westbrook, Greeley, Nabke & Swinbourne, 1991), the modulatory roles of E and P on the

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GABAergic system may underlie the reductions in anxiety behaviors seen in hormone-treated and naturally cycling rats.

The intact rats were influenced more by having been pre-exposed to shock than by their hormonal state, whereas OVX rats seemed to be affected more by hormonal state than pre-exposure to shock. Perhaps the variations in hormone levels within a phase of the estrus cycle (Butcher, Collins & Fugo, 1974; Freeman, 1994) accounts for this difference. Although rats were assumed to be in a particular phase based on the vaginal cells observed, the hormonal milieu, for example, during estrus, changes throughout the phase: Levels of E and P start out high, but are very low by the end of the phase. A possible difference in E levels in gonadally intact rats considered to be in estrus may have caused variability in the results obtained within this group. Alternately, perhaps the effects of exogeneous E and P are greater than the effects of endogenous E and P.

The importance of pre-exposure to shock was shown clearly in the present study, and confirms the importance of context in fear conditioning. Previous research has shown that male rats re-exposed to a chamber where they were previously shocked show increased freezing and passive avoidance (Cole & Jones, 1995; Fanselow & Helmstetter, 1988; Harris & Westbrook, 2001; Melik, Babar-Melik, Ozgunen & Binokay, 2000), but there is a paucity of research investigating the modulation of conditioned freezing by E and P. Similarly, there is a lack of research investigating the effects of ovarian hormones on cued conditioned fear, such as when an animal is re-exposed to a tone or light previously paired with footshock. Given the anxiolytic properties of E and P, it is likely that they modulate many forms of learned associations resulting in conditioned fears. Elucidating the potential modulatory effects of E and P on conditioned fear could have

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clinical implications. For example, does the phase of the menstrual cycle a woman is in affect the development or expression of phobias?

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CHAPTER 3:

HORMONAL STATE DURING CONDITIONED FEAR TRAINING AND TESTING DIFFERENTIALLY ALTERS BEHAVIORAL MEASURES OF ANXIETY IN THE FEMALE RAT

Behavioral differences in stress reactivity exist across the estrus cycle. Relative to rats in estrus and proestrus, rats in diestrus are less active in an open field (Burke & Broadhurst, 1966), exhibit decreased open-arm exploration in the elevated plus maze (Mora, Dussaubat & Diaz-Veliz, 1996), and have impaired acquisition of conditioned avoidance responding (Diaz-Veliz, Urresta, Dussaubat & Mora, 1994; Shors, Lewczyk, Pacynski, Matthews & Pickett, 1998; Sfikakis, Spyraki, Sitara & Varonos, 1978). Frye, Petralia and Rhodes (2000) found that female rats exhibit lower levels of stress reactivity during proestrus and estrus rats relative to females in diestrus or male rats. Together, these data suggest that rats are most reactive to stress during diestrus, when circulating estrogen (E) and progesterone (P) levels are relatively low. Similarly, OVX rats treated with estradiol benzoate (EB) and/or P show more exploration of the open arms in a plus maze (Mora et al., 1996), and a higher response rate in a conflict situation (Roderiguez-Sierra, Howard, Pollard & Hendricks, 1984) than OVX rats not given hormone replacement. Furthermore, EB-treated OVX rats exposed to 21 days of restraint stress also showed more exploration of inner sectors of an open field than untreated OVX rats (Bowman, Ferguson & Luine, 2002). Intact diestrus rats spent less time in the open arms of an elevated plus-maze than rats in proestrus, but this difference was abolished when diestrus rats were given EB (Marcondes, Miguel, Melo & Spadari-Bratfisch, 2001). Together, those data suggest that female rats experience higher stress reactivity in the absence of ovarian hormones.

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Viau and Meaney (1991) investigated the mechanisms underlying behavioral differences in stress reactivity as a function of ovarian hormones. Hormonal responses to restraint stress were enhanced during proestrus, as indicated by higher levels of adrenocorticotrophin (ACTH) and corticosterone (CORT); higher levels of ACTH and CORT were also observed in OVX, EB-treated rats relative to untreated OVX rats, or those treated with EB and P. This finding suggests that the actions of E account for the reduction in stress reactivity. This hypothesis was supported by data from Isgor et al (2003), who demonstrated that rats given infusions of the E receptor (ER) antagonist ICI-182,780 to the PVN had decreased CORT responses following 15 minutes of restraint stress. It was suggested that ERß in the PVN modulates the hypothalamic-pituitary-adrenal (HPA)-axis response to stress because adrenalectomy reduced the expression of ERß but not ERα mRNA in the PVN.

Estrogen may also exert an anxiolytic effect by modifying serotonergic (5-HT) transmission (e.g. Bowman et al., 2002; Williams & Uphouse, 1989). This could occur, for example, by increasing 5-HT receptors in the hypothalamus, preoptic area, and amygdala, where a single dose of EB in OVX rats caused an immediate decrease in 5-HT1 receptors, only to be followed 48-72 hours later with an increase in these receptors (Biegon & McEwan, 1982). Acute administration of EB downregulates 5-HT_{1A} receptor mRNA in the limbic system of female rats (Osterlund & Hurd, 1998), and a reduction in 5-HT_{1A} receptor binding in medial amygdala and hippocampus was found with 2 weeks of chronic EB (Osterlund, Halldin & Hurd, 2000). E also increases P receptors (Pfaff & McEwan, 1983) and oxytocin binding in the ventromedial hypothalamus (VMH) and central amygdala (CEA; McCarthy, McDonald, Brooks & Goldman, 1996). The CEA has

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been implicated in the control of conditioned fear (Koo, Han & Kim, 2004; Pare, Quirk & Ledoux, 2004; Phillips & LeDoux, 1992; Samson & Pare, 2005). Bale et al. (2001) demonstrated that oxytocin receptor expression in the CEA was anxiolytic, and had no effect on female sex behavior, whereas the opposite was true in the VMH, suggesting that the CEA involvement in conditioned fear may be mediated in part by oxytocin binding.

Rats exposed to a chamber where they were previously shocked show increased freezing and passive avoidance (e.g. Cole & Jones, 1995; Feldon & Weiner, 1989), and differences exist in conditioned fear responding as a function of sex. For example, male rats show more freezing in response to both contextual and conditioned stimulus cues that predict foot shock than do female rats (Pryce, Lehmann & Feldon, 1999). Differences have also been detected between male and female rats on a variety of behavioral measures of stress reactivity following inescapable shock. Male rats exposed to the elevated plus maze following inescapable shock showed less arm entries and reared less than did females exposed to the same inescapable shock (Steenbergen, Heinsbroek, van Haaren & van de Poll, 1990). The same sex-related sensitivity following inescapable shock has been observed in shuttle-box performance (Steenbergen, Heinsbroek, van Haaren & van de Poll, 1989), passive avoidance (van Haaren & van de Poll, 1984), and open field behaviour (Heisenbroek, van Haaren & van de Poll, 1988). These data suggest that male rats are more reactive than female rats. However, very few studies have examined conditioned fear across the estrus cycle in female rats. Conditioned fear can enhance morphine-induced analgesia (Stock et al., 2001), and this effect is enhanced during diestrus compared to estrus.

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Taken together, the data showing differences in stress reactivity as a function of estrus cycle phase, along with those indicating differences in conditioned fear between males and females, suggest that differences in conditioned fear in female rats may be observed as a function of ovarian hormone level. The results of the previous chapter show that there are decreases in anxiety behaviors on the elevated plus maze in OVX rats that are treated with EB+P. The goal of the present study was to expand and elucidate these findings, by investigating whether hormonal state, context-specific conditioned fear, or the combined influence of those factors, would alter stress reactivity in female rats. It was hypothesized that rats that were treated with EB+P would have a reduction in anxiety measures, and that this would be augmented in rats that had repeated pairings of EB+P and stress. It was also hypothesized that rats treated with EB+P would have higher levels of contextual conditioned fear.

Materials and methods

Animals and surgery

Female Long-Evans hooded rats (Charles River, St-Constant, QC; 200g-250 g) were housed in groups of two in plastic cages (36 X 26 X 19 cm) in a colony room maintained on a reverse 12:12 hour light/dark cycle (lights off at 08:00), and at a constant temperature of 21°C, and food and water were continuously available. Ovariectomies were performed via bilateral lumbar incisions under ketamine hydrochloride (50 mg / ml) and xylazine hydrochloride (4 mg / ml), mixed at a ratio of 4:3 respectively, and injected

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intraperitoneally in a volume of 1 ml / kg of body weight. Animals were given a week to recover prior to beginning experimental training sessions.

Hormone treatment and training sessions

Rats were randomly assigned to either the hormone or the vehicle training group. For rats in the hormone group, subcutaneous injections of EB (10 μg/0.1 cc of sesame oil) were administered 48 h before each behavioral training or testing session, and P (500 μg/0.1 cc of sesame oil) 4 h before a session. The vehicle group was injected with 0.1 cc of oil 48 h and 4 h before a session. Rats were further randomly assigned to receive either stress or no stress. All sessions took place during the middle third of the dark phase of the light/dark cycle. During each training session, rats were placed in a 30 X 30 Plexiglas box with horizontal metal rods placed 5 cm above the floor. Rats in the shock condition were left in the box for a 5-minute period, following which a series of 11 0.8 mA foot shocks were delivered at 30 s intervals through the metal rods. Rats in the no shock condition were left in the box for 15 minutes. Each rat was subjected to 5 training trials, at 4-day intervals, always under the same hormone and shock condition.

Experiment 1: Effects of hormone condition

Four days after the fifth training trial, rats were given a battery of behavioral tests of stress reactivity. Within each hormonal and stress condition, rats were randomly assigned to the same or different hormone condition on the test day, such that rats trained

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in the EB+P condition were given either EB+P or O+O on the test day, and rats trained in the O+O condition were given either O+O or a priming dose of EB 96 hours prior to testing, followed by EB 48 hours and P 4 hours before the test. There were between 8 and 10 rats per hormone x same or different x stress condition. Four to eight rats were tested each day, and testing took a maximum of 45 minutes for each rat. The order of testing was the same for all rats.

Anticipation: Rats were placed in the shock chamber for 5 minutes prior to foot shock during each training day. On the test day, rats were placed in the same chamber for a 5 minute period. The time that the rat spent freezing was measured on training day 1 and on the test day.

Open field: The open field test was conducted in a Plexiglas enclosure (60 X 60 X 60 cm) with an elevated metal grid floor. The floor of the apparatus was divided into 10 peripheral and 2 central squares. Rats were placed in the box for 5 minutes, and the number of peripheral and central crossings was recorded, as well as the proportion of crossings that were central.

Elevated-plus maze: The elevated-plus maze was a dark Plexiglas T-maze, elevated 70 cm off the ground, and comprised of two open arms (10 X 50 X 1cm) facing each other, two closed arms opposite from each other (10 X 50 X 20 cm), and a central platform (10 X 10 cm; Pellow & File, 1986). Rats were placed on the central platform of the maze facing the point where the open and closed arms meet. The number of closed and open arm entries, latency to enter the closed and open arms, and time spent on the open and closed arms was recorded over a 5 minute period.

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Social interaction: The chamber used for the social interaction test (File & Hyde, 1978) was identical to that used for the open field test. Each rat was placed at one end of the box, and an ovariectomized non-hormone treated rat was placed at the opposite end. The time the animals spent in interaction over a 10-minute period was recorded. Interactions included any form of contact between the 2 animals, such as sniffing, mounting, biting, and grooming.

Defensive burying: The defensive burying test (Gallo & Smith, 1993) was conducted in a Plexiglas box (40 X 40 X 30 cm), the floor of which was covered in 3 cm of the same commercial bedding material covering the floor of the rats' home cages. A 5cm shock-probe designed to deliver shock upon contact protruded from the back wall of the apparatus 2 cm above the bedding material. The rat was placed in the box, and received a 1.6mA shock each time she made contact with the probe throughout a 15 minute period. Measures obtained were the latency to contact the probe, the number of contact-induced shocks, the latency to begin burying following the first shock, the duration of burying, and the amount of time spent immobile following the first shock.

Experiment 2: Effect of conditioned fear

OVX rats were randomly assigned to one of the two hormonal conditions, EB+P or O+O, and hormones were administered as described above. All rats were trained with 5 minutes in the anticipation chamber, followed by 10 minutes of shock, as described above. On the test day, rats were given the same hormonal regimen as they were given on the training days. Within each hormonal condition, half of the rats were randomly

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assigned to expectancy or no expectancy condition. Rats in the expectancy condition were tested as described above. This also served as a replication of Experiment 1. Rats in the no expectancy condition were not exposed to the shock chamber on the test day. They were removed from their home cages, and behavioral testing began immediately with the open field. There were between 8 and 11 rats in each of the hormone x stress x anticipation conditions.

Statistical analyses

For each experiment, data were ranked-ordered to reduce variance (Conover, 1999) and two- or three-way between-groups analyses of variance (ANOVAs) were conducted for the ranks on each measure using SPSS (SPSS, Inc., Chicago, IL). Only significant results are reported. For each significant effect with more than two means, Fisher LSD posthoc tests were conducted on the individual means. Differences were considered significant at $P<.05$.

Results

Experiment 1: Effects of hormonal condition

Anticipatory freezing

As illustrated in Figure 1, a main effect was of shock was found for the amount of time spent freezing ($F(1,66) = 226.982$, $P < .001$), where rats that were shocked during the training period spent significantly more time freezing than did rats trained without

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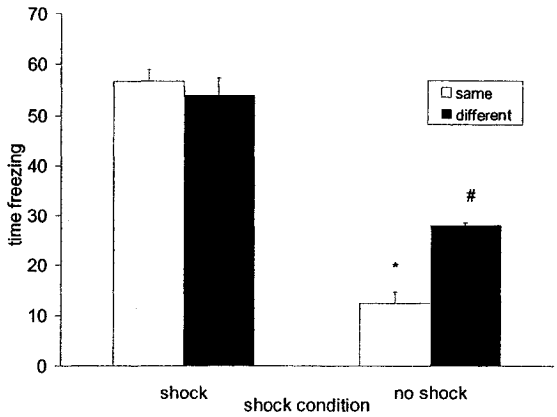

Figure 1. Time spent freezing during the 5 minute anticipatory period on the test day as a function of shock condition, and whether the hormone condition was the same or different from during the training phase (ranked scores). * No shock, same < all other groups; # no shock, different < shock, same and shock, different, $p < .05$.

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shock. A main effect of same or different hormonal state between training and testing was also detected ($F(1,66) = 761.922$, $P < .01$). Rats that were trained and tested in the same hormonal state froze less than rats that were trained and tested in different hormonal states. A significant 2-way interaction between stress and whether the hormone treatment was the same or different during training and testing was found for the amount of time spent freezing during the 5 minute anticipatory period on the test day ($F(1,66) = 15.804$, $P < 0.001$). Post hoc analyses revealed that rats that were not stressed and trained in the same hormone condition as that in which they were tested froze for longer than all other groups, and that rats that were trained and tested in different hormone states and not stressed froze for less time than stressed rats tested in the same and different hormone states from training.

Open field

As illustrated in Figure 2a, a main effect of hormone during testing was observed ($F(1,67) = 32.386$, $P < .001$), with animals tested with EB+P making more central crossings than animals tested with O+O. A significant 2-way interaction was found in the number of central crossings made in the open field test as a function of hormone on the test day and whether the hormone condition was the same or different from that during training ($F(1,67) = 10.626$, $P < .01$). Post-hoc tests revealed that the differences lay between animals trained and tested with EB+P and those trained with EB+P and tested with O+O; those trained and tested with EB+P and those trained with O+O and tested with EB+P; and those trained and tested with EB+P and those trained and tested with O+O.

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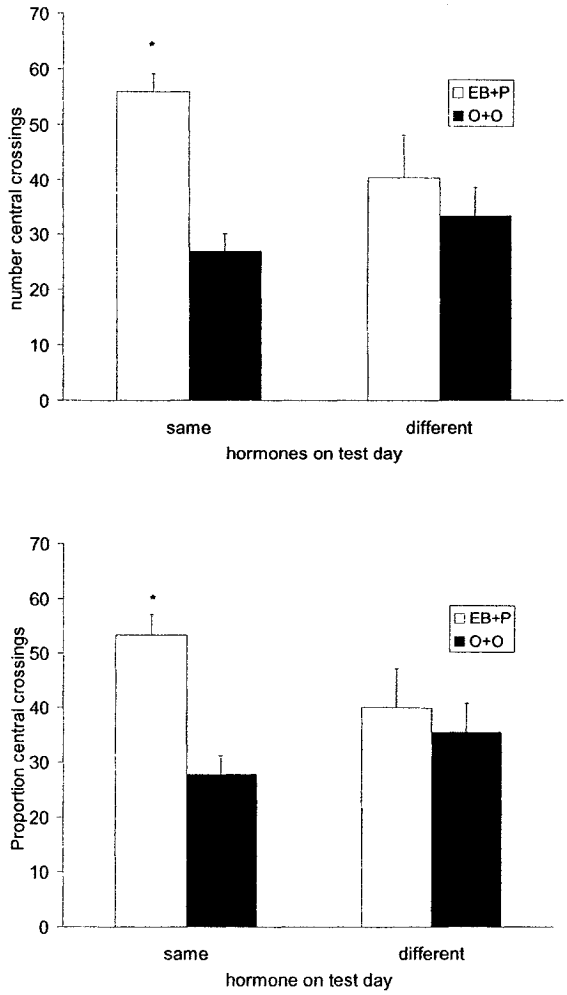

Figure 2. Open field test data (ranked scores). 2a. (top) number of central crossings as a function of hormone condition on the test day, and whether the hormone condition was the same or different from the training days. * EB+P, same > O+O, different; EB+P, different; O+O, same, $p < .05$. 2b. (bottom) Proportion of crossings that were central as a function of hormone condition on the test day, and whether the hormone condition was the same or different from the training phase. * E+P same > O+O same, O+O different, $p < .05$.

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A main effect of hormone was observed for the number of peripheral crossings. The number was higher in rats tested with EB+P (M = 47.203, SD = 19.174) than rats tested with O+O (M = 29.039, SD = 20.599, $F(1,67) = 14.857$, $P < .001$).

The proportion of crossings that were central was recorded by dividing the number of central crossings by the total number of crossings. As illustrated in Figure 2b, a significant interaction was found between the hormonal state on the test day and whether the hormonal condition was the same during training and testing or different ($F(1,67) = 8.184$, $P < .01$). Post-hoc tests revealed that rats that were trained and tested in the presence of EB+P had a significantly higher proportion of central crossings than those that were trained and tested in the presence of O+O. Post-hoc tests also revealed that rats that were trained and tested with EB+P had a higher proportion of central crossings than rats trained with EB+P and tested with O+O.

Elevated plus maze

As illustrated in Figure 3a, there was a main effect of shock on latency to enter the open arm ($F(1,67) = 8.177$, $P < .01$), where shocked animals had a larger latency than did non-shocked animals. There was a significant interaction between shock and whether the hormonal state was the same or different during training and testing on the latency to enter the open arm ($F(1,67) = 7.697$, $P < .01$). Post-hoc tests revealed that rats that were tested in different a hormone condition than training and shocked had a higher latency to enter the open arm than did rats trained and tested in a different hormone condition and not shocked.

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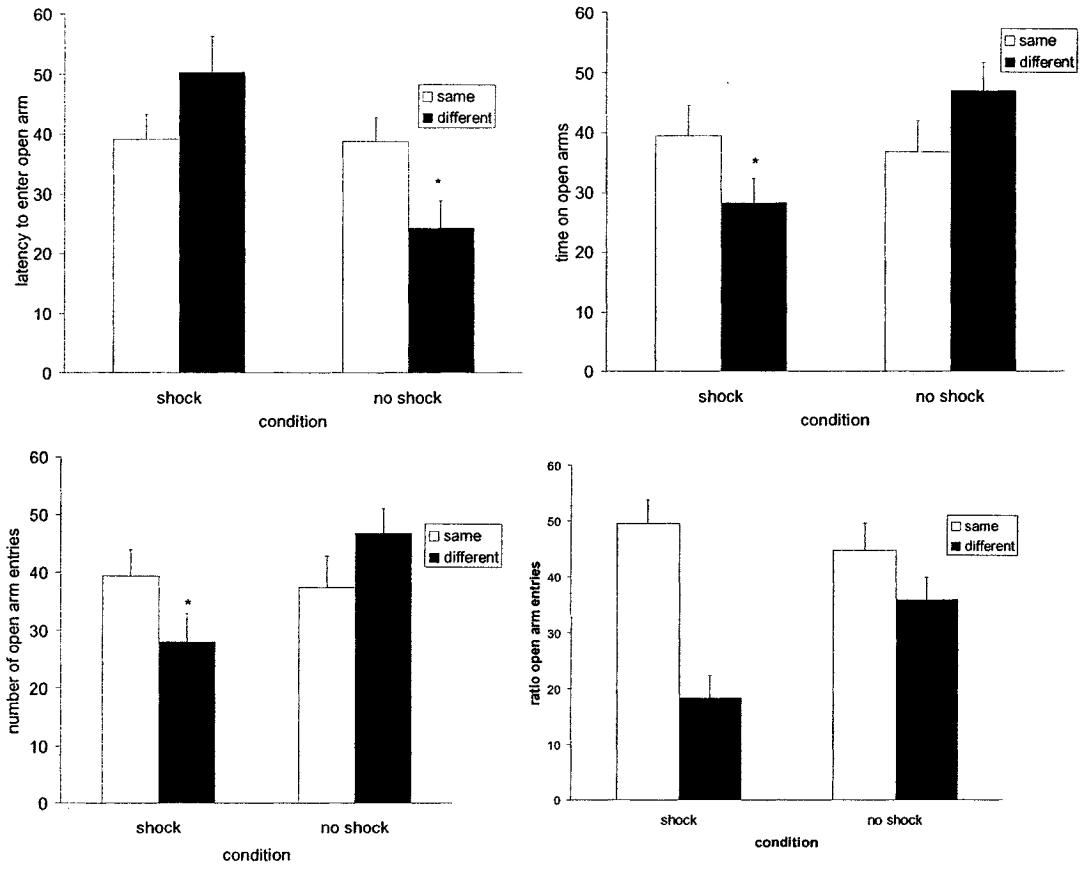

Figure 3. Elevated Plus Maze (ranked scores). 3a. (top left) latency to enter the open arm as a function of shock, and sameor different hormone condition during training and testing. * shock, different > no shock, different, p< .05. 3b. (top right) time spent on the open arms as a function of shock, and hormone same or different. * shock, different < no shock, different, p< .05. 3c. (bottom left) number of open arm entries as a function of shock, and hormone same or different. * shock, different < no shock, different, p< .05. 3d. (bottom right) ration of open arm entries as a function of shock and same or different. * shock, different < shock, same; no shock, different; no shock, same, p< .05.

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The ANOVA detected a significant main effect of hormones on test day ($F(1,67) = 6.787$, $P < .01$), where rats tested in EB+P spent more time on the open arm than did rats tested without hormones. As illustrated in Figure 3b, there was a significant interaction between shock and whether the hormonal state was the same or different during training and testing on the time spent on open arms $F(1,67) = 5.874$, $P < .05$). Post-hoc tests revealed that shocked rats that were tested in the opposite hormone condition from training spent less time on the open arms than non-shocked rats trained and tested in the opposite hormone condition.

The ANOVA detected a significant main effect of hormonal state on test day for time spent in the closed arms of the elevated plus maze ($F(1,67) = 9.964$, $P < .01$). Rats tested without hormones (M = 30.514, SD = 18.500) spent more time in the closed arms than did rats treated with EB+P on the test day (M = 45.289, SD = 22.502). As illustrated in Figure 3c, the ANOVA detected a significant interaction between shock and whether the hormone conditions during training and testing were the same or different on the number of open arm entries ($F(1,67) = 4.662$, $P < .05$). Fisher's LSD revealed that shocked rats that were trained and tested in different hormone conditions made less open arm entries than did non-shocked rats trained and tested in different hormone conditions. Figure 3d illustrates the ratio of arm entries. The ANOVA detected a significant interaction between same and different and shock ($F(1,67) = 6.936$, $P < .01$). Post hoc tests revealed that rats that were tested in the different, no shock group had a significantly lower ratio of open arm entries than did rats in the other groups. A main effect of same or different was also found ($F(1,67) = 20.456$, $P < .001$), rats that were not shocked had a higher ratio of open arm entries than those that were shocked.

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Social interaction

As can be seen in Figure 4, the ANOVA detected a significant interaction between hormone on the test day and whether hormone condition was the same as that during training ($F(1,67) = 13.169$, $P<.001$). Post-hoc tests revealed that rats trained and tested in EB+P spent more time interacting than rats that were trained in O+O and tested in EB+P. Rats trained in EB+P and tested in O+O spent more time interacting than rats trained in O+O and tested in EB+P.

Defensive burying

The ANOVA detected a main effect of hormone during testing in the number of contact-induced shocks ($F(1,62) = 6.778$, $P<.05$). Rats treated with EB+P (M = 41.757, SD = 19.436) made more contacts overall than rats treated with O+O (M = 28.485, SD = 18.902).

As illustrated in Figure 5a, a significant interaction was found in the latency to begin burying the probe as a function of hormone condition on the test day and whether this hormone condition was the same or different from that during training. ($F(1,57) = 4.190$, $P < .05$). Fisher LSD revealed that rats trained and tested in O+O had a shorter latency to begin to bury the probe than did rats trained in E+P and tested in O+O.

As illustrated in Figure 5b, a significant interaction was detected between the hormone condition on the test day and shock in the amount of time spent burying after the first shock ($F(1,60) = 19.052$, $P < .001$). O+O, shocked rats spent less time burying than EB+P, shocked rats, or O+O, non-shocked rats, and EB+P, non-shocked rats spent more time burying than O+O, non-shocked rats.

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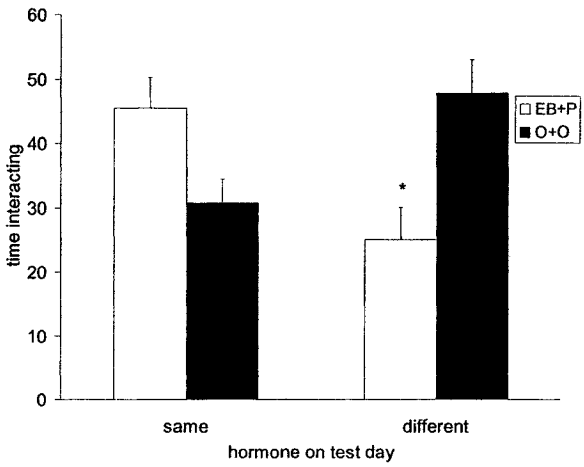

Figure 4. Time spent interacting on the Social Interaction test as a function of hormone condition, and whether it was the same or different during training and testing (ranked scores). * EB+P, different < EB+P, same and O+O, different, $p < .05$.

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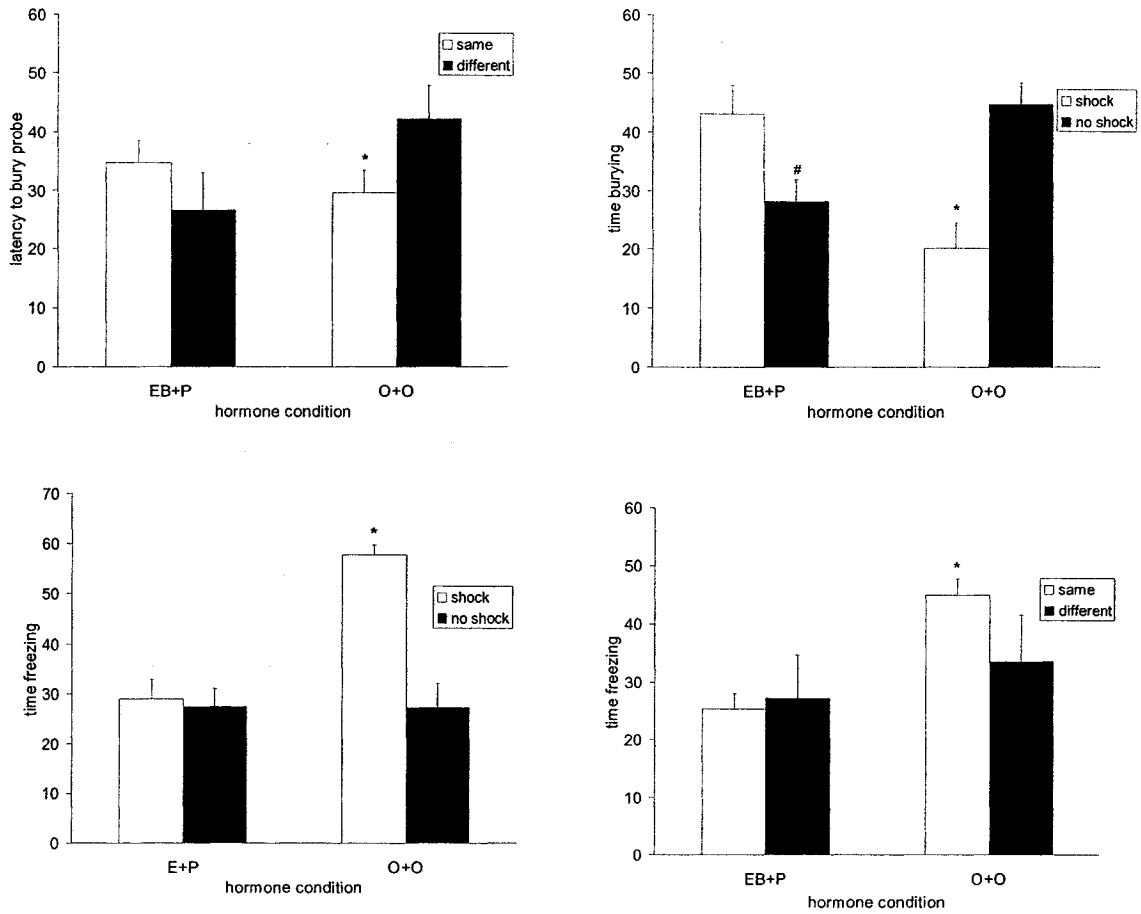

Figure 5. Defensive Burying test (ranked scores). 5a. (top left) Latency to bury the probe as a function of hormone condition on the test day, and whether the hormone condition was the same during training and testing. * O+O, same < O+O, different, $p < .05$. 5b. (top right) Time spent burying as a function of stress and hormone condition on the test day. * O+O shock < E+P, shock; O+O no shock, $p < .05$ # O+O no shock > E+P no shock, $p < .05$. 5c. (bottom left) Time freezing following the first contact-induced shock as a function of hormone condition on the test day and stress or no stress during training. *

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O+O shock > O+O no shock; E+P shock; E+P no shock, $p < .05$. 5d. (bottom right) Time freezing following the first contact-induced shock as a function of hormone condition on the test day and shock. * O+O, same > E+P same; E+P different, $p < .05$.

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As illustrated in Figure 5c, there were differences in the amount of time spent immobile following the first contact-induced shock as a function of hormone and shock. The ANOVA detected a main effect of hormones on the test day ($F(1,60) = 12.096$, $P < .001$), where rats tested with O+O froze for more time than did rats tested with EB+P. A main effect of shock was also detected ($F(1,60) = 25.481$, $P < .001$), where shocked rats spent more time freezing than non-shocked rats. The ANOVA also detected a significant interaction between hormone during testing and shock ($F(1,60) = 22.849$, $P < .001$). O+O shocked rats froze more than EB+P, shocked rats; EB+P, non-shocked rats; and O+O, non-shocked rats. As seen in Figure 5d, a significant interaction between hormone condition on the test day and whether the hormone condition during training and testing were the same was also detected ($F(1,60) = 9.013$, $P < .01$). Rats trained and tested in O+O spent less time freezing than those trained in O+O and tested in EB+P; or those trained and tested in EB+P. A significant 3-way interaction ($F(1,60) = 9.648$, $P < .01$) was also found. Non-shocked rats that were trained in EB+P and tested in O+O spent less time immobile than rats trained and tested with O+O and shocked; trained with EB+P and tested with O+O and shocked, or trained and tested with O+O and not shocked. Rats trained and tested with EB+P and shocked were immobile for less time than those trained and tested with O+O and shocked.

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Experiment 2: Effect of conditioned fear

Open field

A two-way between groups ANOVA was performed on the variable of latency to enter the central portion of the open field. This measure was obtained only for those rats that were not exposed to the shock chamber on the test day. There were no differences found in these rats as a function of shock or hormone condition.

Elevated plus maze

A significant main effect of hormone was also discovered for the amount of time spent on open arms ($F(1,69) = 4.631$, $P < .05$). EB+P rats (M = 44.110, SD = 23.884) spent more time on the open arms than did O+O rats (M = 33.181, SD = 22.370). As illustrated in Figure 6a, a significant 2-way interaction was discovered for the amount of time spent on open arms as a function of shock and anticipation ($F(1,69) = 4.620$, $P < .05$). Post hoc tests revealed that no shock, no anticipation rats spent more time on the open arms than did no shock, anticipation rats.

As illustrated in Figure 6b, a significant 2-way interaction was found for the amount of time spent in the closed arms as a function of shock and anticipation ($F(1,70) = 7.203$, $P < .01$). Post hoc analyses revealed that the no shock, anticipation rats spent more time in closed arms than the no shock, no anticipation rats.

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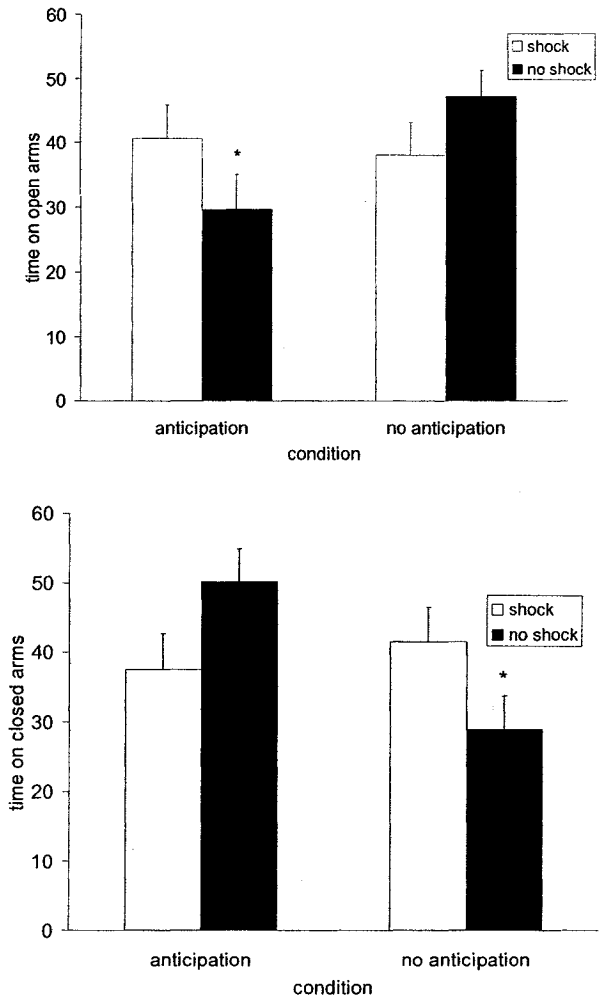

Figure 6. Elevated Plus Maze (ranked scores). 6a. (top) Time spent on the open arms as a function of anticipation and shock. * No shock, anticipation < no shock, no anticipation, $p < .05$. 5b. (bottom) Time spent on the closed arms as a function of anticipation and shock * no shock, anticipation > no shock, no anticipation, $p < .05$.

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Social interaction

The ANOVA detected a significant 3-way interaction of hormone, shock, and anticipation on the amount of time spent involved in social interaction ($F(1,70) = 4.667$, $P < .05$). Post-hoc tests revealed that the significant difference lay between the EB+P treated rats that were shocked and exposed to anticipation (M = 54.250, SD = 22.168), and the EB+P shocked, and non-anticipation rats (M = 20.450, SD = 19.799). Significant 2-way interactions were also found. Figure 7a illustrates the interaction between hormone and anticipation ($F(7.863$, $P < .01$). Rats treated with EB+P and anticipation interacted for more time than did rats treated with EB+P and not given anticipation. Figure 7b illustrates a second 2-way interaction, between shock and anticipation ($F(1,70) = 4.002$, $P < .05$). No shock, no anticipation rats spent more time interacting than no shock, anticipation rats.

Defensive burying

The ANOVA detected a significant main effect of hormone in the number of contact-induced shocks ($F(1,70) = 8.441$, $P < .01$). O+O rats (M = 46.297, SD = 21.840) made significantly more contact-induced shocks than EB+P rats (M = 33.366, SD = 21.857).

As illustrated in Figure 8a, the ANOVA detected a significant interaction between hormones and anticipation in the amount of time spent immobile following the first contact-induced shock ($F(1,70) = 4.507$, $P < .05$). Post-hoc tests revealed that the EB+P, no anticipation rats froze for longer than did the EB+P, anticipation and the O+O, anticipation rats.

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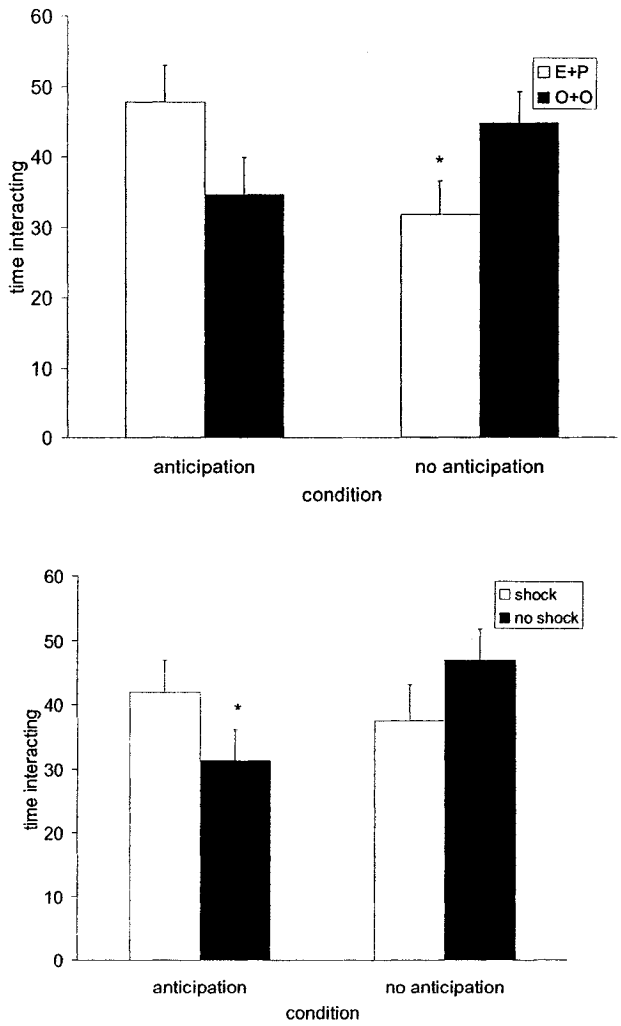

Figure 7. Social interaction (ranked scores). 7a. (top) Time spent interacting as a function of anticipation and hormone condition on test day. * E+P, no anticipation < E+P, anticipation, $p < .05$. 7b. (bottom) Time spent interacting as a function of shock and anticipation. * No shock, anticipation < no shock, no anticipation, $p < .05$.

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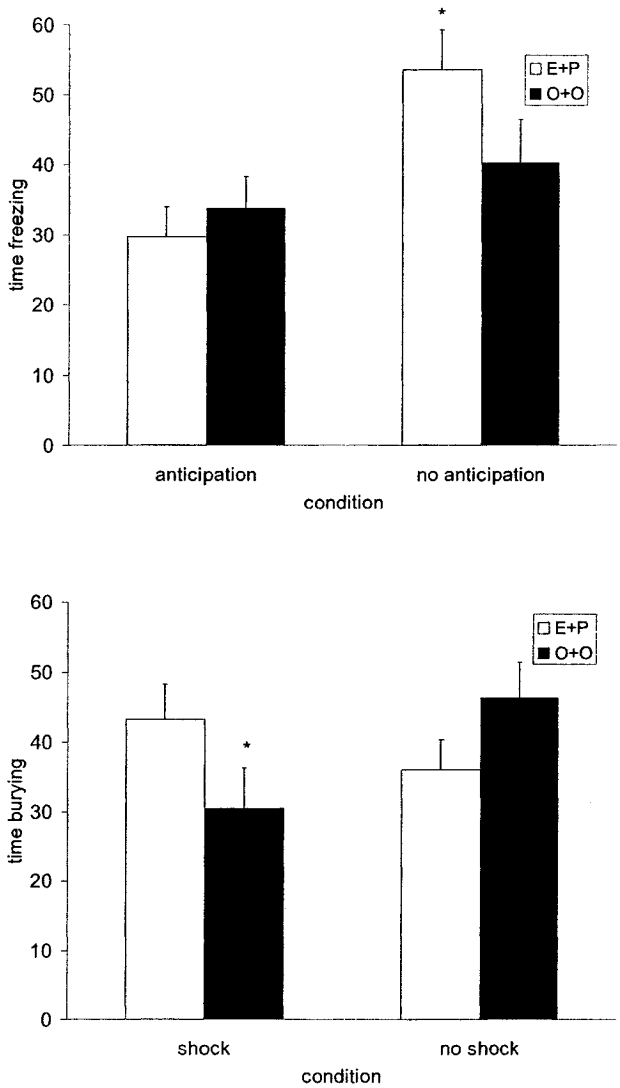

Figure 8. Defensive burying (ranked scores). 8a. (top) Time spent freezing after the first contact-induced shock as a function of hormone condition on the test day and anticipation. * E+P, no anticipation > E+P, anticipation; O+O, anticipation, $p < .05$. 7b. (bottom) Time spent burying the probe after the first shock as a function of hormone on the test day and shock. * O+O, shock < O+O, no shock, $p < .05$.

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A significant 3-way interaction was found on the amount of time spent burying the probe after the first contact-induced shock ($F(1,70) = 4.718$, $P < .05$). Post-hoc analyses revealed that O+O, shock, anticipation rats buried for less time than E+P, shock, anticipation and O+O, no shock, anticipation rats. As seen in Figure 8b, a 2-way interaction was detected between hormone and shock ($F(1,70) = 5.046$, $P < .05$). Post-hoc analyses revealed that O+O shock rats buried for less time than O+O, no shock rats.

Discussion

The present study examined the relative importance of hormonal state and anticipation of inescapable foot shock in anxiety reactivity in the female rat. Overall, the results indicate that rats that were tested under the influence of EB+P were less reactive to stress than rats that were tested in the absence of these hormones. This was observed as rats tested with EB+P having a higher proportion of central crossings in the open field than rats tested without these hormones, spending more time in the open arm of the elevated plus maze, and making more contacts with the probe in the defensive burying test. These findings suggestive of higher anxiety in the absence of EB+P is consistent with previous reports showing increased stress reactivity in rats tested in diestrus relative to those tested in proestrus or estrus (e.g. Burke & Broadhurst, 1966; Diaz-Veliz et al., 1994; Mora et al., 1996; Sfikakis et al., 1978; Shors et al., 1998), and in OVX rats relative to those treated with EB+P (e.g. Bowman et al., 2002; Mora et al, 1996; Roderiguez-Sierra et al., 1984; Stock et al., 2001).

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There is some evidence to suggest that placement of the EB+P rats in the chamber where they were previously shocked served to decrease anxiety relative to rats treated with EB+P and not exposed to the shock chamber. In the social interaction test, rats tested with EB+P and exposed to the shock chamber on the test day interacted for more time than did rats tested with EB+P. These rats also froze for a shorter period of time following shock in the defensive burying test than rats exposed to the shock chamber on the test day. This difference in reactivity as a function of pre-exposure to the shock chamber was not found in O+O treated rats. Perhaps the EB+P rats that were expecting shock showed extinction after only one trial. Previous research indicated that OVX rats treated with EB showed extinction more quickly in a fear-potentiated startle task than OVX rats not treated with hormones (Hiroi & Neumaier, 2006).

Rats did not react with more anxiety if they were trained and tested in the same or a different hormonal state, suggesting that rats may not have discriminated between hormonal states. Discrimination has been found in female rats as a function of ovarian hormones only when the levels of EB and/or P are higher than naturally occurring levels (Ebner, Richardson & Riccio, 1981; Gorzalka, Wilkie & Hanson, 1995; Stewart, Krebs & Kaczender, 1967), or when a task that is more sensitive, such as conditioned flavor aversion, is used (Costanzo, Riccio & Kissinger, 1995). Perhaps a higher dose of ovarian hormones in the present study would have allowed a difference as a function of hormonal state during training to be observed.

Overall, hormone condition on test day was the most significant factor affecting anxiety reactivity, and rats tested in the absence of hormones were more anxious than those tested in the presence of hormones. This pattern of increased anxiety in the OVX

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rats relative to the hormone-treated rats is similar to the results reported by Frye et al. (2000) for rats in diestrus versus proestrus. Not all measures resulted in increased anxiety for non-hormone treated rats, however, which is also consistent with past research (e.g Frye et al., 2000; Ramos, Berton, Mormede & Chaouloff, 1997; Ramos & Mormede, 1998), and may indicate that some tests are more sensitive than others in detecting differences in anxiety as a function of training and hormonal state. Perhaps tests such as the black and white box (Chaouloff, 1994), and the emergence test (Frye et al., 2000) would reflect differences in anxiety as a function of hormonal state. It is also possible that resulting differences in anxiety would be more robust if testing conditions were altered to enhance the salience of the cues predicting the footshock. Phillips and LeDoux (1992) demonstrated that rats trained to expect footshock when a cue, such as a tone, was presented had stronger conditioning that was more resistant to extinction than rats trained to expect footshock in the presence of contextual cues. Similarly, perhaps animals in our paradigm would show stronger conditioning if a tone was paired with the shock. The relative importance of context may also vary as a function of hormonal status. Sava & Markus (2005) demonstrated differences in the water maze task as a function of estrus phase, where estrus rats found the platform more quickly than did proestrus rats. The researchers suggest that when E is high, rats tend to use more extramaze cues, essentially focusing on many cues simultaneously rather than using a few specific cues, making it more difficult for them to find the hidden platform.

There are many possible explanations for how EB and P may be acting to decrease anxiety. Treatment with EB increases the number of binding sites for ^{3}H-muscimol, ^{3}H-diazepam, and ^{35}S-t-butylbicylophosphorothionate on the GABA-A

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receptor (Perez, Zucchi & Maggi, 1988), and the anxiolytic actions of allopregnanolone on the GABA-A receptor are estrogen-dependent (Laconi, Casteller, Gariulo, Bregonzio & Carbrera, 2001). Marcondes et al. (2001) demonstrated that treating diestrus rats with EB resulted in a reduction of anxiety to the same level as proestrus rats, suggesting that the differences in anxiety across the estrus cycle are modulated by estradiol. Sfikakis et al. (2002) also showed decreased serotonergic activity in the hippocampus and hypothalamus-preoptic area in chronically-stressed proestrous rats relative to chronically stressed diestrous rats. These authors suggested that the decrease may be linked to the increase in 5-HT transporter (SERT) binding sites in the forebrain as a result of increased EB priming.

Binding to ER also produces an increase in P receptors (Pfaff & McEwan, 1983), and 5-HT receptors in the hypothalamus, preoptic area, and amygdala (Biegon & McEwan, 1982), effects that may mediate E's anxiolytic properties. Progestins have been shown to increase GABA binding (Canonaco, Tavolaro & Maggi, 1993), and evidence suggests that progesterone exerts its anxiolytic effects through actions of its neurosteroid allopregnanolone at GABA-A receptors (e.g. Bitran, Shiekh & McLeod, 1995; Bitran & Dowd, 1996; Frye & Duncan, 1994; Picazo & Fernandez-Guasti, 1995; Wilson, 1992). Frye & Walf (2004) demonstrated that EB or P administered to the amygdala reduced anxiety in OVX rats. Moreover, recent research suggests that the metabolism of P to 3α,5α-THP in the amygdala is necessary for the anxiolytic effects of P to occur (Walf, Sumida & Frye, 2005). These data may provide some indication of how the CEA is involved in the control of conditioned fear (Koo, Han & Kim, 2004; Pare, Quirk & Ledoux, 2004; Phillips & LeDoux, 1992; Samson & Pare, 2005). The current findings

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suggest that animals treated with EB+P show a reduction in anticipatory freezing, as well as other measures of anxiety, providing some support for a role of these hormones in the expression of conditioned fear.

Although Stock et al. (2001) showed that conditioned fear and morphine-induced analgesia are enhanced during diestrus, no researchers have specifically investigated the role of hormones in conditioned fear. The results of the present study support previous findings that absence or low levels of ovarian hormones are associated with increased anxiety (e.g. Bowman et al., 2002; Frye et al., 2000; Mora et al., 1996), and extend these findings to suggest that the differences in anxiety as a function of hormones can be signaled by internal, hormonal state cues, as well as external, contextual cues. It is possible that this signaling occurs in the amygdala, a site associated with conditioned fear and that has a binds estrogen (Pfaff & Keiner, 1973).

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CHAPTER 4:

DIFFERENTIAL EFFECTS OF DIAZEPAM AND FLUOXETINE ON BEHAVIORAL MEASURES OF ANXIETY IN FEMALE RATS

Measures of anxiety in the rat, such as freezing (e.g. Blanchard and Blanchard, 1969; Bolles and Grossen, 1970), can be displayed conditionally when rats are placed in a chamber where they previously received inescapable footshock (e.g. Cole and Jones, 1995; Fanselow, 1980; Feldon and Weiner, 1989). The association between a particular environment (conditioned stimulus, or CS) and an aversive event (unconditioned stimulus, or US) is termed contextual fear conditioning, and the conditioned fear paradigm has been used as a model for the acquired fear seen in many anxiety disorders in humans (e.g. Davis, Myers, Chahatwal & Ressler, 2006; Izquierdo, Cammarota, Vianna & Bevilaqua, 2004). Evidence suggests that contextual conditioned fear involves neural systems within the basolateral (BLA) and central amygdala (Akirav, Raizel & Maroun, 2006; Keeley et al., 2006; LeDoux, 2000), and the dorsal hippocampus (e.g. Blanchard et al., 1970; Frankland et al., 1998; Maren et al., 1997). Afferents from the hippocampus to the BLA are thought to be required for contextual fear conditioning (Philips & LeDoux, 1992). Damage to the dorsal and/or ventral hippocampus impairs contextual fear conditioning but not auditory fear conditioning (Kim & Fanselow, 1992; Maren, Aharonov & Fanselow, 1997; Philips & Ledoux, 1994; Richmond et al., 1999), suggesting that the hippocampus is necessary for the context to be stored in, and later retrieved from, memory. Lesions to the amygdala impair both acquisition and expression of contextual freezing (e.g. Blanchard & Blanchard, 1972; Calandreau, Desmedt, Decorte & Jaffard, 2005; Cousens & Otto, 1998), and it is believed that the amgydala is more

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liberally involved in the CS-US association and memory consolidation of emotional experiences (Kim & Jung, 2006; Malin & McGaugh, 2005).

Selective serotonin reuptake inhibitors (SSRIs) are often the treatment of choice for anxiety disorders in humans (e.g. den Boer, Westenberg, Kamerbeek, Verhoeven & Kahn, 1987). The effects of these drugs on both the acquisition and testing phases of conditioned fear in rodents have been investigated. Hashimoto, Inoue & Koyama (1996) exposed rats to a shock chamber where they received a series of inescapable footshock for 5 minutes. Twenty-four hours later, the rats were exposed to the same chamber 30 minutes after an injection of citalopram, a SSRI. Freezing in the chamber was significantly reduced in those rats that had been injected with citalopram compared to those injected with saline. Using the same paradigm, Inoue et al. (1996) treated male rats with citalopram during the acquisition phase of conditioned fear, and 24 hours later exposed the rats to the shock chamber. The rats that had been given citalopram during the acquisition phase showed a reduction in freezing relative to those that were not given the SSRIs during acquisition. Burghardt et al. (2004) examined the effects of acute (day of shock training) and chronic (daily for 22 days prior to shock training) injections of citalopram on the acquisition of auditory fear conditioning, and found that when rats were exposed to the tone in the chamber on the test day, 24 hours after the training period, freezing was increased in rats treated acutely, and decreased in those treated chronically with citalopram. The difference between the findings of Inoue et al. (1996) and Burghardt et al. (2004) may reflect differences between contextual and auditory fear conditioning. As suggested by the authors, contextual conditioning involves the processing of complex sensory information, requiring the amygdala and the hippocampus, whereas auditory

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conditioning involves processing of more discrete sensory information, which requires the amygdala and not the hippocampus (Kim & Fanselow, 1992).

Conditioned fear is also altered by benzodiazepines, anxiolytics that exert their effects through augmentation of $GABA_A$ receptor binding (e.g. Cole & Jones, 1995; Fanselow & Helstetter, 1988; Harris & Westbrook, 2001; Inoue et al., 1996; Waddington & Olley, 1977; Westbrook, Greeley, Nabke & Swinbourne, 1991). For example, Fanselow & Helstetter (1988) injected rats with midazolam during either the acquisition or testing phase of conditioned fear, and found that freezing during the testing phase was reduced in rats injected with midazolam relative to those injected with saline, regardless of whether the midazolam was injected during acquisition or testing. Westbrook et al. (1991) injected male rats with midazolam during the acquisition phase of a task where rats were exposed to a hotplate. On the test day, rats that had been injected with midazolam took longer to step down from the hotplate than those that had been injected with saline, suggesting an anxiolytic effect of midazolam during acquisition.

A sex difference has been found in conditioned fear, in that male rats show more conditioned freezing than do intact female rats (Kosten, Miserendino, Bombace, Lee & Kim, 2005; Maren et al., 1994). In OVX rats, treatment with estradiol benzoate (EB) reduces contextual fear conditioning, as measured by freezing during re-exposure to the shock chamber (Altemus, Conrad, Dolan & McEwen, 1998; Gupta, Sen, Diepenhorst, Rudick & Maren, 2001). It has been shown, using measures of activity such as the open field test and the elevated plus maze, and conditioned avoidance, that anxiety is decreased during proestrus and estrus relative to diestrus (Burke & Broadhurst, 1966; Diaz-Veliz, Urresta, Dussaubat & Mora, 1994; Frye, Petralia and Rhodes, 2000; Mora,

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Dussaubat & Diaz-Veliz, 1996; Shors, Lewczyk, Pacynski, Matthews & Pickett, 1998; Sfikakis, Spyraki, Sitara & Varonos, 1978; Zuluaga et al., 2005). Late proestrus and estrus are characterized by high levels of estrogen (E) and progesterone (P; Freeman, 1994). In OVX rats anxiety on a variety of behavioral measures is decreased in when EB and P are administered (e.g. Bowman, Ferguson & Luine, 2002; Marcondes, Miguel, Melo & Spadari-Bratfisch, 2001; Mora et al., 1996; Roderiguez-Sierra et al., 1984). Marcondes et al. (2001) demonstrated that treating diestrus rats with EB resulted in a reduction of anxiety to the same level as proestrus rats, suggesting that the differences in anxiety across the estrus cycle are modulated by E. E may modulate anxiety by increasing P receptors (Pfaff & McEwen, 1983), serotonin (5-HT) receptors (Bigeon and McEwen, 1982), and $GABA_A$ receptors (Herbison and Fenelon, 1995) in the hypothalamus, preoptic area, and amygdala.

Although changes in both pre- and post-synaptic 5-HT tone have been found as a function of estrus cycle phase (Bethea, 1993; Farmer, Isakson, Coy & Renner, 1996; Gundlah, Simon & Auerbach, 1998), there is little research investigating the effectiveness of SSRIs in reducing anxiety as a function of hormonal state. Taylor et al (2004) found no differences in open field or elevated plus maze behaviors as a function of estrogen and fluoxetine treatment. However, SSRIs remain a treatment for premenstrual dysphoric disorder in women, suggesting that these drugs may somehow interact with circulating levels of estrogen and/or progesterone (Pearlstein, 2002).

In female rats, there may be an interaction between hormonal state and the effectiveness of benzodiazepines in reducing anxiety. Using a conflict-operant paradigm, Molina-Hernandez, Contreras & Tellez-Alcantara (2001) demonstrated that female rats

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tested under the influence of diazepam during estrus and proestrus showed an increase in immediate punished reinforcement, suggesting lower levels of anxiety, than female rats treated with diazepam and tested during diestrus. In another study, OVX rats treated with EB+P and diazepam and tested in the elevated plus maze showed less anxiety than when the rats were treated with diazepam but not given hormones (Bitran, Hilvers & Kellogg, 1991).

In this study we examined the effects of diazepam or fluoxetine in OVX rats treated with EB+P, on conditioned freezing behavior, activity in an open field, and the elevated plus maze. It was hypothesized that treatment with diazepam would result in a decrease in anxiety behaviors, and that this decrease would be larger in rats treated with EB+P. Similarly, diazepam in EB+P treated rats was expected to cause a reduction in contextual conditioned fear.

Methods

Animals and surgery

Sixty female Long-Evans hooded rats (Charles River, St-Constant, QC; 200g-250g) were housed in groups of two in plastic cages (36 X 26 X 19 cm) in a colony room maintained on a reverse 12:12 hour light / dark cycle (lights off at 08:00), and at a constant temperature of 21°C, and food and water were continuously available. Ovariectomies were performed via bilateral lumbar incisions under ketamine hydrochloride (50 mg / ml) and xylazine hydrochloride (4 mg / ml), mixed at a ratio of

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4:3 respectively, and injected intraperitoneally in a volume of 1 ml / kg of body weight. Animals were given a week to recover prior to beginning experimental training sessions.

Hormone and drug treatments

Rats were randomly assigned to either the hormone or the vehicle training group. For rats in the hormone group, subcutaneous injections of EB (10 μg / 0.1 cc of sesame oil) were administered 48 h before each behavioral training or testing session, and P (500 μg / 0.1 cc of sesame oil) 4 h before a session. The vehicle group was injected with 0.1 cc of oil 48 h and 4 h before a session. Rats were further randomly assigned to one of three drug conditions: Saline, Diazepam (10 mg/kg), or Fluoxetine (10 mg/kg). Thus there were 6 groups with 10 rats in each group.

Training sessions

All sessions took place during the middle third of the dark phase of the light/dark cycle. During each training session, rats were placed in a 30 X 30 Plexiglas box with horizontal metal rods placed 5 cm above the floor. Rats were left in the box for a 5-minute period, after which a series of 11 0.8 mA foot shocks were delivered at 30 s intervals through the metal rods. Each rat was subjected to 5 training trials at 4-day intervals, always under the same hormone and shock condition. Four days after the fifth training trial, rats completed behavioral testing. The testing took place in the same order for each rat.

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Anticipation: On the test day, as on the training days, rats were placed in the same chamber for a 5-min period. Freezing was defined as the rat staying immobile, all paws on the grid floor, and was measured in seconds during the initial 5 minute period of exposure to the shock chamber on both the 1st training trial day, and the test day.

Open field: The open field test was conducted in a Plexiglas enclosure (60 X 60 X 60 cm) with an elevated metal grid floor. The floor of the apparatus was divided into 10 peripheral and 2 central squares. Rats were placed in the box for 5 minutes, and the number of peripheral and central crossings was recorded, as well as the proportion of crossings that were central.

Elevated-plus maze: The elevated-plus maze was a dark Plexiglas X-maze, elevated 70 cm off the ground, and comprised of two open arms (10 X 50 X 1cm) facing each other, two closed arms opposite from each other (10 X 50 X 20 cm), and a central platform (10 X 10 cm; Pellow & File, 1986). Rats were placed on the central platform of the maze facing the point where the open and closed arms meet. The number of closed and open arm entries, latency to enter the closed and open arms, and time spent on the open and closed arms was recorded over a 5-min period.

Statistical analyses

To measure differences on behavioral measures as a function of diazepam and saline a series of 2X2 between group analysis of variance (ANOVAs) were conducted, with the 2 variables being hormone condition (E+P and O+O) and drug treatment. The same set of analyses was conducted on the behavioral measures with fluoxetine and

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saline as the drug treatment conditions. Where appropriate, post-hoc tests were conducted using Fisher's LSD. Differences were considered significant at the $P < .05$ level.

Results

Anticipation

As shown in Figure 1, no significant differences were found in the amount of time rats spent freezing on the test day as a function of treatment with fluoxetine or diazepam, or hormone.

Open field

Fluoxetine

Figure 2A shows the group means for the number of peripheral crossings on the open field. The ANOVA found a significant difference as a function of hormone ($F(1,36) = 5.403$, $P<0.05$). There were no differences detected as a function of drug condition, and no interaction was found.

Figure 3A shows the group means for the latency to enter the central portion of the open field. The ANOVA detected a significant difference as a function of hormone ($F(1,36) = 6.526$, $P<0.05$). There were no differences detected as a function of drug condition, and no interaction was found.

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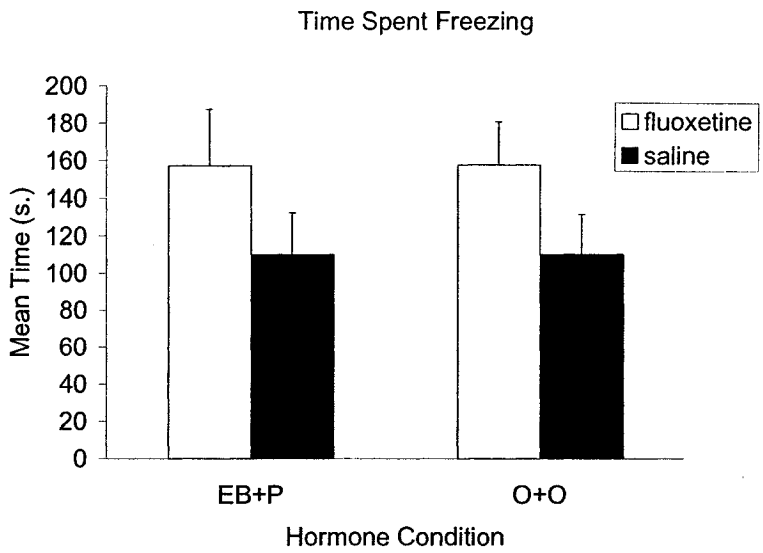

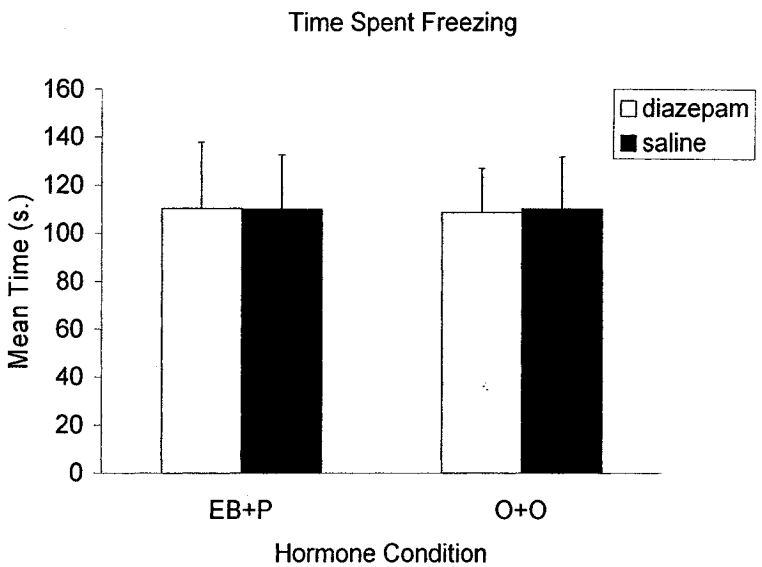

Figure1. Time spent freezing as a function of hormone and drug treatment. Figure 1a (top) illustrates fluoxetine vs. saline. Figure 1b (bottom). illustrates diazepam vs. saline.

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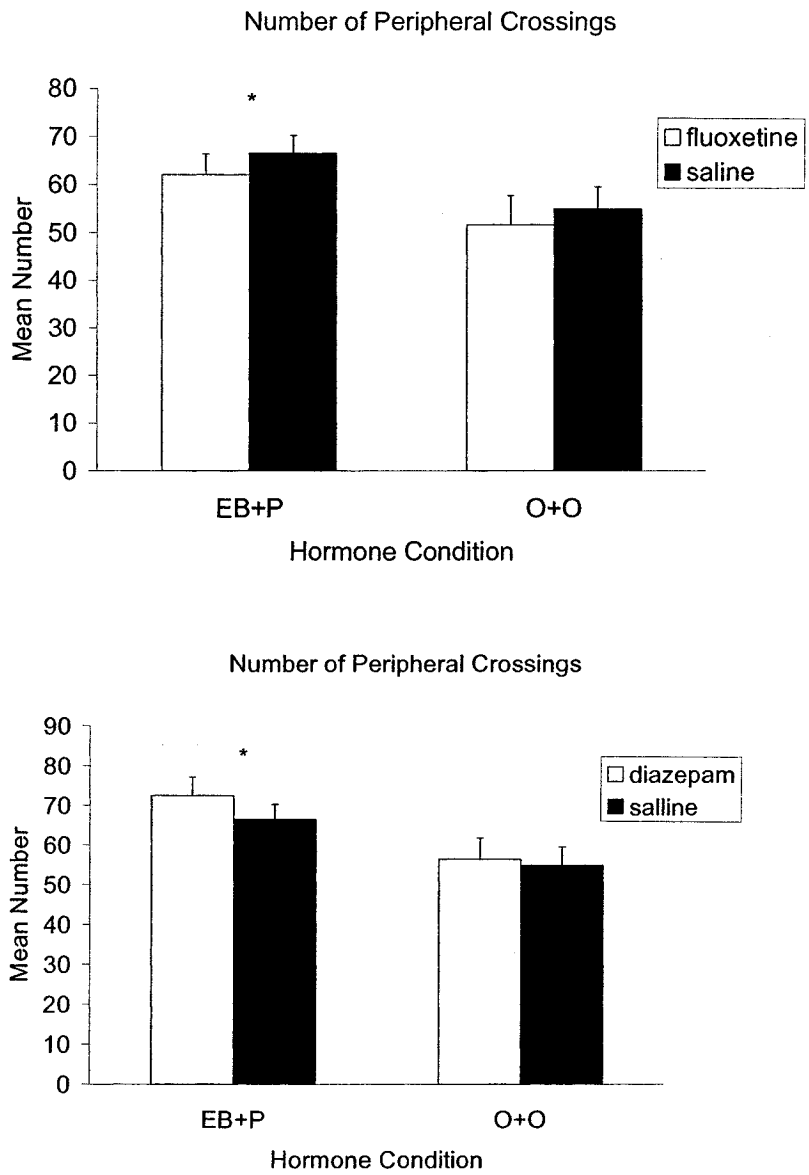

Figure 2. Number of peripheral crossings as a function of hormone and drug treatment. Figure 2a (top) illustrates fluoxetine vs. saline. * EB+P>O+O,P<0.05. Figure 2b (bottom) illustrates diazepam vs. saline. * EB+P>O+O,P<0.05.

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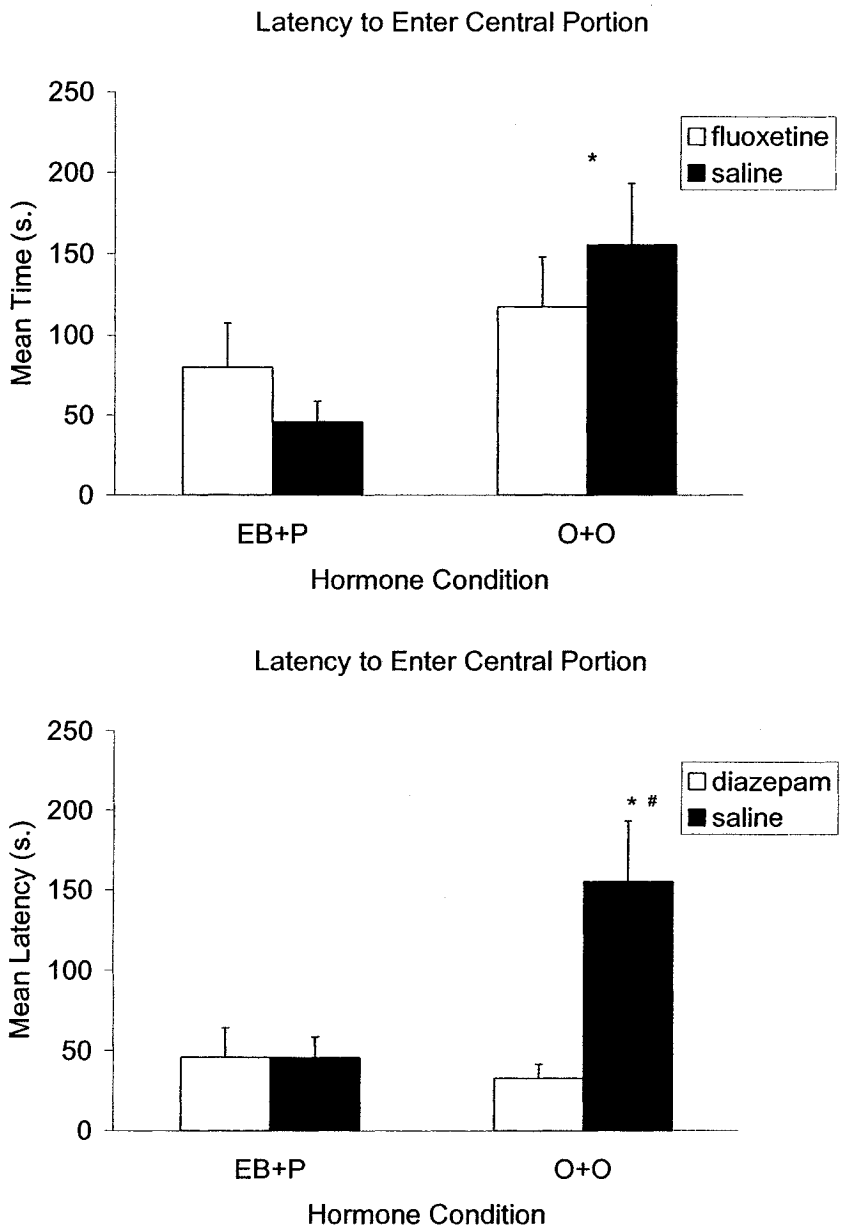

Figure 3. Latency to enter the central portion of the open field as a function of hormone and drug treamtent. Figure 3a (top) illustrates fluoxetine vs. saline. * O+O>EB+P, $P<0.05$. Figure 3b (bottom) illustrates diazepam vs. saline. * saline>diazepam,$P<0.05$, # O+O, saline>O+O,diazepam; EB+P, saline; EB+P, diazepam, $P<0.05$.

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No differences were found between the groups in the latency to begin moving around the peripheral portion of the open field, and no differences were found between the groups on the number of central crossings made (Figure 4A).

Diazepam

Figure 2B shows the group means for the number of peripheral crossings on the open field. The ANOVA found a significant difference as a function of hormone ($F(1,34) = 9.072$, $P<0.01$). Rats treated with EB+P made more crossings than untreated rats. There was no main effect of drug, and no interaction.

Figure 3B shows the group means for the latency to enter the central portion of the open field. The ANOVA detected a significant interaction ($F(1,34) = 7.033$, $P<0.05$). Posthoc comparisons revealed that O+O rats given saline had a higher latency than all other groups. There was also a main effect of drug ($F(1,34) = 6.987$, $P<0.05$), where rats treated with diazepam took less time to enter the central portion of the open field than rats injected with saline. There was no main effect of hormone.

Figure 4B shows the group means for the number of central crossings in the open field. The ANOVA detected a significant difference as a function of hormone ($F(1,34) = 6.614$, $P<0.05$). Rats treated with EB+P made more central crossings than those not given hormones.

No differences were found in the latency for rats to begin to move around the periphery of the open field as a function of hormone or drug.

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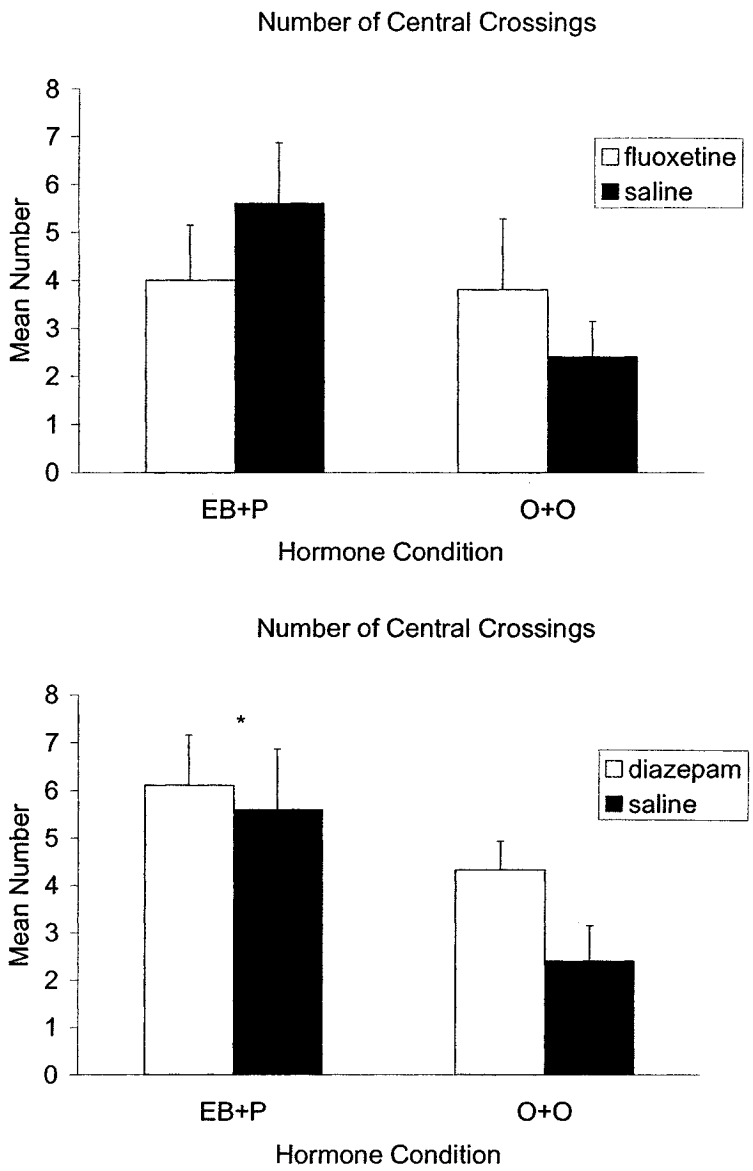

Figure 4. Number of central crossings made as a function of hormone and drug treatment. Figure 4a (top) illustrates fluoxetine vs. saline. Figure 4b (bottom) illustrates diazepam vs. saline. * EB+P>O+O,$P<0.05$.

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Elevated-plus maze

Fluoxetine

Figure 5A shows the group means for the amount of time spent in the open arms. The ANOVA found a significant main effect of drug ($F(1,36) = 5.282$, $P<0.05$). Rats treated with fluoxetine spent less time in the open arms of the elevated plus maze than did rats injected with saline. There was no effect of hormone, and no interaction was detected.

Figure 6A shows the group means for the amount of time spent in the closed arms of the elevated plus maze. The ANOVA found a significant main effect of drug ($F(1,36) = 4.954$, $P<0.05$). Rats treated with fluoxetine spent more time in the closed arms than did rats treated with saline. There was no main effect of hormone, and no interaction was detected on the amount of time spent in the closed arms.

No significant differences were found between the groups as a function of hormone or drug on the latency to enter the open or closed arms.

Diazepam

No significant differences were found on the variables of time spent in the open (Figure 5B) or closed arms (Figure 6B) of the plus maze or the latency to enter the arms as a function of hormone or drug condition.

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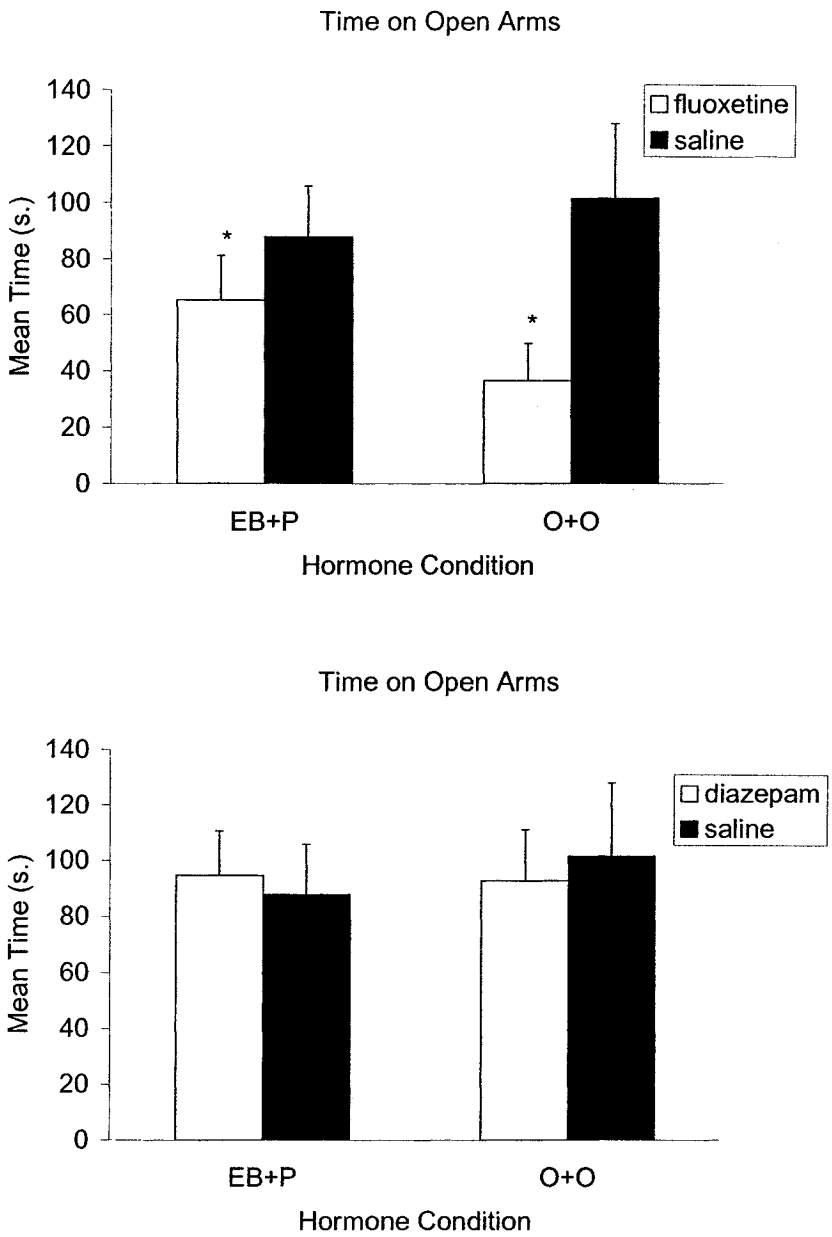

Figure 5. Time spent in the open arms of the elevated plus maze as a function of hormone and drug treatment. Figure 5a (top) illustrates fluoxetine vs. saline. * fluoxetine<saline, $P<0.05$. Figure 5b (bottom) illustrates diazepam vs. saline.

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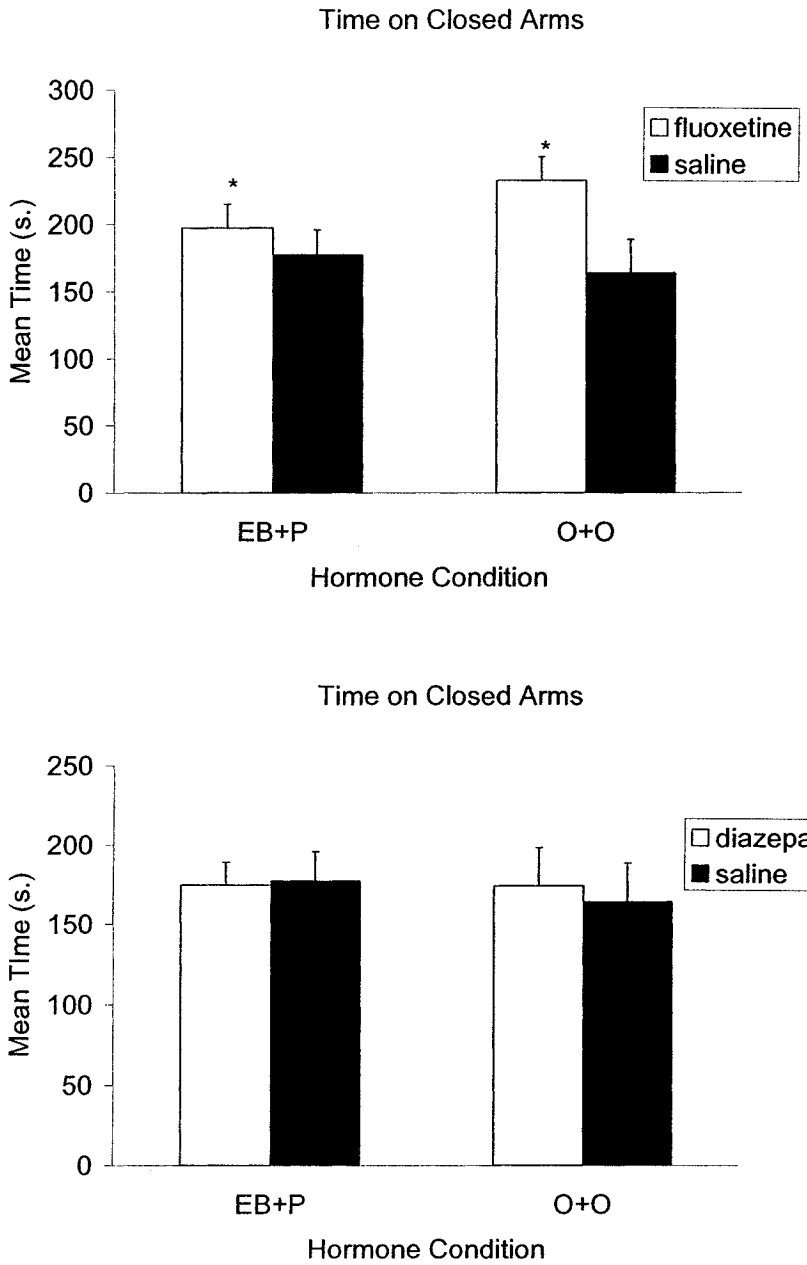

Figure 6. Time spent in the open arms of the elevated plus maze as a function of hormone and drug treatment. Figure 6a (top) illustrates fluoxetine vs. saline. * fluoxetine>saline, $P<0.05$. Figure 6b (bottom) illustrates diazepam vs. saline.

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Discussion

Results of the present study indicate that OVX rats treated with EB+P show less anxiety than those not given hormone replacement. This was inferred by the shorter latency to enter the central portion, and more central and peripheral crossings overall in the open field. These results are consistent with previous findings that rats treated with EB+P exhibit less anxiety than untreated rats on the radial maze (Bowman et al., 2002); open field tests (Bowman et al., 2002; Smith, Schramek & Pfaus, 2006); elevated plus maze (Mora et al., 1996; Smith et al., 2006); passive avoidance task (Mora et al., 1996), and in a conflict resolution situation (Roderiguez-Sierra et al., 1984). In the current study, rats tested with diazepam had a shorter latency to enter the central portion of the open field compared to rats tested without diazepam, and rats tested in the absence of both diazepam and EB+P took longer to enter the central portion. This suggests that diazepam has a greater effect on reducing anxiety when used in the presence of ovarian hormones. In intact females, diazepam can reduce anxiety on the elevated plus maze in rats tested in estrus, but not in diestrus (Nomikos & Spyraki, 1988), providing further evidence for a combined anxiolytic effect of ovarian hormones and diazepam. In fact, progestins have been shown to increase GABA binding in the basolateral amygdala and the dorsolateral septal nucleus (Canonaco, Tavolaro & Maggi, 1993), and evidence suggests that progesterone exerts its anxiolytic effects through actions of its neurosteroid allopregnanolone at $GABA_A$ receptors (e.g. Bitran, Shiekh & McLeod, 1995; Bitran & Dowd, 1996; Frye & Duncan, 1994; Picazo & Fernandez-Guasti, 1995; Wilson, 1992). Allopregnanolone acts in a similar manner to benzodiazepines on $GABA_A$ receptors,

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causing inhibition through enhanced Cl- currents (Gee, Cahng, Brinton & McEwen, 1987; Majewska, Harrison, Schwartz, Barker & Paul, 1986). Interestingly, the anxiolytic actions of allopregnanolone on the $GABA_A$ receptor are estrogen-dependent (Laconi, Casteller, Gariulo, Bregonzio & Carbrera, 2001). Estrogen has other, more direct effects on GABA in the hippocampus, where it has been shown to decrease GABAergic inhibition (Murphy, Cole, Greenberger & Segal, 1998). There is an increase in c-Fos in the hippocampus with the conditioned fear paradigm (Radulovic, Kammermeier & Spiess, 1998), and it is known that there are both E receptor subtypes α and β in the hippocampus (Shughrue, Lane & Merchenthaler, 1997; Su et al, 2001).

In the present study, acute fluoxetine treatment had either an anxiogenic effect or no effect on measures of anxiety. Rats treated with fluoxetine spent significantly less time in the open arms and more time in the closed arms of the elevated plus maze compared to rats injected with saline, although there were no differences on measures obtained from the open field test or during conditioned freezing. Previous research suggests that acute treatment with fluoxetine does not produce anxiolytic effects, but may actually cause an increase in anxiety, and that chronic treatment with fluoxetine is necessary for it to produce anxiolytic effects (e.g. Griebel, 1995). Chronic fluoxetine (24 days) reduced anxiety behaviors in the open field test, whereas subchronic fluoxetine (4 days) did not (Dulawa, Holick, Gundersen & Hen, 2004); rats demonstrated a reduction in anxiety on the unstable elevated plus maze with chronic, but not acute, treatment with fluoxetine (Jones, King & Duxon, 2002); chronic, but not acute, treatment with fluoxetine caused an impairment of one-way escape in the elevated T-maze, suggesting lowered levels of anxiety (Poltronieri, Zangrossi & Viana, 2003); and acute, but not chronic, fluoxetine

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caused an increase in anxiogenic behaviors such as head-dipping and end-arm activity in the elevated plus maze (Silva & Branda, 2000). The difference in behaviors as a function of whether administration was acute or chronic suggests that different mechanisms underlie the effects. It appears that acute administration of fluoxetine causes an immediate activation of 5-HT_{1A} autoreceptors in hippocampal and hypothalamic tissue (Blier & de Montigny, 1987; Maswood, Stewart & Uphouse, 1995; Sprouse & Aghajanian, 1987), which results in a decrease in firing of 5-HT neurons (Wong, Bymaster & Engleman, 1995). However, after 2-3 weeks of fluoxetine treatment, there is a desensitization of 5-HT_{1A} autoreceptors (Hjorth & Auerbach, 1996) which results in an increase of extracellular concentrations of 5-HT (Dong, de Montigny & Blier, 1998; Haddjeri, Ortermann, de Montigny & Blier, 1999), and a decrease in anxiety.

5-HT has been demonstrated to have effects on GABA transmission throughout the brain. Chronic administration of fluoxetine results in a down-regulation of GABA transmission in prefrontal cortical pyramidal neurons (Cai, Flores-Hernandez, Feng & Yan, 2002; Zhou & Hablitz, 1999; Zhong & Yan, 2004), and higher levels of presynaptic 5-HT correlate with reduced GABA release in the amygdala (Kishimoto, Koyama & Akaike, 2000; Koyama, Kubo, Rhee & Akaike, 1999; Krezel et al., 2001). Ovarian hormones have effects on both 5-HT and GABA systems. Administration of 17β-estradiol has been shown to downregulate 5-HT_{1A} receptor mRNA (Osterlund, Halldin & Hurd, 2000), and lower levels of extracellular 5-HT are found in the hypothalamus during estrus relative to diestrus rats (Gundlah et al., 1998). EB+P decrease serotonin turnover in the ventromedial hypothalamus of OVX rats, and injections of P decrease extracellular 5-HT in the hypothalamus of OVX rats (Farmer et al., 1996). More recently, increased 5-

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HT tone has been found in the dorsal raphe nucleus of proestrus and estrus relative to OVX rats, and bicuculline and picrotoxin, $GABA_A$ antagonists, infused into the DRN resulted in a greater decrease in 5-HT efflux in OVX rats, suggesting that in the DRN, GABA-mediated feedback of 5-HT neurons increases with higher circulating levels of E and P (Felton & Auerbach, 2004). Together, these findings suggest that levels and actions of GABA and 5-HT throughout the brain are mediated in part by E and P.

It has been demonstrated that EB increases binding of [^{3}H]muscimol to the $GABA_A$ receptor in the CA1 region of the hippocampus (Schumacher, Corini & McEwen, 1989). Further, 2 weeks after ovariectomy, injections of EB increased mRNA levels of glutamic acid decarboxylase (GAD), the rate-limiting enzyme for GABA synthesis, in the hippocampus (Weiland, 1992). Similarly, ovariectomy caused a decrease in GAD65-immunoreactive cell population in the CA1 pyramidal layer of the hippocampus, and EB increased the GAD65-immunoreactive cell population (Nakamura, Rosell, Akama & McEwen, 2004). Together, these results suggest that E mediates the synthesis of GABA through its actions on GAD expression. E also regulates $GABA_A$ receptor subunits (Weiland & Orchinik, 1995), and it induces P receptors in the CA1 region of the hippocampus (Parsons, Rainbow, MacLusky & McEwen, 1982), and P may affect GABA binding through direct action on the GABA receptor (Gee, Chang, Brinton & McEwen, 1987; Majewska, Harrison, Schwartz, Barker & Paul, 1986), where it modulates the $GABA_A$ receptor such that withdrawal from P decreases the anxiolytic effects of benzodiazepines (Moran, Goldberg & Smith, 1998), and exogenous administration of P elicits anxiolytic effects by potentiating GABA receptor function (Mayo, Vallee, Darnaudery & Le Moal, 1999). The results of these studies suggest that

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lower levels of E and P would result in a decrease in GABA synthesis as well as $GABA_A$ receptor binding, and, as a result, an increase in anxiety. This notion is supported by the current results of enhanced anxiolytic effects of diazepam in rats treated with E and P.

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CHAPTER 5

GENERAL DISCUSSION

The overall goal of this thesis was to elucidate the behavioral differences in response to stressors and the anxiety that occurs in response to cues that predict stressors, as a function of ovarian hormones in female rats. The experiments described in this thesis confirm previous findings that OVX or diestrous rats have higher levels of anxiety overall. Although there was little support found for the hypothesis that rats that are trained to expect shock in a particular hormonal state will experience higher levels of anxiety when in this same hormonal state, re-exposing EB+P treated rats to the context where they had previously experienced shock decreased their anxiety behaviors, suggesting that they are less affected by contextual conditioned fear than are OVX rats. This suggests that the impact of stress is modulated in part by ovarian hormones; as well, ovarian hormones modulate the expression of anxiety. Some ideas of underlying mechanisms are provided by the finding that diazepam reduced the anxiety behavior seen in EB+P, but not OVX, rats, as well, the main effect of E on test day performance suggest that it is E, more than P, that accounts for the reduction in anxiety seen in the current, and in previous studies, of OVX rats treated with EB+P.

The data found in this thesis provide some support for the idea that there is a shift in attention and focus as a function of ovarian hormone status. In fact, higher levels of E are associated with increased sexual receptivity in rodents, primates, and women. It is also associated with heightened arousal, as defined by greater alertness to sensory stimuli and greater emotional reactivity in female rodents (Pfaff et al., 2002) and humans (Krug

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et al., 1994; 2000; Little & Zahn, 1974). Interestingly, researchers now suggest that this enhanced arousal is specific to stimuli that are more emotionally significant to females, in particular, sexual stimuli (i.e. Lacreuse et al., 2006). This would explain previous reports that rats treated with EB+P tend to be more active, and at the same time have a decreased behavioral response to stress. It may be that the increased activity is to aid the female rat in seeking out, and engaging in, copulation.

Changes also occur in stress reactivity during pregnancy, a time of fluctuating hormone levels. Generally, rats are found to have lower levels of experimental anxiety on day 14 to 17 of gestation, a time when P levels are higher, and higher levels of anxiety on day 21 of gestation, when P levels are low (Picazo & Fernandez-Guasti, 1993; Zuluaga et al., 2005). It is believed that the higher levels of P during these times cause increased stimulation at GABA receptors, accounting for the reduction in anxiety (Picazo & Fernandez-Guasti, 1993). It would be interesting to provide pregnant rats with different stressful and sexual stimuli at various points during gestation to determine differences in valence of these stimuli as a function of different ovarian hormone levels.

A variety of different behavioral measures were used in this thesis, and although some tests were sensitive to differences in pre-esposure to stress and/or different hormonal treatment, others were not. Stress is not a unidimensional construct, and it may be that different tests measure different aspects of anxiety (e.g. Belzung, Pineau, Beuzen & Misslin, 1994; Lister, 1990; Ramos, Berton, Mormede & Chaouloff, 1997). In a factor analysis study using different behavioral tests of anxiety, Ramos et al. (1997) demonstrated 3 factors of anxiety. The first, which included entries into the central portion of the open field and onto the open arms of the elevated plus maze was thought to

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reflect approach/avoidance; the second was a measure of locomotion, and included total number of arm entries in the elevated plus maze and total number of quadrant crossings in the open field. Measures of social interaction comprised the third factor. In the current thesis, anticipation of stress caused variations in approach/avoidance, and these differed depending on hormone treatment. Significant differences were also found in the amount of time spent burying or freezing after contacting the electrified probe in the defensive burying test. Rats that were previously shocked spent more time freezing after contact with the probe, whereas those that were not pre-shocked buried the probe. It would be interesting to examine the responses made by rats that were pre-stressed with something other than shock, such as predator odors or unconditionally aversive odors such as cadaverine.

Proposed mechanisms underlying an ovarian hormone-induced reduction in anxiety

There are several mechanisms that may underlie the reduction in anxiety seen in rats treated with ovarian hormones. There may be direct anxiolytic actions of E and/or P, or there may be E- and/or P-induced actions on two specific neurotransmitter systems, GABA and serotonin.

Two subtypes of E receptors (ERs) have been identified: ERα in 1966 (Toft & Gorski, 1966), and, more recently, ERβ, in 1996 (Kuiper, Enmark, Pelto-Huikko, Nilsson & Gustafson, 1996). These receptors have been found throughout the brain, in the medial amygdala, bed nucleus of the stria terminalis, and the preoptic area (Shughrue, Lane & Merchenthaler, 1999). E has many varied effects throughout the brain, for example, in the hippocampus, E has been shown to increase dentritic growth and synaptic plasticity, and

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to enhance neuronal excitability (Foy et al, 1999; Good, Day & Muir, 1999; Terasawa & Timiras, 1968), whereas E in the amygdala has been shown to reduce neuronal excitability (Edwards, Burnham, Mendonca, Bowlby & MacLusky, 1999; Terasawa & Timiras, 1968). In the hippocampus, there is a predominance of ERβ (Shughrue et al., 1999). A recent review of studies investigating the different roles of ERβ suggests that it is the primary receptor involved in the behavioral expression of anxiety (Bodo & Rissman, 2006). It has been hypothesized that regulation of the GABA synthesizing enzyme GAD by ERs may modulate GABA A activity (McCarthy, Kaufman, Brooks, Pfaff & Schwartz-Giblin, 1995; Murphy, Cole, Greenberger & Segal, 1998), thus modulating the behavioral expression of anxiety.

In one study, ERβ-deficient mice showed increased anxiety in the open field and elevated plus maze tests, whereas ERα-deficient mice did not (Krezel, Dupont, Krust, Champon & Chapman, 2001), suggesting that it is ERβ that modulates this effect. Furthermore, the results of that study show that when GABA A receptor function is blocked in the amygdala of normal mice, there is an increase in anxiety to the same level as the ERβ-deficient mice. The authors thus suggest that ERβ affects transcription and post-translational modifications of the GABA A receptor subunits.

Together, these studies suggest a mechanism underlying the reduction in anxiety as a function of E and P. E activates GAD to make GABA, as well, it activates GABA A receptor subunits. P increases GABA binding at GABA A receptors by allosterically changing the binding subunit to a high-affinity state. Together, these hormones thus cause an increase in GABA, as well as an increase in binding at GABA A receptor subunits, resulting, in turn, in a decrease in behavioral anxiety. A recent study suggests that E and

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GABA in the VMH work to sustain sexual responding in female rats by inhibiting the glutamate-induced shutdown of these behaviors caused by vagino-cervical stimulation (Georgescu & Pfaus, 2006). Perhaps GABA and glutamate work in a similar fashion to shift the rat's focus from sexual to stressful stimuli as a function of E. This type of shift may be occurring in the amygdala. Preliminary data suggest that there is an increase in Fos expression in the CA in OVX rats exposed to a conditioned fear chamber relative to OVX rats that are treated with EB+P during both the training and the testing phase (Smith, Schramek & Pfaus, 2002). Although more research is needed to elucidate the nature of these Fos cells, it is possible that when EB+P is present, there is an opposite effect in the CA than in the VMH, resulting in a decrease in the valence of stressful stimuli, and vice versa when EB+P are not administered.

As discussed throughout this thesis, although certain areas, in particular the BLA, dorsal raphe nucleus (e.g. Abrams et al., 2005), and hippocampus, are known to be associated with anxiety and stress reactivity, less is known about how ovarian hormones may be affected GABAergic systems within these areas. It is possible that E and/or P affect GABA expression and/or function within any or all of these areas to modulate the behavioral expression of anxiety across ovarian hormone states.

This proposed mechanism may also underlie the findings in Chapter 4 of decreased anxiety in rats treated with EB+P and diazepam. Benzodiazepines cause increased binding at GABA receptors, having the same effect of a decrease in anxiety. Thus, the combined effects of increased GABA binding and receptor activity as a function of hormone treatment, paired with increased GABA as a function of diazepam treatment, may have produced a more pronounced decrease in anxiety.

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It was hypothesized throughtout this thesis that changes in anxiety reactivity could also occur as a result of changes in the activity of 5-HT. No support for this was found in the present thesis as administration of fluoxetine did not alter stress reactivity or behavioral anxiety. Although changes in 5-HT receptor density and activation occur in response to fluctuations of circulating estrogen (Fink et al., 1996), and in turn alter female sexual responding (Mendelson, 1992), these changes may not underlie the differences observed in response to stress or stimuli associated with stress.

There is some support for the idea that attention changes as a function of ovarian hormones. Studies in women have suggested a differential pattern of neuronal activation as a function of arousal or response to emotional stimuli across the menstrual cycle (e.g. Goldstein et al., 2005; Protopopescu et al., 2005). These researchers demonstrated an attenuation in brain activity in areas involved in the hypothalamic-pituitary-adrenal axis during ovulation, as well as increased activation during the follicular phase. Further, when women were exposed to negative valence/high arousal stimuli, there were greater magnitude of changes in blood oxygenation levels during the early follicular phase than during the midcycle in the cAmy, paraventricular and ventromedial hypothalamic nuclei, hippocampus, orbitofrontal cortex, anterior cingulated cortex, and peripeduncular nucleus of the brainstem, all of which are areas involved in the stress response (Goldstein et al., 2005). Orbitofrontal cortex activity was increased premenstrually and decreased postmenstrually upon presentation of emotionally negative stimuli (Protopopescu et al., 2005), again showing differences in brain activation as a function of hormonal state.

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Clinical implications

The hypothesis of E and P actions at GABA receptors underlying changes in anxiety reactivity may explain some of the changes in anxiety seen in women premenstrually. Although researchers have not found a clear link between E or P levels and increased reports of premenstrual anxiety in women, there may be a dysfunction at the level of E and P effects on the GABAergic system. Furthermore, these effects may occur much earlier than the premenstrual phase. For example, evidence suggests that the symptoms of PMS may be due to events occurring during the follicular, rather than the luteal phase. Schmidt et al. (1991) demonstrated that truncating the late luteal phase of the menstrual cycle in women with PMS with mifepristone, a progesterone antagonist, did not alter the timing or the severity of symptoms, suggesting that if endocrine changes are responsible for premenstrual symptoms, these changes are probably not occurring during the luteal phase. In another study, women with and without PMS were given monthly injections of the GnRH antagonist leuprolide acetate, or placebo, for 3 months (Schmidt, Nieman, Danaceau, Adams & Rubinow, 1998). GnRH antagonists suppress ovarian function by inhibiting the release of gonadotrophins, and have been shown to improve symptoms of irritability, tension, breast tenderness, and bloating (Brown, Ling, Anderson, Farmer & Arheart, 1994; Muse, Cetel, Futterman & Yen, 1984). Symptoms such as sadness, food cravings, irritability, bloating, breast pain, and anxiety were eliminated in 10 of the 18 women with PMS who received leuprolide (Schmidt et al., 1998). In a second phase of the study, the women who had responded to leuprolide were given E or P, and leuprolide, allowing the effects of E and P to be assessed in women whose symptoms seem to be linked to ovarian hormones. In all women, symptoms of

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sadness, anxiety, bloating, impaired function and irritability returned. Furthermore, these symptoms returned 1 to 2 weeks after the hormones were first given, suggesting that changes in E or P secretion during the follicular phase may be linked to later expression of symptoms during the late luteal phase. Perhaps, in a similar fashion, E is interacting with GABA during an earlier part of the ovulatory cycle to produce the heightened anxiety seen in women suffering from premenstrual symptoms.

Limitations

In the current thesis, brain regions involved in anxiety reactivity as a function of hormonal state were not explored. Previous studies suggest activation of a variety of brain areas during anxiety behaviors, including, among others, the hippocampus (e.g. Walf & Frye, 2006), amygdala (e.g. Abrams, Johnson, Hollis & Lowry, 2004; Walf & Frye, 2006), and dorsal raphe nucleus (e.g. Lowry, Johnson, Hay-Schmidt, Mikkelsen & Shekhar, 2005; Rosen, 2004). An important future direction would be to explore differential brain activation during anxiety in rats in different hormonal states. This could be done using Fos studies, or lesion studies. Preliminary studies suggest higher levels of Fos in OVX rats re-exposed to a conditioned fear chamber than in EB+P treated rats (Smith et al., 2002), but this finding needs to be validated and expanded, and the nature of these Fos cells to be determined. Of particular interest to explore would be the amydgala and hippocampus. Both of these areas contain ERs (Walf & Frye, 2006), and both have been implicated in conditioned fear (e.g. Kim & Fanselow, 1992; Maren, Aharonov & Fanselow, 1997), and anxiety (Walf & Frye, 2006). Perhaps differences in

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hormone levels would produce observable changes in Fos expression following exposure to a context previously paired with conditioned fear.

Although the present thesis revealed little evidence for contextual conditioned fear as a function of stress reactivity resulting from ovarian hormone levels, it would be premature to conclude that differences do not exist. Perhaps adding a tone or light to the chamber would serve to increase the strength of the conditioning, and perhaps the previously discussed differences in attention to stimuli across the estrus cycle would differentially influence conditioning.

Conclusion

The results of this thesis suggest that there is increased anxiety in OVX or diestrus rats relative to EB+P treated or estrus rats, and that EB+P rats are less affected by contextual conditioned fear than O+O rats. There was some evidence to suggest that E actions on GABA may modulate this reduction in anxiety in the EB+P treated rats. The underlying mechanisms of this modulation and the brain regions where it may be occurring remain to be determined.

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