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The Effect of Gonadal Hormones on Agonistic Behavior in Previously Defeated Female and Male Syrian Hamsters

Matia B. Solomon
Georgia State University

THE EFFECT OF GONADAL HORMONES ON AGONISTIC BEHAVIOR IN PREVIOUSLY DEFEATED FEMALE AND MALE SYRIAN HAMSTERS

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Under the Direction of Kim L. Huhman

ABSTRACT

Following social defeat, male hamsters exhibit behavioral changes characterized by a breakdown of normal territorial aggression and an increase in submissive/defensive behaviors in the presence of a non-aggressive intruder (NAI). We have termed this phenomenon conditioned defeat (CD). By contrast, only a small subset of defeated females exhibit submissive/defensive behavior in the presence of a NAI. We hypothesized that fluctuations in gonadal hormones might contribute to differences in the display of submissive behavior in intact female hamsters. Following social defeat, proestrous females (higher endogenous estradiol) were more likely to display conditioned defeat compared with diestrous 1 (lower endogenous estradiol) females. This finding suggests that there is an estrous cycle-dependent fluctuation in the display of CD in female hamsters and suggests that increased estradiol might contribute to increased submissive behavior. We then demonstrated that ovariectomized females given estradiol prior to CD testing exhibited significantly higher submissive behavior in the presence of a NAI suggesting that estradiol increases the expression of CD in female hamsters. We have also shown that castrated males that were singly housed for four weeks displayed

significantly more submissive behavior than did their intact counterparts. Interestingly, castrated and intact males that were singly housed for 10 days prior to behavioral testing displayed similar behavior during CD testing.

Together these data suggest that androgens and isolation modulate the display of CD in male hamsters. Finally, we examined brain activation following CD testing in defeated males and females (in diestrus 1 and proestrus). Defeated male and proestrous females exhibited increased Fos activation in the dorsal lateral septum and hypothalamic paraventricular nucleus relative to defeated diestrous 1 females. Diestrous 1 females exhibited increased Fos expression in the lateral bed nucleus of the stria terminalis compared with both defeated groups. Collectively, these data suggest that gonadal hormones and duration of individual housing modulate the display of CD in female and male hamsters and that those animals which display CD exhibit differences in patterns of neuronal activation than do those that do not display CD.

INDEX WORDS: Estrous cycle, Estradiol, Aggression, Submission, Social Stress, Hypothalamic-Pituitary Adrenal Axis (HPA-axis), Social Defeat

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Matia B. Solomon

Major Professor:	Kim L. Huhman
Committee:	H. Elliott Albers
	Timothy Bartness
	Marise Parent
	Mark Wilson

Electronic Version Approved:

Office of Graduate Studies

College of Arts and Sciences

Georgia State University

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17- β Estradiol	**E_2**
Adrenocorticotropin	**ACTH**
Analysis of Variance	**ANOVA**
Basolateral amygdala	**BLA**
Bed nucleus of stria terminalis	**BNST**
cAMP response element-binding protein	**CREB**
Central amygdala	**CeA**
Conditioned Defeat	**CD**
Corticotropin-releasing hormone	**CRH**
Cortisol	**CORT**
Dihydrotestosterone	**DHT**
Epinephrine	**E**
Estrogen	**E_2**
Gonadotropin releasing hormone	**GNRH**
Hypothalamic-Pituitary Adrenal Axis	**HPA-axis**
Hypothalamic-Pituitary Gonadal Axis	**HPG-axis**
Immediate Early Gene	**IEG**
Lateral hypothalamus	**LH**
Lateral septum	**LS**
Light/Dark Cycle	**L/D Cycle**
Locus coeruleus	**LC**

Luteneizing Hormone	**LH**
Medial amygdala	**MeA**
Medial Preoptic Nucleus	**MPOA**
Messenger ribonucleic acid	**Mrna**
Multivariate Analysis of Variance	**MANOVA**
Natural Killer Cells	**NK cells**
N-methyl-D aspartate	**NMDA**
Non-Aggressive Intruder	**NAI**
Norepinephrine	**NE**
Ovariectomy	**OVX**
Paraventricular nucleus of the hypothalamus	**PVN**
Progesterone	**P**
Serotonin	**5-HT**
Standard Error of the Mean	**SEM**
Sympathetic Nervous System	**SNS**
Testosterone	**T**
γ –aminobutyric acid	**GABA**

Chapter 1

GENERAL INTRODUCTION

Stress is a natural part of our daily lives. For some, stressors may be physical or psychological in nature; however, regardless of the source of stress, the overall effect is a similar stress response as evidenced by activation of two systems: sympathetic nervous system (SNS) and the hypothalamic-pituitary adrenal axis (HPA-axis) (Miller & O'Callaghan, 2002) The HPA-axis, in particular, has been studied in detail in terms of its contribution to the development of certain stress-related psychopathologies (e.g., depression, anxiety), and glucocorticoids, the end products of this system, can have multiple effects on a number of cellular and phsyiolgical processes in humans (Makino et al., 2002). Typically, glucocorticoids exert a negative feedback at the level of the hypothalamus and the pituitary, which terminates the stress response. This system is ideal for responding to short term or acute stressors; however, constant activation of the HPA-axis can lead to a number of physiological changes including increased risk for cardiovascular disease, hypertension and increased visceral adiposity (as reviewed in: Tsigos & Chrousos, 2002). Because stress has been associated with a number of deleterious conditions in humans; many preclinical models have been used in order to investigate the maladaptive costs of stress on the brain, physiology and behavior.

Animal Models of Stress

A variety of paradigms have been employed in order to elucidate the underlying neuroendocrine factors that may be associated with stress. Many of these paradigms involve the use of various stressors like footshock, forced swim stress or restraint. Following exposure to these stressors, the experimenter typically measures the animal's hormonal and/or behavioral responses. Although these models have contributed significantly to our overall understanding of the SNS and HPA-axes, they are not stressors that are likely to be encountered by rodents or humans. In order to investigate the underlying neurobiological mechanisms that may be associated with stress-related conditions, it is important to utilize more naturalistic stressors. One stressor that many rodents and humans experience on a daily basis is social conflict (Bjorkqvist, 2001). As a result, numerous laboratories have used social conflict as a means to measure stress-induced changes in both physiology and behavior.

In general, social conflict or agonistic behavior is studied between conspecifics. The term agonistic behavior refers to all behaviors that are displayed during social conflict including aggression (e.g., attack, chase), submission/defense (e.g., flight, rearing) and communication (e.g. scent marking) (for review: Albers et al., 2002). Social conflict can be observed in a number of ways, but the most common method is the resident/intruder paradigm, whereby an intruder is placed into the home cage of another animal. The intruder is typically defeated by the resident aggressor. Following the agonistic encounter, the hormonal and or behavioral impact of this interaction is often studied in both the loser and winner. Most research suggests that the loser and not the

winner, displays many of the behavioral and or physiological changes that are often associated with stress-related psychopathologies in humans.

Physiological and Behavioral Consequences of Social Defeat

Social defeat induces a spectrum of changes in physiology and behavior in a number of species. Defeated animals often display many of the same hallmark hormonal and behavioral changes that are seen in chronically stressed humans. For example, defeated rats, tree shrews, marmosets and mice display increased activation of both the SNS and HPA-axis as illustrated by elevated norepinephrine (NE), epinephrine (E) cortisol and ACTH levels (Stefanski, 2000; Haller et al., 1999; Gould et al., 1998; von Holst, 1972, Fuchs & Flugge, 2002). Social defeat results in decreased immunity as indicated by reduced B, helper T and NK cell counts in male rats and mice (Stefanski et al., 1996a; Stefanski et al., 1996b; Stefanski, 1997; Engler et al., 2005; Devoino et al., 2003). Stress-induced suppression of the hypothalamic-pituitary gonadal axis (HPG-axis) has also been observed in male rats (Stefanski, 2000). In addition, defeated mice exhibit decreased hippocampal and amygdala neurotrophic expression in comparison with their non-defeated counterparts (Pizarro et al., 2004). Further, following either a brief or chronic agonistic encounter, defeated tree shrews exhibit decreased cell production in the dentate gyrus, an effect which is not seen in their non-stressed counterparts (Fuchs & Flugge, 1998).

Defeated animals also show changes in behavior that are not observed in their non-stressed control or dominant counterparts. Following social defeat, animals display increased anxiety-like behavior on several measures of anxiety including the open-field

and elevated plus maze. Following a previous defeat episode, rats and mice display a reduction in locomotor and exploratory behavior in the open-field and elevated plus maze (Meerlo et al., 1996; Kudryatvtseva et al., 1991; Avgustinovich et al., 1997). A reduction in normal territorial aggression and an increase in avoidance and defensive behavior have been reported in male mice following defeat (Lumley et al., 1999; Lumley et al., 2000; Siegfried et al., 1984). In humans, depression is often associated with anhedonia, withdrawal from peers, decreased food consumption and increased drug use (Hankin, 2005). Defeated rodents also exhibit similar responses as indicated by decreased preference for sweet solutions (Rygula et al., 2005), decreased social interaction (Kudryavtseva et al., 1991) and decreased locomotion (Meerlo et al., 1996; Meerlo et al., 1997). Subordination stress also triggers reduction in both food intake and body weight in rats and mice (Stefanski, 1997; Meerlo et al., 1996; Meerlo et al., 1997, Kudryavtseva et al., 19991). Finally, defeated male rats acquire cocaine self-administration in less time than non-defeated males (Tidey & Mizcek, 1997). Taken together these data clearly demonstrate that social defeat is a potent stressor capable of producing a multitude of changes in both physiology and behavior across species.

Social Defeat and Females

Much of the knowledge concerning the adverse effects of social defeat is obtained from male subjects. Social defeat has been shown to induce a number of behavioral changes that are suggestive of anxiety or depression. Given the fact that females are more likely to suffer from these stress-related psychopathologies (Earls, 1987; Hankin, 2005), it would be ideal to examine the effects of defeat in both sexes. One species that has

been studied in detail with respects to the behavioral and hormonal changes that may be associated with subordination stress in females is cynomolgus monkeys (*Macaca fascicularis*). Non-human primates are a highly suitable species with which to study sex differences in response to stress because of their similarity to humans in terms of central nervous system and reproductive system functioning (Dukelow, 1977; Shively et al., 1986). Social hierarchies are characteristic of this species, resulting in dominant and subordinate members. Subordinate members have been reported to display elevated cortisol levels (Shively, 1998), decreased ovarian function (Adams et al., 1985; Shively et al., 1997), coronary artery atherosclerosis (Adams et al., 1985; Shively et al., 1990) and dyslipidemia (Hamm et al., 1983). Further, these females also display decreased body weight and fewer social interactions (Shively et al., 1997; Shively et al., 2005). These data clearly demonstrate that subordination is a major stressor in these females. Although non-human primates are closely related to humans, there are some caveats to studying these animals. For many, working with non-human primates may be difficult due to several factors including cost and possible health care concerns and for these reasons many laboratories opt for smaller non-human animals.

Under normal circumstances, many female rodents do not display high levels of agonistic behavior, making it difficult to study sex differences in the behavioral and hormonal responses to social defeat in these animals. There are however, alternative methods of studying agonistic behavior in female rodents. For example, female rodents will display high levels of aggression toward conspecifics in defense of their young (Sgoifo et al., 1995; Erskine et al., 1979) or following mediobasal hypothalamic lesions (Haller et al., 1999). One study that examined sex differences in the response to social

defeat found that defeated males exhibited decreased body weight and increased corticosterone levels, whereas these same changes were not apparent in female rats. Although, social defeat was not a major stressor in female rats, social instability caused a significant reduction in body weight, increased corticosterone levels and induced thymus involution and adrenal hypertrophy in these females suggesting that social instability is a better stressor for female rats (Haller et al., 1999).

Syrian Hamsters and Agonistic Behavior

Syrian hamsters (*Mesocricetus auratus*) are a highly territorial species that will readily defend their home cage against conspecifics (Payne & Swanson, 1970; Lerwill & Markings, 1971). Unlike many other rodents where only the male displays high levels of agonistic behavior, both male and female Syrian hamsters will engage in high levels of aggressive behavior, making them an ideal species for studying agonistic behavior. Numerous studies investigating social conflict in Syrian hamsters have identified several factors that may be associated with an increased likelihood of success during agonistic interactions. These factors include gender, body weight, social experience and the environment in which the agonistic encounter occurs.

For many species, males are more aggressive than females; however, in Syrian hamsters the opposite is true. Sexually unreceptive females are highly aggressive and will often dominate their male counterparts (Payne & Swanson, 1970; Tiefer, 1970). Body weight is also a significant indicator of success with heavier individuals often dominating their smaller counterparts. With male-male interactions, approximately 60% of the time the smaller male will be defeated by the larger opponent (Vandenbergh, 1971;

Payne & Swanson, 1970). This percentage is even higher in females with approximately 70% of the heavier opponents becoming victorious over the smaller one (Drickamer & Vandenbergh, 1973). Female hamsters are typically heavier than males which may in part explain their dominance; however, even when hamsters are weight matched, females are still victorious (Marques & Valenstein, 1977). An animal's prior social experience has also been associated with success during social conflict. For example, isolated males tend to be more aggressive than group-housed animals (Grelk et al., 1974; Brain, 1972; Payne et al., 1984). In fact, group-housed hamsters are often used as stimulus animals because they display low levels of aggressive behavior toward conspecifics (Potegal et al., 1996; Huhman et al., 2003; Jasnow et al., 1999). Finally, the environment in which the encounter occurs is important with resident hamsters often dominating intruders (Murphy & Schneider, 1970; Murphy, 1976). Because both male and female hamsters will spontaneously display aggressive behavior, many laboratories have examined the role of gonadal hormones on the regulation of aggression in this species.

Gonadal Hormones

Briefly, steroid hormones are synthesized from cholesterol and are often referred to in relation to the organ in which they are produced. In males, testicular hormones or androgens include testosterone (T) and dihydrotestosterone (DHT), while in females ovarian hormones include estrogens (E_2) and progesterone (P). The presence of androgens or E_2 is not exclusive in males and females. Females also secrete T from the ovaries; likewise, the testes also produce some amount of E_2. Although the gonads are the primary source of the hormones, the adrenal cortex also produces some amount of

androgens and P in response to stress (review see: Becker et al., 1992). Collectively, these hormones exert powerful effects on both the central and peripheral nervous systems, thereby resulting in a multitude of behavioral changes in rodents that may include either increased or decreased agonistic behavior.

Gonadal Hormones and Aggression in Male Hamsters

In many species, males are more aggressive than females. It is generally accepted that androgens are responsible for this increased aggression in male rodents. Following castration, male mice and rats display reduced aggression, while T administration restores aggression (Albert et al., 1986; Tolman & King, 1956; Bevan et al., 1958). Further evidence for a positive correlation between androgens and aggression comes from subordinate rodents. Numerous studies have shown that following repeated defeats, subordinate males often display decreased T levels in comparison with dominants or non-stressed controls (Hardy et al., 2002; Kraus et al., 1999; Blanchard et al., 1995).

The role of androgens and E_2 in the display of aggression in male hamsters is not without dispute. Some laboratories report decreases in aggression following castration (Payne & Swanson, 1971; refs), while others report no differences in aggressive behavior between castrated and intact males towards either restrained or freely moving conspecifics (Potegal et al., 1980; Whitsett, 1975; Tiefer, 1970). In one study where castrated males were given T, DHT or androstenidone (a precursor to T), all males that were given these androgens were more aggressive than those given oil; although, androdstenidone was found to be the most effective in increasing aggression (Payne, 1974). As mentioned previously, female hamsters are more aggressive than male

hamsters. In order to assess the role of ovarian hormones in aggression in male hamsters, Payne & Swanson (1971) gave castrated males ovarian tissue from an adult female hamster. They found that castrated males with ovarian transplants were significantly more aggressive than intact males and intact males were significantly more aggressive than castrated males. These data suggest that androgens may exert their effects on aggression via E_2 receptors.

Manipulation of photoperiod length provides an alternative method for studying the effect of gondal hormones on the display of aggression in hamsters. Exposure to short photoperiod (< 12.5 hr of light/day) causes gonadal regression in male hamsters and a subsequent decrease in circulating testicular hormones (Albers, Rowland and Ferris, 1991). If the display of aggression in Syrian hamsters were totally dependent upon the concentration of testicular hormones then one would expect that males with higher androgen concentrations to be more aggressive than those with lower amounts of androgens. Despite lower concentrations of testicular hormones, male hamsters individually housed in short photoperiods display increased aggressive behavior (Garrett & Campbell, 1980; Jasnow et al., 2002). These data suggest that the display of aggression in male hamsters housed under short period conditions is independent of circulating testicular hormones. Overall, some of the discrepancies across studies, concerning the role of gonadal hormones on the display of aggression in male hamsters may be due to several factors such as: characteristics of the opponent (i.e., intact vs. hormonally-treated vs. castrated), the environment (neutral cage vs. animal's home cage) and hormonal administration (acute vs. repeated vs. chronic).

Estrous Cycle

Female rodents have a 4-5 day estrous cycle. The days of the estrous cycle are characterized based on their relation to the day of ovulation. Diestrus 1 or metestrus is the day following ovulation and on the morning of distrus 1 there is a brief increase in P levels followed by a subsequent fall in P later in the afternoon (Figueiredo et al., 2002; Smith et al., 1975; Ridley & Greenwald, 1975). E_2 levels remain relatively low during diestrus 1, while diestrus 2 is characterized by steadily rising levels of E_2 due to the onset of follicular activity and low levels of P. Proestrus is the day preceding ovulation and is characterized by a morning peak of E_2 followed by and an afternoon peak in P levels. All of the hormonal events that are necessary to induce ovulation occur on the evening of proestrus (i.e., GNRH and LH surge). Finally, estrus is the day of vaginal ovulation and is characterized by low levels of E_2 and moderate levels of P. (for review see Becker et al., 1992).

The numerous ways in which the term estrus is used in the literature can be quite confusing. For example, behavioral estrus or "heat" refers to the period when females exhibit sexual receptivity or lordosis. On the other hand, vaginal estrus refers to the period of ovulation. Sexual receptivity or lordosis has been reported to occur during late proestrus after the surge in LH and progesterone levels and persists to the estrus phase of the cycle; as a consequence, some refer to the period of sexual receptivity as proestrus/estrus in rats (Melchoir et al., 2004; Ho et al., 2001; Olsson et al., 2003). Therefore, if females are tested early on during proestrus (before the LH and progesterone surge), they should not display lordosis, while females tested later in the evening will exhibit lordosis.

In female rodents, the estrous cycle is closely tied to the light/dark cycle (L/D cycle) (Fitzgerald & Zucker, 1976; Alleva et al., 1971). Many of the discrepancies regarding when the period of sexually receptivity occurs can be overcome by stating the length of the L/D cycle (i.e., 14:10) as well as the time in relation to lights on or off in which hormones and behavior are measured. We define the morning or "early" part of the proestrus phase of the cycle with light offset. For example, during the proestrous phase on a 14:10 L/D cycle, with lights out at 0800h and lights on at 1800h, the morning of proestrous would begin at 0800 h. The afternoon would occur around 1500 h with the late evening of proestrus characterized by the LH surge followed by the progesterone surge occurring between approximately 0300 and 0500 h (lights are on). The morning of estrus is then defined as the next time that the lights go off at 0800 h.

In female hamsters, some studies report sexual receptivity during proestrus (Takahashi & Lisk, 1983; 1984; Ciaccio et al., 1979) while others report sexual receptivity during the estrus (Floody & Pfaff, 1977). The study conducted by Ciaccio and colleagues (1979) was the only study to specifically state that females during the evening of proestrus exhibit lordosis. This is particularly important because a novice to the field might assume that all proestrous females will exhibit lordosis when, in fact, lordosis is only seen during late proestrus and estrus. Proestrous females in our studies are tested within the first 3 hours after lights out, which is well before the surge of progesterone and LH; therefore, none of our proestrous females exhibit lordosis. Our laboratory, along with others (Wise, 1974), report the presence of a thin, stringy vaginal discharge as the period of sexual receptivity.

The problem in the literature is not so much when females display lordosis (i.e., proestrus vs. estrus), but more the lack of reporting on when females are tested with respect to the L/D cycle. Despite the inconsistencies as to which day signifies the day of sexual receptivity, there is a general agreement that hormonal fluctuations over the course of the estrous cycle can exert widespread effects on cellular and hormonal processes as well as behavior in females.

Gonadal Hormones and Aggression in Female Hamsters

Many female rodents do not typically display high levels of aggressive behavior. Some females do, however, display more aggression at times that are associated with fluctuations in circulating ovarian hormones, namely during the estrous cycle, pregnancy or lactation. There have been reports of increased aggression in some mice and rats during the estrous cycle towards female intruders (Davis and Marler, 2003; Davis and Marler, 2004; Olsson et al., 2003; Melchior et al., 2004). For example, some diestrous 1 and diestrous 2 Wistar rats display increased estrous cycle-related aggression which can be eliminated by ovariectomy and reinstated with administration of estrogen and progesterone that closely mimics the fluctuations within the estrous cycle (Ho et al., 2001). Pregnant and lactating females are also notoriously aggressive towards intruder conspecifics. The intensity of the aggression seen in lactating females depends on several factors including the age and size of the opponent, environment in which the aggressive interaction takes place (i.e., home or opponent cage) as well as time spent with pups. More specifically, larger opponents are reported to be attacked less often than smaller ones, while lactating females that are tested in their home cage are more aggressive in

defense of young than those tested outside of their cages. Further, an increase in time spent away from pups is associated with a decrease in maternal aggression in some mice (see review: Lonstein and Gammie, 2002; Neuman, 2003).

Unlike female mice and rats that only occasionally display aggression, female Syrian hamsters, when not in estrus, are highly aggressive towards male or female conspecifics (Payne & Swanson, 1970; Tiefer, 1970; Floody & Pfaff, 1977; however see, Takahashi & Lisk, 1983). There have been varying reports on the levels of aggression in female hamsters over the estrous cycle with some studies reporting higher levels of aggression during diestrus 1 (Ciaccio et al., 1979), others reporting higher levels of aggression during proestrus (Wise, 1974) and still others reporting no differences in aggression among the three days not associated with sexual receptivity (Payne & Swanson, 1970). Aggression is not only observed across the estrous cycle in female hamsters, as both pregnant and lactating females hamsters also display high levels of aggressive behavior (Wise, 1974; Siegel et al., 1983). Overall, the differences in the amount of aggression observed in female hamsters across studies might be due to the sex of the opponent as well as the hormonal characteristic of the opponent (i.e., intact vs. ovariectomized).

The effects of exogenous administration of gonadal hormones on the display of aggression in female hamsters have yielded mixed results. For example, E_2 has been shown to increase (Kislak & Beach, 1955), decrease (Carter et al., 1973; Payne & Swanson, 1971) or have no effect on aggression (Meisel et al., 1990; Meisel & Sterner, 1990). Similarly, progesterone has been shown to increase (Payne & Swanson, 1971), decrease (Fraile et al., 1987) or have no effect on aggression in female hamsters (Meisel

et al., 1988). Although administration of these hormones alone has produced conflicting results on the expression of aggression in female hamsters, sequential administration of E_2 and P has consistently been shown to decrease aggression and induce sexual receptivity towards either male or female conspecifics (Floody & Pfaff, 1977; Meisel et al., 1990; Meisel & Sterner, 1990). Similar to male hamsters, exposure to short photoperiods increases aggression in female hamsters (Fleming et al., 1988; Elliott & Nunez, 1992). Female hamsters exposed to short photoperiods no longer have estrous cycles and exhibit decreased estradiol levels (Albers, Moline & Moore-Ede, 1984; Moline, Albers, & Moore-Ede, 1986) suggesting that the increase in aggression seen in these females is independent of circulating estradiol. Much work has been done investigating the hormonal correlates of aggression in both male and female hamsters; however there are other aspects of agonistic behavior that have been largely ignored in Syrian hamsters like submissive behavior.

Submissive/Defensive Behavior in Hamsters

While we know much about the hormonal and environmental factors that may be associated with aggression and dominance in Syrian hamsters, much less is known about submissive behavior. For a number of years, we have been investigating the hormonal correlates of social defeat. We have found that defeated male hamsters display a number of physiological changes that include increased HPA-axis activation as evidenced by increased plasma cortisol (Huhman et al., 1991; Huhman et al., 1992; Jasnow et al., 2001) and adrenocorticotropin (ACTH) (Huhman et al., 1992; Huhman et al., 1995) levels. Social defeat also suppresses humoral immunity (Jasnow et al., 2001), increases β-

endorphin (Huhman et al., 1991; Huhman et al., 1992) and decreases testosterone and prolactin levels (Huhman et al., 1991; Huhman et al., 1995). Social defeat not only induces numerous hormonal changes in male hamsters, but it can also create profound alterations in their agonistic behavior. Following defeat, male hamsters no longer display normal territorial aggression and instead display increased submissive behavior in the presence of a smaller intruder (Potegal et al., 1993); we have termed this phenomenon conditioned defeat (CD).

Conditioned Defeat in Male Hamsters

During CD acquisition, the experimental animal is placed into the home cage of a larger aggressive hamster. During this agonistic encounter, the experimental animal typically displays submissive behavior in the presence of the resident hamster as characterized by fleeing, rearing and tail lifts. CD acquisition or training has been done in a series of 5 min-trials (Faruzzi et al., 2005; Huhman et al., 2003; Potegal et al., 1993) or in a single 15-min encounter (Jasnow et al., 2004a; Jasnow et al., 2004b; Cooper et al., 2005). On the following day, during a subsequent test encounter with a smaller non-aggressive intruder (NAI), the previously defeated hamster no longer displays normal territorial aggression but instead displays increased submissive behavior. This is particularly important because during the CD testing period, the defeated hamsters remain in their own home cages and are no longer in a "threatening" situation. The behavioral changes (i.e., increased submissive/defensive behavior and lack of territorial aggression) following defeat are profound and long lasting and can persist for up to one month even though the animals are never attacked again (Huhman et al, 2003). Learning

to avoid a previously aversive stimulus is an adaptive mechanism that can prolong the survival of a species. However, at a certain point the memory of a previous stressful experience (i.e., defeat) may become maladaptive resulting in abnormal behavior and unnecessary activation of the hypothalamic-adrenal-pituitary axis (HPA-axis). In humans, this inability to overcome previously stressful experiences may lead to the development of certain stress-related psychopathologies like generalized anxiety, depression or post-traumatic stress disorder (Albucher & Liberzon, 2002).

We have begun to explore the effect of various hormones on the acquisition and expression of CD in discrete brain regions. For instance, non-selective CRH receptor antagonists as well as selective receptor antagonists for the type-II receptors block the expression of CD when administered centrally or within the bed nucleus of the stria terminalis (Jasnow et al., 1999; Jasnow et al., 2004; Cooper et al., 2005). Intra-amygdala infusions of a GABA receptor agonist reduce both the acquisition and expression of CD (Jasnow & Huhman, 2001). Blockade of NMDA receptors within the amygdala also reduces both the acquisition and expression of CD (Jasnow et al., 2004). Overexpression of CREB (a transcription factor important in mediating synaptic plasticity and memory) within the amygdala enhances the acquisition, but not the expression, of CD in male hamsters (Jasnow et al., 2005). We also have preliminary data that suggests that blockade of Trk receptors (receptors for neurotrophic factors important for synaptic plasticity and memory) within the amygdala decreases the acquisition but not the expression of CD in male hamsters (Lin & Huhman, unpublished observations). Further, blockade of serotonin receptors within the dorsal raphe reduces both the acquisition and expression of CD in male hamsters (Cooper & Huhman, in preparation). Based on these

findings from our laboratory, we have considerable knowledge concerning the role of various hormones and neurotransmitters in the regulation of CD in male hamsters; however, we do not know as much about the behavioral or hormonal responses to social defeat in female hamsters.

Conditioned Defeat and Female Hamsters

While social defeat appears to induce long-lasting changes in agonistic behavior in male hamsters as characterized by decrements in normal territorial aggression, this effect is not apparent in female hamsters. We have shown that following defeat only a small portion of female hamsters exhibit increases in submissive behavior in the presence of a NAI (Huhman et al., 2003; Solomon, 2003). Further, we have demonstrated that whereas approximately 70% of defeated male hamsters maintain CD for at least one month, all defeated female hamsters quickly regain normal territorial aggression by the second test encounter (Huhman et al., 2003). One possible mechanism that could explain this sex difference in the behavioral response to social defeat in Syrian hamsters might be differences in HPA-axis activation. Activation of this system has been associated with social defeat and we, (Huhman et al., 1992; Huhman et al., 1995) along with others (Pich et al., 1993; Buwalda et al., 1999) have shown that defeated males exhibit increases in ACTH levels. It has been hypothesized that ACTH feeds back to subsequently alter future behavior by increasing submissiveness in male rodents (Leshner & Politch, 1979; Roche & Leshner, 1979). Thus it is possible that female hamsters do not display similar increases in ACTH levels following defeat and this may account for the lower levels of submissive behavior observed in these females. Instead, we found

that following a defeat encounter, females display increased plasma ACTH levels (Huhman et al., 2003). Based on these findings, the HPA-axis does not appear to account for the sex differences in the display of CD in Syrian hamsters; on the other hand, gonadal hormones might play a role in these sex differences.

Gonadal hormones have been shown to influence the expression of aggression in both male and female Syrian hamsters, and it is likely that they modulate the display of submissive behavior in these animals, as well. We investigated the role of various hormones on the display of CD in female hamsters (Faruzzi et al., 2005). We found that females administered chronic administration of E_2 and T displayed significantly less submissive behavior following social defeat than did females given DHT, P or blank capsules. Because T, but not the non-aromatizable androgen DHT, decreased the display of submissive behavior in defeated female hamsters, we concluded that decreased submissive behavior in females treated with T was due to the aromatization of T into E_2.

Gonadal Hormones and Stress-Related Psychopathologies

Females are more likely to suffer from anxiety and depression (Earls, 1987). Because of this sex-related discrepancy in the development in these mood disorders, many scientists have investigated the role of gonadal hormones such as E_2 and P, in addition to neuroactive steroids such as allopregnanolone, in the etiology of these psychopathogies; however, this review focuses on the role of E_2 in anxiety and depression in female rodents. E_2 exert widespread effects on both the periphery as well as the brain and subsequently interact with a variety of systems, thereby, altering behavior and physiology. One system that appears to be under direct regulation of E_2, is the HPA-axis.

A key regulator of the HPA-axis is corticotropin releasing hormone (CRH). Briefly, CRH stimulates anterior pituitary corticotrophs which release ACTH into the circulatory system. Circulating ACTH regulates glucocorticoid secretion (corticosterone for many rodents, cortisol for hamsters and humans) from the adrenal cortex and catecholamines from the adrenal medulla (Kalantaridou et al., 2004). CRH has been implicated in the development of stress-related disorders like depression and notably there is an estrogen response element on the promoter region of the CRH gene (Vamvakopoulos & Chorous, 1993). These data suggest that CRH is under direct transcriptional regulation by E_2 and this might explain the increased incidences of stress-related psychopathologies in females. In accordance with this hypothesis, females with higher E_2 levels exhibit greater HPA-axis activity (as measured by elevated corticosterone) in comparison with females with lower levels (Ogle & Kitay, 1977; Rhodes et al., 2004; Handa et al., 1994; Viau & Meaney, 1991). Further, ovariectomy reduces corticosterone levels under basal and stress conditions in intact female rats (Seale et al., 2004). Although it is clear that E_2 have some effect on anxiety or depressive-like behavior in female rodents, the findings are varied in terms of whether this hormone is anxiolytic or anxiogenic. For example, some report that E_2 is anxiolytic (Leret et al., 1994; Frye et al., 2000; Bitran et al., 1993), while others report that it is anxiogenic (Diaz-Veliz et al., 1989; Diaz-Veliz et al., 1991: Morgan & Pfaff, 2001). These mixed findings might be in part due to differences in the behavioral assays or differences in hormonal regimens (i.e., acute or chronic).

Androgens, on the other hand, have been shown to have an inhibitory effect on the HPA-axis (Viau, 2002). Castrated male rats exhibit higher corticosterone levels under basal and stressed conditions in comparison with intact males (Seale et al., 2004).

In addition, intact male rats show reduced ACTH responses to acute restraint stress in comparison with castrated rats (Viau & Meaney, 1996). In general, androgens appear to be anxiolytic in male rodents. For example, it has been shown that castrated male rats have decreased activity in the open field (suggestive of increased anxiety-like behavior) and increased fear in response to shock in comparison with intact males (Frye & Seliga, 2001; Fernandez-Guasti & Martinez-Mota, 2003; Bitran et al., 1993). Administration of T to castrated males can increase activity in the open field and decrease fear in response to shock (Frye & Seliga, 2001; Fernandez-Guasti & Martinez-Mota, 2003). In another study, T administration reduced burying in the defensive burying behavior test in male rats (decreased burying is suggestive of decreased anxiety) (Fernandez-Guasti & Martinez-Mota, 2005). Further, King and colleagues (2005) found that long-term deficits of testosterone resulted in increased fear responses and fear-induced enhancement of analgesia in male rats. Taken together, these data suggest that the HPA and HPG axes may interact to produce differing effects with the onset of stress-related psychopathologies in females vs. males. Unfortunately, much of the work concerning the role of gonadal hormones on the development of stress-related disorders in rodents has been done in rats and mice, as a result there is considerably less work dedicated to this area in hamsters.

Neural Substrates Associated with Social Stress

Several approaches including immunocytochemistry have been used in order to determine what brain regions are associated with social stress. One commonly used tool to identify changes in brain activity in defeated animals is c-fos or its protein product c-

Fos. C-fos is an immediate early gene that is used as a marker for neuronal activity. A benefit of using this immediate early gene (IEG) is that it does not assume any particular neuronal phenotype, and because of this, c-Fos is used in combination with other immunocytochemical antibodies in order to determine the phenotype of neurons. (see Sundquist & Nisenbaum, 2005). Typically, most neurons express very little c-Fos activity, but stress can cause a rapid increase in Fos expression (Herman & Cullinan, 1992; Verbalis, 1991). For the purposes of this review, fos indicates mRNA, while Fos indicates the protein product.

Much work has been done concerning the hormonal correlates of stress; however, hormones exert their effects on the central nervous system within discrete brain regions to alter behavior and/or physiology in rodents and humans. The amydala, in particular, is a key neural region in the regulation of the HPA-axis and is involved in the expression of fear and anxiety in numerous species (Walker et al., 2003; Schulkin et al., 2005.). The amygdala is comprised of several subnuclei, including the central (CeA), medial (MeA) and basolateral complex (which includes the lateral, basolateral and basomedial nuclei) (Swanson & Petrovitch, 1998). The BLA receives sensory input from cortical and thalamic structures and projects to the CeA and bed nucleus of the stria terminalis (BNST) (Amaral & Inasausti, 1992). The BNST, although anatomically distinct from the CeA, is functionally similar to the CeA in that they project to similar brainstem and hypothalamic structures that are responsible for the neuroendocrine and behavioral responses to stress (Davis et al., 1994). The CeA is thought to be more involved with conditioned fear (i.e., stimulus specific), whereas the BNST is thought to be involved with anxiety or unconditioned fear (Davis & Shi, 1999).

Various studies including rats, mice and hamsters have been used in order to map brain activation in animals following an acute defeat encounter. Martinez and colleagues (1998) found that following an acute agonistic encounter, defeated male rats had significantly higher c-fos mRNA levels in lateral septum (LS), BNST, lateral preoptic area, paraventricular nucleus of the hypothalamus (PVN), medial amygdala (MeA) CeA and dorsal raphe nucleus in comparison with non-defeated controls, whereas in mice Matsuda et al., (1996) and Miczek et al., 1999 only found increased c-fos mRNA in the dorsomedial hypothalamic nucleus and the ventrolateral central gray, respectively. Acutely defeated male hamsters exhibited elevated c-fos mRNA expression in LS, BNST, medial preoptic area (MPOA), PVN, corticomedial amygdala, MeA, CeA, central gray and dorsal raphe nucleus in comparison with non-defeated controls (Kollack-Walker, 1997).

Other studies have also examined the effects of social stress on c-fos mRNA expression in animals that were acutely defeated versus those that were repeatedly defeated. Martinez et al., (1998) found that following chronic stress male rats continued to exhibit increased c-fos expression in the BNST, PVN and MeA, but no longer exhibited c-fos expression in the LH and CeA, suggesting habituation in certain areas. Chronically defeated male hamsters displayed decreased fos expression in certain areas like PVN, CeA and LS but continued to exhbit increased c-fos in the dorsal raphe and locus coeruleus (LC) (Kollack-Walker et al., 1999). On the other hand, male mice subjected to chronic social stress display increased c-fos expression in various regions including, but not limited to, the LS, hippocampus, MeA, BLA and dorsal raphe (Matsuda et al., 1996). These differences in patterns of neuronal activation in chronically

stressed animals may be related to the intensity and duration of the encounters as well as species differences.

With the exception of the Kollack-Walker study in 1997, the aforementioned studies failed to examine neuronal activity in the dominant animal. Examining c-fos expression in both the dominant as well as the subordinate animal allows one to differentiate between brain regions that are activated following agonistic interactions in general versus those that are activated only following defeat. The investigators in the Kollack-Walker (1997) study found that dominant male hamsters had significantly higher c-fos expression only in the supraoptic nucleus of the hypothalamus in comparison with subordinates and controls. The subordinates on the other hand, exhibited increased c-fos expression in brain regions more commonly associated with stress including the LS, BNST, CeA, dorsal periaqueductal gray, dorsal raphe and LC. Other studies that have examined c-Fos expression in dominants and controls have found that dominant animals exhibit elevated c-Fos expression in several brain regions, some of which include posterolateral BNST, medial part of the posterodorsal MeA, caudal part of the MPOA, and the dorsal periaqueductual gray (Veening et al., 2005). Gammie & Nelson (2001) found that aggressive mice had significantly greater c-Fos expression in the BNST, MPOA, PVN, cortical amygdala and MeA in comparison with non-aggressive mice. Further, Halasz et al, 2002, found that aggressive rats exhibited increased c-Fos expression in the LS, medial septum, BNST, anterior hypothalamus, MeA, and periaqueductal gray relative to controls. Some studies examining Fos expression in male hamsters following an aggressive encounter have found that male hamsters have increased Fos expression in the MeA, ventrolateral hypothalamus and the dorsalateral

periaqueductal gray or central gray (Delville et al., 2000), while others that have examined Fos expression in female hamsters following an aggressive encounter have found greater activation in the corticomedial amygdala (Potegal et al., 1996) or MeA and MPOA (Meisel et al., 1995).

Indeed, studies that have examined Fos expression in subordinates vs. controls or dominants vs. controls have revealed increased Fos activation in both groups relative to controls. In order to further distinguish between brain regions that are commonly activated during agonistic interactions or those regions that are activated due to social defeat, it is imperative to include dominants, subordinates and controls within the same study. Overall, the data suggest that many of the brain regions that are commonly associated with stress are activated in both dominant and subordinate animals. One common shortcoming in many of these studies is that they do not distinguish between subnuclei within a particular brain region. For example, many studies report increased activation within the BNST; however the BNST is comprised of several sub-regions which may be important for different behaviors. More specifically, the anterolateral BNST has been involved with activation of the HPA-axis (Gray et al., 1993; Herman et al., 1994), while the posterior medial BNST has been implicated in the regulation of mating behavior (Kollack-Walker & Newman, 1997; Fernandez-Fewell & Meredith, 1994). Collectively, these data suggest a need for more studies that include dominants, subordinates and controls within the same study, as well as greater specificity concerning exactly which subnuclei are assessed in a given brain area.

Scope of Thesis

As it stands, the majority of studies examining the hormonal and behavioral effects of social stress have included only male subjects. The data are lacking with respect to the effects of social stress in females, because few female rodents will typically display aggression. Social defeat is such a potent stressor in rodents, non-human primates and humans; therefore, investigating the underlying neuronal and hormonal mechanisms that are associated with this stressor may prove useful in elucidating the putative factors associated with the onset of stress-related psychopathologies.

Syrian hamsters are a useful species with which to study the sex differences in the behavioral responses to social defeat because both male and female hamsters will display high levels of aggressive behavior toward intruders. Following defeat, male hamsters display conditioned defeat which is characterized by a breakdown in normal territorial aggression and subsequent increase in submissive/defensive behavior in the presence of a smaller intruder conspecific. We believe that conditioned defeat is an ethologically relevant model for studying stress-related disorders. In fact, many of the same factors (i.e., CRH, GABA, 5HT) that are associated with depression and/or anxiety have been shown to be involved with either the acquisition or expression of conditioned defeat in male hamsters. Despite much of the knowledge that we have concerning the neural hormonal mechanisms underlying conditioned defeat in male hamsters, we know very little about the role of gonadal hormones in the regulation of conditioned defeat in male and female Syrian hamsters. Unlike males, defeated females typically do not display conditioned defeat; in fact, we have reported that the majority of defeated females maintain normal territorial aggression. One caveat of our initial study examining the

effects of social defeat in female hamsters is that we did not control for the phase of the estrous cycle. Gonadal hormones have been shown to modulate a number of behaviors including depression and anxiety, and it might be likely that we missed an important effect in these females. Similarly, we have not examined the effect of gonadal hormones on the display of conditioned defeat in male hamsters. It is established that gonadal hormones exert their effects within discrete brain regions, which might in part explain some sex differences in the response to certain stressful stimuli. Further, there have been several reports concerning the role of discrete brain regions including the LS, BNST, PVN and certain subnuclei of the amygdala in social defeat. Therefore, the overall goal of this thesis is to determine the effect of gonadal hormones on the display of conditioned defeat in male and female Syrian hamsters and to determine whether there are differences in patterns of neuronal activation (as measured through c-Fos immunoreactivity) between animals that display CD versus those that do not display CD.

Specific Aims

Specific Aim 1

Female Syrian hamsters are notoriously aggressive animals that will readily attack both male and female conspecifics (Payne & Swanson, 1970). Many studies have reported that aggression towards intruder conspecifics varies over the course of the estrous cycle in female hamsters (Takahashi & Lisk, 1983; Ciaccio et al., 1979; Takahashi & Lisk, 1984; Wise, 1974). Because aggression has been reported to change as a function of the estrous cycle it might also be likely that submissive behavior also changes over the estrous cycle as well. In an earlier unpublished study, we found that

proestrous females tested during the early part of proestrus exhibited conditioned defeat. Because the early part of proestrus is characterized by higher endogenous E_2 levels, we hypothesized that higher E_2 levels might be responsible for this increased submissive behavior following defeat. In addition, if circulating levels of gonadal hormones are responsible for the estrous cycle-related changes then proestrous females with higher endogenous levels of hormones should respond differently to social defeat than proestrous females with lower levels of these hormones. Therefore, the present study tested the hypothesis that the display of submissive behavior in defeated females is estrous-cycle dependent and that higher endogenous levels of E_2 would increase the display of submissive behavior in defeated females. Because P has been associated with a decrease in anxiety-like behavior in female rodents (Toufexis et al., 2004), we further tested the hypothesis that females with higher endogenous levels of P would be less likely to display CD than would females with lower endogenous levels of P.

Specific Aim 2

E_2 facilitate various forms of memory (i.e., fear) (Jasnow et al., 2005). In addition, it has been reported that E_2 has a stimulatory effect on the HPA-axis, which might explain sex differences in the prevalence of stress-related disorders (Viau & Meaney, 1991; Rhodes et al., 2004). We have previously examined the role of chronic hormone administration on the display of conditioned defeat in female hamsters, and we have found that females given chronic 4 week treatment of E_2 and T were less likely to display CD in comparison with females given DHT, P or blank capsules (Faruzzi et al., 2005). We believed that the increased length of individual housing (i.e., 4 weeks vs. 10

days) as well as potential changes in receptor sensitivity to gonadal hormones in females treated with chronic vs. acute hormones might result in differing effects in the behavioral responses to social defeat in female hamsters. Further, because the hormones were "on board" both during training and testing it was difficult to differentiate the importance of these hormones on the acquisition and/or expression of conditioned defeat. Therefore, the present study tested the hypothesis that acute E_2 administration would enhance the acquisition and expression of conditioned defeat in ovariectomized female hamsters. We tested this hypothesis by giving female hamsters systemic administration of varying doses of E_2 either before conditioned defeat training or before conditioned defeat testing.

Specific Aim 3

While E_2 has been shown to facilitate fear and anxiety-like behavior in females, androgens such as T and DHT are reported to be anxiolytic (Edinger & Frye, 2005; King et al., 2005; Fernandez-Guasti & Martinez-Mota, 2005). Despite much of the knowledge concerning the role of various neurotransmitters and hormones on the display of conditioned defeat in male hamsters, we do not know the role of gonadal hormones on the regulation of conditioned defeat. Given the fact that gonadal hormones are known to interact with other hormones (i.e., CRH) that we know are involved in the expression of conditioned defeat, it seems likely that they are also involved in conditioned defeat. If androgens are anxiolytic, then it might be likely that removal of these hormones through castration might result in enhanced conditioned defeat in male hamsters. The present studies tested the hypothesis that androgens would decrease the expression of conditioned defeat in male hamsters and that the length of individual housing might also influence the

display of conditioned defeat in male hamsters. Male hamsters were either castrated or sham-operated and individually housed for either 4 weeks or 10 days prior to behavioral training and on the following day were tested for conditioned defeat. In a final experiment, castrated male hamsters were treated with chronic administration of DHT, T and E and underwent defeat training and were later tested for conditioned defeat.

Specific Aim 4

Studies that have examined neuronal activity in male rodents following social stress report increased brain activity in discrete regions including the LS, BNST, PVN as well as several subnuclei within the amygdala (Kollack-Walker et al., 1997; Martinez et al., 1998). Mapping the brain areas that are associated with this phenomenon in both sexes may lead to a greater understanding of the neural circuitry that may be involved in the regulation of conditioned defeat in Syrian hamsters. Therefore the present study was designed to access neuronal activation in male and female hamsters following social defeat. We hypothesized that hamsters that display CD would exhibit increased neuronal activity localized to brain regions that are more commonly associated with stress-related behaviors; on the other hand, hamsters that do not display CD would not exhibit similar patterns of neuronal activity in these brain regions. We measured c-Fos activation in male and female hamsters in specific phases of the estrous cycle in order to assess if the sex difference in the behavioral response to social defeat might be due to differences in neuronal activation in the aforementioned brain regions.

Chapter 2

AGONISTIC BEHAVIOR VARIES OVER THE ESTROUS CYCLE IN PREVIOUSLY DEFEATED AND NON-DEFEATED FEMALE HAMSTERS

Abstract

We have reported that there is a sex difference in the behavioral response to social defeat in hamsters (Huhman et al., 2003). While previously defeated male hamsters fail to display normal territorial aggression and instead display submissive/defensive behavior in the presence of a non-aggressive intruder (NAI), a phenomenon that we have termed conditioned defeat (CD), only a small portion of previously defeated females exhibit CD in the presence of a NAI. It is not known if there is an effect of the estrous cycle on the display of CD in female hamsters. In Experiment 1, we tested the hypothesis that CD varies over the course of the estrous cycle and found that previously defeated female hamsters tested on diestrus 2 and proestrus were more likely to exhibit CD than were females tested on diestrus 1 and estrus. In Experiment 2, we found that regardless of hormonal status, non-defeated females failed to display any submissive behaviors in the presence of NAI and instead displayed normal territorial aggression, indicating that the cyclic effect on submission is dependent on a previous defeat encounter. In Experiment 3, male, diestrous 1 and proestrous female hamsters experienced social defeat and were tested 4 days later (to ensure that the females were trained and tested in the same hormonal state). All males exhibited CD four days after defeat training, whereas females trained and tested in diestrus 1 displayed normal territorial aggression in the presence of a NAI. Proestrous females displayed relatively low amounts of submissive behaviors, but they did not display normal territorial

aggression, suggesting that the previous defeat encounter did alter their behavior somewhat. Taken together, these data suggest that the display of submissive behavior in female hamsters is dependent on both the previous social experience as well as the hormonal status of the animal at the time of the behavioral testing.

Introduction

A variety of nonsocial stress models (e.g., restraint, footshock or swim stress) have been employed in order to elucidate the relationship between stress and subsequent behavioral responses. These models have contributed significantly to our understanding of the neurobiology of stress; however, they do not resemble natural stressors for most animals. Regardless of the type of stressor used, one goal of these studies is to gain a better understanding of the relationship between stress and the development of stress-related disorders in humans. Many laboratories utilize social stressors (i.e., social defeat) because they are deemed ethologically relevant stressors.

One of the more popular social stress models is the resident/intruder paradigm which has been studied extensively in a variety of species. This paradigm involves introducing an animal (intruder) into the home cage of another animal (resident), wherein the intruder is usually defeated. Defeated animals exhibit a myriad of responses including, but not limited to, suppressed immune function (Jasnow et al., 2001; Bailey et al., 2004; Engler et al., 2004), decreased food intake and body weight (Meerlo et al., 1996a), decreased exploratory behavior (Meerlo, et al., 1996b; Meerlo, 1997), as well as alterations in neurochemical systems such as the glutamatergic (Krugers et al., 1993), GABAergic (Miller et al., 1987), and serotonergic systems (Berton et al., 1999).

Syrian hamsters are a territorial species that, when singly housed under laboratory conditions, will readily attack an intruder (Nowack & Paradiso, 1983). However, after being defeated, male hamsters will subsequently fail to display normal territorial aggression even in the presence of a smaller, non-aggressive intruder (Potegal et al., 1993; Huhman et al., 2003). An animal is said to display conditioned defeat (CD) if it exhibits submissive and defensive behaviors and no aggressive behavior in the presence of a non-aggressive intruder. We have studied the neurobiology of CD in male hamsters (Jasnow & Huhman, 2001; Jasnow et al., 1999; Jasnow et al., 2004a; Jasnow et al., 2004b; Whitten et al., 2001; Cooper & Huhman, 2005) and we have shown that the effects of an initial defeat are profound and long lasting (Huhman et al., 2003). Although there are extensive data on the effects of social defeat in males, there is little information on the effect of social defeat in females.

Exploring sex differences in the behavioral response to social defeat may lead to a better understanding of how gonadal hormones may interact with the hypothalamic-pituitary-adrenal axis (HPA-axis) to produce subsequent changes in physiology and behavior. This is particularly important because clinical data indicate that women have a higher incidence of stress-related disorders such as anxiety or depression. Moreover, the occurrence of these disorders increases during periods of hormonal fluctuation as seen during the premenstrual or perimenopausal stages (Burt et al., 1998, Hsiao et al., 2004, McLeod et al., 1993; Soares and Cohen, 2001).

Studying sex differences in the response to social defeat has proven to be difficult in many rodents, particularly because females do not usually form dominate/subordinate relationships with their female counterparts. In fact, female rats and mice typically

display little aggression except in defense of pups (for review, see: Lonstein & Gammie, 2002) or when experimental manipulations, such as lesions are used to induce aggression (Haller et al., 1999). Furthermore, female rats and mice do not typically respond to defeat in the same manner as do males. For example, Haller and colleagues (1999) found that following defeat, male rats experienced decreased weight gain, adrenal hypertrophy and increased corticosterone secretion, whereas these same physiological changes were not found in defeated females. In contrast, because both male and female hamsters will spontaneously display aggressive behavior and they exhibit similar HPA-axis responsivity following a social defeat encounter (Huhman et al., 2003), hamsters present a unique opportunity to explore sex differences in the response to social defeat.

When not in behavioral estrus, female hamsters will readily attack a male or female conspecific; in fact, female hamsters often dominate their male opponents (Payne & Swanson, 1970; Brain, 1972). Several studies have investigated agonistic behavior in cycling female hamsters. All of these studies have focused on the role of ovarian hormones on aggressive behavior in non-defeated female hamsters (for review, see Albers et al., 2002). There have been conflicting results as to when non-defeated female hamsters exhibit higher levels of aggression with some studies reporting higher levels of aggression on diestrus 1 (Ciaccio et al., 1979), some reporting higher levels on proestrus (Wise, 1974), and still others reporting higher levels during the estrus phase of the cycle (Takahashi & Lisk, 1983). Thus, there are inconsistencies among studies and all of these published studies have neglected to examine agonistic behavior other than offensive aggression (i.e., submission and defense).

Recently, we reported that only a small number of females experiencing social defeat subsequently display submissive behavior and even then, the behavioral change is not as long lasting as it is in males (Huhman et al., 2003). Because some females exhibited CD while others displayed aggressive behavior and the animals were tested on different days of the estrous cycle, we hypothesized that the different behavioral responses to social defeat were the result of varying hormonal status on the day of the behavioral training and/or testing. In Experiment 1 we asked: **Is there an estrous-cycle dependent effect on the display of submissive behavior in previously defeated female hamsters?** It is known that aggression varies over the cycle but it is not known whether submissive behavior also varies over the estrous cycle in non-defeated females. In Experiment 2 we asked**: Is there an estrous-cycle dependent effect on the display of submissive behavior in non-defeated female hamsters?** Finally, in Experiment 1 females were trained and tested in different phases of the hormonal cycle making it difficult to determine if the increase in submissive behavior seen in some previously defeated females was attributable to the hormonal status of the female at the time of training or at the time of testing. Therefore, in Experiment 3 we asked**: Do females that are trained and tested during the same phase of the estrous cycle behave similarly to females that are trained and tested during different days of the estrous cycle?**

Materials and Methods

Animals and Housing Conditions

Adult female and male Syrian hamsters (*Mesocricetus auratus*) weighing 120-130g (10 weeks old) at the beginning of the experiment were obtained from Charles

River Laboratories. Hamsters were housed individually for two weeks in a temperature-controlled colony room on a 14:10 hr light: dark cycle with lights off at 0800 h. Additional singly-housed male and ovariectomized female hamsters weighing 150-190 g (> 6 months old) were used as resident aggressors during defeat training. Intact group-housed females and male hamsters (five per cage) weighing 100-110 g (7 weeks old) were used as non-aggressive intruders (NAI) during conditioned defeat testing. Previous data from our laboratory have shown that there are no differences in the display of agonistic behavior in female hamsters that are paired with intact versus ovariectomized female stimulus hamsters during conditioned defeat testing (Huhman *et al*., 2003). All animals were housed in polycarbonate cages (20x40x20 cm) with wire mesh tops, corn cob bedding and cotton nesting materials. Food and water were available ad libitum. All procedures and protocols were approved by the Georgia State University Institutional Animal Care and Use Committee.

Determination of Estrous Cycle

Female hamsters have a consistent four-day ovulatory cycle. At least two weeks prior to behavioral testing all experimental animals were monitored daily between 0800 and 0900h for determination of estrous cycle, and only females that had consistent four day estrous cycles were used in the study. Briefly a cotton swab was placed against the vaginal area. A thin, stringy vaginal discharge signified vaginal estrus (estrus) and the period of sexual receptivity (Wise, 1974), with subsequent days being defined as diestrus 1, diestrus 2, and proestrus.

Conditioned Defeat Training

On the day of training, female hamsters were transported in their home cages from the colony room to the behavioral testing room where they were placed into the home cage of a resident aggressor for four, five-minute training trials. The females were paired with a different aggressor for each training trial. Training began at 0800 h with 2 h intervals between each 5 minute training trial. During the behavioral training session, experienced observers ensured that all experimental animals were attacked by the aggressors and that all of the experimental animals displayed submissive/defensive behavior and no aggressive behavior toward any of the resident aggressors.

Conditioned Defeat Testing

All testing for conditioned defeat was completed during the first 2 h of the dark phase of the LD cycle. A resident/intruder pairing was used in which a NAI was placed into the home cage of each experimental animal for 5 minutes. Female NAIs were screened to ensure that they did not display lordosis and then they were randomly paired with the experimental animals. Videotapes of the testing sessions were scored using the Observer for Windows, version 3.0 (Noldus Information Technology B.V., Wageningen, The Netherlands). The following classes of behaviors were recorded as total duration in seconds. 1) Non-social: locomotor/exploratory, self-groom, nesting, feeding, pouching, sleeping, lordosis; 2) Social: attend, approach, investigate, sniff, touching nose; 3) Submissive/Defensive: upright and side defense, tail lift, flee, full submissive posture; 4) Aggressive: upright and side offense, chase, attack, bite. The videotapes were scored by

two experienced observers that were blind to the experimental condition of each hamster. Inter-rater reliability was greater than 90%.

Experiment 1:

Estrous (n=9), diestrous 1 (n=9), diestrous 2 (n=15), and proestrous (n=8) females were defeated four times for five minutes by a larger resident aggressor (CD training) in the aggressor's home cage. Twenty-four hours following training, females were paired for five minutes with a NAI (CD testing) in their own home cage. Thus, groups of females were trained in either diestrus 1, diestrus 2, proestrus, and estrus and subsequently tested in diestrus 2, proestrus, estrus, and diestrus 1, respectively.

Experiment 2:

Non-defeated female hamsters in estrous (n=8), diestrous 1 (n=9), diestrous 2 (n=9), proestrous (n=8) were paired for five minutes with a NAI in their own home cage.

Experiment 3:

Diestrous 1 (n=4), proestrous (n=5) and male (n=5) hamsters were defeated four times for five minutes by a larger resident aggressor (CD training) in the aggressor's home cage. Four days later, hamsters were paired in their own home cage for five minutes with a NAI (CD testing). Thus, females were trained in either diestrus 1 or proestrus and later tested in that same state of the estrous cycle. The behavioral procedures for these experiments are depicted in Figure 1.1.

Hormonal Assays

Immediately after the final agonistic encounter in Experiment 3, trunk blood was collected from the animal after rapid decapitation. Blood was collected and refrigerated

overnight. Samples were spun in a refrigerated centrifuge, and serum aliquots were aspirated and stored in polypropylene microcentrifuge tubes at -20˚C until measured by radioimmunoassay. Circulating levels of estradiol and progesterone were measured using a commercially prepared kit from Diagnostic Systems Laboratories, Webster TX (DSL 4300 for estradiol and DSL 3900 for progesterone) as described in (Faruzzi et al., 2005). Both assays were validated with hamster serum. Intraassay reliability was 3% for estradiol and 2% for progesterone and all samples were run in a single assay for each hormone.

Statistical Analyses

Normally distributed behavioral data (duration of aggressive, submissive/defensive, social and nonsocial behaviors in sec) for Experiments 1, 2 and 3 were analyzed using a between subjects MANOVA with day of the estrous cycle as the between-subjects factor. Statistically significant differences were furthered analyzed using Tukey-Kramer multiple comparison post hoc test to compare group differences. Aggression scores in Experiment 2 were non-normally distributed, thus the assumption of homogeneity of variance (Levene's Test for Equality of Variances) was violated. In this case, a Kruskal-Wallis test was used to compare total duration of aggressive behavior by phase of the estrous cycle. Individual group differences were then determined using Mann-Whitney U (two-tailed) test. Hormonal data for estradiol and progesterone were analyzed using independent sample t-tests. Statistical significance for all analyses was ascribed at $p<0.05$.

Results

In Experiment 1, there was a significant difference in the display of submissive behavior in previously defeated females depending upon the day of the estrous cycle ($F(3,37)= 7.11$, $p<0.01$). Specifically, diestrous 2 and proestrous females displayed significantly more submissive behavior than did diestrous 1 and estrous females. There were also significant differences in the display of aggressive behavior in previously defeated females ($F(3,37)=7.27$, $p <0.01$). Previously defeated females tested on diestrus 1 displayed significantly more aggressive behavior in the presence of a NAI than did previously defeated females tested on all other days. Moreover, there was a significant difference in nonsocial behavior ($F(3,37)=11.39$, $p<0.01$) with defeated females tested on estrus displaying significantly more nonsocial behavior in comparison to all other groups. It is important to note that these females spent a significant portion of their time displaying lordosis in the presence of the NAI, despite the fact that they were not approached by the NAI (causing us to consider this behavior "nonsocial"). There was no significant difference in social behavior among any of the groups ($F(3,37)= 1.61$, $p >0.05$). The results of Experiment 1 are summarized in Fig. 1.2.

In Experiment 2, there was no significant difference in the display of submissive behavior of non-defeated female hamsters in the presence of a NAI ($F(3,30)=.64$, $p > 0.05$). Regardless of the day of the estrous cycle, non-defeated female hamsters did not display submissive behavior. A Kruskal –Wallis test revealed a significant difference in aggressive behavior among non-defeated female hamsters across the day of the cycle (χ^2 (4, $N=34)=21.35$, $p<0.01$), with non-defeated females tested on diestrus 1 exhibiting more aggressive behavior than those tested on diestrus 2 and estrus. There was no

significant difference in the display of social behavior among any of the groups regardless of the phase of the estrous cycle ($F(3,30)=.65$, $p > 0.05$). Lastly, there was a significant difference in the display of nonsocial behavior among groups ($F(3,30)=14.61$, $p<0.05$) with females in estrus displaying significantly more nonsocial behavior, namely lordosis, in comparison to all other groups. The results of Experiment 2 are summarized in Fig. 1.3.

In Experiment 3, there was a significant difference in the display of submissive behavior in previously defeated hamsters when tested 4 days after CD training ($F(2,11)=14.30$, $p<0.01$). Previously defeated male hamsters displayed significantly more submissive behavior in the presence of a NAI than did proestrous and diestrous 1 female hamsters. Although proestrous females had a higher mean of submissive behaviors relative to diestrous 1 females, there was not a statistically significant difference between these two groups. ANOVA revealed a significant difference in aggressive behavior ($F(2,11)=47.61$,$p<0.01$), with diestrous 1 females displaying significantly more aggressive behavior than did proestrous females and male hamsters. Both defeated proestrus females and male hamsters displayed similarly low levels of aggressive behavior in the presence of a NAI. Furthermore, there was a significant difference in social behavior among groups ($F(2,11)=6.47$, $p<0.05$). Diestrous 1 females were more social compared with proestrous females and male hamsters. However, there were no differences in social behaviors between proestrous females and male hamsters. In addition, there was a significant difference in nonsocial behavior ($F(2,11)=29.20$,$p<0.01$), with defeated diestrous 1 females displaying significantly less nonsocial behavior than did proestrous females and male hamsters and proestrous

females displaying significantly higher levels of nonsocial behavior in comparison with males. Moreover, previously defeated males displayed significantly less nonsocial behaviors relative to proestrous females. The behavioral findings in Experiment 3 are summarized in Fig. 1.4. Defeated proestrous females had significantly higher estradiol levels relative to diestrous 1 females $t(7) = -4.163$, $p<0.05$. Finally, there was a trend towards previously defeated diestrous 1 females having higher progesterone levels relative to previously defeated proestrous females $t(7) = 2.25$, $p=.059$. The hormonal results for Experiment 3 are summarized in Figs. 1.5a and 1.5b.

Discussion

Previously, we have shown that only a small portion of defeated females display CD (Huhman et al., 2003); here, we extend that finding to demonstrate that females in some phases of the estrous cycle will display CD. The results of the present study support our hypothesis that the display of conditioned defeat (CD) varies with the ovulatory cycle. In Experiment 1, previously defeated female hamsters tested on diestrus 2 and proestrus were significantly more likely to show increased submissive behavior in the presence of a NAI than were female hamsters tested on diestrus I and estrus. In Experiment 2, non-defeated females (excluding estrous female hamsters) regardless of the day of the estrous cycle, did not display any submissive behaviors and instead displayed normal territorial aggression in the presence of a NAI. This suggests that the variation in submissive behavior observed in defeated hamsters in Experiment 1 is not due to an underlying cyclic variation in submissive behavior in non-defeated female hamsters. In Experiment 3, male and female hamsters were trained and then tested four

days later in the presence of a NAI. This timing was used to ensure that all females were trained and tested within the same phase of the estrous cycle. In this experiment, the males served as a positive control to indicate the degree of conditioned defeat that could be expected in males four days after defeat training. All of the defeated male hamsters displayed high levels of submissive behavior at testing. By contrast, females trained and tested on diestrus 1 did not display any submissive behavior but instead displayed normal territorial aggression. Females trained and tested in proestrus displayed low levels of submissive behavior relative to males, but they also did not display normal territorial aggression, suggesting that the previous defeat encounter did alter agonistic behavior in these females.

The data from the current study indicate that circulating gonadal steroids may alter submissive behavior in female hamsters depending on their social experience. In Experiment 1, previously defeated females that were trained on diestrus 1 and tested on diestrus 2 and trained on diestrus 2 and tested on Proestrus were more likely to exhibit CD. In the diestrous 2 and proestrous phases of the ovarian cycle, female hamsters have similar, rising estradiol levels (Saidapur & Greenwald, 1978). Our experimental manipulations during proestrus occured before the afternoon proestrus peak in estradiol levels (Solomon & Huhman, unpublished observations). Therefore, it is possible that the similar behavioral response (i.e., increased submissive behavior) seen in some diestrous 2 and proestrous females is due to similar, rising estradiol levels. Furthermore, previously defeated females tested during diestrus 1 displayed no CD and instead displayed normal territorial aggression while previously defeated females tested during estrus displayed neither aggression nor submission but instead displayed lordosis. Thus, a previous defeat

encounter did not produce subsequent alterations in agonistic behavior in female hamsters tested in diestrus 1 and estrus. Taken together, these data suggest that the defeat-induced changes in agonistic behavior in female hamsters are estrous-cycle dependent.

One might argue that the variation in the display of behavior seen during testing in previously defeated females may reflect differences in the defeat training; that is, females in some phases of the hormonal cycle might be attacked differently than females during other phases of the cycle. Although we did not videotape the defeat training, we did carefully observe the initial defeats, and it appeared that all females were similarly defeated. During the defeat training, resident aggressors routinely attacked the subjects regardless of the phase of the ovarian cycle. All of the experimental animals displayed high amounts of submissive behavior and none displayed aggressive behavior toward the resident aggressors. In later experiments, we have analyzed the behavior of the resident aggressors towards females in various phases of the cycle and have found that there are no differences in aggressive, submissive, social or nonsocial behaviors exhibited by the resident aggressors (Solomon & Huhman, in preparation). These findings imply that the difference in submissive behavior among hormonal groups seen during testing is not due to differences in the resident aggressor's behavior toward the experimental animal during the defeat training.

One difficulty in interpretation of the data in Experiment 1 is the fact that females were defeated and tested in different hormonal states. Therefore, it is impossible to determine whether it is the hormonal state in which they are defeated or in which they are tested, or both, that accounts for the behavioral changes. To address this issue in

Experiment 3, we trained females in diestrus 1 or proestrus and waited 4 days to test them. We found that females trained and tested in diestrus 1 displayed no submissive behavior but instead displayed normal territorial aggression in the presence of a NAI. On the other hand, females trained and tested in proestrus displayed some submissive behaviors and did not display normal territorial aggression. Consistent with our previous findings (Huhman et al., 2003), the previously defeated male hamsters maintain CD. Thus, social defeat altered subsequent agonistic behavior only in previously defeated male hamsters and proestrous female hamsters.

Based on these findings, it appears that the display of agonistic behavior in females is likely due to a combination of their social history and the hormonal status of the female at the time of testing. Two findings suggest that the hormonal status of the female at the time of testing and not at training is the more important determinant of behavior. First, females trained in estrus and later tested in diestrus 1 behave similarly (i.e., display no submissive behavior and normal aggressive behavior) to females trained and tested in diestrus 1. Next, females trained in diestrus 2 and later tested in proestrus behave similarly to females trained and tested in proestrus (i.e., display some submissive behavior and no aggressive behavior). If the hormonal status of the female at the time of training was more important, then females defeated during estrus and subsequently tested in diestrus 1 would not behave similarly to those defeated in diestrus 1 and later tested in diestrus 1. Our previous study (Huhman et al., 2003) demonstrated that females do not maintain CD for the same length of time as do male hamsters; in fact by the second behavioral testing session the majority of previously defeated females do not display CD

and instead display normal territorial aggression. Thus, the rapid dissipation of CD in females makes this question difficult to answer in intact females.

The hormonal data obtained in Experiment 3 suggests a role for gonadal hormones in the display of CD in female hamsters. Although there was no difference in submissive behavior between defeated proestrous and diestrous 1 females, there was a significant difference in avoidance behavior between these groups. Proestrous females spent more time avoiding the intruder conspecific, while diestrous 1 females displayed normal territorial aggression. Defeated, proestrous females had significantly higher levels of estradiol in comparison with diestrous 1 females, suggesting that higher levels of estradiol in these females may be responsible for increased avoidance behavior and decreased aggressive behavior. These findings are incongruent with a recent study from our laboratory. Previously, we found that females given chronic treatment of estradiol or testosterone were significantly less likely to display submissive behavior following social defeat in comparison with females given chronic progesterone, dihydrotestosterone, or blank capsules (Faruzzi et al., 2005). From this study, we concluded that estrogen may be an important hormonal factor in the reduction of submissive behavior in defeated female hamsters. There are important methodological differences between the past and current studies. In our previous study, females were ovariectomized and given chronic (4 week) treatment of various hormones. In the current study, we examined cycling females. These inconsistencies in the data suggest that gonadal hormones may produce varying, even opposite effects on behavior depending on the hormonal condition of the animal (i.e., intact vs. chronically hormonally replaced). Future studies will investigate the role of acute hormone replacement on the display of CD in female hamsters.

Non-defeated female hamsters, when not in behavioral estrus, consistently displayed territorial aggression. This finding is consistent with past studies which all report high levels of aggressive behavior in non-defeated female hamsters across the estrous cycle (Wise, 1974; Ciaccio et al., 1979; Takahashi & Lisk, 1983; Takahashi & Lisk, 1984). There was no cyclic variation in submissive behavior in non-defeated female hamsters. Thus, the increase in submission observed on diestrus 2 and proestrus in Experiment 1 does not appear to be due to an underlying increase in submissive behavior across the estrous cycle in non-defeated females. Overall, these findings demonstrate both a sex and estrous cycle difference in the display of conditioned defeat in Syrian hamsters and warrants future studies aimed at determining the role of gonadal hormones on this phenomenon in both male and female Syrian hamsters.

Figure 1.1. Schematic design for Experiments 1, 2 and 3. (**A**) In Experiment 1, females in determined stages of the estrous cycle were trained (paired with resident aggressor) and then tested the next day (paired with non-aggressive intruder (NAI). (**B**) In Experiment 2, non-defeated females in determined stages of the estrous cycle were tested with a non-aggressive intruder (NAI). (**C**) In Experiment 3, males and females in determined stages of the estrous cycle were trained and then tested 4 days later with a NAI.

Figure 1.2. Average duration (mean +/-SEM in seconds) of submissive/defensive, aggressive, social and nonsocial behavior in previously defeated female hamsters tested in diestrus 1(n=9), diestrus 2(n=9), proestrus (n=15), and estrus(n=8) phases of the cycle with a non-aggressive intruder (CD Testing). Analyses were run for each specific behavior; therefore, differences are compared only within one category of behavior and not between behavioral categories. Non-shared letters depict significant differences ($p<0.05$) as determined by Tukey post hoc analyses.

Figure 1.3. Average duration (mean +/-SEM in seconds) of submissive/defensive, aggressive, social and nonsocial behavior in non-defeated female hamsters tested in diestrus 1(n=9), diestrus 2(n=9), proestrus(n=8) , and estrus(n=8) phases of the cycle during a test encounter with a non-aggressive intruder. Analyses were run for each specific behavior; therefore, differences are compared only within one category of

behavior and not between behavioral categories. Non-shared letters depict significant differences ($p<0.05$) as determined by Tukey post hoc analyses for nonsocial behavior, significant differences for aggressive behavior were further analyzed using a Mann-Whitney U test.

Figure 1.4. Average duration (mean +/-SEM in seconds) of submissive/defensive, aggressive, social and nonsocial behavior in defeated female hamsters in diestrus 1 (n=4) and proestrus (n=5) and male hamsters (n=5) tested with a non-aggressive intruder four days after an initial defeat. Analyses were run for each specific behavior; therefore, differences are compared only within one category of behavior and not between behavioral categories. Non-shared letters depict significant differences ($p<0.05$) as determined by Tukey post hoc analyses.

Figure 1.5 (**a**) Serum levels of estradiol and progesterone (**b**) in defeated diestrous 1 and proestrous female hamsters in Experiment 3 following a subsequent test with a non-aggressive intruder (CD Testing). Data are presented as (mean +/-SEM). * indicates statistical significance, $p<0.05$.

1.1

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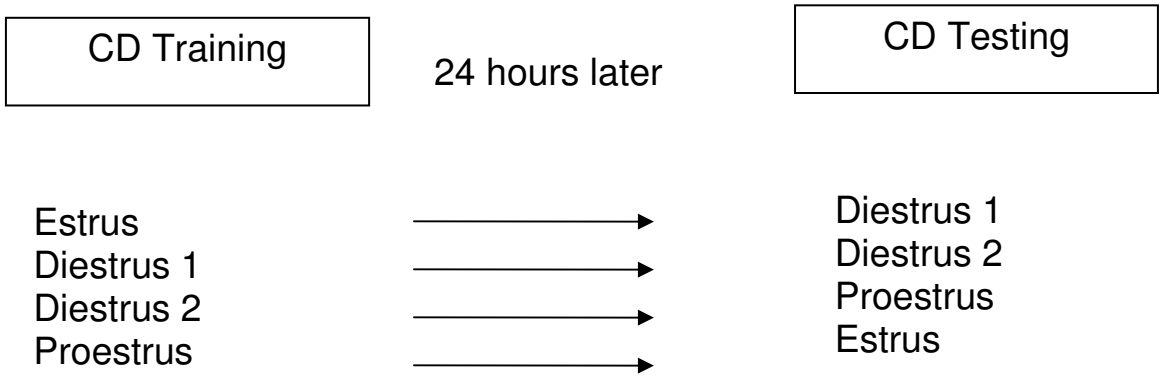
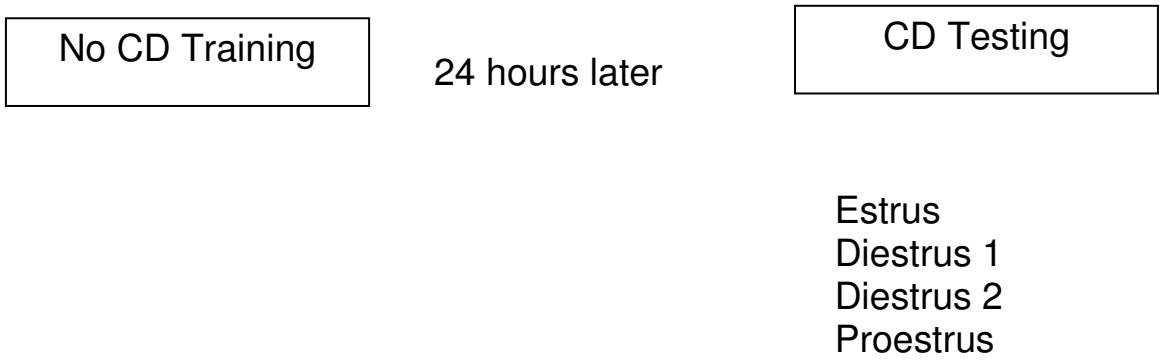
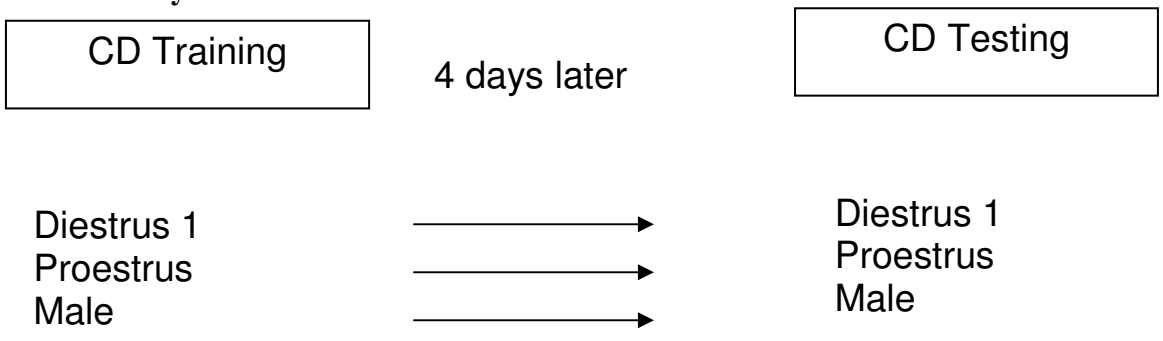


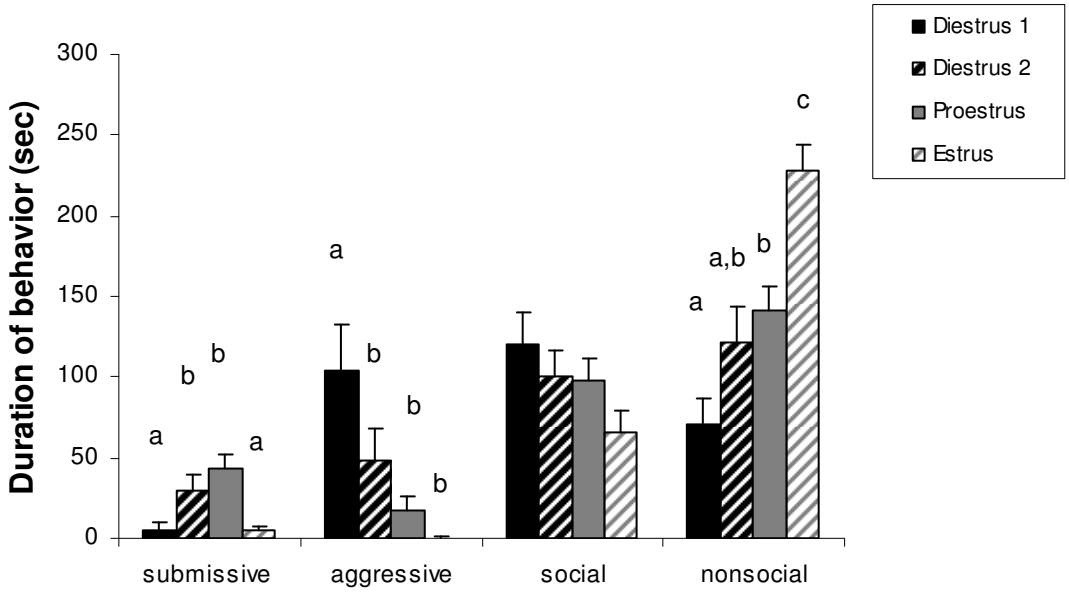
B. Non-Defeated Females



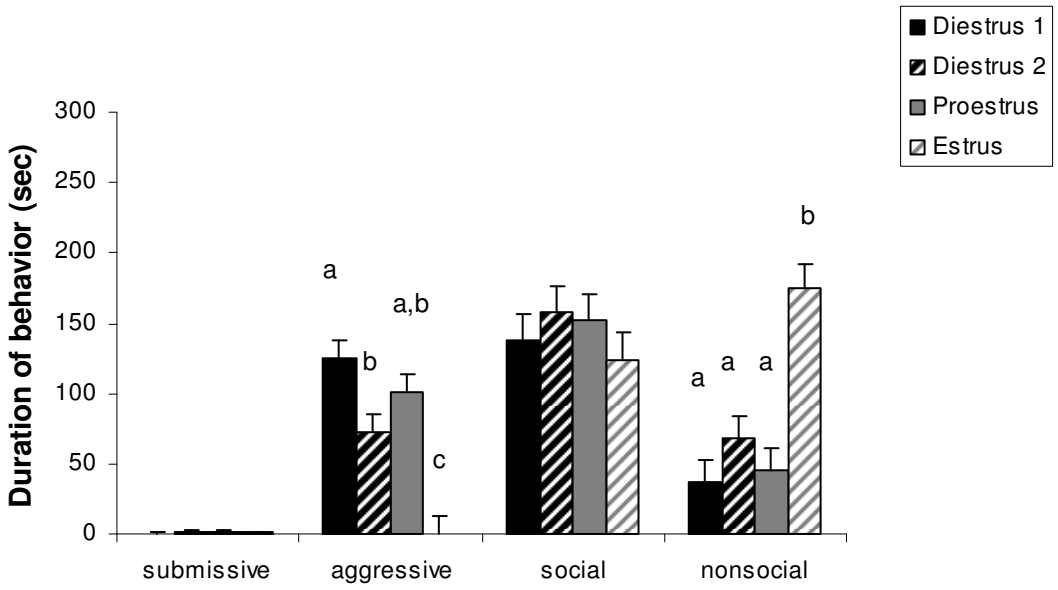
C. Previously Defeated Females and Males



1.2


Diestrus 1
Diestrus 2
Proestrus
Estrus
Duration of behavior (sec)
300
250
200
150
100
50
0
a
b
b
a
a
b
b
b
a
a,b
b
c
submissive
aggressive
social
nonsocial

1.3


Diestrus 1
Diestrus 2
Proestrus
Estrus
Duration of behavior (sec)
300
250
200
150
100
50
0
a
b
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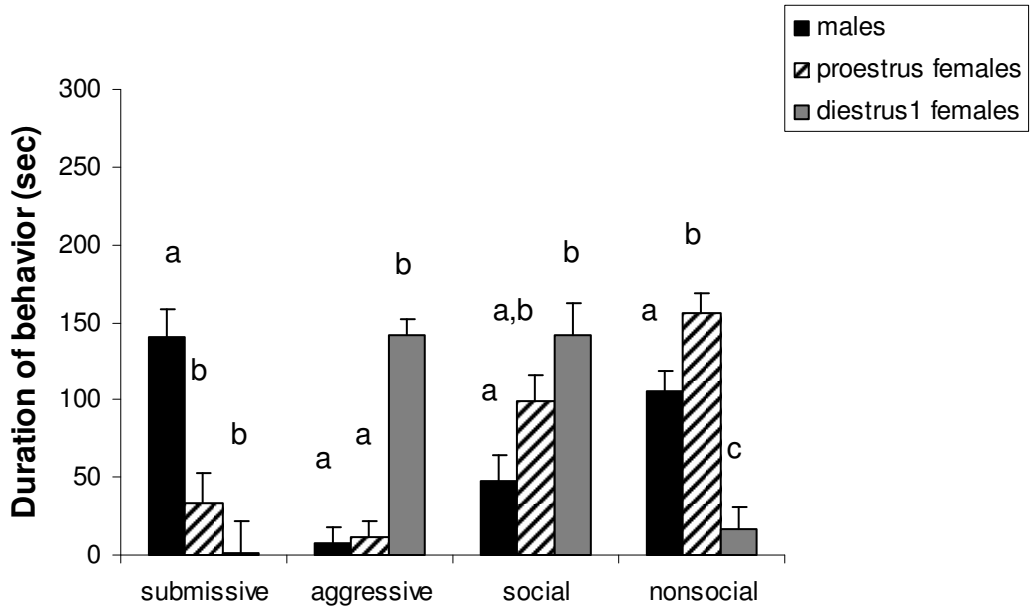
c
a
a
a
b
submissive
aggressive
social
nonsocial

1.4


males
proestrus females
diestrus1 females
Duration of behavior (sec)
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250
200
150
100
50
0
a
b
b
a
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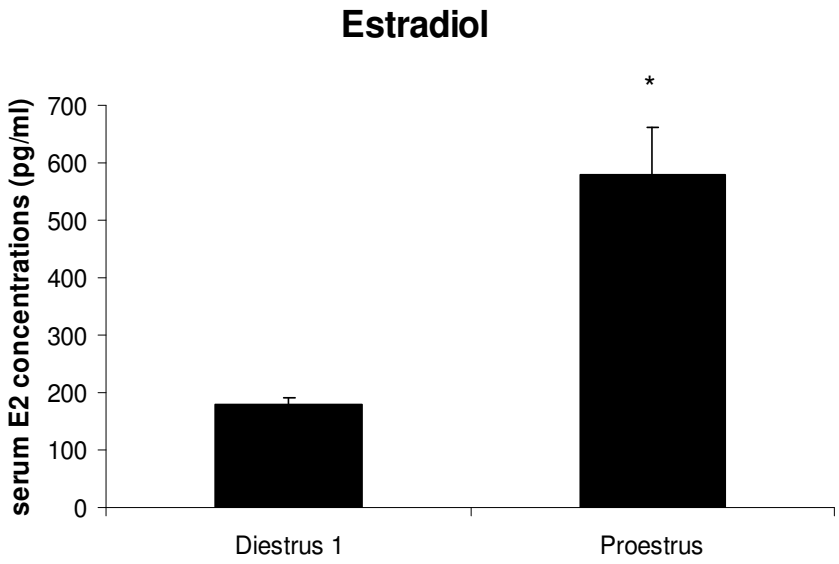
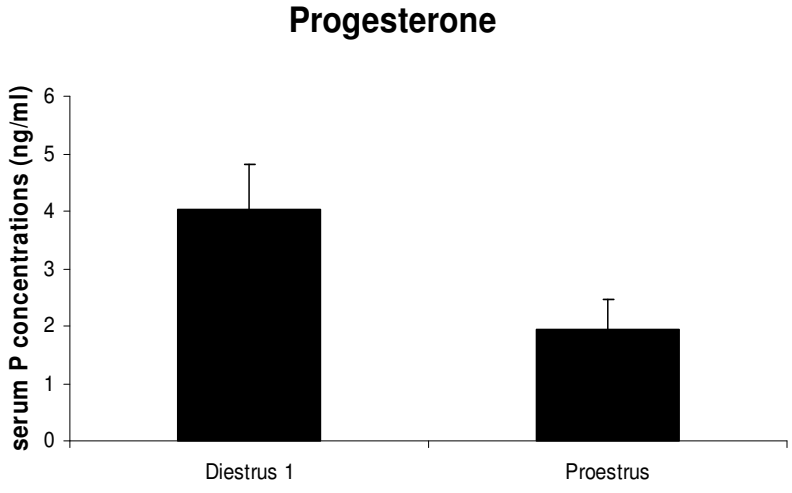
b
c
submissive
aggressive
social
nonsocial

1.5a


Estradiol
serum E2 concentrations (pg/ml)
700
600
500
400
300
200
100
0
*
Diestrus 1
Proestrus

1.5b


Progesterone
serum P concentrations (ng/ml)
6
5
4
3
2
1
0
Diestrus 1
Proestrus

Chapter 3

BEHAVIORAL AND HORMONAL PROFILES IN DEFEATED PROESTROUS FEMALE HAMSTERS: A TIME COURSE STUDY

Abstract

Previously, we have shown that there is an estrous-cycle dependent effect on the display of conditioned defeat in female hamsters. More specifically, following social defeat, proestrous females no longer display normal territorial aggression and instead display submissive/defensive behavior in the presence of a non-aggressive intruder. On the other hand, diestrous 1 females continue to exhibit territorial aggression and do not display submissive behavior, despite a previous defeat. We hypothesized that the differences in the behavioral response to social defeat between proestrous and diestrous 1 females may be due to differences in concentrations of circulating estrogen (E_2) and progesterone (P). During the proestrous phase of the ovulatory cycle, there are marked fluctuations in circulating concentrations of both E_2 and P, and these fluctuations in hormones may in turn influence behavioral responses to social defeat. Therefore, the purpose of the present study was to determine whether there were differences in the behavioral response to social defeat in proestrous females at specific time points of the Light/Dark (LD) cycle correlated with differing levels of E_2 and P. All defeated proestrous females, regardless of the time of testing, displayed CD. There were no differences in serum E_2 levels among any of the groups; however, there were we significant differences in P levels among groups. Thus, the time window chosen was not significantly long to observe a marked drop in E_2 in order to test the hypothesis that

submissiveness would vary with changes in E_2. The present data do suggest that changes in P are not associated with changes in agonistic behavior following defeat, however.

Introduction

Social defeat is a potent stressor known to provoke changes in both physiology and behavior in a number of species (for review, see: Tamashiro et al., 2005). It has been established that exposure to prolonged stressors is associated with an increased likelihood to develop certain stress-related psychopathologies such as anxiety disorders and depression (Meyer et al., 2001; Makino et al., 2002; Nemeroff, 1998). Clinical data indicate that females are more likely to suffer from these stress-related psychopathologies (Kessler & Walters, 1998; Earls, 1987). Furthermore, these disorders are more apparent during periods of marked hormonal fluctuation (i.e., premenstrual and postpartum) (Burt et al., 1998; Soares & Cohen, 2001), thus it has been suggested that estrogens (E_2) and progesterone (P) may play a role in the etiology of these disorders.

Ovarian hormones (i.e., E_2 and P) enter the general circulatory system and exert widespread effects on target areas including the brain and pituitary, which in turn coordinate many aspects of reproductive behavior. Female rodents have a 4-5 day estrous cycle that may be likened to the female human ovarian cycle. The days of the estrous cycle are characterized relative to their levels of circulating gonadal hormones and to the day of ovulation. The proestrous phase of the cycle is often referred to as the most eventful day of the estrous cycle, as the hormonal milieu necessary for ovulation occurs on this day. The afternoon of proestrus is characterized by high E_2 and steadily rising P levels, whereas the late evening" of proestrus is characterized by high P and falling E_2 levels (Saidapur & Greenwald, 1978; Saidapur & Greenwald, 1979). Because

E_2 and P reach their peak at different points during proestrus, the time point at which one measures behavior and/or hormones is critical.

There is a general agreement that E_2 and P influence the display of anxiety and depressive-like behaviors in female rodents; however, the direction and magnitude of these hormonally-induced influences are mixed. For example, some preclinical data indicate that exogenous administration of E_2 and P produces anxiolytic or antidepressant-like effects in rodents (Leret et al., 1994; Nomikos & Spyraki, 1998; Frye & Walf, 2004; Frye et al., 2000; Bitran et al., 1993), while others report that administration of E_2 produces anxiogenic-like effects on behavior (Diaz-Veliz et al., 1989; Diaz-Veliz et al., 1991; Morgan & Pfaff, 2001; Morgan & Pfaff, 2002). Some of the discrepancies may be due to differences in the timing of hormone administration (i.e., acute vs. chronic) or the hormonal dosage. Although several laboratories report a decrease in anxiety-like behavior in proestrous female rodents on several behavioral indices of anxiety, there is some inconsistency as to which gonadal hormone may be responsible for this reduction. Some studies report that the anxiolytic behavior observed in proestrous female rodents are due to increased E_2 (Mora et al., 1996; Marcondes et al., 2001; Diaz-Veliz et al., 1997); while others report that the anxiolytic effects seen in proestrous female rodents are due, at least in part, to increased P and/or its metabolites possibly through their interaction with the $GABA_A$ receptor (Zuluaga et al., 2005; Zimmerberg et al., 2005; Frye et al., 2000).

A preclinical model of potential value for studying the neurobiological basis of stress-related disorders such as anxiety or depression is conditioned defeat (CD). This phenomenon occurs after a hamster has experienced social defeat. As a result of the

previous stressful episode, the defeated hamster no longer displays normal territorial aggression and instead displays increased submissive behavior, even in the presence of a smaller, non-aggresssive intruder. The behavioral response to social defeat in males is profound and long-lasting (Huhman et al., 2003). Defeated males typically display behaviors that are suggestive of anxiety or depression. For example, defeated male rodents display decreased locomotor behavior (Meerlo et al., 1997), decreased social interaction (Potegal et al., 1993; Siegfried et al., 1984), anhedonia (Rygula et al., 2005) as well as decreases in food intake and body mass (Meerlo et al., 1996) in comparison with their non-defeated counterparts.

Recently, we have been exploring the behavioral responses to social defeat in female hamsters. We have demonstrated that defeated females tested in diestrus 1 (a period characterized by relatively low levels of E_2 and moderate P levels) do not display CD, whereas previously defeated females tested in the proestrous phase of the cycle (a period characterized by rapidly changing levels of E_2 and P) will typically display CD (Solomon & Huhman, in preparation). We hypothesized that the observed behavioral differences following social defeat between diestrous 1 and proestrous females may be due to differences in concentrations of E_2 and P. More specifically, we hypothesized that high E_2 levels and low but rising, P levels in defeated proestrous females may result in increased submissive behavior and a decrease in aggressive behavior during CD testing. Likewise, the decreased E_2 levels and higher P levels in diestrous 1 females in comparison with proestrous females, may result in decreased submissive behavior and a subsequent increase in aggressive behavior. Although, not commonly reported there are

reports of two peaks in P levels, one during early diestrus 1 and again in late proestrus (Figueiredo et al., 2002; Smith et al., 1975).

As mentioned previously, there are marked variations in circulating concentrations of both E_2 and P throughout the day of proestrus. With this in mind, and based on the experimental design of a previous study, (Saidapur & Greenwald, 1978) we decided to test female hamsters throughout the day of proestrus at time points that would be characterized by relatively high and low levels of these hormones. If the behavioral differences observed following social defeat in female hamsters were the result of differences in hormone concentrations, then females in the proestrous phase of the cycle should behave differently during CD testing as the gonadal hormones change. We hypothesized that proestrous females with higher levels of E_2 would be more likely to display CD than would proestrous females with lower levels of E_2 and proestrous females with higher levels of P would be less likely to display CD than would proestrous females with lower P levels.

Materials and Methods:

Animals and Housing Conditions

Adult female Syrian hamsters (*Mesocricetus auratus*) weighing 110-120g (approximately10 weeks old) at the beginning of the experiment were obtained from Charles River Laboratories. Hamsters were housed individually for two weeks in a temperature-controlled colony room on a 14:10 hr L/D cycle with lights off at 0800 h. Additional singly-housed, ovariectomized female hamsters weighing 150-190 g (> 6 months old) were used as resident aggressors during defeat training. Intact group-housed female hamsters (five per cage) weighing 100-110 g (7 weeks old) were used as

non-aggressive intruders (NAI) during conditioned defeat testing. All animals were housed in polycarbonate cages (20x40x20 cm) with wire mesh tops, corn cob bedding and cotton nesting materials. Food and water were available ad libitum. All procedures and protocols were approved by the Georgia State University Institutional Animal Care and Use Committee.

Determination of Estrous Cycle

At least two weeks prior to behavioral testing all experimental animals were monitored daily between 0900 and 0930h for determination of estrous cycle, and only females that had consistent four day estrous cycles were used in the study. Briefly, a cotton swab was placed against the vaginal area. A thin, stringy vaginal discharge signified vaginal estrus (Estrus) and the period of sexual receptivity (Wise, 1974), with subsequent days being defined as diestrus 1, diestrus 2, and proestrus.

Conditioned Defeat Training

On the day of training, female hamsters in the diestrus 2 phase of the cycle were transported in their home cages from the colony room to the behavioral testing room where they were placed into the home cage of a resident aggressor for four, five-minute training trials. The females were paired with a different aggressor for each training trial. Training began at 0900 h with 1 h intervals between each 5 minute training trial. During the behavioral training session, experienced observers ensured that all experimental animals were attacked by the aggressors and that all of the experimental animals

displayed submissive/defensive behavior and no aggressive behavior toward the resident aggressors.

Conditioned Defeat Testing

A resident/intruder pairing was used in which a NAI was placed into the home cage of each experimental animal for 5 minutes. Female NAIs were screened to ensure that they did not display lordosis, and then they were randomly paired with the experimental animals. Videotapes of the testing sessions were scored using the Observer for Windows, version 3.0 (Noldus Information Technology B.V., Wageningen, The Netherlands). The following classes of behaviors were recorded as total duration in seconds. 1) Non-social: locomotor/exploratory, self-groom, nesting, feeding, pouching, sleeping, lordosis; 2) Social: attend, approach, investigate, sniff, touching nose; 3) Submissive/Defensive: upright and side defense, tail lift, flee, full submissive posture; 4) Aggressive: upright and side offense, chase, attack, bite. The videotapes were scored by an experienced observer who was blind to the experimental condition of each hamster.

<u>Experimental Groups:</u>

Based on the time points outlined in Saidapur & Greenwald (1978), we tested females for conditioned defeat at specific times during the LD cycle on the day of proestrus. In accord with this study E_2 levels should peak between approximately 0100 and 0200 h, while P levels should peak between 0400 and 0500 h. All females were defeated during the diestrous 2 phase of the cycle four times for five minutes by a larger resident aggressor (CD training). On the following day, females were paired for five

minutes with a NAI (CD testing) in their own home cage. Proestrous females were sacrificed at 1000 h (n=5), 1500 h (n=8), 0100 h (n=8), or 0400 h (n=8).

Hormonal Assays

Immediately after the final agonistic encounter, trunk blood was collected after rapid decapitation. Blood was collected and refrigerated overnight. Samples were spun in a refrigerated centrifuge, and serum aliquots were aspirated and stored in polypropylene microcentrifuge tubes at -20˚C until measured by radioimmunoassay. Circulating levels of E_2 and P were measured in duplicate using a commercially prepared kit from Diagnostic Systems Laboratories, Webster TX (DSL 4300 for estradiol and DSL 3900 for progesterone) as described in Faruzzi et al., (2005). Both assays were validated with hamster serum. Intra-assay reliability for both E_2 and P were less than 10% and all samples were run in a single assay for each hormone.

Statistical Analyses

Behavioral data were analyzed using multivariate analyses of variance (MANOVA) with time of day as the independent variable and aggressive, submissive/defensive, social and nonsocial behaviors as dependent variables. Hormonal data for E_2 and P were analyzed using one-way analysis of variance (ANOVA) with time of day as the independent variable and E_2 or P levels as dependent variables. Statistically significant differences for all tests were analyzed using Tukey-Kramer multiple

comparison post hoc test to compare group differences and significance for all analyses was ascribed at $p<0.05$.

Results

There was no significant main effect of time on submissive, ($F(3,28)=.170$, $p>0.05$) aggressive, ($F(3,28)=1.145$, $p>0.05$) social ($F(3,28)=1.726$, $p>0.05$) or nonsocial ($F(3,28)=2.336$, $p>0.05$) behaviors; thus, all previously defeated proestrous females regardless of time, responded similarly to social defeat. One female from Group A (1000 h) was eliminated from the hormonal analyses due to an insufficient amount of serum. ANOVA revealed no significant main effect of time on serum E_2 levels, ($F(3,23)=2.463$, $p>0.05$). That is, females regardless of the time tested, had similar levels of E_2. There was, however, a significant main effect of time on serum P levels ($F(3, 23)=14.89$, $p<0.01$) with Group D (0400 h) having significantly higher serum P levels in comparison with all other groups. The hormonal analyses are summarized in Figures 2.1a and 2.1b. The behavioral analyses are summarized in Figure 2.2.

Discussion

We have demonstrated that there is a difference across the estrous cycle in the display of conditioned defeat (CD) in female hamsters (Solomon & Huhman, in preparation). Defeated proestrous females will display CD, whereas defeated diestrous 1 females will not display CD, but will instead display normal territorial aggression. During proestrus, depending upon the time in the LD cycle, there may be marked differences in the concentrations of both E_2 and P (Saidapur & Greenwald, 1978). The present study was designed to test the hypothesis that defeated proestrous females with

higher levels of E_2 and lower levels of P will display CD, while proestrous females with lower levels of E_2 and higher levels of P will not display CD.

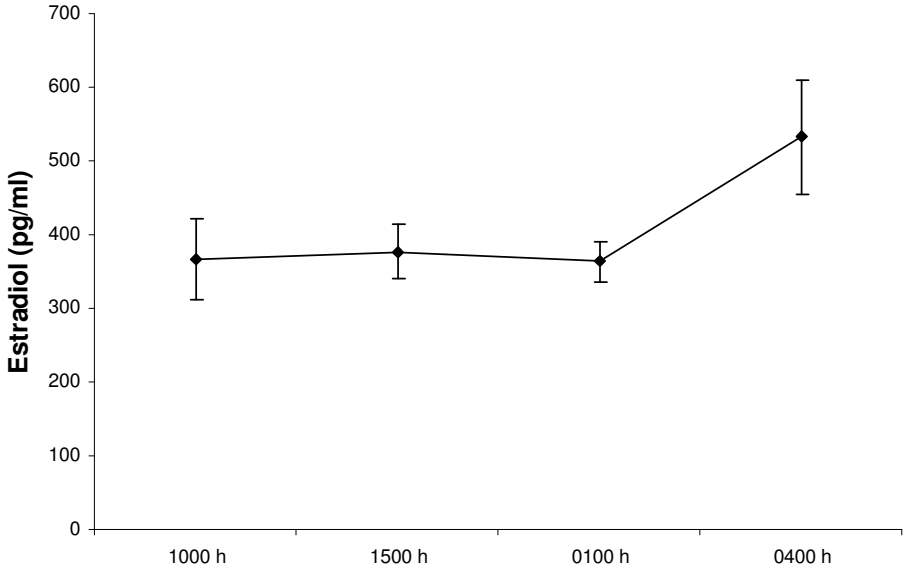
In the current study all defeated proestrous females, regardless of the time during the LD cycle, displayed CD. That is, they did not display normal territorial aggression and instead displayed some submissive/defensive behavior. The similarities in the display of CD may be due to the fact that there were no differences in serum concentrations of E_2 among any of the groups. Based upon the results of a past study (Sadipur & Greenwald, 1978), we had expected that the chosen time points would be characterized by varying levels of E_2. Surprisingly, we did not observe a significant decrease in E_2 levels. It is possible that the previous defeat episode in diestrus 2 may have subsequently altered the normal hormonal pattern of E_2 in our female hamsters. In fact, it has been shown that female rats stressed in diestrus 2 through the use of either swim stress or footshock exhibit sustained elevations in E_2 that persist for at least 24 hours (Shors et al., 1999). This may explain the increased levels of E_2 levels throughout proestrus in our previously defeated females.

It is interesting that we did not observe any differences in serum hormonal concentrations with respect to E_2, but that we did observe differences in P levels. Because there were no behavioral differences in the response to social defeat in defeated proestrous females, it appears that the circulating levels of P do not influence the behavioral response to social defeat in these females.

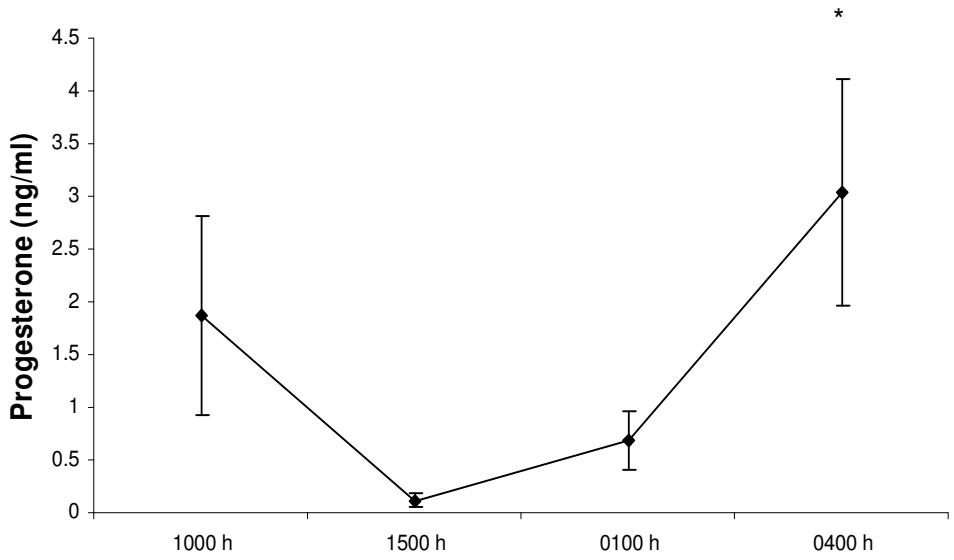
Figure 2.1. **(a)** Serum levels of estradiol and progesterone (**b**) in defeated proestrous females following a subsequent test with a non-aggressive intruder (CD testing). There were no significant differences in estradiol levels among any of the groups. * indicates statistical difference in progesterone levels among groups, $p<0.05$. Data are represented as (mean +/-SEM).

Figure 2.2. Average duration (mean +/-SEM in seconds) of submissive/defensive, aggressive, social and nonsocial behaviors in defeated proestrous females tested at 1000 h (n=5), 1500 h (n=8), 0100 h (n=8), and 0400 h (n=8) with a non-aggressive intruder (CD testing). Analyses were run for each specific behavior; therefore differences are compared only within one category of behavior and not between behavioral categories. There were no significant differences among any of the groups.

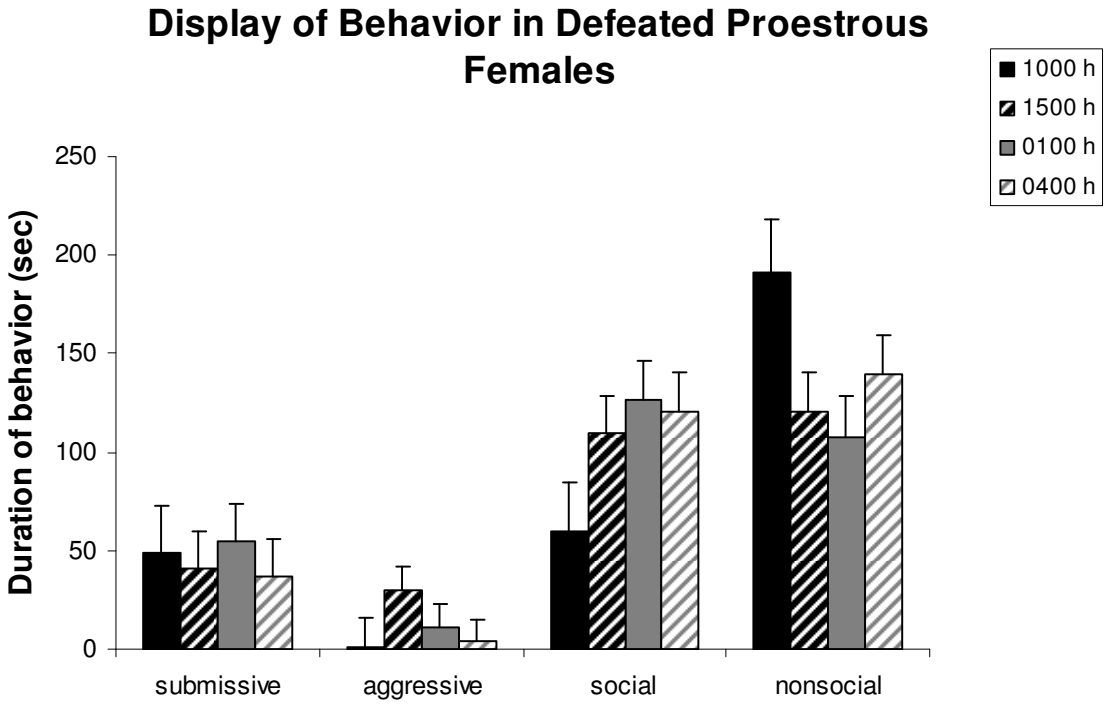
2.1a


Estradiol (pg/ml)
700
600
500
400
300
200
100
0
1000 h
1500 h
0100 h
0400 h

2.1b


Progesterone (ng/ml)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
1000 h
1500 h
0100 h
0400 h

2.2


Display of Behavior in Defeated Proestrous Females
1000 h
1500 h
0100 h
0400 h
Duration of behavior (sec)
250
200
150
100
50
0
submissive
aggressive
social
nonsocial

Chapter 4

THE EFFECTS OF ACUTE ESTROGEN ADMINISTRATION ON THE ACQUISITION AND EXPRESSION OF CONDITIONED DEFEAT IN FEMALE HAMSTERS

Abstract

Estrogens (E_2) have been shown to facilitate various types of memory (i.e., fear). Conditioned defeat (CD) is one example of an ethologically relevant model of fear learning and memory. Following social defeat, proestrous females display increased submissive behavior in the presence of a non-aggressive intruder (NAI), whereas diestrous 1 females display normal territorial aggression. It was hypothesized that the increased submissive behavior (which is suggestive of enhanced memory) in proestrous females, might be due to higher E_2 levels at the time of testing. Therefore, the purpose of the following study was to determine if acute E_2 administration would enhance the acquisition and expression of conditioned defeat (CD) in female hamsters. In Experiment 1, prior to defeat training, ovariectomized females were given either 0, 10 or 20 μg of E_2. On the following day, conditioned defeat was examined. We found that defeated females, regardless of hormone dosage, displayed CD (i.e., increased submissive and no territorial aggression) in the presence of a NAI. In Experiment 2, ovariectomized females underwent defeat training and on the following day they were administered 0, 10 or 20 μg of E2, prior to CD testing. We found that defeated females given the highest dosage of E_2 prior to CD testing, exhibited significantly higher submissive behavior in the presence of a NAI in comparison with those given 0 or 10 μg of E_2. Non-defeatead females in both Experiments 1 and 2 displayed normal territorial aggression, regardless of hormonal dosage, suggesting that E_2 administration alone does not alter normal agonistic behavior

in female hamsters. These data suggest that E_2 can enhance the expression, but not the acquisition of CD in a dose-dependent manner.

Introduction

In an unfamiliar territory, an animal's ability to learn to avoid a previously aversive stimulus (e.g., predator) can help prevent injury and prolong the animal's life. Likewise, the ability to remember the location of rewards, (e.g., food) is equally beneficial. In short, memories are adaptive processes that can protect and ensure the survival of a species; however, not all memories are adaptive. For some individuals, the memory of an aversive event can be overwhelming and can interfere with their ability to re-adapt to a safe or "non-threatening" environment. In humans, an inability to overcome a previously stressful event can lead to the development of certain stress-related psychopathologies like anxiety disorders or depression (as reviewed in Grillon et al., 1996).

For a number of years, our laboratory has studied a phenomenon that we have termed conditioned defeat (CD) in male Syrian hamsters (Potegal et al., 1993; Jasnow et al., 1999; Jasnow and Huhman, 2001; Jasnow et al., 2004a; Jasnow et al., 2004b). Syrian hamsters are solitary, aggressive animals that will attack intruding conspecifics. However, following social defeat, male hamsters no longer display normal territorial aggression and will instead display submissive/defensive behavior in the presence of a non-aggressive intruder. We have shown that this decrement in normal territorial aggression is highly reproducible and will persist for an extended period of time, even in the absence of a threatening opponent (Huhman et al., 2003). That is, the memory of the previously stressful episode (i.e., defeat) interferes with the hamster's ability to re-adapt

to a safe environment. Thus, we believe that CD is a powerful phenomenon and may serve as an ethologically relevant model for studying certain stress-related psychopathologies.

We have reported that there are important sex differences in the behavioral responses to social defeat in Syrian hamsters. Unlike males, the majority of defeated females do not display submissive behavior and instead display normal territorial aggression in the presence of a non-aggressive intruder (Huhman et al., 2003). In this study, there was, however, a small subset of females that displayed mild CD. Because we did not control for the estrous cycle, we hypothesized that the differences in the behavioral response to social defeat in some females may be due to the hormonal status (i.e., estrous cycle) of that female. Therefore, we examined the display of CD across the estrous cycle. We found that females trained (defeated) in diestrus 2 and later tested in proestrus (when E_2 levels are highest) were more likely to display CD (Solomon & Huhman, in preparation). However, because females were trained and tested during different hormonal states, we were uncertain if the hormonal status at the time of training or testing was responsible for the different behavioral responses to social defeat in some females.

In order to address this issue, we trained and tested females in the same hormonal state (i.e., diestrus 1 or proestrus). Again, we found that females trained and tested in diestrus 1 did not display CD and instead displayed normal territorial aggression. On the other hand, females trained and tested in proestrus did not display normal territorial aggression, indicating that the previous defeat encounter altered their behavior. In short, females that were trained in different hormonal states (i.e., estrus and diestrus 2) and later

tested in diestrus 1 and proestrus respectively, behaved similarly to females that were trained and tested in diestrus 1 and proestrus. These data suggest that the hormonal status at the time of testing, and not necessarily training, was responsible for the different behavioral responses to social defeat observed in cycling females. In addition, these data also suggest that high or rising endogenous E_2 levels as seen in proestrous females during testing, might be associated with increased submissive behavior and decreased aggressive behavior following defeat. Relatedly, the lower endogenous E_2 levels in diestrous females may be associated with increased aggressive behavior and decreased submissive behavior.

Based on our previous data with intact females, we hypothesized that both the previous defeat encounter and the hormonal status of the female at the time of testing, and not training, were important in the display of increased submissive behavior (i.e., CD) in female hamsters. In the present study, we examined the effect of acute E_2 administration on both the acquisition and expression of CD in female hamsters. In Experiment 1, we tested the hypothesis that E_2 administration would enhance the acquisition of CD in female hamsters. In Experiment 2, we tested the hypothesis that E_2 administration would enhance the expression of CD in female hamsters. The data presented here suggest that E_2 administration enhances the expression, but not the acquisition of CD in female hamsters.

Methods

Animals and Housing Conditions

Adult female Syrian hamsters (*Mesocricetus auratus*) weighing 110-120g (approximately10 weeks old) at the beginning of the experiment were obtained from Charles River Laboratories. Hamsters were housed individually for two weeks in a temperature-controlled colony room on a 14:10 hr light: dark cycle with lights off at 0900 h. Additional singly-housed, ovariectomized female hamsters weighing 150-190 g (> 6 months old) were used as resident aggressors during defeat training. Intact group-housed female hamsters (five per cage) weighing 100-110 g (7 weeks old) were used as non-aggressive intruders (NAI) during conditioned defeat testing. All animals were housed in polycarbonate cages (20x40x20 cm) with wire mesh tops, corn cob bedding and cotton nesting materials. Food and water were available ad libitum. All procedures and protocols were approved by the Georgia State University Institutional Animal Care and Use Committee.

Surgery and Hormone Replacement

Hamsters were anesthetized deeply with sodium pentobarbitol (90mg/kg) and were ovariectomized 10 days prior to behavioral testing. Ovariectomized hamsters were subcutaneously administered vehicle (safflower oil, 0.2cc) or 17-β estradiol benzoate: (Sigma) (10 μg/0.1cc or 20 μg/0.2cc) either before defeat training or conditioned defeat testing. These hormonal dosages was chosen because it has been used in other models of fear learning and memory (Walf and Frye, 2005; Rhodes and Frye, 2004; Frye and Rhodes, 2002; Leuner et al., 2004).

CD Training

On the day of training, female hamsters were transported in their home cages from the colony room to the behavioral testing room where they were placed into the home cage of a resident aggressor for four, five-minute training trials. The females were paired with a different aggressor for each training trial. Training began at 0900 h with 1 h intervals between each 5 minute training trial. During the behavioral training session, experienced observers ensured that all experimental animals were attacked by the aggressors and that all of the experimental animals displayed submissive/defensive behavior and no aggressive behavior toward the resident aggressors.

CD Testing

All testing for conditioned defeat was completed during the first 2 h of the dark phase of the LD cycle. A resident/intruder pairing was used in which a non-aggressive intruder (NAI) was placed into the home cage of each experimental animal for 5 minutes. Female NAIs were screened to ensure that they did not display lordosis and then they were randomly paired with the experimental animals. Videotapes of the testing sessions were scored using the Observer for Windows, version 5.0 (Noldus Information Technology B.V., Wageningen, The Netherlands). The following classes of behaviors were recorded as total duration in seconds. 1) Non-social: locomotor/exploratory, self-groom, nesting, feeding, pouching, sleeping, lordosis; 2) Social: attend, approach, investigate, sniff, touching nose; 3) Submissive/Defensive: upright and side defense, tail

lift, flee, full submissive posture; 4) Aggressive: upright and side offense, chase, attack, bite.

Hormonal Assays

Immediately after the final agonistic encounter, trunk blood was collected after rapid decapitation. Blood was collected and refrigerated overnight. Samples were spun in a refrigerated centrifuge, and serum aliquots were aspirated and stored in polypropylene microcentrifuge tubes at -20˚C until measured by radioimmunoassay. Circulating levels of estradiol were measured in duplicate using a commercially prepared kit from Diagnostic Systems Laboratories, Webster TX (DSL 4300 for estradiol) as described in (Faruzzi et al., 2005). The estradiol assay was validated with hamster serum. Intra-assay reliability was 2% and inter-assay reliability was 6%.

Experiment 1a:

Female hamsters (n=23) were weight matched and randomly assigned to either defeat vehicle (n=7), defeat 10 μg E_2 (n=8) or defeat 20 μg E_2 (n=7). On Day 1, animals were injected with E_2 or vehicle, 4 hours prior to defeat training (paired with a resident aggressor). On Day 2, females were paired in their own home cage with a non-aggressive intruder (NAI).

Experiment 1b:

Non-defeated female hamsters (n=21) were weight matched and randomly assigned to either vehicle (n=7), 10 μg E_2 (n=7) or 20 μg E_2 (n=7). On Day 1, animals

were injected with either E_2 or vehicle and remained in their home cages. On Day 2, females were paired in their own home cage with a NAI.

Experiment 2a:

Female hamsters (n=29) were weight matched and randomly assigned to either vehicle (n=10), 10 μg E_2 (n=9) or 20 μg E_2 (n=10). On Day 1, animals were defeated in the home cage of a resident aggressor. On Day 2, animals were injected with either E_2 or vehicle, 4 hours prior to CD testing (paired with a NAI).

Experiment 2b:

Non-defeated female hamsters (n=15) were weight matched and randomly assigned to either vehicle (n=4), 10 μg E_2 (n=5) or 20 μg E_2 (n=6). On Day 1, animals remained in their home cages. On Day 2, animals were injected with either E_2 or vehicle 4 hours prior to being tested with an NAI in their own home cage. The methodological design for all experiments is depicted in Figure 3.1.

Statistical Analyses

Behavioral data were analyzed using multivariate analyses of variance (MANOVA) with hormonal dosage as the independent variable and aggressive, submissive/defensive, social and nonsocial behaviors as dependent variables. Hormonal data for estradiol were analyzed using one-way analysis of variance (ANOVA) with hormonal dosage as the independent variable and estradiol as the dependent variable.

Statistically significant differences for all tests were analyzed using Fisher's (LSD) post hoc tests. Statistical significance for all analyses was ascribed at $p<0.05$.

Results

In Experiment 1a, there was no effect of pre-training E_2 administration on submissive ($F(2,19)=.03$, $p > 0.05$), aggressive ($F(2,19)=1.05$, $p > 0.05$), social ($F(2,19)=.11$, $p > 0.05$) or nonsocial ($F(2,19)=.79$, $p > 0.05$) behaviors. That is, all defeated females, regardless of hormonal dosage displayed moderate submissive behavior and little to no aggressive behavior in the presence of a NAI (See Figure 3.2a). E_2 administration produced significant increases in serum E_2 levels in defeated females ($F(1,19)=88.67$, $p < 0.01$) with $0 < 10 < 20$ μg (See Figure 3.3a).

In Experiment 1b, there was no effect of hormone on submissive ($F(2,18)=.69$, $p > 0.05$), aggressive ($F(2,18)=.23$, $p > 0.05$), social ($F(2,18)=.14$, $p > 0.05$) or nonsocial ($F(2,18)=.14$, $p > 0.05$) behaviors. All non-defeated females displayed normal territorial aggression and little to no submissive behavior (See Figure 3.2b). E_2 administration produced significant increases in serum E_2 levels in non-defeated females ($F(1,18)=59.39$, $p < 0.01$). More specifically, non-defeated females administered vehicle had significantly lower E_2 levels compared with those given 10 or 20 μg of E_2 (See Figure 3.3b).

In Experiment 2a, there was a significant effect of pretesting E_2 administration on the display of submissive ($F(2,26)=3.91$, $p < 0.05$) behavior in defeated females. Defeated females administered the highest dosage of E_2, displayed significantly higher submissive behavior in the presence of a NAI compared with those administered 0 or 10 μg E_2. There was however, no effect of pretesting E_2 administration on aggressive

($F(2,26)=2.76$, $p > 0.05$), social ($F(2,26)=1.21$, $p > 0.05$) or nonsocial ($F(2,26)=1.76$, $p > 0.05$) behaviors (See Figure 3.4a). Administration of E_2 prior to testing produced significant increases in serum E_2 levels in defeated females ($F(2,26)=121.43$, $p <0.01$) with 0 <10 <20 μg (See Figure 3.5a).

In Experiment 2b, there was no effect of hormone on submissive ($F(2,12)=.53$, $p > 0.05$), aggressive ($F(2,12)=.80$, $p > 0.05$), social ($F(2,12)=.16$, $p > 0.05$) or nonsocial ($F(2,12)=1.26$, $p > 0.05$) behaviors. All non-defeated females displayed normal territorial aggression and little to no submissive behavior in the presence of a NAI (See Figure 3.4b). E_2 administration prior to testing, produced significant increases in serum E_2 levels ($F(2,13)=16.15$, $p < 0.01$). Non-defeated females administered vehicle had significantly less serum E_2 levels relative to females given 10 or 20 μg E_2 (See Figure 3.5b).

Discussion

The present data are one of the first to examine how both pretraining and pretesting administration of E_2 affects the subsequent behavior of females exposed to a naturalistic stressor like social defeat. The purpose of this study was to examine the effects of acute E_2 administration on both the acquisition and expression of CD in female hamsters. Our previous data using intact females, suggested that higher E_2 at the time of testing, as seen in defeated proestrous females, was associated with increased submissive behavior. On the other hand, lower E_2, as seen in diestrous 1 females, might be associated with decreased submissive behavior (Solomon & Huhman, in preparation). Based on these findings, we hypothesized that both the experience of defeat and the hormonal

status of the female at the time of testing were important factors in the display of CD in female hamsters.

The findings from Experiment 1 indicate that E_2 administration prior to defeat training does not enhance the acquisition of CD in female hamsters. In Experiment 1, all defeated females regardless of hormone dosage, displayed moderate levels of submissive behavior and little to no aggressive behavior when tested with a non-aggressive intruder (NAI). Defeated females given the highest dosage of E_2 (i.e., 20 μg) prior to CD testing, on the other hand, displayed significantly more submissive behavior compared with those receiving either 10 μg or vehicle. This duration of submissive behavior was actually much higher than usually observed in female hamsters (Faruzzi et al., 2005; Solomon, 2003; Solomon & Huhman, in preparation). These data suggest that acute E_2 administration enhances the expression of CD in a dose-dependent fashion. The mean increase in submissive behavior seen in defeated females given 20 μg of E_2 prior to testing was not due to a generalized effect of the hormone on submissive behavior because non-defeated females given this same dosage prior to testing displayed normal territorial aggression in the presence of a NAI. The fact that E_2 increased submissive behavior (which is suggestive of increased fear) in defeated females is consistent with previous data which suggest that E_2 facilitate fear learning or anxiety-like behavior (Jasnow et al., 2005; Morgan & Pfaff, 2001; Morgan & Pfaff, 2002; Diaz-Veliz et al., 1991; Gibbs et al., 1998).

All non-defeated females, regardless of hormone dosage or timing of E_2 administration (pretraining or pretesting), exhibited normal territorial aggression and very little submissive behavior in the presence of a NAI. Thus, E_2 administration alone did not

produce changes in agonistic behavior in the absence of social defeat. This is consistent with previous findings which show that E_2 does not alter aggression levels relative to vehicle (Meisel et al., 1990; Meisel & Sterner, 1990). The data presented here, extend these previous studies and demonstrate that E_2 administration alone does not alter other aspects of agonistic behavior, namely, submission.

In the present study, pretraining administration of 10 and 20 μg of E_2, yielded mean levels of 127 pg/ml and 351 pg/ml in defeated females and 230 pg/ml and 249 pg/ml in non-defeated females, respectively. However, pretesting administration of E_2 produced mean levels of 394 pg/ml and 611pg/ml in defeated females and 670 pg/ml and 833 pg/ml in non-defeated females, respectively. The difference in circulating E_2 in pretraining vs. pretesting groups is due to the time at which the hormone was administered prior to sample collection (i.e., ˜28 hrs. vs. ˜5 hrs).

An important issue that is raised by the current data is that the behavioral effects of gonadal steroids may be quite different depending on the hormonal replacement regimen used. We have recently reported that females that were ovariectomized, given chronic estrogen replacement and tested approximately one month later actually exhibited significantly less submissive behavior following social defeat (Faruzzi et al., 2005). This effect is opposite of that obtained in the present experiments. There are a number of possibilities to explain this inconsistency. To begin with the length of time that the animals were housed prior to training and testing might be an important factor. For example, in the past study, females were singly housed 4 weeks prior to training and testing compared with 10 days in the present study. Further, in the Faruzzi study females were trained and tested in the same hormonal state, whereas our data involving intact

females and those administered acute E_2, were trained and tested in different hormonal states. In addition, there may be significant changes in receptor distribution as well as receptor sensitivity to various endogenous hormones which may in turn alter behavioral responses to social defeat, depending upon the time post-ovariectomy. In short, there may be several factors that may be attributable for the varying effects of E_2 (depending on dosage) on the behavior of defeated females.

The enhancing effect of E_2 on the expression, but not the acquisition of CD in female hamsters suggests that E_2 does not affect the encoding of the memory of defeat. There are different phases of memory which include encoding, storage and retrieval and it might be likely that E_2 affects other aspects of memory like consolidation. In order to test this, we could give post-training injections of E_2, and determine if females given post-training injections of this hormone exhibit higher levels of submissive behavior in the presence of a NAI in comparison with those given vehicle. It might seem likely that the increase in submissive behavior in females given the highest dosage of E_2 is due to some generalized effect of the hormone on submissive behavior. However, we are certain that this is not the case, because non-defeated females given this same dosage exhibited normal territorial aggression and little to no submissive behavior. This finding instead suggests an interaction of social history and hormones on subsequent behavior in female hamsters. Future studies will be conducted in order to further examine the relationship between social defeat and hormones and to determine the role of E_2 on other aspects of memory (i.e., consolidation) related to conditioned defeat in female hamsters.

Figure 3.1. Schematic design for study. In Experiment 1, females were injected with 17-β estradiol benzoate or safflower oil, four hours prior to defeat training (paired with resident aggressor). On the following day, they were paired with a non-aggressive intruder in their own home cage. In Experiment 3, females underwent defeat training (paired with a resident aggressor). On the following day, they were injected with 17-β estradiol benzoate or safflower oil, four hours prior to behavioral testing (paired with non-aggressive intruder).

Figure 3.2. (**a**) (**CD Acquisition**) Average duration (mean +/-SEM in seconds) of submissive/defensive, aggressive, social and nonsocial behavior in defeated and non-defeated (**b**) female hamsters tested with a non-aggressive intruder. Analyses were run for each specific behavior; therefore, differences are compared only within one category of behavior and not between behavioral categories.

Figure 3.3. (**a**) (**CD Acquisition**) Serum levels of estradiol in defeated and non-defeated (**b**) female hamsters following a test encounter with a non-aggressive intruder. Non-shared letters depict statistical significance, ($p < 0.05$). Data are presented as (mean +/-SEM). E_2 administration was given approximately 28 hours prior to sample collection.

Figure 3.4. (a) (**CD Expression**) Average duration (mean +/-SEM in seconds) of submissive/defensive, aggressive, social and nonsocial behavior in defeated (Figure 4A) and non-defeated (**Figure 4B**) female hamsters tested with a non-aggressive intruder.

Analyses were run for each specific behavior; therefore, differences are compared only within one category of behavior and not between behavioral categories. Non-shared letters depict statistical significance, ($p < 0.05$).

Figure 3.5. (**a**) (**CD Expression**) Serum levels of estradiol in defeated (Figure 5A) and non-defeated (**b**) female hamsters following a test encounter with a non-aggressive intruder. Non-shared letters depict significant differences ($p < 0.05$). Data are presented as (mean +/-SEM). E_2 administration was given approximately 5 hours before sample collection.

3.1
Experiment 1: CD Acquisition

Day 1

Group A: (Defeat)

Inject → 4 hours later → CD Training

Group B: (No Defeat)

Inject → 4 hours later → Remain in home cage

Day 2

Groups A & B: → CD Testing

Experiment 2: CD Expression

Day 1

Group A: (Defeat)

CD Training

Group B: (No Defeat)

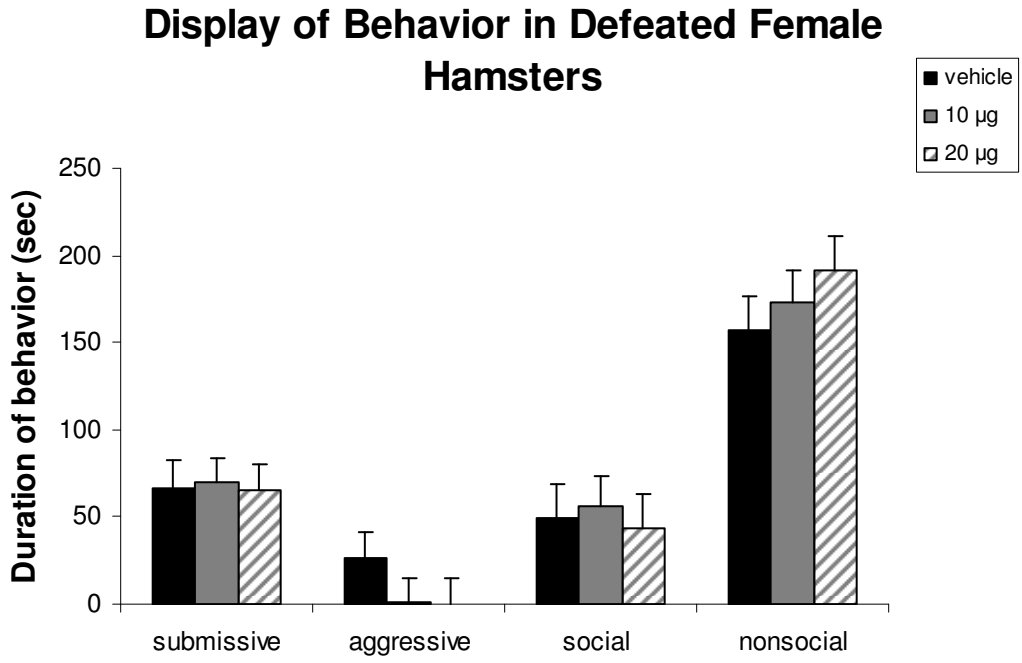
Remain in home cage

Day 2

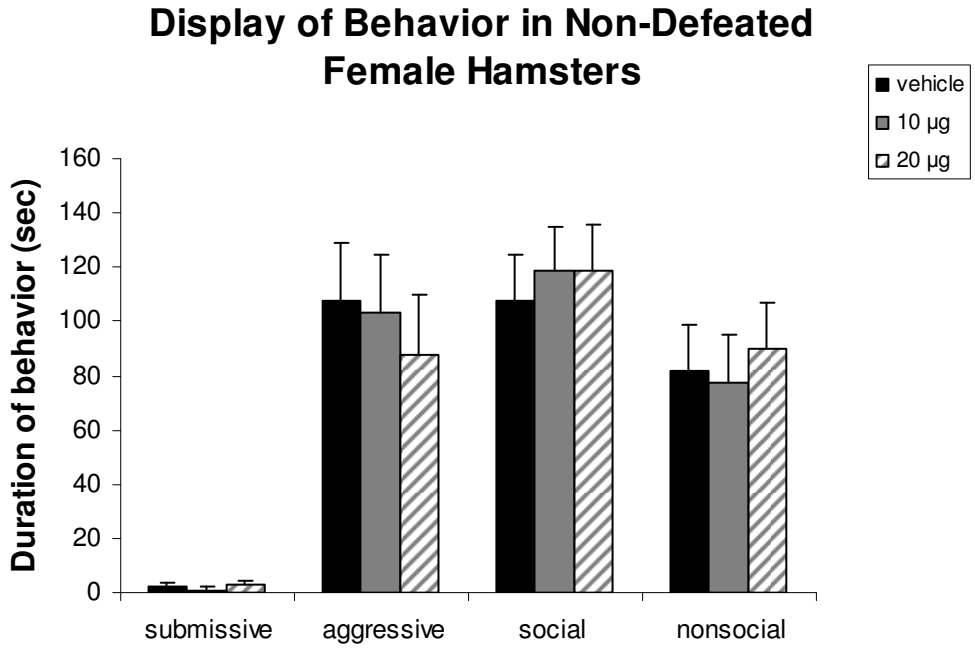
Groups A & B:

Inject → 4 hours later → CD Testing

3.2a


Display of Behavior in Defeated Female Hamsters
vehicle
10 µg
20 µg
Duration of behavior (sec)
250
200
150
100
50
0
submissive
aggressive
social
nonsocial

3.2b


Display of Behavior in Non-Defeated Female Hamsters
vehicle
10 µg
20 µg
Duration of behavior (sec)
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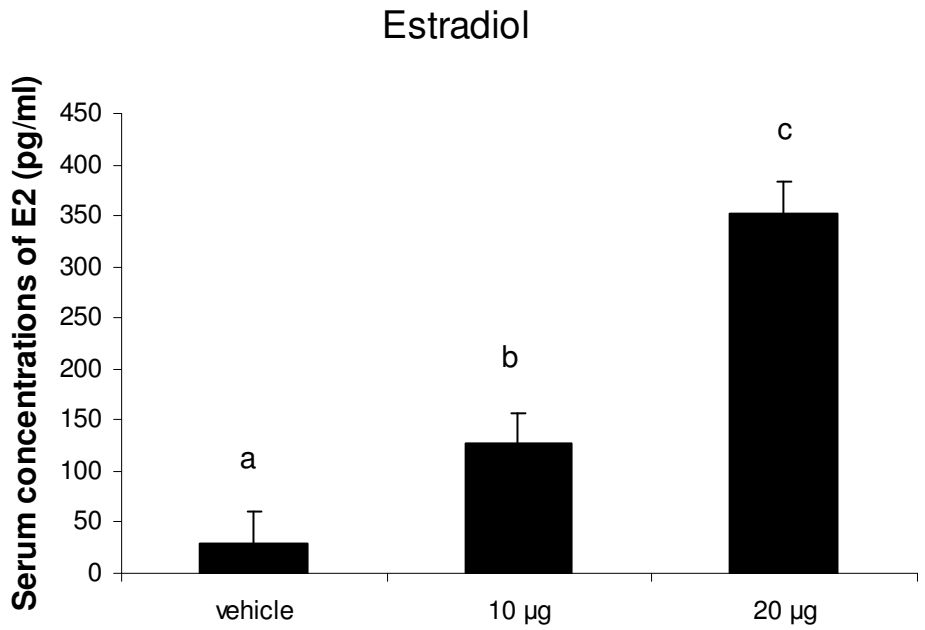
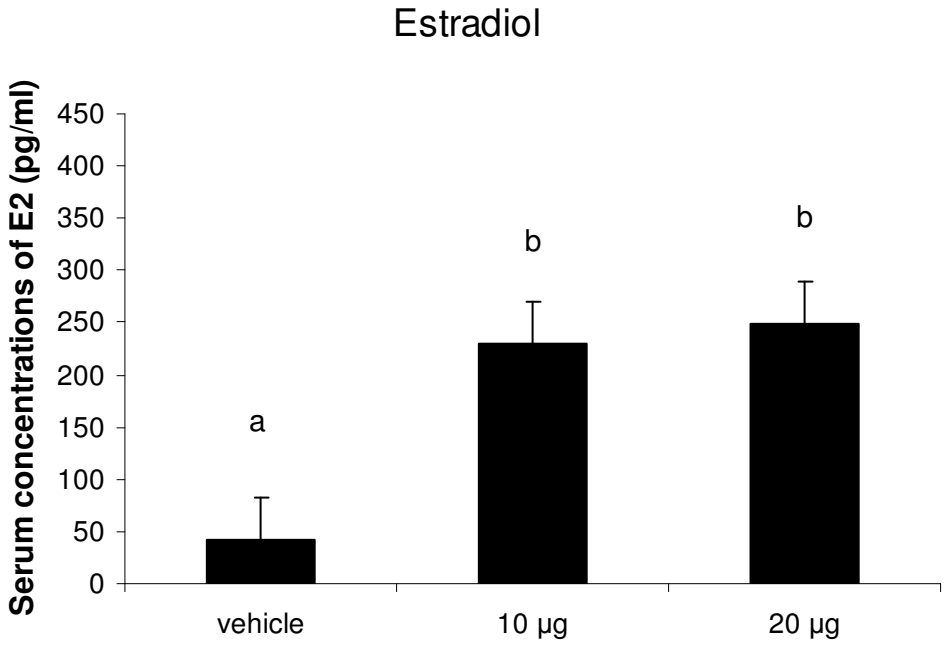
140
120
100
80
60
40
20
0
submissive
aggressive
social
nonsocial

3.3a


Estradiol
Serum concentrations of E2 (pg/ml)
450
400
350
300
250
200
150
100
50
0
a
b
c
vehicle
10 µg
20 µg

3.3b


Estradiol
Serum concentrations of E2 (pg/ml)
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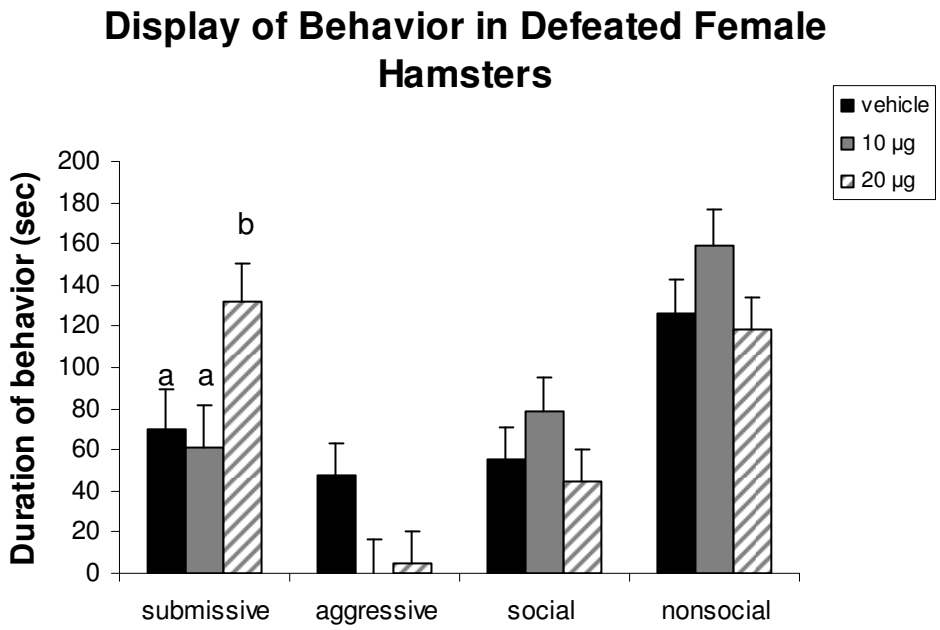
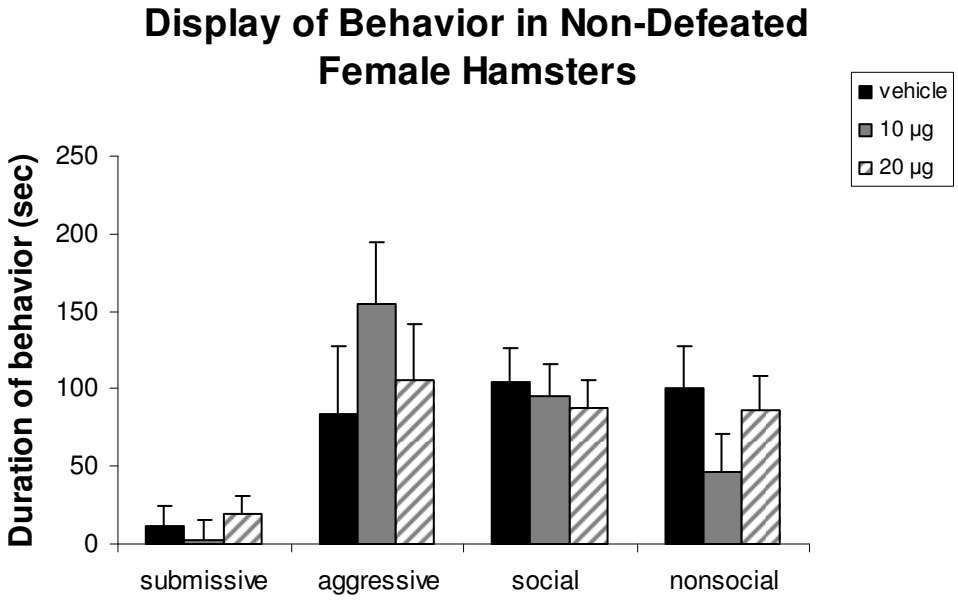
400
350
300
250
200
150
100
50
0
a
b
b
vehicle
10 µg
20 µg

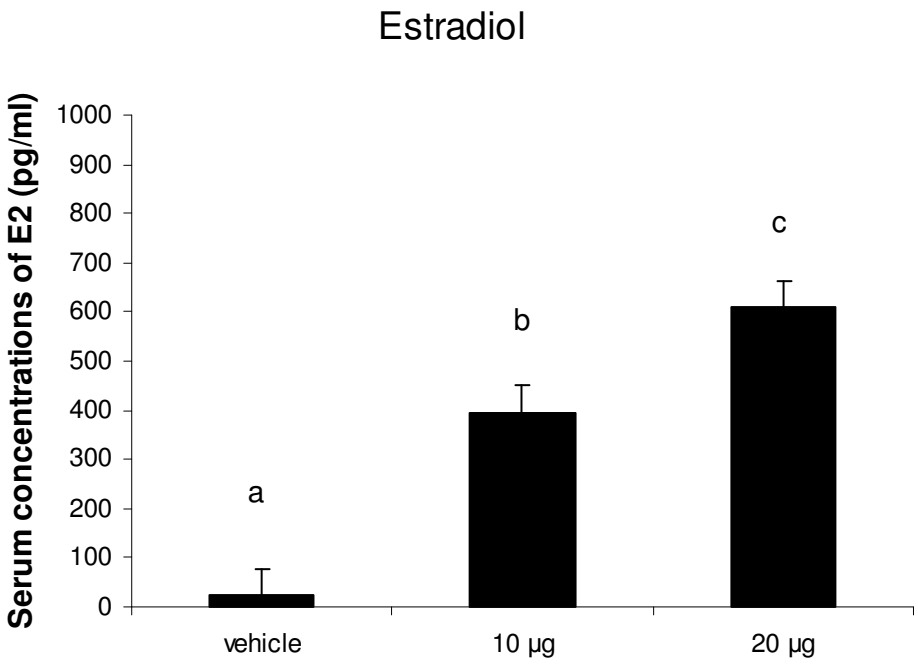
3.4a


Display of Behavior in Defeated Female Hamsters
vehicle
10 µg
20 µg
Duration of behavior (sec)
200
180
160
140
120
100
80
60
40
20
0
a
a
b
submissive
aggressive
social
nonsocial

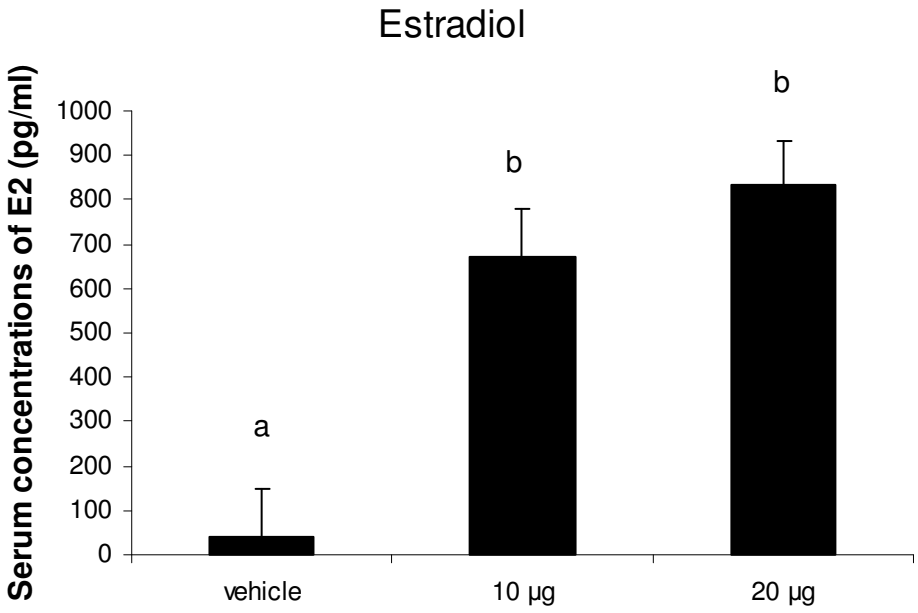
3.4b


Display of Behavior in Non-Defeated Female Hamsters
vehicle
10 µg
20 µg
Duration of behavior (sec)
250
200
150
100
50
0
submissive
aggressive
social
nonsocial

3.5a


Estradiol
Serum concentrations of E2 (pg/ml)
1000
900
800
700
600
500
400
300
200
100
0
a
b
c
vehicle
10 µg
20 µg

3.5b


Estradiol
Serum concentrations of E2 (pg/ml)
1000
900
800
700
600
500
400
300
200
100
0
a
b
b
vehicle
10 µg
20 µg

Chapter 5

THE EFFECTS OF GONADAL HORMONES ON THE DISPLAY OF CONDITIONED DEFEAT IN MALE HAMSTERS

Abstract

It has been widely reported that gonadal hormones (e.g., testosterone, estrogen) influence the display of aggression in Syrian hamsters; conversely, much less is known about whether gonadal hormones modulate the display of submissive/defensive behavior in these animals. Following social defeat, male hamsters no longer display normal territorial aggression and instead display submissive/defensive behavior in the presence of a smaller opponent, a phenomenon we have termed conditioned defeat (CD). It is not know if gonadal hormones modulate this increased submissive behavior seen in male hamsters. The purpose of the present study was to examine the effects of gonadal hormones on the display of CD in male hamsters. In Experiment 1, following four weeks of single housing, sham-operated males displayed significantly less submissive behavior following defeat than did their castrated counterparts. In Experiment 2, by contrast, castrated and sham-operated males that were singly housed for only 10 days displayed similar behavioral responses to social defeat. Finally, in Experiment 3, animals that received exogenous administration of testosterone and dihdyrotestosterone displayed a trend toward less submissive behavior ($p = 0.06$) in the presence of a smaller opponent, compared with those receiving estradiol and cholesterol capsules. Taken together, these data suggest that androgens and isolation can modulate CD in male hamsters.

Introduction

Agonistic behavior includes all behaviors that are exhibited during social conflict, including aggressive, submissive/defensive and communicative (e.g., scent marking) behaviors. Unlike rats where agonistic behavior has been studied almost exclusively in males, agonistic behavior has been widely studied in both male and female Syrian hamsters (Whitsett, 1975; Grelk et al., 1974; Payne, 1973; Payne & Swanson, 1971; Payne, 1974; Wise, 1974; Takahashi & Lisk, 1984; Meisel et al., 1988). Aggression is one form of agonistic behavior that has been extensively studied in Syrian hamsters. While various measures (e.g., lesions, electric shocks) have been used to stimulate aggression in other rodent species, Syrian hamsters readily attack intruder conspecifics with little intervention from the experimenter.

Several factors have been shown to influence the outcome of social conflict in Syrian hamsters. For example, body weight, environment and gonadal hormones have all been shown to be key predictors of success during agonistic interactions. In male-male interactions over 60% of the time, heavier males will dominate smaller opponents (Vandenbergh, 1971; Drickamer et al., 1973). The environment in which the agonistic encounters take place is also an important factor in predicting success. More specifically, Syrian hamsters are often more aggressive when defending their own home cage than when placed in a neutral area (Murphy & Schneider, 1970; Murphy, 1976), although it has been shown that Syrian hamsters are still aggressive when paired outside of their home cages (Payne & Swanson, 1970). In addition, gonadal hormones have also been shown to influence the likelihood of "winning." For instance, it has been reported that

intact male hamsters are more aggressive and often defeat their castrated counterparts (Vandenbergh, 1971; Payne & Swanson, 1971, 1972).

In many species, androgens facilitate the display of aggression in males. Castration in both rats and mice has been shown to reduce aggression, while androgen replacement restores aggression in both species (Albert et al., 1986; Christie & Barfield, 1979; Bartke et al., 1973; Bevan et al., 1958; Tolman & King, 1956). Likewise, in Syrian hamsters there have been reports of castration decreasing aggression with androgen replacement increasing aggression (Payne & Swanson, 1971; Payne, 1973; Grelk et al., 1974; Drickamer et al., 1973). However, there have also been some reports that demonstrate that the display of aggression in adult Syrian hamsters is independent of gonadal hormones (Whitsett, 1975; Tiefer, 1970; Garrett & Campbell, 1980). Several variables may account for these varied effects of gonadal hormones on aggression in male hamsters, including time since castration, age of animals, characteristics of the opponent (i.e., castrated vs. intact), environment (i.e., home cage vs. neutral arena) as well as the measure used to assess aggression (e.g., latency to attack and duration of aggressive behavior).

While much attention has been paid to the environmental and hormonal factors that may be associated with "winning," our laboratory is interested in the behavioral and hormonal consequences of "losing." We study a phenomenon, termed conditioned defeat (CD) in Syrian hamsters. During the CD acquisition period, the experimental animal is defeated by a larger, resident aggressor. On the following day (CD testing), the previously defeated animal displays increased submissive/defensive behavior when paired with a smaller opponent. This increase in submissive behavior is particularly

notable because the experimental animal is in its own home cage and is heavier than the intruder. As mentioned previously, both environment (i.e., home cage) and increased body weight are key predictors in the success of agonistic encounters, but following defeat, the influence of these factors diminishes.

A variety of hormones have been associated with losing agonistic encounters. For example, we have shown that defeated animals display increased activation of the hypothalamic-pituitary adrenal axis (HPA-axis) as evidenced by elevated ACTH and cortisol levels (Huhman et al., 1990, 1992). Following repeated defeats, male hamsters show suppression of hypothalamic-pituitary gonadal axis (HPG-axis) as demonstrated by decreased testosterone levels in comparison with their dominant counterparts (Huhman et al., 1991). In addition, we have also explored the role of several neurochemical signals that may be associated with CD in male hamsters. Administration of corticotropin-releasing hormone (CRH) into discrete brain regions has been shown to increase both the acquisition and expression of CD in male hamsters (Faruzzi & Huhman, unpublished observations). On the other hand, administration of GABA, CRH antagonists as well as the NMDA antagonist, AP5, has been shown to reduce the expression of CD in males (Jasnow et al., 1999; Jasnow & Huhman, 2001; Cooper & Huhman, 2005; Jasnow et al., 2004a,b). Thus, we have information concerning the role of various hormones and peptides on the display of CD in male hamsters.

Despite our understanding of the role of various neurotransmitters and hormones on the display of CD, we have not examined the effect of gonadal hormones on this phenomenon in male hamsters. Because androgens have been shown to influence the display of aggression in male hamsters, it seems possible that these hormones also play a

role in submissive/defensive behavior. The present study was designed to assess the effect of gonadal hormones on the display of CD in male hamsters. In experiment 1, male hamsters were castrated or sham-operated and singly housed for four weeks prior to social defeat and submissive behavior was observed 24 hours later in the presence of a non-aggressive intruder. Because isolation for longer periods has been associated with increased aggression in Syrian hamsters (Wise, 1974; Brain, 1972), we wanted to look at the effect of gonadal hormones on the display of CD in castrated male hamsters that were housed for a shorter period of time. Therefore, in experiment 2 male hamsters were castrated or sham-operated and singly housed for 10 days prior to behavioral testing. Finally, in experiment 3, we examined the effect of exogenous administration of testosterone, dihydrotestosterone or estradiol on CD in castrated males.

Methods

Subjects

Adult male Syrian hamsters (*Mesocricetus auratus*, Charles River Laboratories) weighed approximately 120-130 g (10 weeks) upon arrival. Male hamsters were individually housed in a temperature-controlled colony room on a 14:10 hr light: dark cycle with lights off at 1100 h. Additional singly-housed male hamsters weighing >180 g (>6 months old) were used as resident aggressors during defeat training. Group-housed male hamsters (five per cage) weighing 110-120 g (7 weeks old) were used as non-aggressive intruders (NAI) during conditioned defeat testing. All animals were housed in polycarbonate cages (20x40x20cm) with wire mesh tops, corn cob bedding and cotton nesting materials. Food and water were available ad libitum. All procedures and

protocols were approved by the Georgia State University Institutional Care and Use Committee.

Surgery/Hormone Replacement

Experiments 1 & 2

All male hamsters were anesthetized deeply with sodium pentobarbitol (90mg/kg). In Experiment 1, hamsters (n=29) were castrated (n=16) or sham-operated (n=13) and were individually housed 4 weeks prior to behavioral testing. In Experiment 2, hamsters (n=27) were castrated (n=11) or sham-operated (n=16) and were individually housed 10 days prior to behavioral testing. All hamsters were defeated one time for 15 minutes by a larger resident aggressor (CD training) in the aggressor's home cage. On the following day, hamsters were paired in their own home cage for five minutes with a non-aggressive intruder (CD testing).

Experiment 3

19 male hamsters were castrated and individually housed for 4 weeks prior to behavioral testing. Immediately following surgery all animals were surgically implanted (subcutaneously) with Silastic capsules containing 17-β estradiol benzoate (E_2) (n=5), dihydrotestosterone (DHT) (n=5), testosterone (T) (n=5) or cholesterol (C) (n=4). All hamsters were defeated for one time for 15 minutes by a larger resident aggressor (CD training) in the aggressor's home cage. On the following day, these hamsters were paired in their own home cage for five minutes with a smaller non-aggressive intruder (CD testing).

A separate group of 15 male hamsters DHT (n=4), T (n=4), E_2 (n=4) and C (n=3) were handled, no defeat controls who were handled and remained in their home cage until testing with a smaller non-aggressive intruder.

Hormonal Capsules

E_2 capsules were made of Silastic Brand medical-grade tubing (0.078in. i.d. x 0.125 in. o.d.), 10 mm in length, sealed with Factor II 6382 RTV Silicone and Elastomer, and filled with 5 mm of 17-β-estradiol (Sigma, cat. number E1024-1G St. Louis, MO). This dosage of E_2 was chosen because it produces similar blood levels to those found in intact male hamsters (unpublished observations). T capsules were made of Silastic tubing (0.078 in. i.d. x 0.125 in. o.d.), 28 mm long and filled 20 mm with testosterone propionate (Sigma, cat. number T-1500, St. Louis, MO). The dose of T was chosen because it produces circulating serum T concentrations similar to that of an intact male Syrian hamster (unpublished observations). DHT (Sigma, cat. number A-8380, St. Louis, MO) and C (Sigma, cat. number C3045-5G, St. Louis, MO.) capsules were 30 mm long and filled 20 mm with DHT. This dosage of DHT was chosen in order to mimic the concentration used in the T capsules.

Conditioned Defeat Training/Testing

All behavioral training and testing took place during the first two hours of the daily light:dark cycle. On the day of conditioned defeat training, male hamsters were transported from the colony room to the behavioral testing room. Conditioned defeat training consisted of a single resident/intruder pairing in which experimental animals were placed into the home cage of a larger resident aggressor for 15 minutes. During the

15 minute defeat session, experimental animals were routinely attacked by the resident aggressors and displayed submissive and defensive behavior towards resident aggressors. On the following day, a similar resident/intruder pairing was used in which the experimental animals were paired in their own home cage with a smaller non-aggressive intruder. All training and testing sessions were recorded on VHS tape, transferred to CD-ROM, and scored by a trained observer blind to the experimental condition using Noldus Observer (version 5) (Noldus Information Technology, Wageningen, Netherlands). The following classes of behaviors were scored as total duration in seconds: 1) Non-social: locomotor/exploratory, self-groom, nesting, feeding, pouching, sleeping; 2) Social: attend, approach, investigate, sniff, touching nose; 3) Submissive/defensive: upright and side defense, tail lift, flee, full submissive posture; 4) Aggressive: upright and side offense, chase, attack, bite.

Hormonal Assays

Nonextracted hamster serum samples were assayed in duplicate for E_2, T, and DHT using commercial radioimmunoassay kits (Active® Estradiol RIA DSL-43100, Active® Testosterone RIA DSL-4000 and Dihydrotestosterone Non Extraction RIA DSL-96100 (Diagnostic Systems Laboratories, Inc., Webster TX), respectively. All kits were validated with hamster serum. E_2, T and DHT intra and inter-assay statistics were 2% and 6% (0.52-1033.76 pg/ml detection range), 3% and 5% (0.01-21.69 ng/ml detection range) and 4% and 7% (1.59-1250.70 pg/ml detection range), respectively. The specificity for the E_2 kit for 17-β estradiol was 100%, with a negligible cross reactivity (< 0.01%) for testosterone. This particular E_2 kit was not validated against DHT; therefore, the cross-reactivity of these two compounds using this kit is unknown.

The specificity for the T kit for T was 100%, with a 5.8% cross reactivity with dihydrotestosterone. This kit had insignificant cross-reactivity with 17-β estradiol (< 0.01%). The specificity for the DHT kit for DHT was 100%, with a 0.6% cross-reactivity for T and a negligible cross reactivity (<0.01%) for E_2.

Statistical Analyses

In experiments 1 and 2, behavioral data were analyzed using a 2-way multivariate analyses of variance (MANOVA) with social experience (i.e., defeat vs. no defeat) and hormonal condition (i.e., castration vs. sham-operated) as the independent variables. Submissive/defensive, aggressive, social and nonsocial behaviors were dependent variables. Hormonal data for experiments 1 and 2 were analyzed using one-way analysis of variance with hormonal condition (i.e., castration vs. sham-operated) as the independent variable and serum concentration of T, DHT and E_2 as the dependent variables. In experiment 3, behavioral data were analyzed using multivariate analyses of variance with social experience (i.e., defeat or no defeat) as the independent variable and submissive/defensive, aggressive, social and nonsocial behaviors as the dependent variables. Hormonal data for experiment 3 were analyzed using a one-way analysis of variance with hormonal treatment as the independent variable and serum concentration of T, DHT and E_2 as the dependent variables. When necessary, statistically significant differences for all tests were further analyzed using Fisher's post hoc analyses, and significance for all analyses was ascribed at $p < 0.05$.

Results

Behavioral Analyses

In experiment 1, one animal from the defeat castrated group was eliminated from behavioral analyses due to injury during CD training. Another animal from the no-defeat castrated group was eliminated because it was attacked by the non-aggressive intruder during CD testing. Hormonal status (i.e., castrated vs. sham-operated) strongly influenced the duration of aggressive behavior of resident aggressor's towards experimental animals that were housed four weeks prior to behavioral testing ($F(1,9)=20.23$, $p < 0.01$). More specifically, resident aggressors were significantly more aggressive during CD training towards sham-operated intruders compared with castrated intruders. There was no difference in the display of submissive/defensive, social or nonsocial behaviors in resident aggressors towards these two groups, ($p > 0.05$) (See Figure 4.1a). Despite the fact that castrated intruders were attacked significantly less than sham-operated animals, there was no difference between these groups in the display of submissive/defensive, aggressive, social or nonsocial behaviors in the presence of a resident aggressor, ($p > 0.05$) (See Figure 4.1b).

During CD testing, social experience (i.e., defeat vs. no-defeat) influenced whether or not male hamsters would display conditioned defeat ($F(1,25)=13.67$, $p < 0.01$) or territorial aggression ($F(1,25)=19.80$, $p < 0.01$). When paired with a non-aggressive intruder, defeated male hamsters were significantly more submissive and significantly less aggressive relative to their non-defeated counterparts. Social and nonsocial behavior did not differ between groups ($p > 0.05$). Hormonal status influenced the display of submissive ($F(1,25)=32.57$, $p < 0.01$) and social ($F(1,25)=17.83$, $p < 0.01$) behaviors in

experimental animals paired with non-aggressive intruders. Castrated males were significantly more submissive and significantly less social than were sham-operated males. Aggressive and nonsocial behavior did not differ between groups ($p > 0.05$). MANOVA revealed a significant interaction of social experience x hormonal status on submissive ($F(1,25)=5.34$, p , 0.05) and social behaviors ($F(1,25)=7.93$, $p < 0.01$). During CD testing castrated males that had been previously defeated were significantly more submissive and less social when compared with all other groups. In addition, castrated males without a previous defeat were significantly more submissive compared with non-defeated sham-operated males. There was no significant interaction of social experience and hormonal status on aggressive and nonsocial behavior, $p > 0.05$. The behavioral results for experiment 1 are summarized in Figure 4.2.

In experiment 2, two animals from the no-defeat castrated group were not included in the behavioral analyses. One animal was not included because it was attacked by a NAI, and the other animal was eliminated because its behavior was not videotaped. We routinely score the CD training period in the event that there is a difference between treatment groups in the level of defeat or in the display of CD. This enables us to determine whether the differences that we observe during CD testing are due to differences in how animals were attacked during the CD training period. In experiment 2, there was no effect of hormonal status on submissive, aggressive, social or nonsocial behavior ($p > 0.05$) in animals that were individually housed 10 days prior to behavioral testing; therefore, we did not score the CD training period. Social experience did however, influence the display of submissive ($F(1,23)=34.61$, $p < 0.01$), aggressive ($F(1,24)=15.23$, $p < 0.01$) and social behavior ($F(1,23)= 7.496$, $p =0.01$) in male hamsters

during CD testing. Again, defeated males were significantly more submissive and significantly less aggressive and less social toward non-aggressive intruders in comparison with their non-defeated counterparts. Non-social behavior did not differ between groups ($p > 0.05$). Finally, there was no significant interaction of social experience and hormonal status on nonsocial behavior in male hamsters in this particular experiment ($p > 0.05$). The behavioral data for experiment 2 are depicted in Figure 4.3.

In experiment 3a, there was no effect of hormone treatment on the display of social and nonsocial behavior in defeated male hamsters ($p > 0.05$). However, there was a strong trend towards an effect of hormone on submissive ($p=0.06$) and aggressive ($p=0.07$) behavior. More specifically, defeated males treated with DHT and T had a lower mean of submissive behavior relative to those treated with E_2 and C capsules. Likewise, the defeated group treated with T had a higher mean of aggressive behavior compared with all other groups. In experiment 3b, there was no effect of hormone treatment on the display of submissive, social and nonsocial behavior ($p > 0.05$) in non-defeated male hamsters. Yet, there was an effect of hormone treatment on aggressive behavior ($F(3,11)=3.71$, $p < 0.05$). Interestingly, non-defeated male hamsters treated with T had significantly lower aggressive behavior in comparison with those treated with E_2 and C. There was no difference in aggressive behavior between males treated with DHT vs. T or those treated with E_2 vs. C, respectively. The behavioral data for experiment 3 are depicted in Figure 4.4.

Hormonal Analyses

In experiment 1, six animals (three from each group) were not included in the hormonal analyses due to an insufficient amount of serum. Castration greatly reduced serum concentrations of T ($F(1,21)=290.32$, $p < 0.01$), DHT ($F(1,21)=163.20$, $p < 0.01$) and E_2 ($F(1,21)=41.46$, $p < 0.01$) in male hamsters that were individually housed 4 weeks prior to behavioral testing (Refer to Table 1). In experiment 2, two animals from the sham-operated group were not included in the DHT analyses due to insufficient amount of serum. Again, castration reduced serum concentrations of T ($F(1,25)=70.54$, $p < 0.01$), DHT ($F(1,23)=38.29$, $p < 0.01$) and E_2 ($F(1,25)=20.98$, $p < 0.01$) (Refer to Table 2). In experiment 3, one animal from the C group was not included in the hormonal analyses due to unusually high amounts of gonadal hormones, which is suggestive of incomplete gonadectomy. Hormonal treatment greatly influenced serum hormonal concentrations. Male hamsters treated with T had significantly higher serum T concentrations compared with those given C capsules ($F(1,13)=28.64$, $p < 0.01$). In addition, males treated with DHT had significantly higher serum DHT concentrations compared with those given C capsules ($F(1,13)=5.06$, $p < 0.05$). Likewise, males treated with E_2 had significantly higher serum E_2 concentrations compared with those given C capsules ($F(1,13)=11.22$, $p < 0.01$ (Refer to Table 3).

Discussion

The data from the current study suggest a unique interaction of testicular hormones and length of individual housing on the reduction of CD in male hamsters. In our past studies, experimental animals are individually housed approximately 10 days prior to behavioral testing and they typically display increased submissive and nonsocial

behaviors, as they are actively avoiding the intruding conspecific. In experiment 1, intact male hamsters that were individually housed for four weeks were very social and followed their opponent closely; on the other hand, their castrated counterparts displayed very little social behavior and spent the majority of their time fleeing from the intruder. In experiment 2, both intact and castrated males that were individually housed 10 days prior to behavioral testing displayed increased submissive behavior and little to no aggressive behavior in the presence of a smaller intruder. Together these data suggest that individual housing for longer periods of time (i.e., 4 weeks) reduces the display of CD in intact male hamsters, while individually housing castrated males for this same amount of time increases the display of CD in male hamsters.

The finding from the current study suggests that neither the presence of hormones or the length of individual housing as individual factors are enough to produce subsequent changes in behavior in defeated hamsters. In both experiments, castrated males had significantly less circulating hormones relative to intact males. However, we only observed a difference between these two groups in the behavioral response to social defeat in the first experiment. If the reduced submissive behavior following defeat in intact males was due to the presence of hormones only, then one should expect that intact males in experiment 2 would also display reduced submissive behavior, but they did not. Similarly, if the reduction in submissive behavior was due to length of isolation alone, then one would expect castrated males in the first experiment to display this same decrease in submissive behavior following social defeat. Together, these data suggest that both the increased time of individual housing and the presence of testicular hormones may interact to decrease the expression of CD in male hamsters.

In both experiments, non-defeated males, regardless of hormonal status, displayed territorial aggression toward an intruder. This finding is consistent with other studies which report that castrated male hamsters will exhibit aggression towards either freely moving or restrained conspecifics (Whitsett, 1975; Potegal et al., 1980). Of particular interest, is the fact that non-defeated castrated, males in experiment 1 displayed more submissive behavior in the presence of a smaller intruder than did the defeated intact males. The castrated males that were singly housed for four weeks prior to behavioral testing appeared to display a striking increase in fear or "anxiety-like" behavior both during CD training and during CD testing despite the fact that these males were attacked significantly less than were intact males. It would be interesting to determine if this increased "anxiety-like" behavior is apparent only during agonistic interactions or if this translates over to more traditional indices of anxiety, like the open field. In fact, there have been some reports that the presence of T in male rodents is anxiolytic (Fernandez-Guasti & Martinez-Mota, 2005; Frye & Seliga, 2001) and that reduced T facilitates enhanced fear responses in male rats as indicated by increased freezing and fear-induced analgesia (King et al., 2005). If this is the case, then it might be likely that long-term withdrawal (i.e., 4 weeks) from endogenous hormones in castrated male hamsters may induce an anxiogenic state in these animals

In experiment 3, we examined the effect of exogenous hormone administration on the display of CD in male hamsters. Castrated males given silastic capsules containing T and DHT were considerably less (though not statistically different) submissive following defeat than were males given E_2 and C. Overall, the defeated males in this experiment displayed little to no aggressive behavior in the presence of a smaller intruder. The

defeated males given T displayed a higher mean increase (though not significant) of aggressive behavior. This finding is somewhat paradoxical, given the fact that normally, intact males do not display aggressive behavior, following defeat. At this point, it is difficult to explain why testicular hormones may produce varied effects on behavior depending upon the hormonal status of the animal (i.e., intact vs. hormonally replaced).

Non-defeated male hamsters, given T, DHT or E did not display any submissive behavior in the presence of a non-aggressive intruder. These data are in accord with a previous study which reported no difference in submissive behavior in male hamsters given various hormones such as T and DHT (Payne, 1974). Surprisingly, males given T actually displayed less aggressive behavior compared with those given other treatments. This finding contrasts with previous findings which report that T increases aggression in male hamsters (Payne, 1974; Grelk et al., 1974). It is not that these males given T were not interested in the non-aggressive intruder; in fact, these males were "overly social" or borderline aggressive. More specifically, they did not display as much aggressive behavior as indicated by attacking or biting, but rather followed the animal very closely. This decrease in aggressive behavior seen in non-defeated males given T is somewhat puzzling, given the fact that defeated males given T displayed some aggression towards the non-aggressive intruder. Clearly, more studies have to be conducted in order to assess the interaction between androgens and social experience on the display of agonistic behavior in male hamsters.

The contrasting effects of hormones and length of individual housing on agonistic behavior in defeated male hamsters might be explained by the varied concentration of circulating androgens in males across the experiments in this study. For example, in the

first and second experiments, T and DHT concentrations in intact males were 6.08 ng/ml and 639.19 pg/ml vs. 3.70 ng/ml and 362.07 pg/ml, respectively. Perhaps isolation for longer periods of time increases androgen levels in male hamsters. Although no data exist that clearly demonstrate that isolation increases androgen concentrations in male hamsters, social isolation increases T concentrations in Balb/C mice (Sayegh et al., 1990). Overall, the hormone treated males had significantly lower levels of androgens in comparison with intact males. Hormone treated males that were individually housed for 4 weeks prior to testing had similar T concentrations (3.37 ng/ml) to males that were individually housed for 10 days (3.70 ng/ml). By contrast, hormone treated males had markedly lower T concentrations compared with intact males that were individually housed for the same amount of time (6.08 ng/ml). Serum DHT concentrations were also considerably lower in hormone replaced males (74.02 pg/ml) compared with intact males in experiment 1 (639.19 pg/ml) and experiment 2 (362.07 pg/ml). Thus, it is conceivable that the varied responses to social defeat seen in males across the three experiments might be due to differences in serum concentrations of these hormones.

It is important to note that hormonal manipulations (i.e., castration) may influence how the experimental animal responds to an opponent, but that it may also alter that animal's stimulus properties (e.g., scent) which in turn may influence how an opponent responds to it. In the first experiment, castrated males were highly submissive during CD training despite the fact that they were attacked significantly less than were their intact counterparts. During CD training, the resident aggressors initially spent more time investigating the genital area of the castrated opponents. The resident aggressors did not attack the castrated males with the same intensity as they did the intact males

suggesting that the resident aggressors were able to distinguish between an intact and hormone-deprived animal. These data are consistent with previous studies which report that castrated males are attacked less than intact males (Evans & Brain, 1974). Perhaps castrated males are viewed as less of a threat than are intact male hamsters, or alternatively perhaps the resident aggressors were spending more time trying to determine the sex of the opponent. Overall, these findings do suggest the need to consider the hormonal status of both the experimental animal as well as its opponents on the display of agonistic behavior. Collectively, these data suggest that the display of CD in male hamsters is independent of circulating testicular hormones and that housing for longer periods of time (i.e., 4 weeks) may reduce the display of CD in intact male hamsters.

Figure 4.1. (a) Average duration (mean +/-SEM in seconds) during a 15- minute training episode of submissive/defensive, aggressive, social and nonsocial behavior of resident aggressors towards sham-operated (n=4) and castrated (n=7) male hamsters that were singly housed for 4 weeks post surgery. MANOVA revealed that resident aggressors were significantly more aggressive towards sham-operated males compared with castrated males (* $p < 0.01$). **(b)** Average duration (mean +/-SEM in seconds) of submissive, aggressive, social and nonsocial behavior of experimental hamsters during a 15-minute training episode with a resident aggressor.

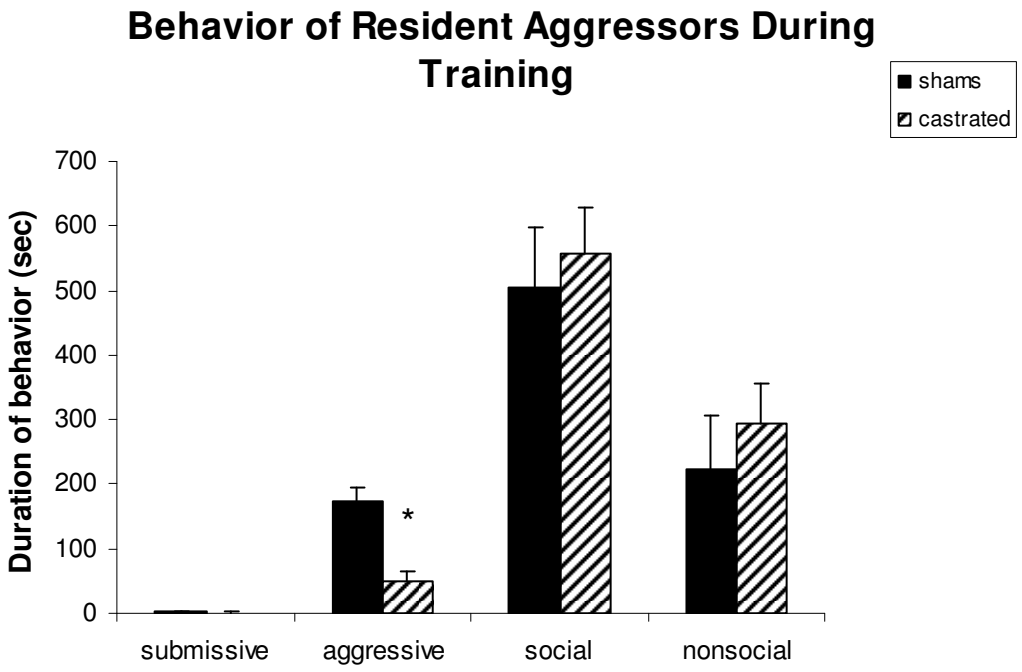
Figure 4.2. Average duration (mean +/-SEM in seconds) of submissive/defensive, aggressive, social and nonsocial behavior in defeated sham-operated (n=6), defeated castrated (n=8), non-defeated sham-operated (n=7) and non-defeated castrated (n=8) male hamsters (that were individually housed for 4 weeks prior to behavioral testing) during testing with a non-aggressive intruder. Analyses were run for each specific behavior; therefore, differences are compared only within one category of behavior and not between behavioral categories. Non-shared letters depict significant difference ($p < 0.05$) as determined by Fisher's post hoc analyses.

Figure 4.3. Average duration (mean +/-SEM in seconds) of submissive/defensive, aggressive, social and nonsocial behavior in defeated sham-operated (n=8), defeated castrated (n=7), non-defeated sham-operated (n=8) and non-defeated castrated, (n=4) male hamsters (that were individually housed for 10 days prior to behavioral testing) during testing with a non-aggressive intruder. Analyses were run for each specific

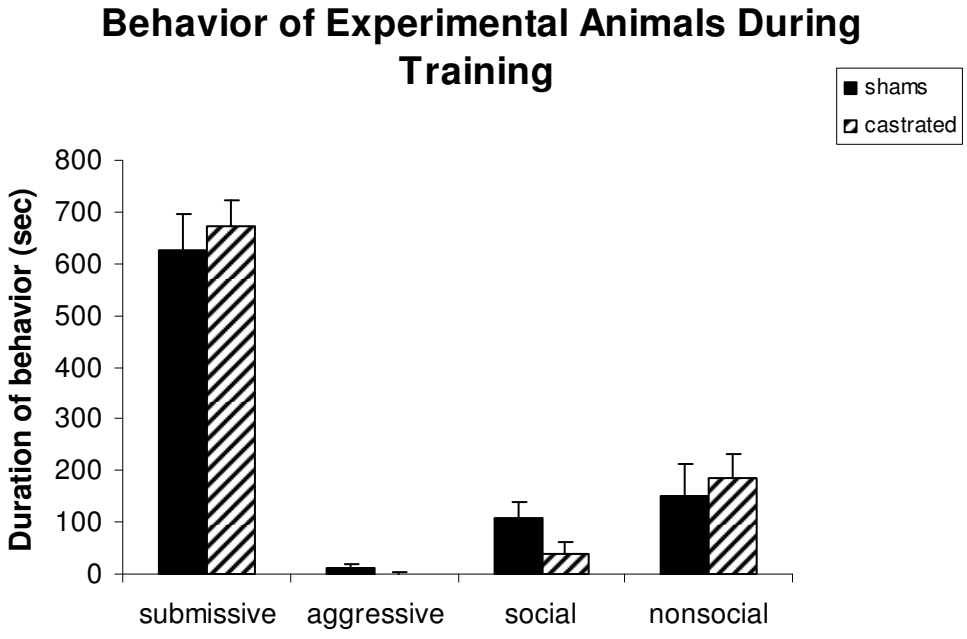
behavior; therefore, differences are compared only within one category of behavior and not between behavioral categories. Non-shared letters depict significant differences ($p < 0.05$) as determined by Fisher's post hoc analyses.

Figure 4.4 (a) Average duration (mean +/-SEM in seconds) of submissive/defensive, aggressive, social and nonsocial behavior in defeated male hamsters administered DHT (n=5), E_2 (n=5), T (n=5) and cholesterol (C) (n=4) and **(b)** non-defeated male hamsters administered DHT (n=4), E (n=4), T (n=4) and C(n=3) during a test encounter with a non-aggressive intruder. Analyses were run for each specific behavior; therefore, differences are compared only within one category of behavior and not between behavioral categories. Non-shared letters depict significant differences ($p < 0.05$) as determined by Fisher's post hoc analyses.

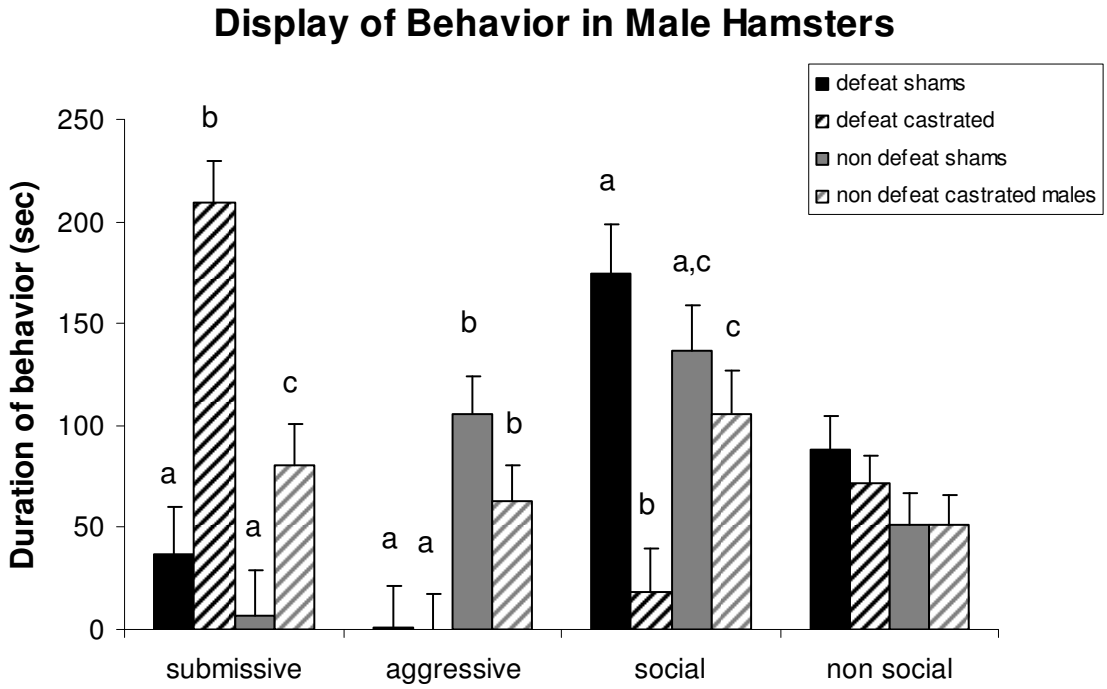
4.1a


Behavior of Resident Aggressors During Training
shams
castrated
Duration of behavior (sec)
700
600
500
400
300
200
100
0
*
submissive
aggressive
social
nonsocial

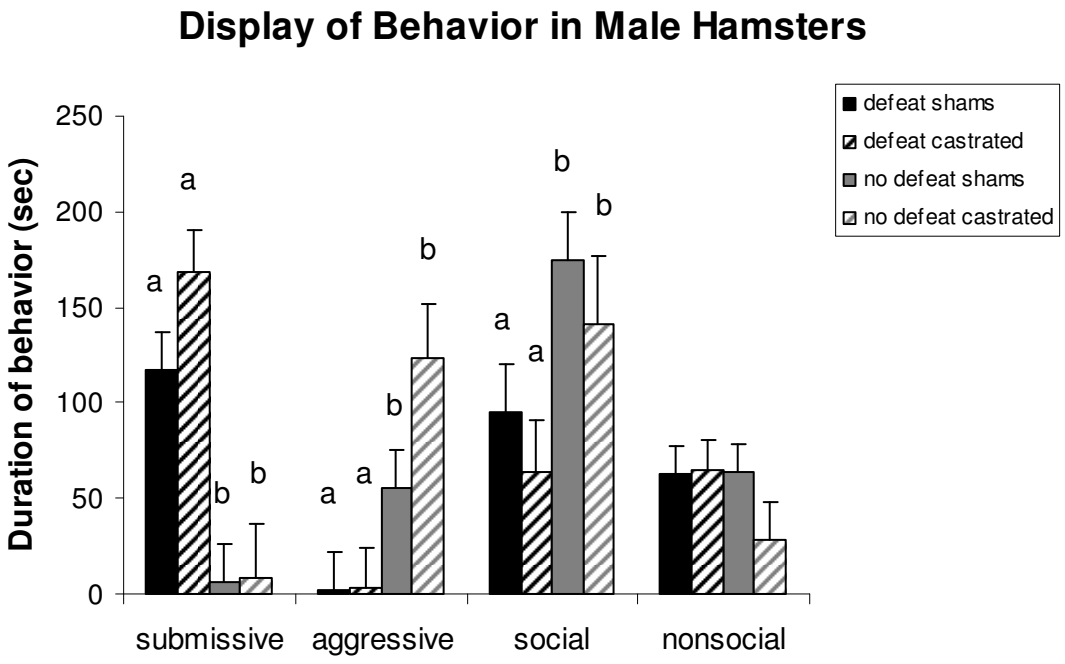
4.1b


Behavior of Experimental Animals During Training
shams
castrated
Duration of behavior (sec)
800
700
600
500
400
300
200
100
0
submissive
aggressive
social
nonsocial

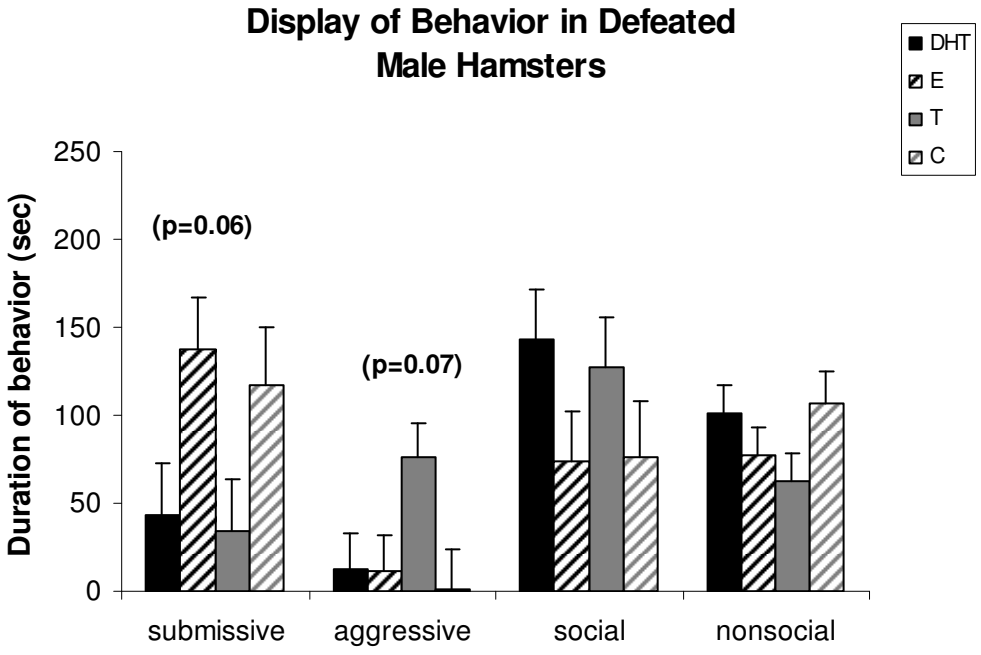
4.2


Display of Behavior in Male Hamsters
defeat shams
defeat castrated
non defeat shams
non defeat castrated males
Duration of behavior (sec)
250
200
150
100
50
0
a
b
a
c
a
a
b
b
a
b
a,c
c
submissive
aggressive
social
non social

4.3


Display of Behavior in Male Hamsters
Duration of behavior (sec)
250
200
150
100
50
0
defeat shams
defeat castrated
no defeat shams
no defeat castrated
a
a
b
b
a
a
b
b
a
a
b
b
submissive
aggressive
social
nonsocial

4.4a


Display of Behavior in Defeated Male Hamsters
DHT
E
T
C
Duration of behavior (sec)
250
200
150
100
50
0
(p=0.06)
(p=0.07)
submissive
aggressive
social
nonsocial

4.4b

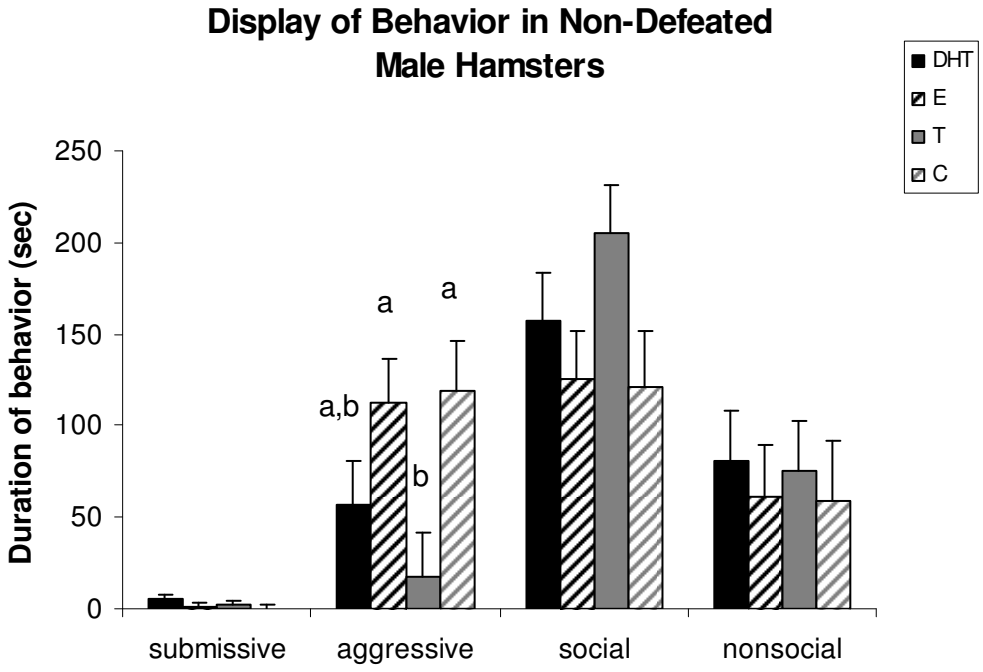

Display of Behavior in Non-Defeated Male Hamsters
DHT
E
T
C
Duration of behavior (sec)
250
200
150
100
50
0
a
a
a,b
b
submissive
aggressive
social
nonsocial

Table 1. Serum levels of T, DHT and E_2 in sham-operated and castrated male hamsters that were individually housed for 4 weeks prior to behavioral testing for Experiment 1. Data are presented as (mean +/-SEM). * indicates statistical significance, $p < 0.01$.

Hormonal Group	n	Serum Levels of Hormone
Castrate T (ng/ml)	12	0.00+/-0*
Sham	11	6.08+/-.37
Castrate DHT (pg/ml)	12	5.51+/-3.11*
Sham	11	639.19+/-51.81
Castrate E (pg/ml)	12	88.74+/-5.55*
Sham	11	162.48+/-10.32

Table 2. Serum levels of T, DHT and E_2 in sham-operated and castrated male hamsters that were individually housed for 10 days prior to behavioral testing for Experiment 2. Data are presented as (mean +/-SEM). * indicates statistical significance, $p < 0.01$.

Hormonal Group	n	Serum Levels of Hormone
Castrate T (ng/ml)	11	.06+/-.05*
Sham	16	3.70+/-.36
Castrate DHT (pq/ml)	11	5.62+/-5.06*
Sham	14	362.07+/-50.67
Castrate E (pg/ml)	11	126.05+/-6.31*
Sham	16	215.61+/-15.53

Table 3. Serum levels of T, DHT and E_2 in hormonally treated male hamsters that were individually housed for 4 weeks prior to behavioral testing for Experiment 3. Analyses were run such that each hormone–treated group is compared against the cholesterol group only for the specific hormone of interest. Data are presented as (mean +/-SEM). * indicates statistical significance, $p < 0.05$.

Hormonal Group		**n**	**T (ng/ml)**	**DHT (pg/ml)**	**E (pg/ml)**
T	Treated	9	3.37+/-.45*		
DHT	Treated	9		74.02+/-16.49*	
E	Treated	9			198.73+/-24.59*
C	Treated	7	.03+/-.02	17.88+/-17.88	87.36+/-16.52

Chapter 6

FOS EXPRESSION IN PREVIOUSLY DEFEATED MALE AND FEMALE HAMSTERS FOLLOWING AN AGONISTIC ENCOUNTER

Abstract

In the present study we examined behavior and Fos expression in previously defeated male, and proestrous and diestrous 1 female hamsters following exposure to a non-aggressive intruder (NAI). In Experiment 1, defeated male and proestrous female hamsters displayed conditioned defeat (CD) which is characterized by an increase in submissive behavior and a decrease in normal territorial aggression. By contrast, diestrous 1 females did not display CD confirming that there is an estrous cycle-dependent effect on the display of CD in female hamsters. Following exposure to a NAI, both defeated male and proestrous females exhibited greater Fos activation in the dorsal lateral septum and paraventricular nucleus of the hypothalamus compared with diestrous 1 females. In addition, the defeated proestrous females exhibited greater Fos activation in the intermediate lateral septum and central amygdala compared with the diestrous 1 females suggesting that these regions might be specific to the behaviors that characterize conditioned defeat. On the other hand, defeated diestrous 1 females displayed greater Fos activation in the lateral bed nucleus of the stria terminalis suggesting that this region might be involved in the maintenance of aggression in diestrous 1 females. Finally, because it is possible that the behavioral differences across the estrous cycle are due to changes in anxiety, we examined behavior in an open field arena in diestrous 1 and proestrous females. We found no effect of hormonal status alone, on locomotion, grooming or rearing in the open field suggesting that differences in conditioned defeat are probably not due to alterations in anxiety-like behavior across the estrous cycle.

Introduction

Social defeat induces marked behavioral and physiological changes in a variety of species. For example, defeated animals exhibit suppressed immune and gonadal functioning (Devoino et al., 2003; Stefanski, 1997; Engler et al., 2005) as well as decreased social interaction with conspecifics (Kudryavtseva et al., 1991). At present, much of the research involving the neural and hormonal mechanisms associated with social defeat in rodents involves male subjects. This is partly the case because few female rodents readily exhibit territorial aggression (for review see Tamashiro, Nguyen & Sakai, 2005). Syrian hamsters are a unique species in that both male and female hamsters will spontaneously display territorial aggression towards intruding conspecifics (Payne & Swanson, 1970; Lerwill & Markings; Tiefer, 1970). Thus, this species presents a valuable opportunity to explore sex differences in the neural, hormonal and behavioral responses to social defeat.

Following social defeat, male hamsters' normal territorial aggression is replaced by increased submissive/defensive behavior in the presence of a smaller intruding conspecific; we have termed this phenomenon conditioned defeat (CD) (Potegal et al., 1993; Cooper & Huhman, 2005; Jasnow, Cooper & Huhman, 2004). CD is maintained in male hamsters for a period of up to one month, even if they are never attacked again (Huhman et al., 2003). Many female hamsters, on the other hand, either do not exhibit CD at all or do not maintain CD as long as do males. In fact, by the second test encounter most, if not all, females regain their normal territorial aggression, indicating that there is a sex difference in the maintenance of CD in Syrian hamsters (Huhman et al., 2003). Since this initial finding, we have examined the effect of the estrous cycle on the

display of CD in female hamsters and have found that proestrous female hamsters are more likely to exhibit CD than are diestrous 1 females (Solomon & Huhman, in preparation). These data indicate that there is also an estrous cycle-dependent effect on the display of CD in this species.

We hypothesized that there would be differences in patterns of neuronal activation as measured by c-Fos (a marker for neuronal activation) between females that displayed CD versus those that did not display CD. Much of the work examining Fos expression in defeated male rodents has found that defeated males exhibit increased Fos activation in a variety of brain regions that are thought to be involved with stress or stress-related behaviors like the lateral septum (Martinez, Phillips & Herbert, 1998; Kollack-Walker, Watson & Akil, 1997; Kollack-Walker, Watson & Akil, 1999), bed nucleus of the stria terminalis (Martinez, Phillips & Herbert, 1998; Kollack-Walker, Watson & Akil, 1999), paraventricular nucleus of the hypothalamus (Matsuda et al., 1996; Kollack-Walker, Watson & Akil, 1997; Kollack-Walker, Watson & Akil, 1999) as well as the amygdala (Martinez, Phillips & Herbert, 1998; Kollack-Walker, Watson & Akil, 1997; Kollack-Walker, Watson & Akil, 1999). One shortcoming of these studies is the failure to include dominant counterparts in the immunocytochemical analyses. The inclusion of this group should allow greater differentiation between brain areas that are involved in agonistic interactions, in general, versus those that are specific to being defeated.

Because we have demonstrated that previously defeated male and proestrous female hamsters display CD while diestrous 1 females do not display CD, we hypothesized that it might be possible to distinguish between brain regions that are

important for the expression of CD versus those that are involved in the maintenance of territorial aggression. The present study was designed to test the hypothesis that previously defeated male and proestrous females that display CD will show greater Fos activation in regions that are associated with stress in comparison with diestrous 1 females which do not display CD. We measured Fos activation in the following brain areas: dorsal, intermediate and ventral lateral septum, anterior lateral and medial bed nucleus of the stria terminalis, paraventricular nucleus of the hypothalamus, and medial, central and basolateral amygdala.

Finally, gonadal hormones are known to influence anxiety-like behavior in female rodents (Frye & Walf, 2002; Frye, Petralia & Rhodes, 2000; Walf & Frye, 2005; Marcondes et al., 2001; Mora, Dussaubat & Diaz-Veliz, 1996). It is possible that the behavioral differences in the response to social defeat observed in diestrous 1 and proestrous females might be due to underlying differences in "anxiety-like" behavior in these females. In order to test this hypothesis, we quantified behavior in an open-field arena, a commonly used apparatus for anxiety. Females in either the diestrous 1 or proestrous phase of the cycle were exposed to bright or dim light conditions and behavior in the open field was monitored.

Methods:

Animals and Housing Conditions

Adult female and male Syrian hamsters (*Mesocricetus auratus*) weighing 120-130g (10 weeks old) at the beginning of the experiment were obtained from Charles River Laboratories. Female and male hamsters were individually housed in separate temperature-controlled colony rooms on a 14:10 hr light: dark cycle with lights off at 0800 h (females) and 1100 h (males). Additional singly-housed male and ovariectomized female hamsters weighing >180 g (>6 months old) were used as resident aggressors during defeat training. Intact group-housed female and male hamsters (five per cage) weighing 100-110 g (7 weeks old) were used as non-aggressive intruders (NAI) during conditioned defeat testing. All animals were housed in polycarbonate cages (20x40x20cm) with wire mesh tops, corn cob bedding and cotton nesting materials. Food and water were available ad libitum. All procedures and protocols were approved by the Georgia State University Institutional Care and Use Committee.

Determination of Estrous Cycle

At least two weeks prior to behavioral testing all female subjects were monitored between 0800 and 0900 for determination of estrous cycle, and only females that had consistent four day estrous cycles were used in the study. Briefly a cotton swab was placed against the vaginal area. A thin, stringy vaginal discharge signified vaginal estrus (Estrus) and the period of sexual receptivity, (Wise, 1974) with subsequent days being defined as diestrus 1, diestrus 2, and proestrus.

Conditioned Defeat Training

On the day of training, male and female hamsters were transported in their home cages from the colony room to the behavioral testing room where they were placed into the home cage of a resident aggressor for four, five-minute training trials. All hamsters were paired with a different aggressor for each training trial. Training began at 0800 h for females and 1100h for males with a 1 h interval between each 5 minute training trial. During the sessions, trained observers ensured that all experimental animals were attacked by the aggressors and that all of the experimental animals displayed submissive/defensive behaviors and no aggressive behaviors toward any of the resident aggressors.

Conditioned Defeat Testing

Behavioral testing began the day following defeat training and all testing was completed during the first 2 h of dark phase of the LD cycle. A resident/intruder pairing was used in which a non-aggressive intruder (NAI) was placed into the home cage of each experimental animal for 5 minutes. Female NAIs were screened to ensure that they did not display lordosis and then they were randomly paired with the experimental animals. Videotapes of the testing sessions were scored using the Observer for Windows, version 3.0 (Noldus Information Techonology B.V., Wageningen, The Netherlands). The following classes of behaviors were recorded as total duration in seconds. 1) Non-social: locomotor/exploratory, self-groom, nesting, feeding, pouching, sleeping, lordosis; 2) Social: attend, approach, investigate, sniff, touching nose; 3) Submissive/Defensive: upright and side defense, tail lift, flee, full submissive posture; 4) Aggressive: upright and side offense, chase, attack, bite. The videotapes were scored by a trained observer that

was experienced in scoring agonistic behavior and was blind to the experimental condition of each hamster.

Experiment 1:
c-Fos Expression in Previously Defeated Male and Female Hamsters

Estrous (n=7), diestrous 2 (n=5) and male (n=7) hamsters were defeated four times for five minutes by a larger resident aggressor (CD Training). Twenty-four hours following training, females and males were paired for five minutes with a NAI (CD Testing) in their own home cage. Thus, groups of females were trained in either estrus or diestrus 2 and subsequently tested in diestrus 1 or proestrus, respectively. In addition, control animals included diestrous 1 (n=4), proestrous (n=4) and male (n=4) hamsters that were handled only and remained in their home cages during the experiment.

Immunocytochemistry

Approximately one hour after defeat testing, all animals were given an overdose of sodium pentobarbital and perfused intercardially with 100 ml of 0.9% saline followed by 200 ml of 4% paraformaldehyde in 0.1M phosphate buffer (PBS) (pH 7.6). Brains were subsequently removed and post-fixed in 4% paraformaldehyde for 24 hours at 4˚C. The brains were transferred into 0.1M PBS containing 30% sucrose for 2 days. Coronal sections were cut at 45 μm with a freezing microtome and collected in alternate sections into three vials that were coded for each animal. Each vial contained 0.05M PBS (pH 7.6). Sections were pretreated with 0.1% sodium borohydride for 20 minutes to remove any residual aldehydes. After this step and all subsequent incubations, sections were rinsed 3 X 15 minutes in PBS. Subsequent incubations were (1) rabbit polyclonal c-Fos antibody (1:5000, Santa Cruz Biotechnology) in Supermix (0.05M solution of PBS

containing 0.25% gelatin and 0.5% Triton X-100) overnight at room temperature; (2) biotinilated goat anti-rabbit (1:500); Vector Laboratories, Burlingame, CA) in supermix for 2 hours at room temperature; (3) avidin-biotin horseradish peroxidase complex (1:1500; Vectastain ABC Elite Kit, Vector Laboratories) in supermix for 2 hours at room temperature; (4) ABC-horseradish peroxidase complex was visualized with 3,3'-diaminobenzidine (Sigma) that was dissolved in a solution containing Tris-NaCl and 0.09% hydrogen peroxide for 15-25 minutes. Sections were mounted on gelatinized slides, allowed to dry, dehydrated with alcohol and Citrosolv and coverslipped.

c-Fos Quantification

Specific brain regions of interest were located using light microscopy by a trained individual using the golden hamster atlas (Morin and Wood, 2001) and local landmarks in each brain section. All counts were unilateral. The lateral septum was divided into three regions - dorsal, intermediate and ventral - at the level where the anterior commisure lies below the lateral ventricle (Bregma 0.9mm). The anterior bed nucleus of the stria terminalis was divided into the lateral and medial subdivisions and surrounded the anterior commisure (Bregma 0.9mm). The PVN was a triangular region beside the dorsal portion of the 3rd ventricle (Bregma -0.9mm). The posterior medial amygdala was lateral to the optic tract (Bregma -1.5mm), while the central amygdala was a circular region dorsolateral to the medial amygdala, and the basolateral amygdala was lateral to the central amygdala (Bregma -1.5mm). Using Bioquant software (R & M Biometrics, Nashville, TN, version BQ-TCW98, 3.50.6NT) a representative section for each brain region was selected which allowed the observer to identify precisely the microscopic

field and neurons to be counted. c-Fos positive nuclei were manually counted and a cell was considered to be labeled with c-Fos if the staining was dark and nuclear.

Experiment 2: Open Field Behavior in Diestrous 1 and Proestrous Female Hamsters

Female hamsters were exposed on different days of the estrous cycle to either bright (diestrous 1 (n=7) or (proestrous (n=9)or dim (diestrous 1 (n=7) or (proestrous (n=8) lighting conditions to assess the effect of light and hormonal status on anxiety-like behavior in female hamsters in an open field arena. The open field was a 91x91x41 cm white acrylic box open on top with a grid on the floor whose squares measure 14.5cm^2. Lighting in the open field during testing was indirect and measured approximately 870-lux (white light for the bright testing conditions) or 14-lux (red light for dim testing conditions). White noise played in the background during all open field testing. Each hamster was tested individually. Animals were placed in the center of the arena and the behavior in the open field was monitored for 5 minutes. Behaviors were videotaped and scored by trained behavior raters using a computerized scoring system ("Hind Sight", developed by Dr. Scott Weiss). These behaviors included locomotion (total number of squares entered) number of central, intermediate and outer square entries, duration of time spent in central, intermediate and outer squares, frequency of rearing, and duration of grooming. An entry was defined by all four paws completely in one square.

Statistical Analyses

In Experiment 1, behavioral data were analyzed using one-way multivariate analyses of variance (MANOVA) with group (i.e., proestrus) as the independent variable

and submissive, aggressive, social and nonsocial as dependent variables. For immunocytochemical anyalses in Experiment 2 differences in c-Fos positive nuclei within each brain region were analyzed using a one-way analysis of variance (ANOVA). In Experiment 2, 2-way ANOVA was used to examine the effect of hormonal status and lighting conditions on behavior in an open field arena. All statistical analyses were followed by Fisher's Least Significant Difference (LSD) post-hoc tests to determine significant differences among groups. Significance for all analyses was ascribed at $p<0.05$.

Results

In Experiment 1, (CD Training) there were no significant differences in the display of submissive, ($F(1,10)=0$, $p>0.05$) aggressive, ($F(1,10)=.319$, $p>0.05$), social ($F(1,10)=1.066$, $p>0.05$) or nonsocial ($F(1,10)=.285$, $p>0.05$) behaviors of the resident aggressors toward experimental female hamsters tested in either estrus or diestrus 2. The behavioral results for CD Training are summarized in Figure 5.1a. For the CD Testing analyses, one previously defeated female hamster from the proestrous group was eliminated from statistical analyses because of an unusually high amount of aggressive behavior that was not consistent with the display of aggressive behavior in the other females of this group and was deemed an outlier (i.e., Z score >3). MANOVA revealed a significant main effect of group on submissive behavior ($F(2,15)=15.33$, $p<0.01$). Previously defeated males displayed significantly more submissive behavior in the presence of a NAI than did defeated females tested in diestrus 1 and proestrus. Proestrous female hamsters displayed significantly more submissive behavior in

comparison with diestrous 1 female hamsters but significantly less submissive behavior compared with defeated male hamsters in the presence of a NAI. There was a significant difference in the display of aggressive behavior depending on group, ($F(2,15)=5.66$, $p<0.05$) in that previously defeated diestrous 1 females displayed significantly more aggressive behavior than did defeated male and proestrous females during CD testing. There were no significant differences in the display of aggressive behavior between defeated male and proestrous female hamsters. All groups displayed similar levels of social behavior in the presence of a NAI, ($F(2,15)=1.843$, $p>0.05$). There was however a significant difference in the display of nonsocial behavior among groups ($F(2,15)=5.70$, $p<0.05$. Previously defeated females tested in proestrus displayed significantly higher nonsocial behavior in comparison with both previously defeated male hamsters and diestrous 1 females. There were no significant differences in nonsocial behavior between previously defeated male and diestrous 1 female hamsters. The behavioral results for CD Testing are summarized in Figure 5.1b.

Immunocytochemistry

Due to background one animal from the defeated male group and one female from the diestrous 1 and proestrous control groups was eliminated from statistical analysis.

Lateral Septum

There was a significant main effect of group on Fos activation within the dorsal lateral septum (LS), ($F(5,25)=10.03$, $p < 0.01$). All control groups and defeated diestrous 1 females exhibited similar Fos activation in this region. In addition, all of these groups exhibited significantly less Fos activation within the dorsal lateral septum compared with both defeated male and proestrous female hamsters. The defeated proestrous females

exhibited significantly higher Fos activation compared with all other groups in the dorsal LS. There was also a significant main effect of group on Fos activation within the intermediate LS ($F(5,25)=12.67$, $p < 0.01$. All control groups exhibited similar Fos activation. Although the defeated males had a higher mean (110.43 vs. 57.66) in comparison with control diestrous 1 females, this did not reach statistical significance. Both control male and proestrous female hamsters had significantly lower Fos activation within the intermediate LS compared with all previously defeated groups. Both defeated male and diestrous 1 female hamsters exhibited similar Fos activation, but significantly lower Fos activation compared with defeated proestrous females. Defeated proestrous females had significantly higher Fos activation within the intermediate LS compared with all other groups. Similar to the dorsal and intermediate LS, there was a significant main effect of group on Fos activation within the ventral LS ($F(5,25=18.84$, $p < 0.01$). All control groups had similar Fos action and all had significantly lower Fos activation compared with the defeated groups. The defeated diestrous 1 and proestrous females had similar Fos activation and both were significantly higher than the defeated male groups. (These data are depicted in Figure 5.2).

Bed nucleus of stria terminalis

There was a significant main effect of group on Fos activation within the anterior lateral bed nucleus of the stria terminalis ($F(5,24)=12.97$, $p < 0.01$). All control groups were similar to one another and all had significantly less Fos activation within this region compared with all defeated groups. Defeated diestrous 1 females had significantly greater Fos activation within this region compared with both defeated male and proestrous females. Both the defeated male and proestrous females hamsters exhibited

similar Fos activation. Although there was a strong trend of group on Fos activation within the medial BNST this did not reach statistical significance ($F(5,24)=2.53$, $p = .057$. Thus all groups despite social history exhibited similar Fos activation within the medial BNST. (The data for the BNST are summarized in Figures 5.3a and 5.3b).

Medial amygdala

There was a significant difference in Fos activation within the medial amygdala (MeA) depending upon group ($F(5,24)=20.22$, $p < 0.01$). All control groups had similar Fos activation within the MeA and all had significantly lower Fos activation compared with defeated groups. (These data are represented in Figure 5.4).

Paraventricular nucleus of the hypothalamus

There was a significant difference in Fos activation within the paraventricular nucleus of the hypothalamus (PVN) depending upon group ($F(5,24)=10.54$, $p < 0.01$). All control groups had similar Fos activation and all had significantly less activation within the PVN compared with defeated male and proestrous females. Defeated diestrous 1 females exhibited similar Fos activation compared with control male hamsters. Defeated proestrous and male hamsters had significantly greater Fos activation compared with all other groups. (Refer to Figure 5.5).

Central Amygdala

There was a significant difference in Fos activation depending upon group ($F(5,23)=7.08$, $p < 0.01$). All control groups exhibited similar Fos activation. The defeated diestrous 1 females had significantly less activation in CeA compared with control diestrous 1 females and defeated proestrous females, but similar activation compared with control male and proestrous female hamsters and defeated male hamsters.

Defeated male and proestrous females exhibited similar Fos activation. Defeated proestrous females had significantly higher Fos activation compared with all groups except defeated males. (These data are depicted in Figure 5.6).

Basolateral Amygdala

There was a significant difference in Fos activation depending upon group ($F(5,24)=3.89$, $p =0.01$. All control groups had similar activation. Male and diestrous 1 control groups were significantly lower than defeated proestrous and diestrous 1 groups, but similar to defeated males. Control proestrous females exhibited similar Fos activation compared with all other groups, except the defeated P females which had significantly higher Fos expression compared with al groups except the defeated diestrous 1 females (These data are represented in Figure 5.7).

In Experiment 2, there was a significant main effect of light ($F(1,27)=23.56$, $p < 0.01$)on open field behavior in hamsters. Female hamsters exposed to bright light regardless of hormonal status, displayed significantly less locomotion in the open field compared with those exposed to dim light (See Figure 5.8). There was also a significant main effect of light on the number of central ($F(1,27)=6.30$, $p < 0.05$), intermediate ($F(1,27)=32.28$, $p <0.01$) and outer ($F(1,27)=33.90$, $p < 0.01$) square entries. Animals that were exposed to bright light entered significantly fewer squares compared with those exposed to dim light (See Figure 5.9a-5.9c). There was however, no difference on duration of time spent in the center, intermediate or outer squares.

Finally, there was no significant main effect of light or hormone alone on frequency rearing or % of time grooming, (data not shown). There was however, a significant interaction of hormone and light on the frequency of rearing ($F(1,27)= 5.61$, p

< 0.05) with diestrous 1 females exposed to bright light having greater frequency of rearing compared with other groups (Figure 5.10).

Discussion

These data are the first to explore Fos activation in male and female hamsters following CD testing. In addition, whereas much of the research investigating anxiety-like behavior in the open field involves female rats and mice, these data are among the few exploring open field behavior in female Syrian hamsters. In Experiment 1, consistent with our previous findings (Solomon & Huhman, in preparation), defeated male and proestrous female hamsters exhibited submissive behavior and a lack of territorial aggression during CD testing. Although both groups displayed CD, males displayed significantly higher levels of submissive behavior indicating that there is a sex difference in the expression of CD. By contrast, diestrous 1 females did not display CD confirming that there is an estrous-cycle dependent effect on the expression of CD in female hamsters.

One might argue that the striking behavioral difference in the response to social defeat in diestrous 1 versus proestrous females might be due to differences in how the resident aggressors (RAs) responded to them during CD training. We carefully analyzed the behavior of the RAs towards both groups and found that they responded similarly in terms of attacks and bites. This finding suggests that the behavioral differences observed between diestrous 1 and proestrous females during CD testing is not due to differences in how the RAs responded to them during CD training.

The increased submissive behavior observed in defeated male hamsters during CD testing is consistent with data from our laboratory (Potegal et al., 1993; Huhman et al., 2003; Cooper & Huhman, 2005; Jasnow et al., 1999). Our initial study which reported that female hamsters do not display CD did not control for the estrous cycle (Huhman et al., 2003). Several studies have explored other aspects of agonistic behavior, like aggression, in non-defeated female rodents across the estrous cycle. For example, some diestrous 1 and diestrous 2 Wistar rats display increased aggression towards intruding conspecifics compared with those in the proestrous phase of the cycle (Melchoir et al., 2004; Olsson et al., 2003). A similar finding of increased aggression during the diestrous phase of the cycle relative to the proestrous phase has been observed in California mice (Davis & Marler, 2003; Davis & Marler, 2004).

Fos Analysis

Lateral Septum

The lateral septum (LS) has been implicated in a variety of behaviors including fear and aggression. For example, septal stimulation diminishes fear or anxiety, while septal lesions induce fear (for review: Sheehan, Chambers & Russel, 2004). Septal lesions have also been shown to markedly increase aggressiveness indicating that this area may also play an important role in the inhibition of aggression (Albert & Walsh, 1982). Within the dorsal LS both defeated male and proestrous female hamsters exhibited greater Fos action compared with both control animals and aggressive diestrous 1 females suggesting that increased activation within this region might be associated with submissive behavior. This finding is consistent with other studies (Kollack-Walker, Watson & Akil, 1997; Kollack-Walker, Watson & Akil, 1999) which found increased

activation in the dorsal LS in submissive male hamsters relative to control males. The defeated proestrous female hamsters exhibited greater Fos activation compared with all other groups within the intermediate LS, but the defeated male hamsters did not exhibit a similar increase in Fos activation within this region, ruling out the possibility that increased activation within the intermediate LS is selective to submissive behavior. This finding is in contrast with other studies which report increased Fos activation within this region in submissive male hamsters relative to controls (Kollack-Walker, Watson & Akil, 1997; Kollack-Walker, Watson & Akil, 1999). All defeated animals exhibited greater Fos activation within the ventral LS compared with control animals suggesting that increased activation within this region is due to exposure to agonistic encounter and not selective to submission or aggression. This finding also contrasts with some studies which report increased Fos activation in the ventral LS in aggressive mice compared with non-aggressive mice (Davis & Marler, 2004) as well as aggressive rats compared with control rats (Halasz et al., 2002). Together, these data suggest that the LS is involved in the modulation of agonistic behavior, but the mixed findings on the role of this region in submission or aggression probably result from differences in social history of the subjects or the species used.

Bed nucleus of stria terminalis

The role of the bed nucleus of stria terminalis (BNST) in fear/stress-related behaviors has been well documented (Lee & Davis; Zhu et al., 2001; Khoshbouei, Cecchi & Morilak, 2002). The anterior lateral portion of the BNST is also known to regulate HPA-axis activity (Gray et al., 1993; Herman, Cullinan, & Watson, 1994). Our finding of increased activation in the lateral BNST in all defeated animals relative to the controls

suggests that exposure to an agonistic encounter, regardless of the behavior exhibited, increases Fos activation within this region. Increased Fos activation within this region in defeated diestrous 1 females relative to both defeated male and proestrous female hamsters, however, suggests that increased activation within this region might be involved in the maintenance of aggression in diestrous 1 females. A similar finding of increased Fos activation within the BNST in aggressive animals has also been reported in aggressive female mice (Davis & Marler, 2004), lactating mice (Gammie & Nelson, 2001; Hasen & Gammie, 2005) and aggressive male hamsters (Delville, Devries & Ferris, 2000). By contrast, one studying examining patterns of activation in dominant males versus males given a chance to copulate with a female found that agonistic interactions in general, increases Fos activation in the anterolateral BNST and mating increases Fos expression in the posterior medial BSNT (Kollack-Walker & Newman, 1995). The authors concluded that activation in the anterolateral BSNT was not specific to submission or aggression. Many factors, may contribute to the difference observed between the Kollack-Walker study and the present including previous social history as well as the length of the agonistic encounter (i.e., 5 min. in the present study vs. 10 min. in their study).

Much of the work concerning the medial BNST has involved the posterior division which has been implicated in behaviors like mating and olfaction (Wood & Swann, 2005). Much less has been reported on the anterior medial division of the BNST, although one study has implicated this region in olfaction (Hairston, Ball & Nelson, 2003). In the present study there were no differences in Fos activation among any of the groups suggesting that this region is not selective to aggression or submission.

Medial amygdala

The medial amygdala (MeA) has been recognized for is role in behaviors such as chemoinvestigation and mating (Newman, 1999). Activation in the posterior MeA in all defeated animals relative to their respective controls suggests that exposure to an agonistic encounter increases Fos activation within this region. This finding is consistent with previous findings which report no difference in Fos activation within the MeA between submissive and aggressive hamsters (Kollack-Walker, Watson & Akil, 1999; Kollack-Walker & Newman, 1995); however, see (Joppa, Meisel & Garner, 1995; Knyshevski et al., 2005; Gammie & Nelson, 2001). The data presented here suggests that activation within the MeA is related to exposure to another conspecific and is not specific to the type of agonistic behavior produced.

Paraventricular nucleus of the hypothalamus

Stress activates the hypothalamic paraventricular nucleus (PVN) resulting in the release of corticotropin releasing hormone (CRH), which is a key regulator of the HPA-axis (Makino, Hashimoto, & Gold, 2002). Thus, the PVN serves as the "stress center." Increased Fos activation in all previously defeated animals relative to their non-defeated counterparts suggests increased HPA-axis activity in animals that are exposed to an agonistic encounter compared with control counterparts. Many data indicate that submissive, but not dominant, hamsters exhibit greater HPA-axis activation as evidenced by increased plasma ACTH and CORT (Huhman et al., 1990; Huhman et al., 1991). Therefore our finding of increased activation in submissive male and proestrous female hamsters relative to aggressive diestrous 1 females following an agonistic encounter supports these findings and suggests that increased activation of the PVN might be

important in the maintenance of submissive behavior in male and proestrous female hamsters.

Central amygdala

The central amygdala (CeA) has been associated with fear behavior in non-human primates and rodents. For example, lesions in the CeA reduce fear behavior in both rodents (Goosens & Maren, 2001) and primates (Kalin, Shelton & Davidson, 2004). Defeated male and proestrous female hamsters exhibited greater Fos activation compared with all other control groups suggesting that increased Fos activation within these groups was related to exposure to an agonistic encounter. Although defeated males were significantly more submissive than were diestrous 1 females they displayed similar Fos activation; however, the defeated proestrous females exhibited greater Fos activation compared with diestrous 1 females. This pattern of activation is difficult to explain, given the fact that the proestrous females were less submissive than were the defeated males. Previous data examining Fos activation within the CeA have found increased Fos activation in submissive rats and hamsters compared with control animals (Martinez et al., 1998; Kollack-Walker, Watson & Akil, 1997; Kollack-Walker et al., 1999) and decreased Fos activation in the CeA in aggressive hamsters relative to non-aggressive hamsters (Knyshevski et al., 2005). Based on these data, increased Fos activation in the CeA might be preferentially involved in increased submissive behavior in proestrous females only.

Basolateral amygdala

The basolateral amygdala (BLA) receives sensory input from cortical and thalamic structures and projects to the CeA and the BNST (Amaral & Inasausti, 1992). The few studies that have examined Fos activation within this region following exposure to an agonistic encounter have found no difference between submissive and control animals (Matsuda et al., 1996; Kollack-Walker, Watson & Akil, 1997). Our findings are consistent with these earlier reports as there were no clear differences in patterns of neuronal activation between defeated male and control animals; however, there was a significant increase in Fos activation between defeated proestrous female hamsters and control animals. Given the fact that the BLA has been associated with fear-related behavior (Everitt et al., 2003; Maren, 2003) it was surprising that there was no difference in Fos activation between submissive male hamsters and control animals.

Open Field Behavior

Finally, in Experiment 2, we examined behavior in the open field in diestrous 1 and proestrous female hamsters in order to determine whether there were differences in anxiety-like behavior over the estrous cycle in a non-social context. We did not find any effect of hormonal status on locomotion, grooming or rearing in the open field arena suggesting that these females display similar anxiety-like behavior in this particular apparatus. This finding, however, contrasts with previous findings in female rats that report reduced anxiety-like behavior in proestrous females compared with diestrous females in the open field (Frye, Petralia & Rhodes, 2000; Frye & Walf, 2002). Consistent with Mora and associates (1996), bright light did increase anxiety-like

behavior in female hamsters as indicated by decreased exploratory behavior in the open field arena validating the sensitivity of this model to known anxiety-producing stimuli in hamsters.

Overall, the data from the present study indicate both a sex and estrous-cycle difference in the display of CD in Syrian hamsters and implicate key neural substrates like dorsal LS, CeA and PVN in maintaining submissive behavior in previously defeated proestrous female hamsters, while the dorsal LS and PVN appear to be important in maintaining submissive behavior in defeated male hamsters. In addition, the data suggest that proestrous and diestrous 1 females exhibit similar behavior outside of a social context in a commonly used apparatus for anxiety indicating that the behavioral differences in the display of CD in these females is not due to a generalized difference in anxiety-like behaviors across the estrous cycle. .

Figure 5.1. (a) Average duration (mean +/-SEM in seconds) during 5-minute training episode of submissive/defensive, aggressive, social and nonsocial behavior of resident aggressors (Experiment 2) towards experimental females tested in estrus (N=7) or diestrus 2 (N=5) (CD Training). **(b).** Average duration (mean +/-SEM in seconds) of submissive/defensive, aggressive, social and nonsocial behavior in previously defeated females hamsters tested in diestrus 1 (N=7) or proestrus (N=4) and male hamsters (N=7) during a subsequent test encounter with a non-aggressive intruder (CD Testing). Analyses were run for each specific behavior; therefore, differences are compared only within one category of behavior and not between behavioral categories. Non-shared letters depict statistical significant differences ($p<0.05$) as determined by Fisher's post hoc analyses.

Figure 5.2. Number of c-Fos positive nuclei in the dorsal **(b)** intermediate and **(c)** ventral lateral septum in male and female hamsters following an agonistic encounter with a non-aggressive intruder. Values are reported as mean +/-SEM. Non-shared letters depict statistical significance ($p <0.05$) as determined by Fisher's post hoc analyses.

Figure 5.3. (a) Number of c-Fos positive nuclei in the lateral and **(b)** medial bed nucleus of the stria terminalis in male and female hamsters following an agonistic encounter with a non-aggressive intruder. Values are reported as mean +/-SEM. Non-shared letters depict statistical significance ($p <0.05$) as determined by Fisher's post hoc analyses.

Figure 5.4. Number of c-Fos positive nuclei in the medial amygdala in male and female hamsters following an agonistic encounter with a non-aggressive intruder. Values are reported as mean +/-SEM. Non-shared letters depict statistical significance ($p < 0.05$) as determined by Fisher's post hoc analyses.

Figure 5.5. Number of c-Fos positive nuclei in the hypothalamic paraventricular nucleus in male and female hamsters following an agonistic encounter with a non-aggressive intruder. Values are reported as mean +/-SEM. Non-shared letters depict statistical significance ($p < 0.05$) as determined by Fisher's post hoc analyses.

Figure 5.6. Number of c-Fos positive nuclei in the central amygdala in male and female hamsters following an agonistic encounter with a non-aggressive intruder. Values are reported as mean +/-SEM. Non-shared letters depict statistical significance ($p < 0.05$) as determined by Fisher's post hoc analyses.

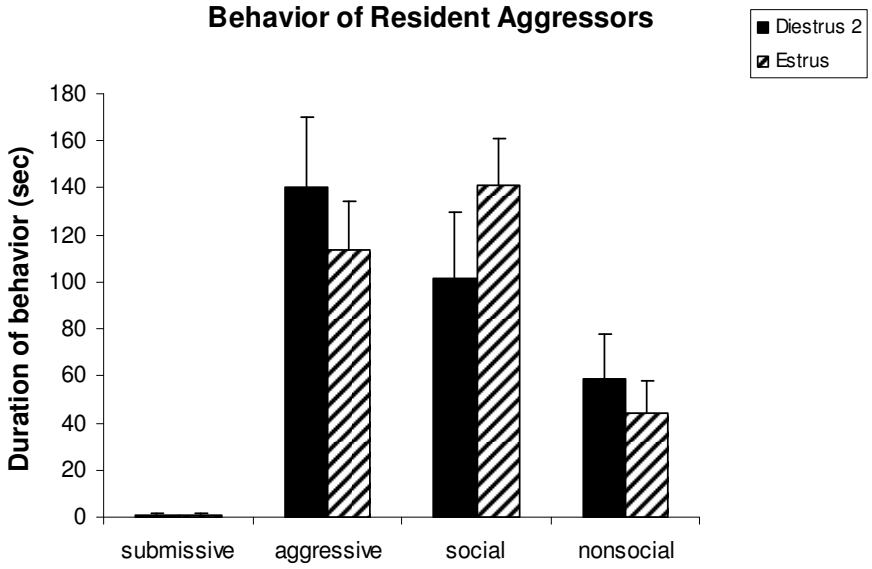
Figure 5.7. Number of c-Fos positive nuclei in the basolateral amygdala in male and female hamsters following an agonistic encounter with a non-aggressive intruder. Non-shared letters depict statistical significance ($p < 0.05$) as determined by Fisher's post hoc analyses.

Figure 5.8. Mean locomotion scores for Experiment 3 in diestrous 1 (N= 14) and proestrous (N=17) female hamsters exposed to either bright or dim light in an open-field arena. (*) indicates animals exposed to dim light had significantly higher locomotion scores than did those exposed to bright light. Values are reported as mean +/-SEM. (*) denotes statistical significance ($p < 0.05$).

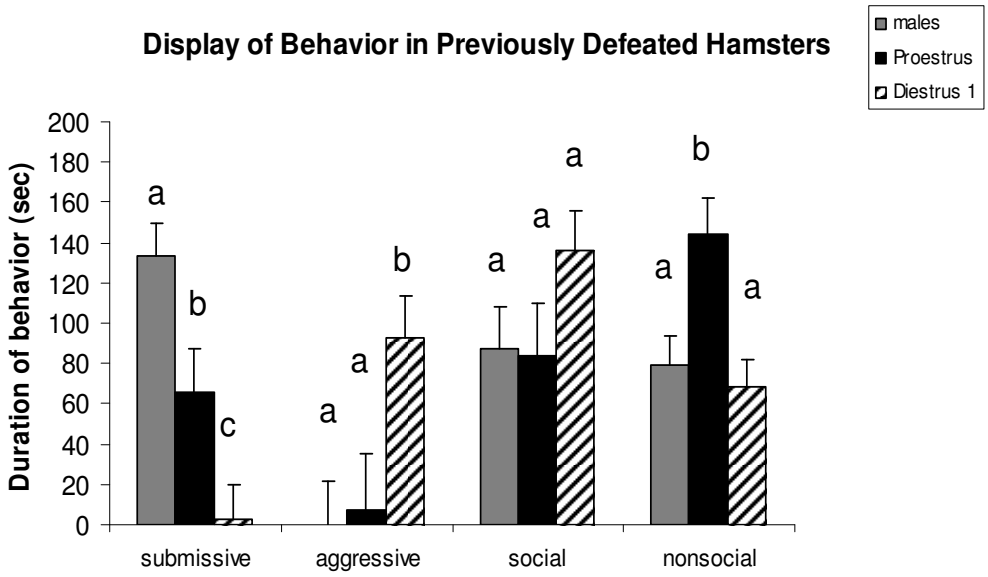
Figure 5.9. **(a)** Number of central **(b)** intermediate **(c)** and outer square entries in the open field of diestrous 1 and proestrous female hamsters exposed to either bright or dim light in an open-field arena. Values are reported as mean +/-SEM. (*) indicates animals exposed to dim light entered more central, intermediate and outer squares than did those exposed to bright light. (*) denotes statistical significance ($p < 0.05$).

Figure 5.10. Frequency of rearing (mean +/-SEM) in diestrous 1 and proestrous female hamsters exposed to either bright or dim light in an open field arena. Non-shared letters depict statistical significance ($p < 0.05$) as determined by Fisher's post hoc analyses.

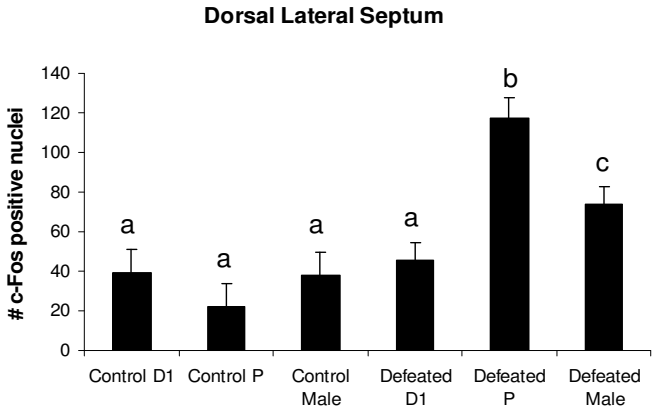
5.1a


Behavior of Resident Aggressors
Diestrus 2
Estrus
Duration of behavior (sec)
180
160
140
120
100
80
60
40
20
0
submissive
aggressive
social
nonsocial

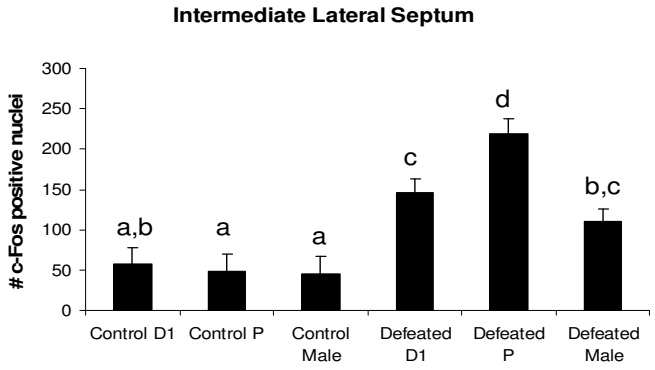
5.1b


Display of Behavior in Previously Defeated Hamsters
males
Proestrus
Diestrus 1
Duration of behavior (sec)
200
180
160
140
120
100
80
60
40
20
0
a
b
c
a
a
b
a
a
a
b
a
a
submissive
aggressive
social
nonsocial

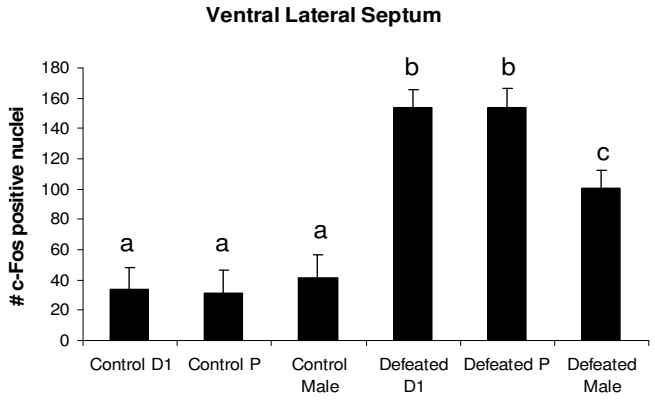
5.2a


Dorsal Lateral Septum
c-Fos positive nuclei
140
120
100
80
60
40
20
0
a
a
a
a
b
c
Control D1
Control P
Control Male
Defeated D1
Defeated P
Defeated Male

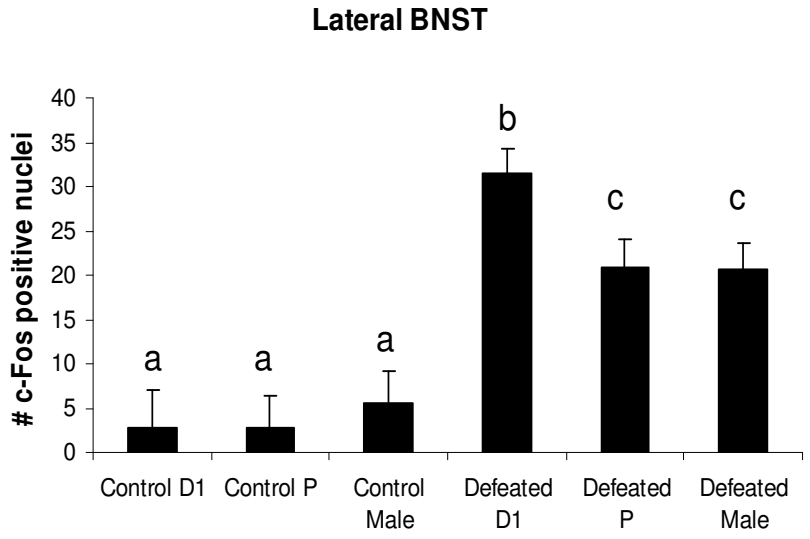
5.2b


Intermediate Lateral Septum
c-Fos positive nuclei
300
250
200
150
100
50
0
a,b
a
a
c
d
b,c
Control D1
Control P
Control Male
Defeated D1
Defeated P
Defeated Male

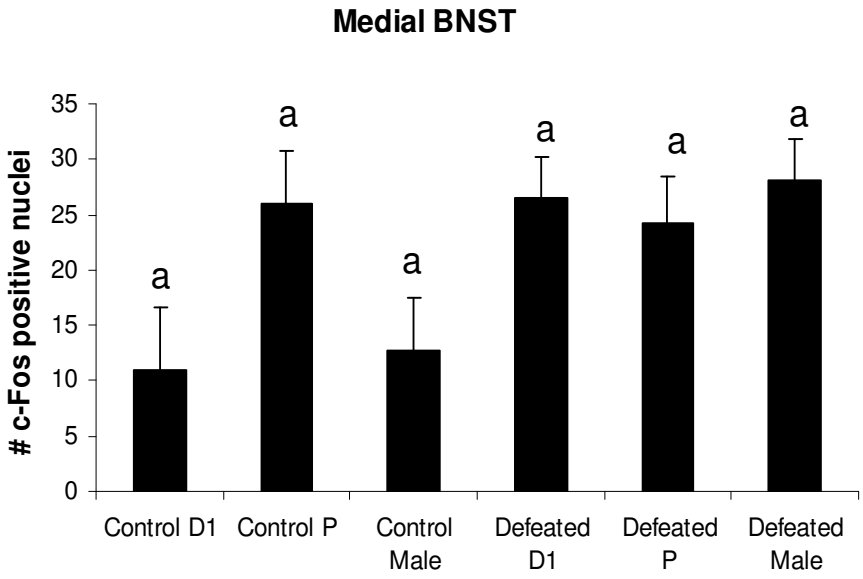
5.2c


Ventral Lateral Septum
c-Fos positive nuclei
180
160
140
120
100
80
60
40
20
0
a
a
a
b
b
c
Control D1
Control P
Control Male
Defeated D1
Defeated P
Defeated Male

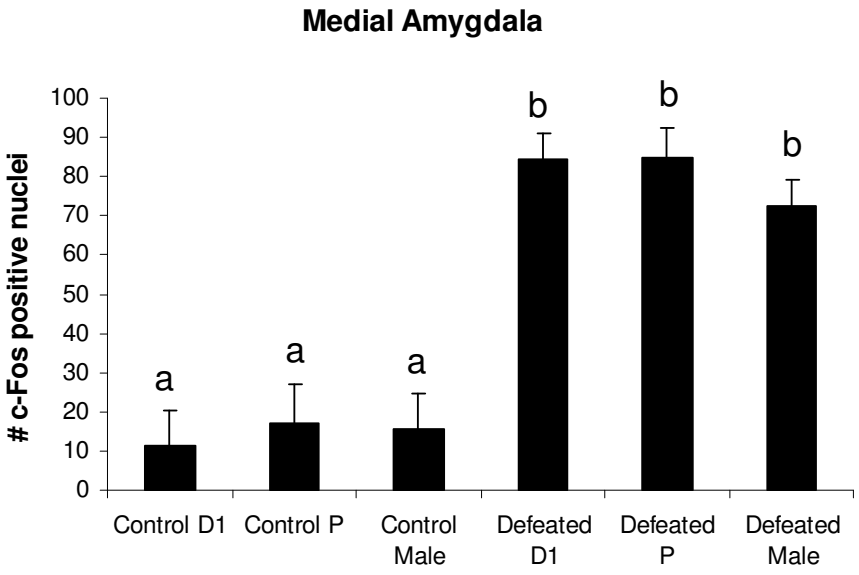
5.3a


Lateral BNST
c-Fos positive nuclei
40
35
30
25
20
15
10
5
0
a
a
a
b
c
c
Control D1
Control P
Control Male
Defeated D1
Defeated P
Defeated Male

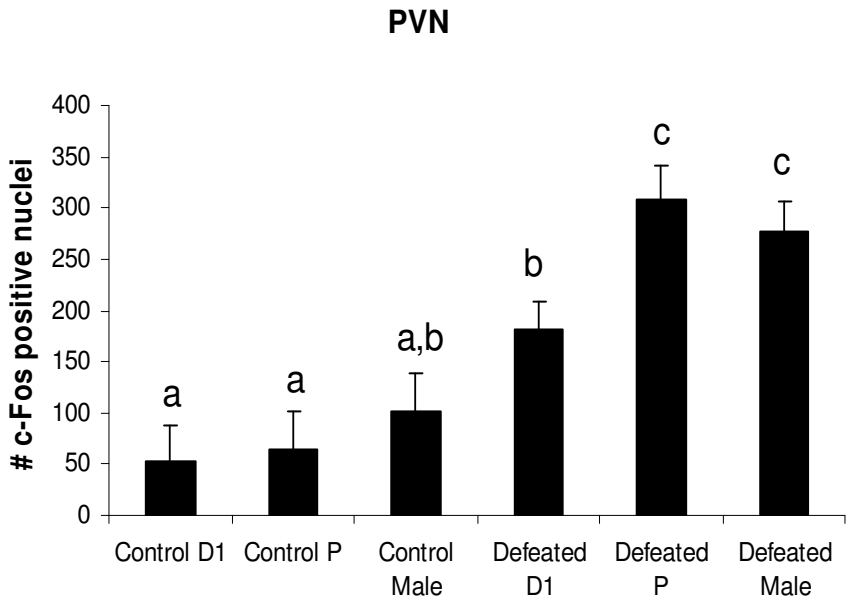
5.3b


Medial BNST
c-Fos positive nuclei
35
30
25
20
15
10
5
0
a
a
a
a
a
a
Control D1
Control P
Control Male
Defeated D1
Defeated P
Defeated Male

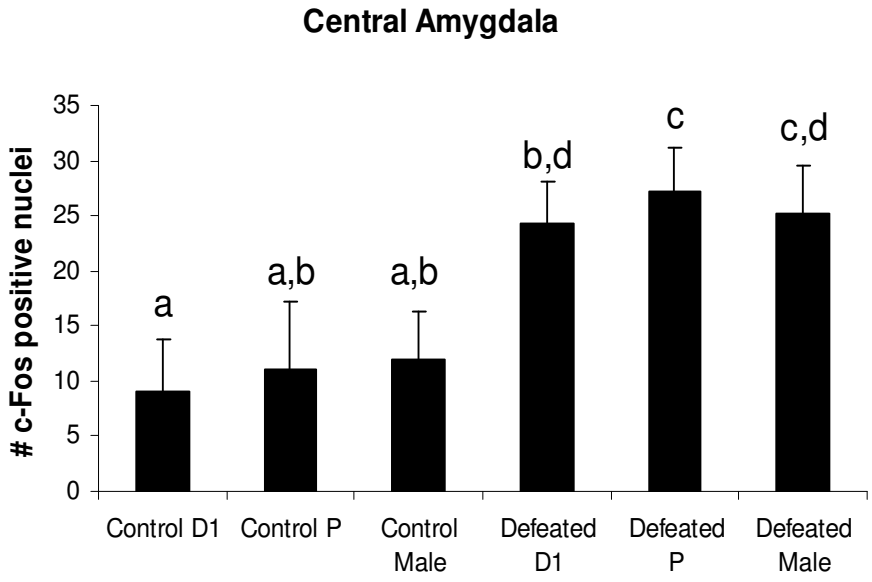
5.4


Medial Amygdala
c-Fos positive nuclei
100
90
80
70
60
50
40
30
20
10
0
a
a
a
b
b
b
Control D1
Control P
Control Male
Defeated D1
Defeated P
Defeated Male

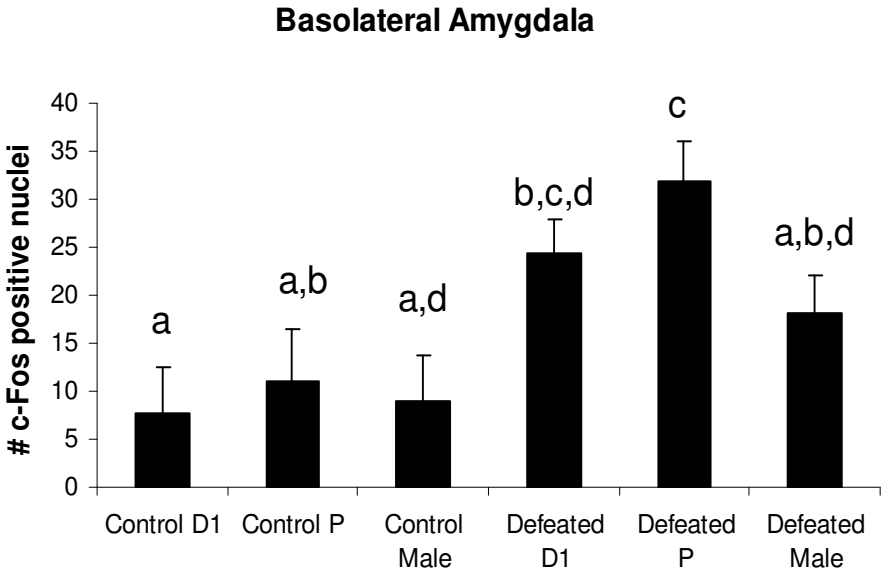
5.5


PVN
c-Fos positive nuclei
400
350
300
250
200
150
100
50
0
a
a
a,b
b
c
c
Control D1
Control P
Control Male
Defeated D1
Defeated P
Defeated Male

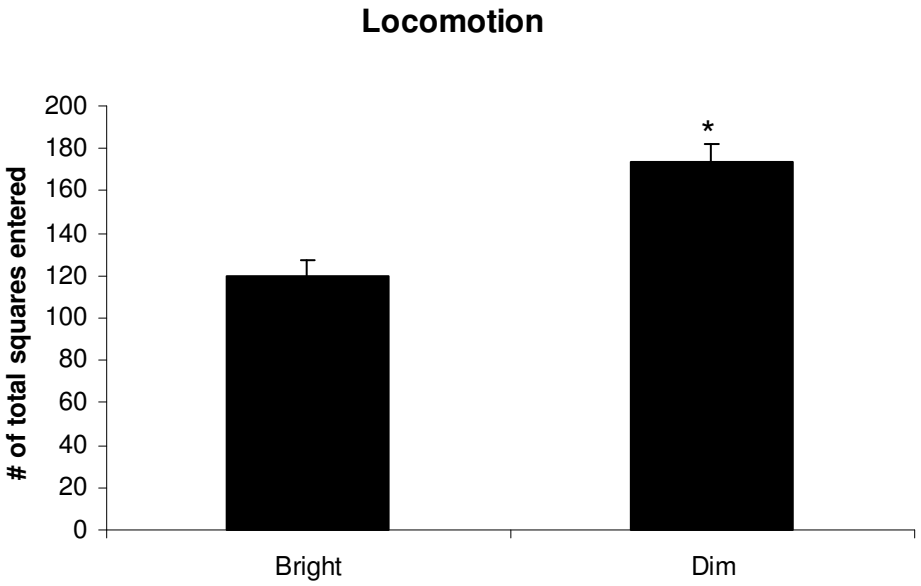
5.6


Central Amygdala
c-Fos positive nuclei
35
30
25
20
15
10
5
0
a
a,b
a,b
b,d
c
c,d
Control D1
Control P
Control Male
Defeated D1
Defeated P
Defeated Male

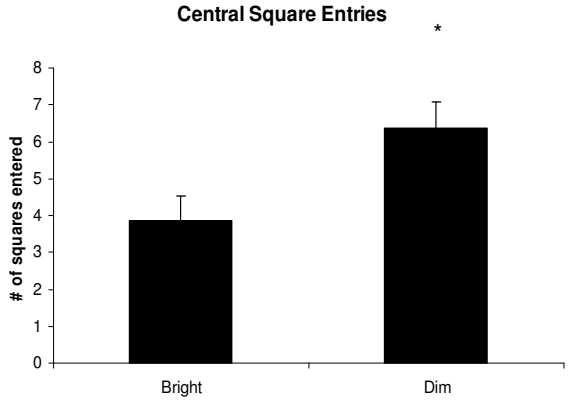
5.7


Basolateral Amygdala
c-Fos positive nuclei
40
35
30
25
20
15
10
5
0
a
a,b
a,d
b,c,d
c
a,b,d
Control D1
Control P
Control Male
Defeated D1
Defeated P
Defeated Male

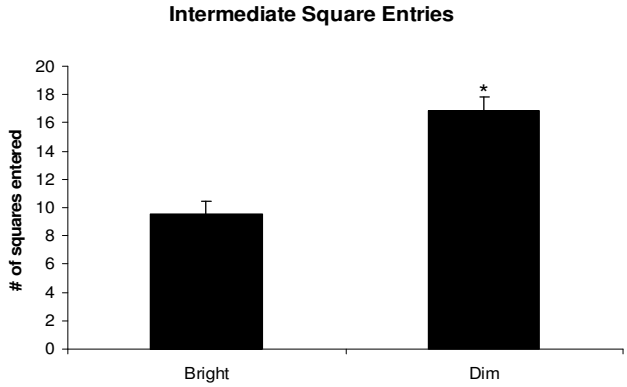
5.8


Locomotion
# of total squares entered
200
180
160
140
120
100
80
60
40
20
0
*
Bright
Dim

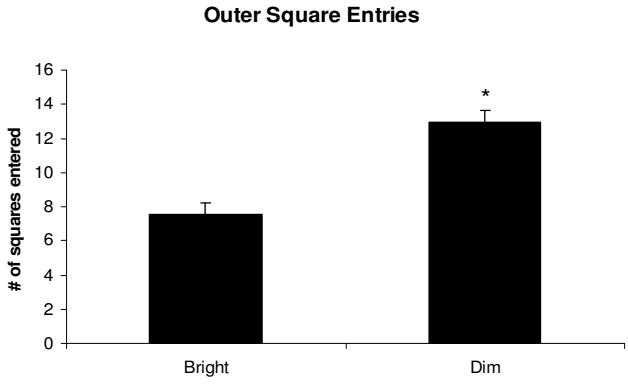
5.9a


Central Square Entries
*
# of squares entered
0
1
2
3
4
5
6
7
8
Bright
Dim

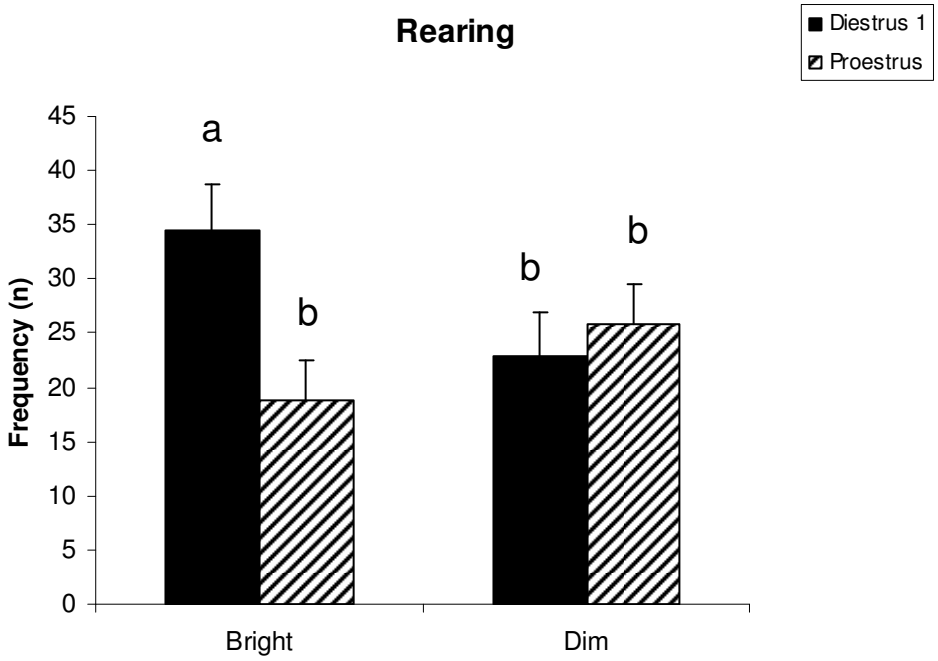
5.9b


Intermediate Square Entries
*
# of squares entered
0
2
4
6
8
10
12
14
16
18
20
Bright
Dim

5.9c


Outer Square Entries
*
# of squares entered
0
2
4
6
8
10
12
14
16
Bright
Dim

5.10


Rearing
Diestrus 1
Proestrus
Frequency (n)
45
40
35
30
25
20
15
10
5
0
a
b
b
b
Bright
Dim

Chapter 7

CONCLUSIONS

The aim of this dissertation was to examine the effects of gonadal hormones on the display of CD in female and male hamsters. The results indicate that the display of CD in females is estrous-cycle dependent. Females that were defeated in diestrus 1 and diestrus 2 and subsequently tested in diestrus 2 and proestrus, respectively, were more likely to display CD than were females tested in other phases in the cycle. Non-defeated females that were tested with a non-aggressive intruder over the course of the estrous cycle did not display any submissive behavior and instead displayed normal territorial aggression. This finding suggests that the increase in submissive behavior seen in defeated diestrous 2 and proestrous females during CD testing is not due to an underlying fluctuation in submissive behavior over the cycle.

Because diestrus 2 and proestrus are characterized by higher endogenous estradiol (E_2) compared with other phases of the cycle, we hypothesized that the higher E_2 levels at the time of testing might contribute to the display of CD in female hamsters. The most dramatic fluctuations in E_2 occur during the proestrous phase of the cycle. The early part of proestrus is characterized by higher E_2 and lower, but rising progesterone (P), with the late part of proestrous having higher P and falling E_2 levels (Saidapur & Greenwald, 1978; Saidapur & Greenwald, 1979). In this study, we tested the hypothesis that proestrous females with higher endogenous E_2 levels would be more likely to display CD than would proestrous females with lower E_2 levels. Unfortunately, we did not observe the expected changes in E_2 over the course of testing, and all previously defeated proestrous females displayed CD. One shortcoming of this study was the failure to

include non-defeated females. The inclusion of this group would have allowed us to determine if exposure to the previous stressor (i.e., social defeat) during diestrus 2 interfered with the normal fluctuations in E_2 during proestrus as all of our previously defeated females displayed elevated E_2 levels throughout testing. In support of the hypothesis that the previous exposure to stress altered estrogen on the following day, one study has found that female rats exposed to footshock stress during diestrus 2 have persistently elevated plasma concentrations of E_2 (Shors et al., 1999). We did observe, however, significant changes in P during the testing period suggesting that changes in P are not associated with changes in agonistic behavior following social defeat. Overall, the findings involving intact females indicate that females with higher endogenous E_2 at the time of testing are more likely to display CD.

Perhaps one of the most important findings of this dissertation is that gonadal steroids can produce varying, even opposite, effects on behavior. Previously, we reported that females given chronic T and E_2 administration were less likely to display CD than were females given DHT, P or blank capsules (Faruzzi et al., 2005). These data suggest that E_2 decreases submissive behavior in previously defeated females. By contrast, in the present study, females with higher endogenous E_2 and those given acute administration of E_2 (at higher doses) at the time of testing were more likely to display CD than were females given lower doses of E_2 or cholesterol. There are several important differences between the past and current studies. Two of these are the length of individual housing (i.e., 4 weeks vs. 10 days) before testing and the timing of hormone replacement (i.e., acute versus chronic). Unfortunately, based on these studies alone it is difficult to determine which factors contribute to the varying effects of hormones on behavior in

female hamsters. A plausible experiment to determine if the varying effects of hormones on conditioned defeat is due to method of hormonal administration would be to give females either acute (i.e., single injection) vs. chronic (i.e., silastic implants) administration of E_2 but individually house them for the same length of time. If there were differences in behavior between these two groups, then it would appear that different methods of hormonal administration resulted in the behavioral differences. On the other hand, if there were no differences in agonistic behavior between females given acute vs. chronic administration of E_2, then this would suggest that length of individual housing is the important factor and future studies varying the length of individual housing could be completed.

The data examining the effects of gonadal hormones in male hamsters do suggest that there is an interaction of testicular hormones and length of isolation on the display of CD. Our data indicate that previously defeated, intact males that were singly housed for 4 weeks prior to behavioral testing displayed significantly less submissive behavior in the presence of a NAI compared with their castrated counterparts. By contrast, despite significant differences in circulating testicular hormones, castrated and intact males that were singly housed for only 10 days displayed similar increases in submissive behavior in the presence of a NAI. Finally, castrated males that were singly housed for 4 weeks and given exogenous administration of T and DHT displayed a strong trend for decreased submissive behavior in the presence of a NAI compared with those given E_2 and cholesterol capsules. Together, these data suggest that testicular hormones and length of isolation can both modulate CD in male hamsters.

The data presented in this dissertation implicate key brain regions in the display of CD in male and female hamsters. Defeated male hamsters exhibited greater Fos activation in the dorsal LS and PVN while defeated proestrous females exhibited greater Fos activation in the dorsal LS, CeA and PVN compared with defeated diestrous 1 females suggesting that these regions might be involved in the display of CD (the production of submission or the inhibition of aggression) in defeated male and proestrous females. On the other hand, diestrous 1 females, which did not display CD, exhibited greater Fos activation in the lateral BNST suggesting that this area might be involved in the maintenance of territorial aggression in these females. Studies investigating the association of certain brain regions with either submission or aggression are highly variable. For example, some studies report increased Fos activation within the LS, CeA, BNST and PVN in submissive animals (Martinez, Phillips & Herbert, 1998; Matsuda et al., 1996; Miczek et al., 1999; Kollack-Walker, Watson & Akil, 1997; Kollack-Walker, Watson & Akil, 1999), while other studies report increases in these same areas in aggressive animals, as well (Davis & Marler, 2004; Halasz et al., 2002; Gammie & Nelson, 2001; Hasen & Gammie, 2005; Delville, Devries, & Ferris, 2005).

The comparison of Fos activation among studies should be carefully considered as many factors other than the behavioral manipulations might account for differences in Fos activation. The variability among studies might be due to several factors including the use of appropriate control groups within a particular study, the length and intensity of the agonistic encounter, species differences, as well as the failure to specifically state which subdivision of a particular brain area is being assessed. Perhaps, it might prove more beneficial to compare findings across studies that use the same species as well as

comparable behavioral testing paradigms. In addition, it is imperative that future studies more carefully document which subdivisions within a given brain region are assessed, as different subdivisions might play discrete roles in agonistic behaviors. Although a great deal of work has been devoted to using c-fos and its protein product Fos as markers for neuronal activation, we must be careful how we interpret these data. The absence of Fos activation within a particular region does not signify a lack of involvement of that area. Neurons might be activated but might express other immediate early genes like c-jun. In addition, the use of Fos as a marker, alone, does not tell us about the phenotype of the neurons that are involved in agonistic behavior in a given region. (for Fos review see: Hoffman & Lyo, 2002).

The findings of this dissertation indicate that gonadal hormones modulate the display of CD in both female and male hamsters. Of particular importance is the finding that the influence of these gonadal hormones on agonistic behavior may be altered by the length of individual housing and the method of hormone replacement. The present data also suggest that discrete brain regions like the CeA might be important in the display of CD in female hamsters and suggest that future studies should investigate the role of this area in the display of CD in female hamsters. Previous data from our laboratory suggests that the amygdala is important in both the acquisition and expression of CD in male hamsters (Jasnow & Huhman, 2001; Jasnow, Cooper & Huhman, 2004). The present data suggest that E_2 is important in the expression of CD in female hamsters and given the fact that the CeA expressed increased Fos activation in previously defeated proestrous females, it would be interesting to determine if the Fos-ir was co-localized with ER-ir within the same neurons in the CeA. If there was, indeed, an abundance of ER-ir neurons

in the CeA, then a plausible next step would be to determine whether blockade of E_2 receptors within CeA region decreases the expression of CD in female hamsters.

It is important to note that in comparison to defeated males, defeated intact females display noticeably less submissive behavior in the presence of a NAI, regardless of hormonal status. Thus, there is also a clear sex difference in the behavioral response to social defeat in Syrian hamsters. This finding is consistent with a recent study which demonstrated that following repeated social stress, female hamsters were less likely to display alterations in the development of agonistic behavior and were quicker to habituate to repeated social stress than were males (Taravosh-Lahn & Delville, 2004). There are very few field data available about the natural environment of Syrian hamsters. Perhaps, a female hamster's ability to habituate to repeated social stress is critical to her ability to defend her home territory and ensure the survival of her pups. A female that exhibits submissive behavior and flees from her territory may be less likely to find a mate, defend a burrow or produce and protect her offspring. By contrast, male hamsters do not have a role in taking care of pups, consequently it may be adaptive for a male that is defeated to flee that territory and move to another, thus avoiding injury.

These data provide a good foundation for other studies aimed at investigating CD in female hamsters. There are however, many important issues that must be addressed before continuing this line of work. One is which is the appropriate hormonal milieu (i.e., intact vs. hormonally replaced or both) to study CD in female hamsters. Few data have explored social defeat in females in part due to the fact that females of many species display little spontaneous agonistic behavior but also because there are many more variables involved with female subjects compared with male subjects. Despite these

difficulties, the field of behavioral neuroendocrinology must forge ahead in designing appropriate experimental designs that will best allow us to study not only sex differences but also estrous cycle-related differences in a variety of behaviors including aggression and submission.

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