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Glucocorticoids and the Development of Agonistic Behavior in Male Golden Hamsters

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Glucocorticoids and the Development of Agonistic Behavior in Male Golden Hamsters

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Dedication

This work is dedicated to all of those who told me I could.

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Glucocorticoids and the Development of Agonistic Behavior in Male Golden Hamsters

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Puberty is a developmental period characterized by behavioral and physiological transitions. In male golden hamsters, agonistic behavior undergoes a pubertal transition from play fighting to adult aggression. Previous studies have shown that this behavioral development is not related to increased activity of the HPG axis. Interestingly, repeated social defeat, a stressor that causes daily increases in cortisol, accelerates the pubertal transition from play fighting to adult aggression. This prompted me to study the relationship between glucocorticoids and the development of agonistic behaviors. I found that in male golden hamsters, basal cortisol levels undergo a gradual two- to three-fold increase during puberty. Post-stress cortisol levels also increase steadily during this time. These new data led me to hypothesize that increases in cortisol levels controlled the pubertal maturation of aggressive behavior. This hypothesis was tested though a series of experiments. First, animals treated with cortisol during during early puberty showed an accelerated transition from play fighting to adult aggression. This developmental acceleration is similar to the one previously observed in repeatedly

subjugated juveniles. Next, the behavioral effects of cortisol were blocked by a co-treatment with a type II corticosteroid receptor antagonist. In addition, repeated injections of a corticosteroid type II receptor agonist mimicked the effects of repeated cortisol injections. These findings were unique in that they are the first to show a facilitating role for stress hormones in the pubertal development of a social behavior. As such, these findings present a novel way in which steroid hormones influence the development of social behaviors.

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Chapter 1: Introduction

Agonistic Behavior

Agonistic behavior is a broad term that includes various responses related to social conflict. Individual agonistic behaviors are frequently described as either offensive or defensive. Both categories of agonistic behavior are comprised of several different response types. For example, offensive responses include attacks, pins, and forms of communicative behaviors such as scent marking. Defensive responses include a number of separate behaviors such as defense postures, submission, avoidance, and risk assessment. Although most vertebrates display some form of agonistic behavior, there is a great deal of variability between species. Moreover, all agonistic behaviors can be modulated by internal factors such as motivation, or endocrine state, or external factors such as environmental agents and social experiences.

Testing Offensive and Defensive Responses in the Laboratory

In a laboratory setting, agonistic responses can be described as offensive or defensive. Defensive responses are typically performed in reaction to the advances of an aggressor. In contrast, individuals initiate offensive responses (Adams, 1979; Blanchard and Blanchard, 1977; 1988). Offensive responses typically involve an active approach, while defensive responses are typically reactions to the advances of an aggressor. In intraspecific conflicts offensive responses are displayed in the form of attacks directed towards specific targets on the body of an opponent. Defensive responses, in turn, consist of specific motions or postures intended to block the aggressor's access to the

targeted region. While both are common forms of agonistic behavior, offensive and defensive responses vary greatly between species. For example, the attacks of adult rats are typically directed towards the back, while adult hamsters focus their attacks on the lower belly or rear of an opponent (Pellis and Pellis, 1987; 1988a).

Offensive responses can be tested using the resident/intruder paradigm in which a smaller and younger conspecific is placed into the home cage of an experimental animal for a predetermined amount of time (Miczek, 1979). The use of smaller and younger intruders is important as it presents an advantage to the experimental animals and favors the display of offensive responses. The use of a more evenly matched intruder may lead to agonistic interactions in which the responses of the experimental animal are both offensive and defensive.

Defensive responses can also be measured using the resident/intruder paradigm. In these experiments, the experimental animal is placed in the home cage of a larger conspecific. In other words, experiments on defense focus on the behaviors of the smaller intruder rather than the larger resident. As a result, experiments that do not control for intruder size would be measuring different types of responses and may produce data that are difficult to interpret.

Agonistic responses can be measured as dependent variables in several ways. Commonly, experiments measure the number of times within the test period that the animal performs the response (frequency) or the amount of time between the start of the test and the first occurrence of the response (response latency). While frequency and latency have been useful, the use of these quantitative variables as principle measure of

agonistic behavior can lead to an impoverished understanding of the topic (Pellis and Pellis, 1998). The uses of qualitative variables, such as attack target, have allowed researchers to expand their understanding of agonistic behavior.

Agonistic Interactions of Golden Hamsters

Golden hamsters (*Mesocricetus auratus*) originate from Northern Syria and have been used for laboratory purposes for nearly 75 years (Seigel, 1985). Though there is a limited amount of field data on the species, golden hamsters are a solitary species (Gatterman et al., 2001; Murphy, 1977). As a result, hamsters can be housed in individual cages in the laboratory, and agonistic behaviors are observed when hamsters are paired together in a home or neutral cage.

During agonistic interactions, the behavioral responses of golden hamsters follow a general sequence (Albers et al., 2002; Floody and Pfaff, 1977). Initially when paired, hamsters undergo a period of olfactory investigation that usually involves snout contact. This period can last for a few minutes. Shortly following, a member of the pair will attack its opponent. Attacks consist of an approach followed by an attempt to bite (Wommack et al. 2003). In hamsters, attacks are not randomly directed towards the opponent. Rather, attacks are usually directed towards a specific body part of the conspecific. (Attack target will be discussed in further detail later). It is important to note that attacks in hamsters are non-injurious, as bite marks are rarely observed on their skin (Blanchard et al., 2003).

During the interaction, additional offensive responses are frequently observed. Victorious hamsters commonly engage in a species-specific form of scent marking known as a flank mark (Dieterlen, 1959; Johnston, 1975a; b). Flank marks are observed when hamsters arch their backs and rub excretions from a sebaceous gland (located near the dorsal flanks) on objects within the environment (Johnston, 1975a; b). Flank marks are used by hamsters to alert others of their presence or to signify dominance (Ferris et al., 1986; 1987; Johnston, 1985). As such, these behaviors are generally grouped into the category of offensive responses.

Defensive responses are typically performed in response to attacks and consist of motions or postures that block access to attack targets. Hamsters typically defend themselves by using their forepaws, either from an upright or supine position. If the defender successfully blocks an attack, the aggressor will usually attempt to bite an undefended region of the defender. Successful bites are usually immediately followed by an audible squeak by the defender. Often the pair separates after the attack, either because the aggressor moves away or the subordinate animal performs an avoidance response, such as a retreat. During the period between attacks, the subordinate hamsters occasionally approach the aggressor with a stretched posture and engage in olfactory investigation or risk assessment (Potegal et al., 1993). Defeated golden hamsters commonly display submissive postures. These responses are performed by defeated hamsters and are commonly displayed in conjunction with defensive responses (Potegal et al., 1993). The most extreme form of submission in hamsters is the on-back submissive posture. This behavior is observed when a submissive hamster remains

motionless and supine for seconds to minutes at a time (Albers et al., 2002). A less extreme submissive response in hamsters is known as the tail up. This response is characterized by an elevated tail and an arched back posture and is only performed by males (Albers et al., 2002).

The Development of Agonistic Behavior

Two common types of agonistic behavior are play fighting and aggression. Although both are forms of agonistic behavior, they can often be distinguished from each other based on consequence and because they are predominantly performed during separate periods of an animal's life. Aggressive behaviors often directly influence an individual's access to fitness enhancing resources such as food, mates, and territory (Archer, 1988). Although play fighting can be important for the development of responses to social challenges and maintenance of dominant/subordinate relationships, its influence on fitness is less direct than aggression (Pellis and Pellis, 1998; Pellis et al., 1993; Van den Berg et al., 1999). Despite the differing outcomes, play fighting and aggression can be separated into offensive and defensive responses and studied similarly within the laboratory.

In most mammalian species, puberty is marked by a transition from play fighting to adult aggression (Fagen, 1981). The earliest forms of agonistic behaviors are play-fighting responses that precede adult typical behaviors (Blanchard et al., 2003; Delville et al., 2003; Pellis, 2002; Pellis et al., 1997; Pellis and Uwaniuk, 2000). The transition from

play fighting to adult aggression commonly occurs over the course of puberty although the patterns of development vary between species.

Rats, the most frequently used species for the study of play fighting, show three distinct phases of behavioral development. Around postnatal day 20 (P-20), rat pups begin engaging in rough play with their littermates, but after weaning, their agonistic behaviors are purely playful for a period of time (around P-30 to P-40). Some data suggest that playful interactions during this time may be rewarding (Burgdorf and Panksepp, 2001). Following P-40, rats again engage in rough play that eventually becomes adult aggression. Nevertheless, play-fighting behaviors never fully disappear from the rat's behavioral repertoire, as subordinate adults initiate play-fighting interactions with dominant adults (Pellis et al., 1993).

Play fighting and adult-typical responses are often qualitatively different and therefore easily distinguishable behavioral responses. For example, offensive responses can be characterized as play fighting or adult-like based on the area of the body targeted in attacks. In rats, play-fighting attacks are directed at the nape of the neck, while adult attacks are directed towards the back (Panksepp, 1981; Pellis and Pellis, 1987; Blanchard, 2003). From P-20 to P-40, the majority of attacks by rats are directed towards the nape of the neck (Pellis and Pellis, 1991; Takahashi and Lore, 1983). After P-40, back-directed attacks become the preponderant offensive response and continue to be throughout adulthood (Beanninger, 1967; Meaney and Stewart, 1981a, Panksepp, 1981; Takahashi and Lore, 1983; Thor and Holloway, 1984).

The defensive responses of rats can also be used to distinguish between play fighting and adult aggression. In a common defensive response (complete rotation), rats rotate along their rostro-caudal axis into a supine posture (Pellis, 2002; Pellis et al., 1992; Pellis and Pellis, 1990; 1997). From this position, they are able to use all of their limbs to block attacks from a conspecific. This form of defense leads to playful interactions and is the most frequently used defensive response during the developmental period that is purely playful, P-25 to P-40 (Pellis, 2002). A second type of defense (partial rotation) is observed when the animal turns it body away from the attacker and supports itself with it's hind limbs while using the forelimbs to deflect attacks (Pellis, 2002; Pellis et al., 1992; Pellis and Pellis, 1997). This form of defense leads to rougher interactions and the predominant defensive response during developmental periods of rough play and adult aggression (Pellis, 2002).

Although the transition from play fighting to adult aggression is well understood in rats, this species does not necessarily represent the typical rodent pattern of development. Similar, but not identical, behavioral transitions occur in other rodent species (Pellis, 2002; Pellis and Pellis, 1998). Two primary factors can be used to compare the development of agonistic behavior between species (Pellis, 2002). First, one can compare the complexity of interactions. Some species, such as rats, show a highly complex interaction between agonistic responses, while mice show more simplified behavioral sequences (Pellis, 2002; Pellis and Pasztor, 1999). The number of distinct developmental periods provides a second useful characteristic for comparing the behavioral development between species. While rats have three distinct periods for the

development of agonistic behavior, golden hamsters show a gradual transition from play fighting to adult aggression and do not show a period devoted to playful social interactions during puberty (Goldman and Swanson, 1975; Pellis, 2002; Pellis and Pellis, 1988a, b; Wommack et al., 2003).

Behavioral Development in Golden Hamsters

In golden hamsters, agonistic behavior undergoes quantitative and qualitative transitions during puberty. The quantitative changes in agonistic behavior are observed through attack frequency. As soon as golden hamsters are capable of coordinated locomotor activity (around P-20), they engage in agonistic behavior with their littermates (Goldman and Swanson, 1975; Schoenfield and Leonard, 1985). Attack frequency peaks between P-30 and P-35 and then steadily declines until early adulthood (P-70) (Goldman and Swanson, 1975; Pellis and Pellis, 1988a; Wommack et al., 2003).

The transition from play fighting to adult aggression, or the qualitative change in agonistic behavior, is based on attack targets. Play-fighting attacks are focused on the head and cheeks of the intruder (Pellis and Pellis, 1988a; b, Wommack et al., 2003). This response type is the predominant form of agonistic behavior in social interactions between littermates and in the first few weeks of puberty. In contrast, adult attacks are directed towards the belly or rear of conspecifics (Pellis and Pellis, 1988a; b; Wommack et al., 2003). This behavioral response is rarely observed during early puberty. Instead, the frequency of belly/rear attacks steadily increases over the course of puberty and is the

predominant form of agonistic behavior during adulthood (Pellis and Pellis, 1988a; Wommack et al., 2003).

The pubertal development of defensive responses is not as well understood in golden hamsters. The available data show that hamsters and rats employ similar defensive strategies. Hamsters defend themselves by either partially rotating away from the attack and into an upright position (upright defenses) or by assuming supine defensive posture (on-back defense) (Pellis and Pellis, 1988a). Juvenile hamsters are more likely to perform on-back defenses, while adults primarily perform upright defenses (Pellis and Pellis, 1988a). Similar to rats, the defensive responses of hamsters are performed during specific periods of behavioral development. However, it is important to remember that the areas of the hamster targeted for attack change during this time period. As defensive tactics adapt to attack targets, the age-dependent expression of defensive responses may simply be due to the types of attacks the hamsters receive. For example, play-fighting attacks usually result in the attacker pinning its opponent on its back (Pellis and Pellis, 1988a). Therefore, the preponderance of on-back defenses during early puberty is likely due to the fact that the animals are being pinned by their opponents.

Sex Differences and Agonistic Behavior

Both hamsters and rats show quantitative sex differences in the development of agonistic behavior (Meaney et al., 1985; Pellis, 2002; Pellis et al., 1997; Taravosh-Lahn and Delville, 2004). Quantitatively, male rats initiate attacks more frequently than females over the course of puberty (Meaney et al., 1985; Olioff and Stewart, 1978; Pellis

and Pellis, 1990; Thor and Holloway, 1984). Male golden hamsters are more likely to attack during early puberty (around P-35) (Guerra et al., 1992). Longitudinal studies of agonistic behavior in golden hamsters have provided a more detailed understanding of sex differences within this species (Taravosh-Lahn and Delville, 2004). Both male and female hamsters show similar levels of attack frequency at the onset of puberty. In males, attack frequency peaks at around P-35 then slowly declines throughout puberty. In contrast, females show similar levels of attack frequency across puberty. As a result, females are more likely to initiate attacks during adulthood (Guerra et al., 1992; Taravosh-Lahn and Delville, 2004).

Rats and hamsters also show qualitative sex differences in the development of agonistic behavior (Pellis, 2002; Pellis et al., 1997; Taravosh-Lahn and Delville, 2004). While male rats show three developmental periods based on defensive responses (partial and complete rotations), female rats show only two. Female rats show complete rotations throughout adulthood and never return to the use of the partial rotation as a defensive tactic (Pellis and Pellis, 1990; Smith et al., 1998). Full rotations lead to more playful interactions this indicating that the behavioral repertoire of adult female rats contains more play-fighting behaviors than that of adult males. A similar finding has been observed in the offensive responses of female golden hamsters (Taravosh-Lahn and Delville, 2004). While the target of attack undergoes a more rapid progression down the rostro-caudal axis in female hamsters than in males, females continue to attack the face and cheeks of intruders well into adulthood. In contrast, male hamsters do not target the face and cheeks of intruders during adulthood (Wommack et al., 2003). Sex differences

in the development and expression of agonistic responses suggest that these behaviors are modulated by gonadal hormones.

Gonadal Steroids and Agonistic Behavior

During adulthood, offensive aggression in male rodents is clearly influenced by androgens (Albers et al., 2002; Simon, 2002). In male golden hamsters, castration during adulthood inhibits aggression, but this effect is reversed by testosterone replacement (Delville et al., 1996; Drickhamer et al., 1973; Grelk et al., 1974; Payne, 1973; Payne, 1974; Potegal et al., 1980; Vandenbergh, 1971). The degree to which castration and testosterone replacement affects aggression varies between studies, yet each presents data supporting a positive relationship between testosterone and aggression. In contrast, a number of other studies in adult golden hamsters have failed to show a link between castration, testosterone replacement, and male aggression (Evans and Brain, 1974; Garrett and Campbell, 1980; Tieffer, 1970; Whitsett, 1975). Theses conflicting results may be due to differences in experimental procedures and design. For example, the type of behavioral test used can have a large impact on the outcome of the data. Specifically, whether experimental animals were paired with an equal or smaller opponent, and if they were tested in their home cage or a neutral arena can greatly affect their aggressive behavior, regardless of testosterone manipulation. The timing of testosterone manipulation and behavioral testing can also have an impact. The effects of castration may not be observable until three weeks following gonadectomy (Miller et al., 1992). Studies that tested aggression within this time window have not found differences

in aggression between intact and castrated males (Romeo et al., 2003). This is possibly due to the persistence of the downstream effects of testosterone on the neural mechanisms controlling aggression. For example, the expression of vasopressin, a neuropeptide shown to facilitate offensive aggression, is testosterone-dependent (Delville et al., 1996; DeVries et al., 1985). Castration decreases vasopressin immunoreactivity within the brain of male rats, but this effect is not observed until three weeks after castration (De Vries et al., 1985).

The relationship between testosterone and aggression is strong but not always absolute. For instance, in many species, males show the highest levels of aggression during the breeding season, when testosterone levels are high (Balthazart, 1983; Crews and Silver, 1985; Wingfield and Marler, 1988). However, male hamsters that are housed in a short-day photoperiod show increased aggression, despite low plasma testosterone levels (Jasnow, 2002). Recent reports have suggested that the increased aggression in short day conditions is mediated by adrenocortical steroids (Demas et al., 2004).

Gonadal Steroids and the Development of Agonistic Behavior

A number of studies have shown that perinatal androgen exposure is critical for the organization of agonistic behavior (Beatty et al., 1981; Meaney et al., 1983; Meaney and Stewart, 1981b; Pellis and McKenna, 1992; Taylor et al., 1986; Thor and Holloway, 1985; Tonjes et al., 1987). Perinatal castration prevents masculinization of the development of play fighting frequency in rats. Furthermore, perinatal testosterone treatment masculinizes the development of play fighting frequency in female rats.

However, the qualitative sex differences in defensive tactics of rats are not fully determined by androgen exposure. Perinatal androgen treatment is not sufficient to alter the transition back to partial rotations in female rats (Smith et al., 1998a; 1998b). The appearance of the male phenotype for defensive behavior in rats requires ovariectomy, either during the perinatal or preweaning period (Pellis 2002; Smith et al., 1998a). This finding indicates that the development of agonistic behavior in rats is dependent on both androgens and ovarian hormones.

The relationship between pubertal changes in testosterone levels and agonistic behavior is not as clear. First, hamsters and rats engage in play fighting prior to the pubertal increases of testosterone levels, suggesting that the onset of agonistic behavior is independent of gonadal steroids (Goldman and Swanson, 1975; Schoenfield and Leonard, 1985; Vomachka and Greenwald, 1979). Additionally, increasing testosterone levels do not appear to be associated with the transition from play fighting to adult aggression. While significant increases in plasma testosterone levels coincide with the transition from play fighting to adult aggression in male golden hamsters, preweaning castration does not affect this behavioral shift (Pellis and Pellis, 1988a; Romeo et al., 2003; Vomachka and Greenwald, 1979; Wommack et al., 2003). Similarly, preweaning gonadectomy in rats does not affect playful defenses outside the dominant/subordinate hierarchy, as castrated and intact juvenile rats show similar levels of play-fighting frequency and are just as likely to perform full rotations (Beatty et al., 1981; Pellis, 2002; Smith et al., 1996). The lack of a clear relationship between pubertal increases in testosterone and the development of agonistic behavior may be similar to castration studies in adults, a result

of experimental procedures. For example, the juvenile hamsters that were gonadectomized one week prior to behavioral testing showed similar levels of aggression as compared to intact males (Romeo et al., 2003). Since this study measured bite location instead of attack target the lack of effect may result from the approach to behavioral observations. Also, as previously mentioned, the lack of effect may be due to androgen-dependent mechanisms that persist following gonadectomy. Studies that manipulate testosterone differently may provide a clearer understanding of the relationship between pubertal changes in gonadal steroids and agonistic behavior. For example, studies in juvenile hamsters have shown that long-term treatment with anabolic androgenic steroids increases aggression (DeLeon et al., 2002a, Melloni et al., 1997).

Adrenal Steroids and the Development of Agonistic Behavior

Adrenal androgens may play a role in the development of agonistic behavior. For instance, juvenile hamsters begin to engage in agonistic behaviors during a period prior to the onset of puberty, but coinciding with adrenarche (Cutler et al., 1978; Goldman and Swanson, 1975; Vomacka and Greenwald, 1979). Puberty, as defined by increasing gonadal steroid levels, begins around P-28 in golden hamsters (Vomachka and Greenwald, 1979). However, individuals engage in agonistic interactions with their littermates as early as P-18 (Goldman and Swanson, 1975). These findings suggest that the onset of agonistic behavior is unrelated to increases in gonadal hormone levels. However, the onset of flank marking (a form of agonistic behavior) coincides with adrenarche, the activation of the hypothalamic-pituitary-adrenal (HPA) axis and a surge

of adrenal androgens and glucocorticoids (Cutler et al., 1978; Ferris et al., 1996). Male and female golden hamsters do not flank mark until P-19, a time when adrenal androgen hormones are on the rise. It is unclear whether the onset of flank marking is mediated by increases in adrenal androgens or glucocorticoids.

In addition to the hypothalamic-pituitary-gonadal (HPG) axis, other neuroendocrine systems undergo pubertal transitions. For example, the HPA activity changes over the course of puberty, although the nature of these changes varies between species. For example, male rats show prolonged adrenocorticotropin releasing hormone (ACTH) release and delayed corticosterone responses during early puberty, yet basal and peak corticosterone levels remain unchanged throughout during this time (Gomez et al., 2002; Romeo et al., 2004; Vazquez, 1998). In contrast, baseline glucocorticoid levels increase gradually throughout puberty in other species, including tree shrews (Van Kampen and Fuchs, 1998) and humans (Elminger et al., 2002; Jonetz-Mentzel et al., 1993; Kiess et al., 1995).

Importantly, glucocorticoids have been shown to enhance offensive aggression. Microinjection of cortisol into the anterior hypothalamus enhances aggression in male golden hamsters (Hayden-Hixson and Ferris, 1991). In addition, glucocorticoids mediate short-day increases in aggression of male siberian hamsters (Demas et al., 2004). However, pubertal changes in the HPA axis and the influence of corticosteroids on behavioral development have yet to be studied.

Environmental Factors Influencing Behavioral Development

Previous studies have examined environmental factors that influence the development of offensive aggression in hamsters such as anabolic steroids (DeLeon et al., 2002a; Melloni, et al., 1997), social stress (Delville, et al., 1998), alcohol (Ferris, et al., 1998), lead exposure (Delville, 1999), and cocaine (Harrison, et al., 2000; DeLeon et al., 2002b; Ricci et al., 2004). For example, social stress caused by repeated exposure to aggressive adults during puberty results in context-dependent changes in agonistic behavior (Delville et al., 1998; Wommack et al., 2003). Repeated subjugation during puberty decreases offensive responses towards intruders of equal size and age but increases aggression towards smaller and younger intruders (Delville et al., 1998). Repeated stress during early puberty also accelerates the transition from play fighting to adult aggression (Wommack et al., 2003). When paired with a smaller and younger intruder, subjugated juveniles show a decreased percentage of play-fighting attacks and an increased percentage of adult-like attacks (Wommack et al., 2003). In this study, group differences in attack frequency are only observed later in development as, subjugated juveniles also display a higher attack frequency when paired with a smaller and younger intruder during early adulthood (Wommack et al., 2003).

The Neural Control of Offensive Aggression

Brain areas that control offensive aggression in male golden hamsters have been identified through a variety of experimental techniques. Studies using either electrical stimulation or lesions have shown that a number of limbic areas, such as the septum, hypothalamus, amygdala, and preoptic area, regulate offensive responses (Bunnell et al., 1970; Hammond and Rowe, 1976; Potegal et al., 1981; 1996; Shipley and Kolb, 1977; Sodetz and Bunnell, 1970). These studies have shown that the contribution to offensive aggression varies from area to area. For example, offensive aggression is facilitated by electrical stimulation of the corticomedial amygdala and inhibited by electrical stimulation of the septum (Potegal et al., 1981; 1996). Neurochemical studies have also implicated the anterior hypothalamus as an important center for the control of offensive responses (Ferris and Potegal, 1988; Potegal and Ferris, 1990). Additionally, retrograde labeling and c-fos immunocytochemistry have added to the understanding of the neural pathways controlling offensive aggression (Delville et al., 2000). The anterior hypothalamus also shows increased density of cyclic AMP binding protein, phosphorylated (pCREB) following offensive aggression (David et al., 2004). Areas such as the anterior hypothalamus, ventrolateral hyhpothalamus, medial amygdala, bed nucleus of the stria terminals, and the dorsolateral part of the midbrain central gray show increase in c-fos immunolabelling specific to offensive aggression (Delville et al., 2000). Moreover, each of these areas is reciprocally connected to the anterior hypothalamus, suggesting that this area is the center of a neural network of offensive aggression in male golden hamsters.

Neurochemically, offensive aggression in male golden hamsters is facilitated by arginine vasopressin (AVP) at the level of the anterior hypothalamus. Microinjections of vasopressin into the hypothalamus facilitate offensive responses in adult male hamsters (Ferris et al., 1997). Moreover, these effects are mediated via the V1A receptor subtype, as microinjections of a selective V1A antagonist in the anterior hypothalamus cause a dose-dependent inhibition of offensive aggression (Ferris and Potegal, 1988). AVP also stimulates aggressive behavior when injected into the ventrolateral hypothalamus (Delville et al., 1996a). Interestingly, AVP receptors in the ventrolateral hypothalamus are testosterone-dependent, as receptor binding significantly decreases after castration (Delville, et al., 1996b).

Serotonin (5HT) has been identified as a neurotransmitter that inhibits offensive aggression at the level of the anterior hypothalamus. Offensive aggression is blocked by either peripheral treatment of Fluoxetine, a 5HT reputake inhibitor, or i.c.v. injections of selective 5HT 1A receptor agonists (Delville et al., 1996a, Ferris et al., 1997; 1999; Joppa et al., 1997). These effects are likely due to an interaction between 5HT and AVP within the anterior hypothalamus (Ferris et al., 1997). Treatment with Fluoxetine blocks AVP-induced aggression (Ferris et al., 1997). 5HT also blocks the effects of AVP at the level of the anterior hypothalamus via the 5HT 1A receptor (Ferris et al., 1999). Additional evidence suggests that offensive aggression is influence by 5HT/AVP interactions within the within the ventrolateral hypothalamus (Delville et al., 1996b).

Recent reports in juvenile male hamster have also implicated dopamine as a neurotransmitter controlling aggression. Juveniles repeatedly treated with low to

moderate doses of cocaine show enhanced aggression towards smaller and younger opponents (Harrison et al., 2000). Moreover, the behavioral effects of cocaine treatment during adolescent may result from affects of the drug on 5HT within the anterior hypothalamus (DeLeon et al., 2002b; Ricci et al., 2004). Additionally, subjugated juveniles that behave more aggressively when paired with a smaller and younger intruder also show increased tyrosine hydroxylase immunoreactivity within the medial amygdala and bed nucleus of the stria terminalis (Delville et al., 1998; Wommack and Delville, 2002). As the medial amygdala and the bed nucleus of the stria terminalis are involved in the control of offensive responses and are connected to the anterior hypothalamus, these areas may affect aggressive behavior through dopamine release (Delville et al., 2000; Potegal et al.,1996).

Even more recent evidence suggests that corticotropin releasing hormone (CRH) is another neurotransmitter system potentially involved in the development of offensive responses in male golden hamsters. CRH fibers are widely observed throughout the brain of male golden hamsters, including limbic areas involved in offensive aggression (Delville et al., 1992). Additionally, oral administration of a CRH type-1 receptor antagonist inhibits aggression in adult male hamsters (Farrokhi et al., 2004). While the potential for CRH to influence offensive aggression in male golden hamsters is promising, additional studies are required to better understand the role of this neuropeptide.

The Biobehavioral Consequences of Social Stress

Repeated social stress influences numerous physiological and behavioral measures. These effects include, immunosuppression, increased anxiety, submissive behavior, decreased testosterone levels, enhanced glucocorticoid synthesis (Blanchard et al., 2002).

The neuroendocrine and behavioral consequences of social stress in adult male golden hamsters are consistent with the findings in other species. For example, social defeat in male golden hamsters activates the HPA axis causing an acute increase in plasma levels of ACTH and cortisol (Huhman et al., 1991). Males that are repeatedly defeated also have elevated basal cortisol levels and decreased testosterone levels (Huhman, et al., 1992). Additionally, repeated social subjugation during adulthood causes a complete inhibition of offensive aggression, along with increased submission, and avoidance (Huhman et al., 1991; 1992; 2003; Jasnow et al., 1999; Potegal et al., 1993). These effects last for more than a month after subjugation (Huhman et al., 2003) and may remain present for the duration of the individual's life. These effects are similar to a condition known as social castration. In African cichlid fish, the HPG axis is more active in territorial males than nonterritorial males, but the activity of this neuroendocrine axis, as well as aggressive behavior, can be reversed by exogenous stimuli, such as social cues (Francis et al., 1993).

The Consequences of Social Stress during Puberty

In contrast to adults, socially subjugated juvenile male hamsters show an enhancement of offensive aggression (Delville et al., 1998; Wommack et al., 2003). This effect is context-dependent, as repeatedly defeated males show increase offensive responses towards smaller and younger intruders and decreased offensive responses towards intruders of equal size and age (Delville et al., 1998). Additionally, repeated social stress during early puberty accelerates that transition from play fighting to adult aggression and increases offensive responses during adulthood (Wommack et al., 2003). This effect stands in direct opposition to the complete inhibition of offensive aggression observed in defeated adult hamsters. As such, the contrasting outcomes of social stress during puberty vs adulthood indicate that the consequences of subjugation can be determined by the age of the animal.

While the endocrine consequences of social defeat are well documented in adult male hamsters, the effects of subjugation on gonadal and adrenal steroids are unknown in juveniles (Blanchard, 2002; Huhman et al., 1991; 1992). Additionally, the neural mechanisms responsible for the enhanced aggression observed in subjugated juveniles have yet to be determined. The unique context-dependent effect on offensive aggression caused by repeated social stress during puberty was associated with increased serotonin innervation and decreased vasopressin content in the anterior hypothalamus (Delville et al., 1998). These results indicate a correlation between neurochemical changes and behavioral inhibition towards intruders of equal size and age, as offensive aggression is inhibited by serotonin and facilitated by vasopressin release in the anterior hypothalamus

(Ferris and Delville, 1994; Ferris et al., 1997; Ferris et al., 1999). However, these findings were inconsistent with the increased aggression displayed by subjugated individuals when in the presence of smaller and younger stimuli. It was previously hypothesized that the enhanced aggression in the presence of smaller and younger intruders was associated with altered expression of vasopressin and/or serotonin receptors (Delville et al., 1998). However, preliminary experiments failed to support this hypothesis (Ferris and Delville, unpublished observations).

Recently, it has been reported that repeated subjugation during puberty causes a site-specific increase of tyrosine hydroxylase immunoreactivity (TH-IR) in dopamine neurons within posterior portion of the medial division of the bed nucleus of the stria terminalis (BSTPM) and the posterodorsal part of the medial amygdala (MePD) (Wommack and Delville, 2002). These areas are interesting for a number or reasons. First, the BSTPM and MePD are both involved in a number of social behaviors, including offensive aggression (Delville et al., 2000; Newman, 1999). Second, these specific neurons are testosterone-dependent (Asmus et al., 1992; Asmus and Newman, 1993) and may be sensitive to the physiological changes associated with puberty (Romeo and Sisk, 2001). Furthermore, the BSTPM and MePD show increased Fos immunoreactivity following social defeat (Kollack-Walker et al, 1999; Martinez et al., 1998). Finally, these areas show stress-induced plasticity that is involved in anxiety behaviors (Pawlak et al., 2003; Roozendaal et al., 1997). Consequently, it is possible that increased TH-IR within the BSTPM and MePD is related to the behavioral effects of social subjugation during puberty

Experimental Overview

The primary goal of this dissertation is to understand the neuroendocrine mechanisms controlling the development of agonistic behavior in male golden hamsters. Previous experiments on the development of offensive aggression have failed to establish a link between gonadal steroids and the transition from play fighting to adult aggression. Other experiments have shown that repeated social stress accelerates the development of offensive responses. In adults, social stress is a potent activator of the HPA axis, but the endocrine effects of defeat during puberty are not as well understood. As data from rats and other species show that the HPA axis changes over the course of puberty, it is possible that the HPA axis changes also undergoes pubertal development in hamsters. As pubertal changes in HPA activity would coincide with the transition from play fighting to adult aggression, changes in stress hormones accelerate behavioral development. Additionally, increasing levels of glucocorticoids could also be responsible for the accelerated behavioral development observed in repeatedly defeated juvenile hamsters. Therefore, the main hypothesis is that the glucocorticoids control the development of agonistic behavior in male golden hamsters. I will investigate this topic by asking the basic questions listed below.

1) How do defensive forms of agonistic behavior change over the course of puberty, and can this aspect of behavioral development be affected by repeated social defeat?

Repeated social stress alters the development of offensive forms of agonistic behavior. However, the effects of social stress on the development of other agonistic

behaviors are unknown. The portion of the dissertation (chapter 2) will attempt to describe pubertal changes in defensive forms of agonistic behavior and test the effects ofrepeated social stress.

2) Does the HPA axis of male golden hamsters change during puberty?

If stress hormones play a role in shaping the development of agonistic behavior, it would be likely that this is a result of developmental changes in the HPA axis. Only a limited number of studies have investigated the possibility that the HPA changes during the course of puberty. These studies, done primarily in rats, have shown that neither basal nor post-stress corticosterone levels change over the course of puberty. However, studies in other species such as humans and tree shrews suggest that glucocorticoid levels increase during puberty. This portion of the dissertation (chapter 3) will test whether or not the HPA axis of male golden hamsters changes during puberty. Additionally, experiments will be conducted to determine the effects of social defeat on stress hormones in juvenile male hamsters. Data from these latter studies should provide insight into the mechanisms by which social stress accelerates the development of offensive responses and could also add to the understanding of the physiological factors guiding behavioral maturation under normal or unstressed, animals.

3) Could increasing cortisol levels affect the pubertal development of offensive responses?

Data from chapter 3 showed that increases in basal and post-stress cortisol levels coincide with the transition from play fighting to adult aggression. Moreover, repeated social stress is a stressor that results in daily increases in plasma cortisol levels and

causes an accelerated transition from play fighting to adult aggression. This portion of the dissertation (chapter 4) will test whether cortisol is a hormone that influences the development of offensive responses. Additionally, experiments will be designed to test the specific role of corticosteroid type II receptors in the development of offensive aggression.

4) What is the neural mechanism that controls the development of offensive responses?

Previously, I found that social stress during early puberty enhances the number of dopamine neurons within the bed nucleus of the stria terminalis and medial amygdala, brain areas involved in offensive aggression. Additionally, other labs have reported that chronic low doses of cocaine during puberty increase aggression in male golden hamsters. As such, it is possible these dopamine neurons are involved in the development of agonistic behavior. However, the development of these neural populations must be better understood in order to determine their role in the development of offensive forms of agonistic behavior. This portion of the dissertation (chapter 5) will investigate the behavior and experience dependent changes in the neural populations to understand if and how they are involved in the development of agonistic behavior.

As results from the first experiments in chapter 5 rejected the hypothesis that dopamine neurons within the medial and extended amygdala are involved in the development of offensive responses, I decided to investigate the role of CRH in the development of agonistic behavior. CRH has recently been shown to influence offensive aggression in male golden hamsters. However, little is understood about how this

neuropeptide changes during puberty. Therefore, additional experiments were conducted in this chapter to determine the developmental changes of CRH innervation of limbic areas involved in offensive aggression. Additionally, CRH innervation was analyzed in the brains of subjugated juveniles to determine whether or not this neuropeptide is involved in the behavioral consequences of repeated social stress.

Chapter 2: The Pubertal Development of Defensive Responses

Introduction

Puberty is marked by various behavioral transitions. Agonistic responses are amongst the behavioral categories that change during this time. Agonistic behavior includes many types of actions observed during a social conflict, such as offensive (e.g. attacks, pins, and flank marks) and defensive responses (e.g. defense, submission, avoidance, and risk assessment)

In male golden hamsters, several studies have focused on the pubertal maturation of offensive responses (Pellis and Pellis, 1988a; b; Wommack et al., 2003; Taravosh-Lahn and Delville, 2004). However, other aspects of agonistic behavior such, as defensive responses, have been less explored in a developmental context. Studies in rats have shown that defensive forms of agonistic behavior change substantially during this developmental period. For example, male rats alter their defense to an opponent's attacks over the course of puberty. During early puberty, a common defensive response of rats is the complete rotation where they rotate along their rostro-caudal axis into a supine posture to deflect the advances of the attacker (Pellis 2002; Pellis et al., 1992; Pellis and Pellis, 1990; 1997). Around mid puberty, the complete rotation is replaced by the partial rotation where rats turn their bodies away from the attacker and support themselves with their hind limbs, while using the forelimbs to deflect attacks (Pellis, 2002; Pellis and Pellis, 1990; 1997; Pellis et al., 1992). This development of defensive responses parallels changes in offensive responses. Juvenile rats attack the nape of an opponent's neck while adults direct their attacks toward an opponent's back (Pellis, 2002; Pellis and Pellis,

1991; Pellis et al., 1992; Takahashi and Lore, 1983). As both offensive and defensiver responses undergo pubertal transitions in rats, it is possible that the same occurs in golden hamsters.

Indeed, some data suggests that juvenile and adult male golden hamsters employ different defensive tactics. Juvenile males paired with equally matched opponents defend themselves from a supine position more frequently than adults (Pellis and Pellis, 1988a). However, these observations were made in a single social context, a contest between two equally matched opponents. It is possible that the responses to a larger, aggressive opponent undergo a separate developmental pattern during puberty. Additionally, the previous experiment did not observe other defensive responses such as submission, avoidance, and risk assessment are regularly performed in a defensive context (Albers et al., 2002; Pellis and Pellis, 1988a).

The maturation of agonistic responses is not solely influenced by internal factors. A few studies show that the development of agonistic behaviors is susceptible to alterations by external factors. For example, chronic exposure to lead during development accelerates the transition from play fighting to adult aggression during puberty (Cervantes et al., 2005). Similarly, repeated exposure to social stress results in an accelerated maturation of offensive responses (Wommack et al., 2003). The effects of social stress are long lasting, as subjugated juveniles are more aggressive than socially naive controls during early adulthood (Wommack et al., 2003).

Socially defeated adult male hamsters show increased submission and risk assessment in male golden hamsters, the effects of social stress on the development of

defensive responses in not understood (Potegal et al., 1993; Huhman et al., 2003). Therefore, it is possible that social during puberty also affects of defensive responses.

The goals of this study are to 1) assess the pubertal development of defensive responses 2) determine whether the maturation of these behaviors can be altered by an external factor such as social stress.

Methods

Animals and Treatment

The animals (male golden hamsters) were bred in the laboratory from a colony that originated from Harlan Sprague Dawley (Indianapolis, IN). Approximately five days after birth, all litters were culled to 6 pups including males and females. On postnatal day 25 (P-25), all animals were weaned and individually housed in plexiglass cages (20x33x13 cm). Within 2 days of weaning, each animal was briefly (a few seconds) observed in the presence of an adult intruder. Individuals that immediately fled from the adult were considered to be inherently fearful (approximately 1 in 12) and were removed from the experiment due to their abnormal responses to a social stimulus. All animals received food and water ad libitum and were housed under a reversed light/dark cycle (14L-10 D, lights off at 9:00am).

Experimental Design

This experiment sought to understand the pubertal development of defensive responses and to determine whether or not this development could be altered by an external factor such as social stress. Social stress alters the development of offensive responses; therefore, it is likely that subjugation also affects the development of

defensive responses (Wommack et al., 2003). On P-28, animals were separated into two groups (Naive and Subjugated). Social subjugation was performed according to a previously described protocol (Delville et al., 1998; Wommack and Delville, 2002). Daily subjugation started on P-28, which coincides with the onset of puberty (Vomachka and Greenwald, 1979), and ended on P-42, near mid puberty. This period was roughly equivalent to the first half of puberty (Delville et al., 2003). Animals in the subjugated group were placed in the home cage of an aggressive adult for 20 minutes daily, while naive animals were placed in a clean and empty cage for the same period of time. Subjugated juveniles were cycled through a group of aggressive adults (n=8) for each subjugation day. Prior to the experiment, adults were tested for offensive aggression by the resident-intruder paradigm (Miczek, 1979). Smaller and younger intruders were placed in the home cage of adult hamsters. The adults used in this experiment were trained fighters selected for their aggressiveness. During repeated subjugation, animals were observed daily while in the home cage of an adult to ensure they were chased and attacked. Daily subjugation and behavioral tests were performed during the second half of the dark phase.

To test for defensive responses, subjugated and naïve animals (n=9-16 per day per group) animals were placed in the home cage of larger adults on various days across puberty. The exposure to a larger, older opponent ensured that the experimental animals would be defeated and that their behavioral responses would be purely defensive.

At the onset of puberty, on P-28, a group of animals that had not been assigned to either the naive or subjugated group were observed for twenty minutes in the home cage

of an aggressive adult. These animals were tested on this day to establish a developmental trajectory. On P-35 (early puberty), P-45 (mid puberty), or P-70 (early adulthood), both naive and subjugated groups were observed for twenty minutes in the home cage of an aggressive adult. Animals in the subjugated group were paired with an unfamiliar opponent to prevent a confound from individual recognition. These test dates were chosen to establish a developmental trajectory and to understand the time course of experimental effects. Each encounter was recorded with a Sony digital video camera for later review with iMovie software. Each experimental animal was only used once, as they were immediately sacrificed after the behavioral encounter and tissue was collected for a separate experiment (data presented in chapter 3).

Defensive Responses

During social conflict, golden hamsters display a number of stereotypical behaviors in response to an aggressor (Albers et al., 2002). Defensive responses can be separated into several distinct categories: defense, submission, avoidance, or risk assessment. During 20-minute exposures to unfamiliar aggressive adults, I observed responses for each individual in these behavioral categories. Defense consisted of upright defenses and was scored when the subjects stood upon their hind legs and oriented their face and forelimbs toward the resident to deflect attacks. Submission consisted of tail-up and on-back submissive displays. Tail-ups and on-backs were scored as submission. During tail-ups, the hamsters maintained a lordosis combined with a raised tail while either moving slowly or standing still. In contrast, on-backs were scored when the juveniles remained motionless and supine (from seconds to minutes at a time)

without using their forelimbs to defend from the attacks of the adults. Avoidant behavior consisted of retreats. Retreats were scored when the juveniles retreated or fled from the adults. Risk assessment consisted of olfactory investigations and were recorded when the subjects actively approached and stretched their neck so that their snout made contact with the adults. This type of behavior has previously been observed in defeated adult hamsters (Potegal et al., 1993). Similar forms of risk assessment behavior have also been observed in mice in the presence of predators (Blanchard et al., 2001). Also recorded was the number of attacks received by individuals during the exposure to the adult. All behaviors were scored in terms of frequency (number/20-minute period). For on-backs and olfactory investigations, durations (seconds/20-minute period) were recorded in addition to frequency.

Data Analysis

Parametric data (e.g. duration of behaviors) were compared between groups over time by 2-way ANOVAs (independent variables: treatment groups and age). Nonparametric data (behavior frequencies) were compared separately with Mann-Whitney tests (two-tailed) for group comparisons, and with Kruskal-Wallis tests followed by Mann-Whitney test for age comparisons (two-tailed). Spearman‘s Rank Correlations were also performed for attacks received and retreats.

Results

Defense and submission did not change over the course of puberty. Similar levels of upright defenses, tail-up, and on-back submissive displays were observed across test

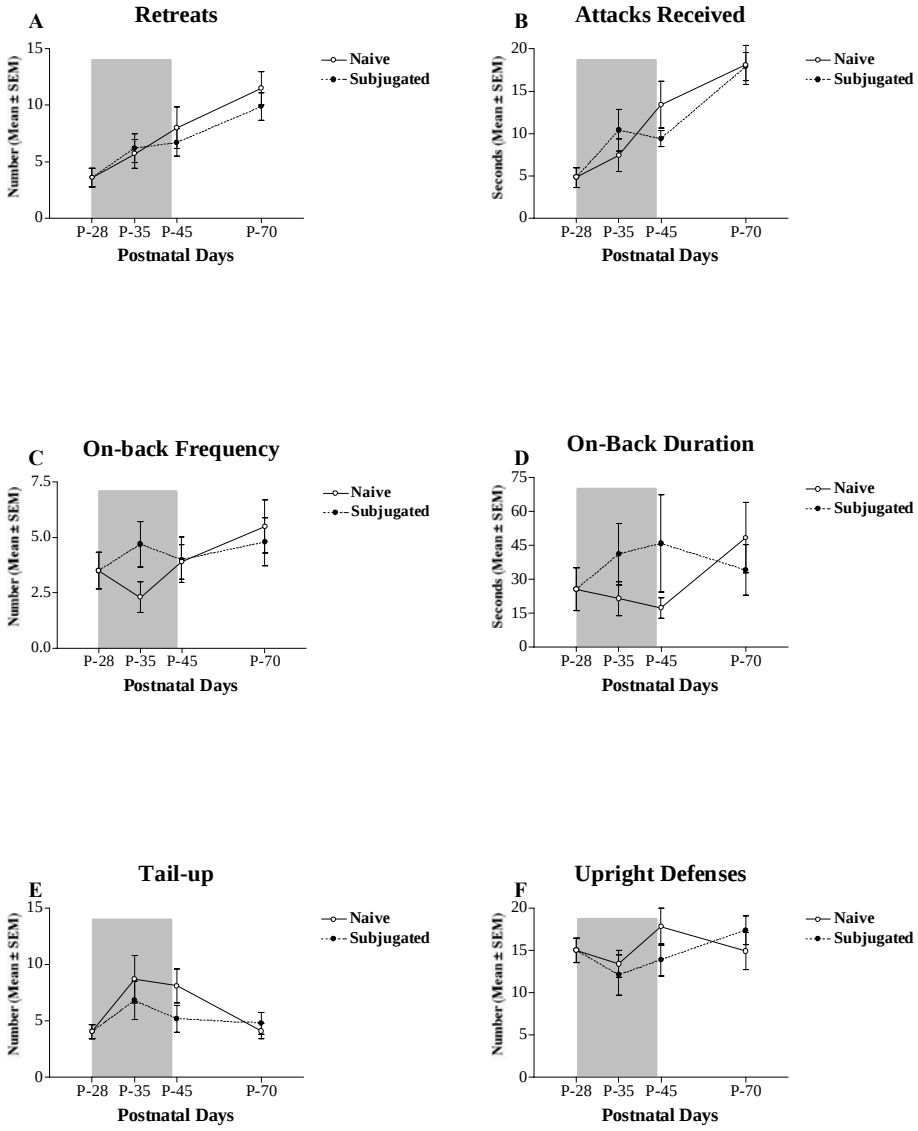
days (Fig. 2.1). These behaviors were not affected by repeated social stress, as no statistically significant group differences were observed for any measure of submission or defense. However, subjugated animals appeared to have a slightly higher frequency of on-back displays on P-35 and longer duration of on-backs on P-35 and P-45, but due to high variability these differences were not statistically significant.

Avoidance increased significantly during puberty, but this change was paralleled by increases in the number of attacks received. The frequency of retreats increased significantly from P-28 to P-70 in both subjugated and naive hamsters [respectively, $H(3)14.7$, $p<0.01$; $H(3)15.5$, $p<0.01$; Kruskal-Wallis]. However, there was no statistically significant difference between these subjugated and naive groups on any test day. In addition, the number of attacks received by the animals increased significantly for both subjugated and naive groups from P-28 to P-70 [respectively, $H(3)26.5$, $p<0.001$; $H(3)19.9$, $p<0.001$;]. Similar to the number of retreats, the number of attacks received did not differ significantly between groups on any test day. However, for both groups across test days, the number of attacks received and retreats were highly correlated ($r = 0.774$, $p<0.001$; Spearman's Rank Correlation).

Although risk assessment did not change over the course of puberty, socially subjugated animals showed a complete inhibition of olfactory investigation during the period of daily subjugation (Fig. 2.2). Subjugated animals performed olfactory investigations less frequently (respectively, $U=8$, $U`=92$, $p<0.01$; $U=16$, $U`=74$, $p<0.05$; Mann-Whitney) and for a shorter duration [$t(18)=4.03$, $p<0.001$; $t(18)=-2.43$, $p<0.05$; Student's t-test] than control animals when tested on P-35 and P-45. This effect was not

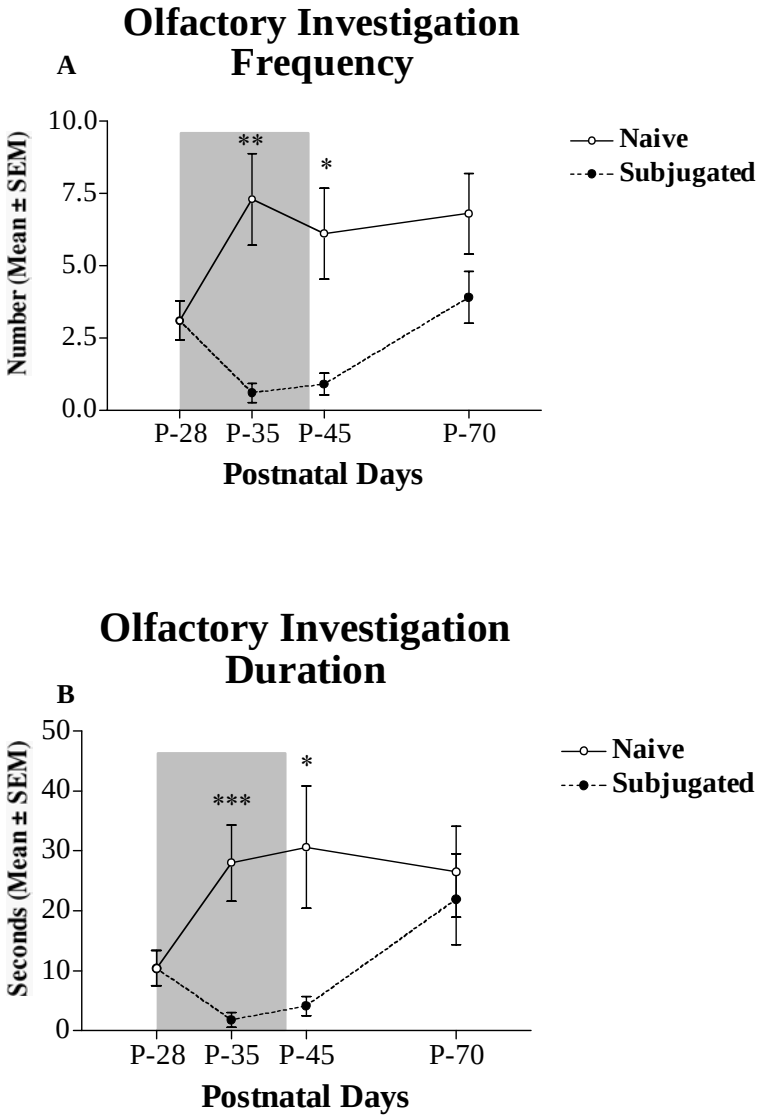
long lasting. On P-70, four weeks after the end of daily subjugation, no statistically significant difference in olfactory investigation frequency ($U=73$, $U`=107$, $p>0.05$) or duration [$t(23)=-0.3$, $p>0.1$] was observed between groups.

Figure 2.1: Defensive Responses



Retreats (A), attacks received (B), number (C) and duration (D) of on-back submissive postures, tail-ups (E) and upright defenses (F) as observed in subjugated and naïve animals during a 20-minute exposure to an aggressive adult. Shaded area represents period of daily subjugation.

Figure 2.2: Risk Assessment



Frequency (A) and duration (B) of olfactory investigations as observed in subjugated and control animals during a 20-minute exposure to an aggressive adult. Shaded area represents the period of daily subjugation. (Mann-Whitney or Student's t-test, two-tailed, *p<0.05, **p<0.01, ***p<0.001). Frequency (A) and duration (B) of olfactory investigations as observed in subjugated and control animals during a 20-minute exposure to an aggressive adult. Shaded area represents the period of daily subjugation. (Mann-Whitney or Student's t-test, two-tailed, *p<0.05, **p<0.01, ***p<0.001).

Discussion

Data from the current study report two major findings. First, the pubertal development of defensive responses was limited to avoidance. While the number of retreats increased gradually during puberty, the frequency of other forms of behavior such as defense, submission, and risk assessment remained constant. Second, social stress during early puberty did not alter the development of defensive responses. Instead, social stress caused a transient increase in risk assessment behavior (olfactory investigation) that was observed only during the period of daily subjugation.

Over the course of puberty, naïve male golden hamsters expressed consistent levels of defense, submission, and risk assessment. These data contrast with reports that male hamsters more frequently display on-back defensive postures during early puberty than during adulthood (Pellis and Pellis, 1988a). These opposing observations can be reconciled by addressing the difference in social conflict. The study that reported more frequent on-backs during early puberty was based on observations from equally matched opponents (Pellis and Pellis, 1988a). As the experimental animals in this study were always paired with larger opponents, it could indicate that the differential use of on-back defenses between juveniles and adults may only be observed when animals are paired with an equally matched opponent. Moreover, these data suggest that the defensive responses to a larger opponent do not change over the course of puberty.

Daily subjugation did not produce any clear difference in defense or submission. Repeatedly subjugated animals showed a higher frequency and duration of on-back submissive responses during the defeat tests on P-35 and P-45. However, these

differences were not statistically significant, possibly due to a high variability in subjugated animals. Previously reported individual differences in on-back responses may account for this high variability (Wommack and Delville, 2003). In contrast, social subjugation during adulthood leads to a significant increase in on-back submissive responses in hamsters (Potegal et al, 1993; Huhman et al., 2003). These observations point to a difference in consequences of social stress during peri-pubertal development.

Social subjugation did not affect avoidance behaviors (e.g. retreats). However, the number of retreats did increase during puberty for both the subjugated and naive group. Interestingly, this developmental increase correlated strongly with increased number of attacks received from the adults. As such, this developmental trend may not solely be attributable to the juveniles, but rather to increased aggression of the adult residents towards larger juvenile intruders. The increased aggression by the residents towards older animals possibly occurred because they were older and were perceived as more of a threat.

During the period of daily subjugation, repeatedly subjugated individuals did not engage in risk assessment (i.e., olfactory investigation) during the defeat tests. These findings are consistent with reports of decreased olfactory investigation in repeatedly subjugated adult hamsters (Potegal et al., 1993; Huhman et al., 2003). Similarly, defeated adult hamsters also showed a learned avoidance to the odor of a familiar winner (Lai and Johnston, 2004). Importantly, subjugated animals in this study were not paired with familiar conspecifics during daily subjugation or on tests days to avoid a confounding effect related to individual recognition. Reduced risk assessment may also

be an effect of repeated social experience and not just social stress. An additional control group of animals that experience a daily non-threatening social stimulus could potentially resolve this issue.

This decreased risk assessment behavior in socially subjugated hamsters may be comparable to learned helplessness in rats. Learned helpless rats fail to escape from electrical shocks after receiving inescapable and unpredictable shocks (Seligman and Beagley, 1975). Similarly to learned helpless rats, social defeat caused a post-stress behavioral inhibition when the aversive stimulus was presented, as the subjugated animals did not approach and investigate the aggressive adults. Interestingly, on P-70, four weeks after the period of daily subjugation, previously subjugated hamsters once again engaged in normal levels of olfactory investigation. In contrast, the behavioral effects of social defeat last more than a month in adult hamsters (Huhman et al, 2003), similar long-term effects have been reported in fear conditioning studies in adult rats (Kim and Fanselow, 1992; Van Dijken et al., 1992). Therefore the current findings show that the consequences of repeated social stress and the capacity to recover from this type of experience changes during peri-pubertal development.

The apparent resiliency that juveniles are capable of showing toward social stress may be ecologically relevant. In areas of high population density, the species would benefit if younger generations were not as susceptible to the negative effects of social stress. The lack of long-lasting negative consequences from social defeat could help to ensure that juveniles are capable of becoming dominant upon reaching adulthood. This

idea is supported by the current study in which subjugated juveniles showed no long-term behavioral inhibitions or adaptations.

In summary, the current study found that, unlike offensive responses, the defensive responses, with the exception of avoidance, do not change over the course of puberty in male golden hamsters. Also, social stress did not affect the development of defensive responses, suggesting that only the maturation of offensive response is susceptible to alteration by external factors. Nevertheless, risk assessment behavior was completely inhibited by social subjugation during early puberty. This effect was only transient indicating that male golden hamsters may be somewhat resilient to the effects of repeated social stress during puberty. As the effects of subjugation in adults are severe and often long lasting, this study suggests that puberty is a unique developmental period when individuals are less vulnerable to the negative effects of social stress.

Chapter 3: Pubertal Development of the HPA Axis

Introduction

During puberty social behaviors undergo transitions from juvenile to adult forms. Although the pubertal development of reproductive behaviors receives the majority of attention, other forms of behavior, such as agonistic responses, also undergo significant transitions during this time. For example, offensive responses in male golden hamsters undergo a transition from play fighting to adult aggression during adolescence (Pellis and Pellis, 1988a; Wommack et al., 2003).

The physiological factors guiding the transition of offensive responses in golden hamsters are poorly understood. Studies manipulating the hypothalamic-pituitary-gonadal (HPG) axis have failed to show a clear relationship between increasing sex steroids and changes in offensive responses (Beatty et al., 1981; Pellis, 2002; Romeo et al., 2003; Smith et al., 1996). However, certain issues such as behavioral analysis and duration between castration and aggression testing make the results of these studies problematic. Experiments manipulating testosterone differently could clarify the relationship between testosterone and the development of offensive responses. Nevertheless, the results of previous studies suggest that the transition from play fighting to adult aggression is not controlled by testosterone.

If one focused on the HPG axis, they would be led to believe that pubertal development of agonistic behavior is guided by nonsteroidal mechanisms. Indeed, such

explanations have been proposed (Sisk and Foster, 2004). However, behavioral development may also be influenced by other steroid systems that change during puberty.

The hypothalamic-pituitary-adrenal (HPA) axis is one such steroid system that potentially influences behavioral development. Recent studies have shown that HPA axis undergoes considerable and species-specific transitions. For example, pre-pubertal male rats show prolonged adrenocorticotropin releasing hormone (ACTH) release and delayed corticosterone responses (Gomez et al., 2002; Romeo et al., 2004; Vazquez, 1998). In this species, baseline HPA activity does not change during puberty (Gomez et al., 2002; Romeo et al., 2004; Vazquez, 1998). In contrast, baseline glucocorticoid levels increase gradually throughout puberty in other species including tree shrews (Van Kampen and Fuchs, 1998) and humans (Elmlinger et al., 2002; Jonetz-Mentzel et al., 1993; Kiess et al., 1995). These data present the possibility that the HPA axis of juvenile male hamsters also changes during puberty.

The transition from play fighting to adult aggression in juvenile hamsters is susceptible to influence from external factors. For example, lead exposure during the perinatal period accelerates the pubertal development of offensive responses in male golden hamsters (Cervantes et al., 2005). Moreover, repeated exposure to social stress during early puberty also accelerates the transition from play fighting to adult aggression in male golden hamsters (Wommack et al., 2003). In adult hamsters, social defeat is a potent activator of the HPA axis, causing acute increases of cortisol and long-term increases in baseline cortisol levels (Huhman et al., 1991; 1992). As such, it is possible that this external factor could influence behavioral development via the HPA axis.

Moreover, as social stress during adulthood decreases testosterone levels, socially subjugate juveniles may also have decreased testosterone levels (Huhman et al., 1992). As such, a careful examination of the consequences of repeated stress during puberty on cortisol and testosterone activity could provide insight into how this experience alters behavioral development and potentially shed light onto the endocrine factors that guide this behavioral transition under normal conditions.

The overall goal of these studies was to clarify the endocrine factors influencing behavioral development. For example, if social subjugation affects the development of offensive responses via cortisol, then social defeat should increase plasma cortisol levels. Additionally, if the HPA axis is involved in the behavioral maturation, cortisol levels (basal and/or post-stress) should undergo changes that coincide with developmental transition from play fighting to adult aggression. Likewise, a longitudinal analysis of testosterone levels in naïve and subjugated animals could potentially clarify the role of this hormone in the development of offensive responses.

Methods

Animals and Treatment

The animals (male golden hamsters) were bred in the laboratory from a colony that originated from Harlan Sprague Dawley (Indianapolis, IN). Approximately five days after birth, all litters were culled to 6 pups including males and females. On postnatal day 25 (P-25), all animals were weaned and individually housed in plexiglass cages (20x33x13 cm). Within 2 days of weaning, each animal was briefly (a few seconds) observed in the presence of an adult intruder. Individuals that immediately fled from the

adult were considered to be inherently fearful (approximately 1 in 12) and were removed from the experiment. All animals received food and water *ad libitum* and were housed under a reversed light/dark cycle (14L-10 D, lights off at 9:00am).

Experiment 1: The Endocrine Consequences of Repeated Social Stress during Puberty

Experimental Design:

The goals of this experiment were to understand how an external factor such as social stress could alter the endocrine profiles of juvenile male hamsters. The first experiment sought to confirm that social defeat increases cortisol levels in juvenile male hamsters. In the second experiment, the development of cortisol and testosterone levels were compared between repeated subjugated animals and socially naïve controls.

Social Subjugation.

On P-28, animals were separated into two groups (Naive and Subjugated). Social subjugation was performed according to a previously described protocol (Delville et al., 1998; Wommack and Delville, 2002). Daily subjugation started on P-28, which coincides with the onset of puberty (Vomachka and Greenwald, 1979), and ended on P-42, near mid puberty. This period was roughly equivalent to the first half of puberty (Delville et al., 2003). Animals in the subjugated group were placed in the home cage of an aggressive adult for 20 minutes daily while naive animals were placed in a clean and empty cage for the same period. Subjugated juveniles were cycled through a group of aggressive adults (n=8) for each subjugation day. Prior to the experiment, adults were tested for offensive aggression by the resident-intruder paradigm (Miczek, 1979).

Smaller and younger intruders were placed in the home cage of adult hamsters. The adults used in this experiment were trained fighters selected for their aggressiveness. During repeated subjugation, animals were observed daily while in the home cage of an adult to ensure they were chased and attacked. Daily subjugation and behavioral tests were performed during the second half of the dark phase.

Cortisol Levels in Defeated Juvenile Hamters.

The goals of this study were to determine the effects of social stress on cortisol levels following a single defeat or after two weeks of daily subjugation. To confirm that social defeat during puberty activated the HPA axis, cortisol levels were assayed from samples collected under basal conditions or following 20 minutes in the home cage of an aggressive adult or in a clean and empty cage on P-28 (n= 7-9 per group). It is important to note that both a single exposure to a clean and empty cage and social defeat cause a stress response in adult hamsters (Huhman et al., 1991; Weinberg and Wong, 1986). As this was the first day of the experiment, the animals of sacrificed on P-28 had not been assigned to either the subjugated or naïve group. An additional set of animals was used to test the ability of social defeat and exposure to a novel cage to increase cortisol levels after 14 consecutive days of treatment. On P-42 subjugated animals were sacrificed under basal conditions or following 20 minutes in the home cage of an unfamiliar adult, while naïve animals were sacrificed under basal conditions or following 20 minutes in a clean and empty cage n=7-9 per group). Separate baseline samples were collected for the naïve and subjugated groups on P-42 because reports in adult hamsters show that social stress can alter baseline cortisol levels.

The Effects of Repeated Defeat on the Development of Cortisol and Testosterone Levels.

To test endocrine effects of subjugation, subjugated and naïve animals were sacrificed under basal conditions or following defeat in the home cage of larger adults on various days across puberty. The exposure to a larger, older opponent ensured that the animals would be defeated and that their behavioral responses would be purely defensive. At the onset of puberty, on P-28, a group of animals that had not been assigned to either the naive were sacrificed under basal conditions or twenty minutes in the home cage of an aggressive adult. These animals were sacrificed on this day to establish a developmental trajectory. On P-35 (early puberty), P-45 (mid puberty), or P-70 (early adulthood), both naive and subjugated groups were sacrificed under basal conditions or for twenty minutes in the home cage of an aggressive adult. The experimental design is summarized in table (3.1). Importantly, animals in the subjugated group were paired with an unfamiliar opponent to prevent a confound of individual recognition. These test dates (P-28, P-35, P-45, and P-70) were chosen to establish a developmental trajectory and to understand the time course of experimental effects.

Table 3.1: Summary of Experimental Design for Basal and Post-Defeat Cortisol

Subjects	**Test Days**			
	P-28	**P-35**	**P-45**	**P-70**
Control [a]	Baseline [c] Post-Defeat [d]	Baseline Post-Defeat	Baseline Post-Defeat	Baseline Post-Defeat
Subjugated [b]		Baseline Post-Defeat	Baseline Post-Defeat	Baseline Post-Defeat
	Early Puberty [e]		**Mid Puberty** [e]	**Adulthood** [e]

[a] Control animals were placed in a clean and empty cage for 20 minutes a day from postnatal day 28 (P-28) to P-42.
[b] Subjugated animals were placed in the home cage of an aggressive adult for 20 minutes a day from P-28 to P-42.
[c] Animals from both the control and subjugated groups were sacrificed under basal conditions on various days during puberty.
[d] Animals from control and subjugated groups were sacrificed after a 20 minutes in the home cage of an aggressive adult on various developmental days.
[e] The postnatal days corresponding to early puberty, mid puberty, and adulthood were determined by testosterone levels, testes weights, and existing literature (Vomacka and Greenwald, 1979).

Cortisol and Testosterone Assays

Plasma cortisol levels were assayed in naive and experimental animals in samples collected under basal conditions (baseline) or immediately following (post-defeat) social defeat on P-28, P-35, P-45, or P-70. Plasma testosterone levels were only assayed in samples collected under basal conditions across all test days and following defeat on P-45. Subgroups of subjugated and naïve animals were sacrificed by rapid decapitation

upon removal from their home cage or immediately following a 20-minute period of social defeat (n=8-16 per group). After decapitation, trunk blood samples were collected and centrifuged at 5,000 rpm for five minutes.

Plasma was saved at –20 °C. All cortisol assays were performed with Cortisol Correlate-EIA™ kits (Assay Designs, Inc., Ann Arbor, MI). The cross reactivity of the antibody supplied with the assay kit was 4.0% for 11-deoxycortisol, 3.6% for progesterone and less than 1% for all other endogenous steroids. The antibody also had a 27.8% cross reactivity with corticosterone; however, corticosterone levels were below 1ng/ml. Testosterone assays were performed with Testosterone Correlate-EIA™ kits (Assay Designs). The cross reactivity for the testosterone antibody supplied with the assay kit was 7.2% for 4-androsten-3, 17-dione, and less than 1% for all other endogenous steroids. Samples were assayed in duplicate from 10µl aliquots. Intraassay variability was 8.8% and interassay variability was 13.6%. For testosterone, intraassay variability was 5.2%, and interassay variability was 10.0%. Plasma levels of testosterone and cortisol were expressed as ng/ml. In addition, the percent increase over baseline was calculated to assess cortisol responses to social defeat. Each post-defeat cortisol level was divided by the average baseline concentration for respective group (subjugated or control) and postnatal day. For example, an individual cortisol level from a member of the control group defeated on P-45 would be divided by the average baseline cortisol level of the P-45 control group, then multiplied by 100.

Data Analysis

Hormone levels and body weights were compared between groups over time by 2-way ANOVAs (independent variables: treatment groups and age) followed by a Fisher's PLSD post hoc test. Post-defeat cortisol and testosterone levels were compared between subjugated and naïve animals on P-45 by Student's t-test (two-tailed). Additionally, basal (for each test day) and post-defeat (for P-45 only) cortisol and testosterone levels were correlated for naïve and subjugated animals by Pearson's Correlation Coefficient.

Results

Cortisol Levels in Defeated Juvenile Hamters.

On P-28, both exposure to an aggressive adult or clean and empty cage resulted in a three-fold increase from baseline plasma cortisol concentrations [$F(2,22)=4.6$, $p<0.05$] (Fig. 3.1 A). The elevations were statistically significant in isolated animals ($p<0.01$, Fisher's PLSD). Following two weeks of daily subjugation, significant differences were again observed between groups [$F(3,27)=3.7$, $p<0.05$]. Exposure to an aggressive adult resulted in a three-fold increase in plasma cortisol ($p<0.05$). However, individuals exposed to a clean and empty cage on this day showed no increase from the cortisol baseline (Fig 3.1 B). Importantlybaseline cortisol concentrations did not differ between groups on P-42.

The Effects of Repeated Defeat on the Development of Cortisol Levels.

Plasma cortisol concentrations were analyzed as baseline levels, post-defeat levels, and percent increase over baseline (Fig. 3.2). Baseline cortisol levels increased gradually during puberty from P-28 to P-70 in both groups [$F(3,58)=8.1$, $p<0.001$,

ANOVA]. This developmental increase was not significantly affected by daily social subjugation [$F(1,58)=0.6$, $p>0.1$] nor was there a group x day interaction [$F(3,58)=1.0$, $p>0.1$]. Post-defeat cortisol levels also increased during puberty in both groups [$F(3,74)=32.2$, $p<0.001$]. Interestingly, repeated subjugation from P-28 to P-42 resulted in a statistically significant group difference for post-defeat cortisol levels [$F(1,74)=7.4$, $p<0.01$] and a statistically significant group x day interaction [$F(3,74)=4.3$, $p<0.01$]. On P-45, subjugated animals showed post-defeat cortisol levels that were increased 2-fold from baseline while naive animals showed a 6-fold increase from baseline. The group difference in post-defeat cortisol levels was statistically significant on this day [$t(18)=3.25$, $p<0.01$]. A group difference in post-defeat cortisol levels was not observed on any other test day.

Percent increase over baseline also changed significantly during puberty [$F(3,74)=5.8$, $p<0.01$]. Significant interactions for group and group x day were also observed [respectively, $F(1,74)=11.4$, $p<0.01$; $F(3,74)=5.8$, $p<0.01$]. On P-28 and P-35, subjugated and naïve animals each showed approximately a 250% increase from baseline following defeat. Throughout puberty percent over baseline remained around 250% in subjugated animals. In contrast, naïve animals showed nearly a 700% increase on P-45 followed by a 350% increase on P-70. The group differences on P-45 and P-70 were statistically significant [respectively, $t(18)=3.7$, $p<0.01$; $t(24)=4.8$, $p<0.001$].

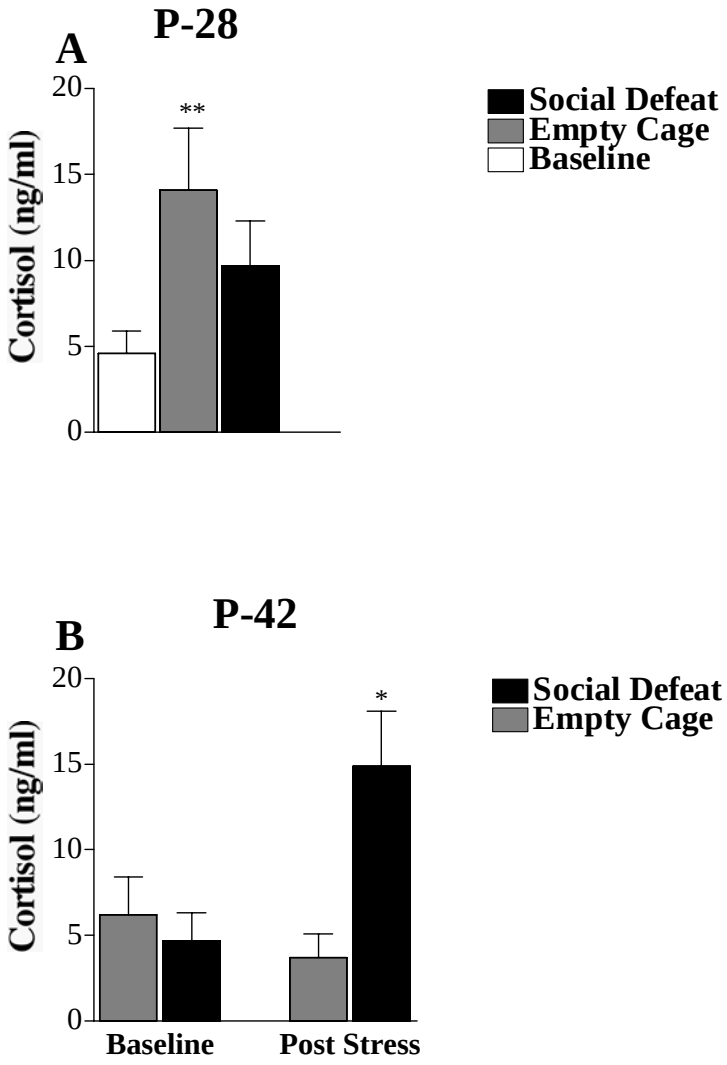
Testosterone and Testes and Body Weights.

During puberty, basal testosterone levels increased gradually in both naive and subjugated hamsters [$F(3,58)=21.5$, $p<0.001$] (Fig. 3.3 A). However, subjugated

juveniles had lower levels of plasma testosterone than naïve animals P-45 [t(14)3.6, $p<0.01$]. This effect did not last. On P-70, plasma testosterone levels did not differ significantly between previously subjugated and socially naive animals [t(14)1.1, $p>0.1$]. In addition, testes and body weights were also recorded and compared between groups in this study. Testes grew from 0.51 ± 0.1g on P-28 to 4.0 ± 0.2g and 3.8 ± 0.4g (in naive and subjugated animals, respectively) on P-70 (Fig 3.3 B). During that time, the animals grew from 49.0 ± 2.8g on P-28 to 109.5 ± 11.1g and 104.2 ± 11.9g (in naive and subjugated animals, respectively) on P-70 (Fig 3.3C). Neither measure was statistically different between groups at any point during the experiment.

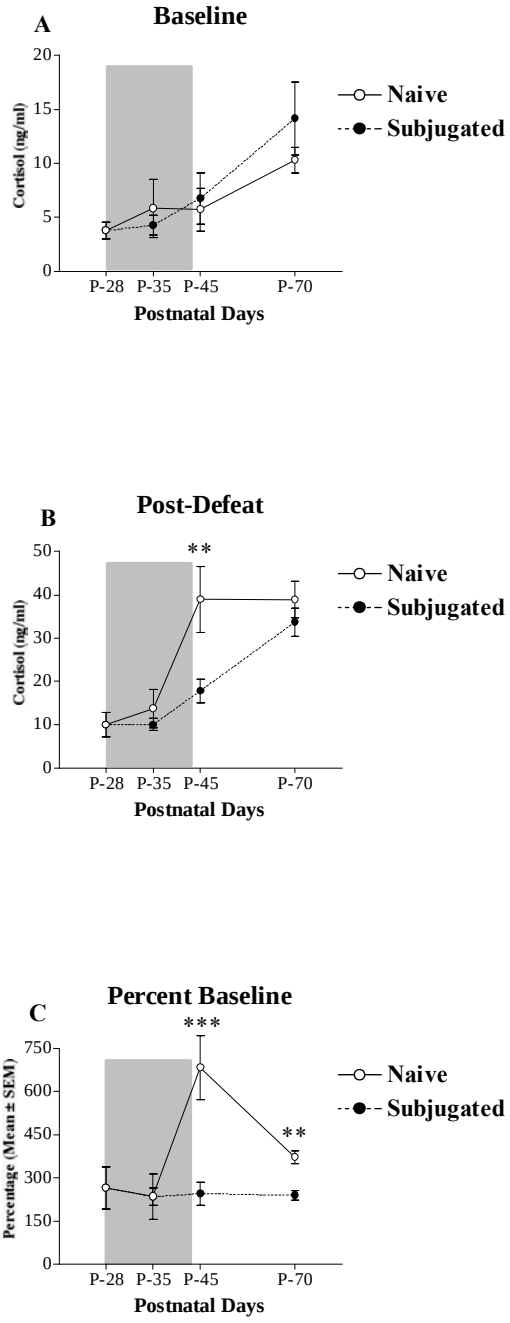
No group differences were observed for post-defeat testosterone levels on P-45 [t(16)=0.1, $p>0.1$] (Fig. 3.4). However, post-defeat testosterone was nearly two-fold lower than baseline in naïve animals, but this difference was not statistically significant [t(15)2.0, $p=0.058$]. Additionally, no significant correlations between basal cortisol and testosterone levels were observed on any test day for both the subjugated and naïve groups (data summarized in table 3.2). Basal cortisol and testosterone were negatively correlated on P-45 in naïve animals (r –0.655), but this correlation was not statistically significant ($p<0.07$). Additionally, no significant correlations were found for post-defeat cortisol and testosterone levels on P-45 for either group.

Figure 3.1: Cortisol Levels following Social Defeat or Exposure Clean and Empty Cage.



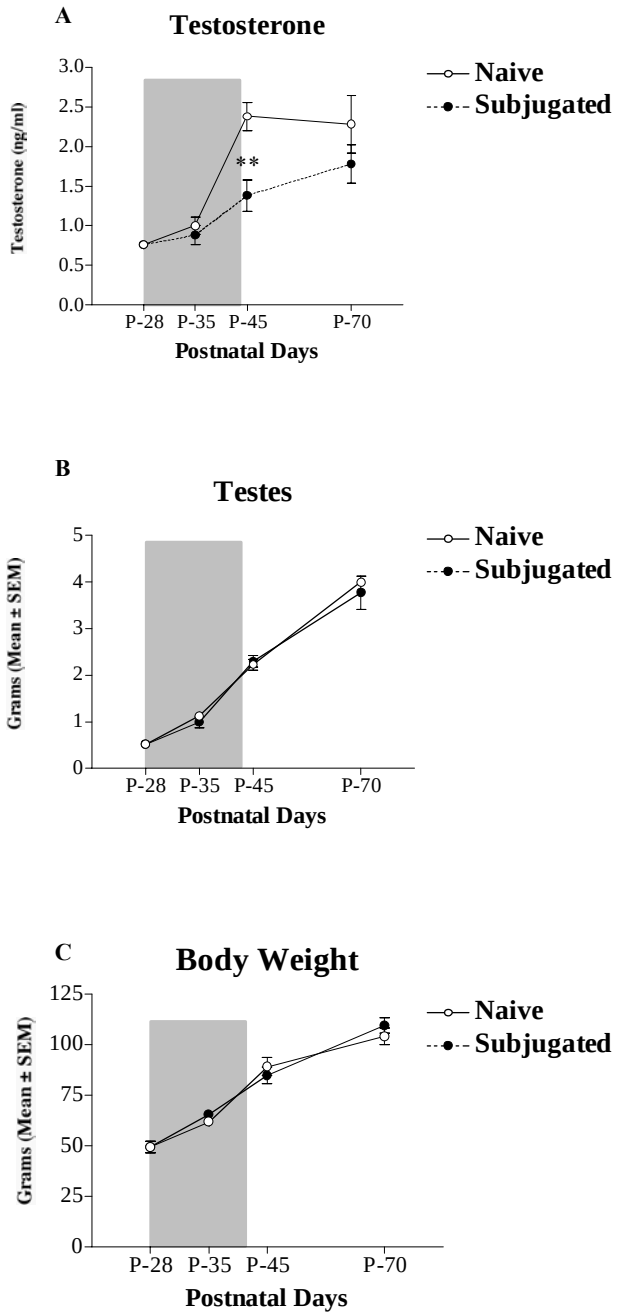
Cortisol levels were assayed in samples collected on under basal conditions (Baseline) or immediately following 20 minutes in the home cage of an aggressive adult (Social Defeat) or a clean and empty cage (Empty Cage) for the first time on postnatal day 28 (P-28) (A) or after 2 weeks of repeated exposure to each condition on P-42 (B). ANOVA followed by Fisher's PLSD, *$p<0.05$, **$p<0.01$.

Figure 3.2: Pubertal Changes in Basal and Post-Defeat Cortisol Levels in Subjugated and Naïve Animals



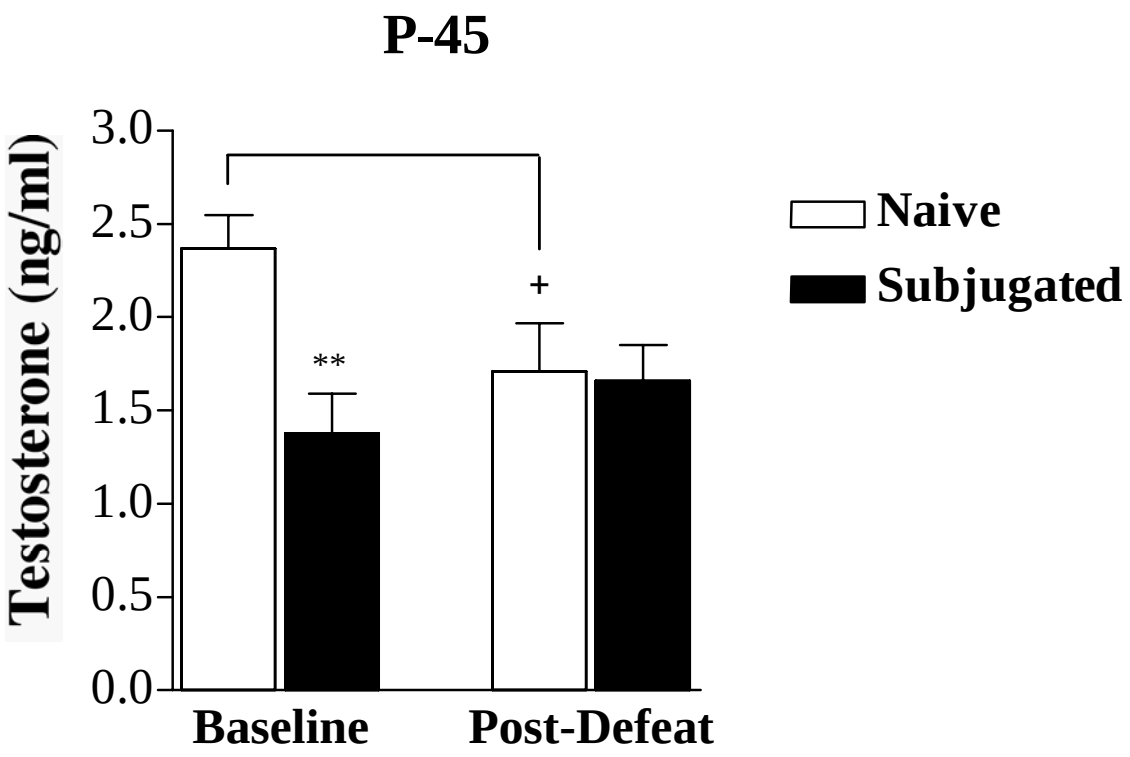
Cortisol levels assayed in subjugated and naive animals under basal conditions (A) following defeat (B) and expressed as percent increase over baseline (C). Shaded area represents the period of daily subjugation. Student's t-test (two-tailed), *p<0.05, **p<0.01, ***P<0.001.

Figure 3.3: Pubertal Changes in Testosterone Levels, Testes Weights, and Body Weights in Subjugated and Naïve Animals



Testosterone levels (A), testes weights (B), and body weights (C) of subjugated and naïve animals across puberty. Testosterone levels were assayed from samples collected under basal conditions. Shaded area represents the period of daily subjugation. Shaded area represents period of daily subjugation. (Student's t-test (two-tailed) **$p<0.01$).

Figure 3.4: Post-Defeat Testosterone Levels



Testosterone levels were assayed in samples collected from subjugated animals under basal conditions (Baseline) or immediately following 20 minutes in the home cage of an aggressive adult (Post-Defeat) on P-45. Student's t-test (two-tailed) + $p<0.06$, ** $p<0.01$.

Table 3.2: Correlations Between Cortisol and Testosterone

	P-28	**P-35**	**P-45**	**P-70**
Naive	-0.142	-0.456	-0.655	-0.422
	p>0.1	p>0.1	p>0.07	p>0.1
Subjugated		-0.546	-0.411	-0.266
		p>0.1	p>0.1	p>0.1

Baseline cortisol and testosterone levels were correlated within groups for each developmental day. Correlations are expressed by Pearson's Correlation Coefficient (*r*).

Experiment 2: Pubertal Development of the HPA Axis

Experimental Design

The following experiments were conducted to confirm that HPA activity increases during puberty in male golden hamsters. In experiment 1, post-defeat cortisol levels increased during puberty. However, this may have resulted differences in subjugation intensity, as older animals receive more attacks than younger animals (data from chapter 2). Therefore, the first goal was to investigate stress responsiveness following a standardized stressor (restraint) on developmental days corresponding to early puberty (P-28), mid puberty (P-45) and adulthood (P-70). These dates were chosen as studies in rats have shown that corticosterone and ACTH levels in responses to restraint stress differ between early puberty, late puberty and adulthood (Gomez et al., 2002; Romeo et al., 2004). Secondly, this experiment compared the density of CRH immunoreactive (CRH-ir) fibers projecting to the median eminence between male hamsters during early puberty, mid puberty, and adulthood.

Cortisol Assays

Plasma cortisol levels were assayed in samples collected immediately following restraint on P-28, P-45, or P-70. Animals were sacrificed by rapid decapitation immediately following a 20-minute period restraint stress (n=7-8/day). Trunk blood samples were collected and centrifuged at 5,000 rpm for five minutes. Sera were saved at –20 °C until assayed using Cortisol Correlate-EIA™ kits (Assay Designs, Inc., Ann Arbor, MI). Samples were assayed in duplicate from 10µl aliquots. Intraassay variability was 5.2%. Interassay variability was not calculated as all samples were used in a single assay. Plasma levels of cortisol were expressed as ng/ml. The cross reactivity of the antibody supplied with the assay kit was 4.0% for 11-deoxycortisol, 3.6% for progesterone, and less than 1% for all other endogenous steroids. The antibody also had a 27.8% cross reactivity with corticosterone; however, corticosterone levels were below 1ng/ml.

CRH Immunocytochemistry

Male golden hamsters were sacrificed by rapid decapitation on P-28, P-45, or P-70. Following sacrifice, brains were collected and fixed by overnight immersion in 10% acrolein in 0.1M KPBS buffer (pH 7.2) at 4°C and later saved in 20% sucrose/KPBS. The brains were then sectioned into 40 µm-thick coronal sections with a freezing rotatory microtome and were stored in a cryoprotectant (Watson et al., 1986) at -20°C until labeled by immunocytochemistry using a previously described protocol (Delville et al., 1992). Briefly, free-floating sections were pretreated in 1% sodium borohydrite (to remove residual aldehydes) followed by a preincubation in a solution containing 20% normal goat serum, 1% hydrogen peroxide, and 0.3% Triton X-100 (respectively, to

block nonspecific labeling, eliminate endogenous peroxidase activity, and permeabilize the tissue). Sections were then incubated in a rabbit polyclonal antibody to Human/Rat CRH (1:6,000; Peninsula Laboratories, Inc., San Carlos, CA), containing 2% normal goat serum and 0.3% Triton X-100 for 48 hours at 4°C. After washing, the sections were incubated in the secondary antibody (biotinylated goat anti-rabbit IgG; 7.5µg/ml; Jackson Immunoresearch Laboratories, West Grove, PA) followed by a tertiary incubation (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA). Between incubations, sections were washed in 0.05M TBS (pH 7.6). Finally, the sections were labeled with with diaminobenzidine (DAB, 0.5mg/ml) and 0.05% hydrogen peroxide. Labeled sections were mounted on gel-coated slides, dehydrated in a series of alcohols, and coverslipped with permount.

The density of CRH immunoreactive fibers projecting into the median eminence (ME) was quantified using NIH Image Software (v. 1.62, NIH, Bethesda, MD). Specifically, CRH fibers passing into the ME were observed in the posterior hypothalamus near the arcuate nucleus en route to the median eminence (Morin and Wood, 2001). This area has been previously described in a study focusing on AVP innervation (Delville et al., 1998). Brain sections containing the posterior hypothalamus were observed through a 20X objective as digitized images recorded by a Cohu CCD camera mounted on a Nikon Microscope and imported to a Macintosh computer using a frame grabber (LG3, Scion Corporation, Frederick, MD). The density of CRH-ir fibers was expressed as size of the area covered by immunoreactive fibers within a standard sample placed over matching zones of the posterior hypothalamus. The sample (a 60 µm

diameter circle) was placed over the digitized area. Measurements were taken after normalizing for background and foreground differences (Delville et al., 1998). Several measurements (n=7) were taken from each side of the brain in consecutive sections and averaged for each individual.

Data Analysis

Post-restraint cortisol levels and CRH fiber densities were compared between age groups by ANOVA, followed by Fisher's PLSD post hoc tests.

Results

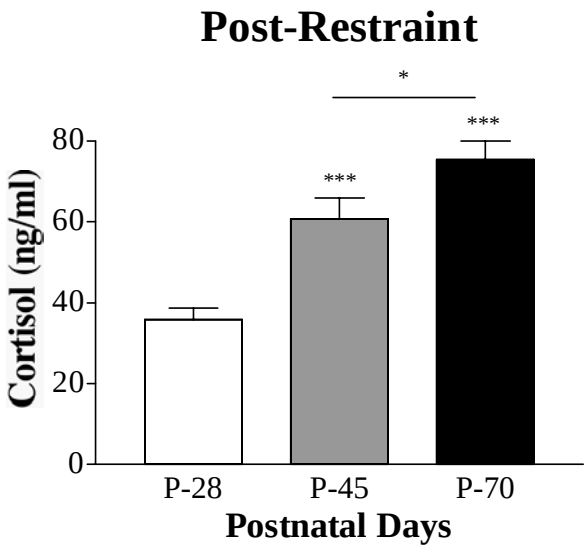
Post-Restraint Cortisol Levels

Stress responsiveness to a standardized stressor (restraint) increased across puberty. Post-restraint cortisol levels increased by approximately two-fold from P-28 to P-70 [$F(2,19)=20.0$, $p<0.001$, ANOVA] (Fig. 3.5). On P-45 and P-70 cortisol levels were higher than on P-28 ($p<0.01$ and $p<0.001$ respectively, Fisher's PLSD). Cortisol levels collected on P-70 were also higher than P-45 levels ($p<0.05$).

CRH Fiber Density

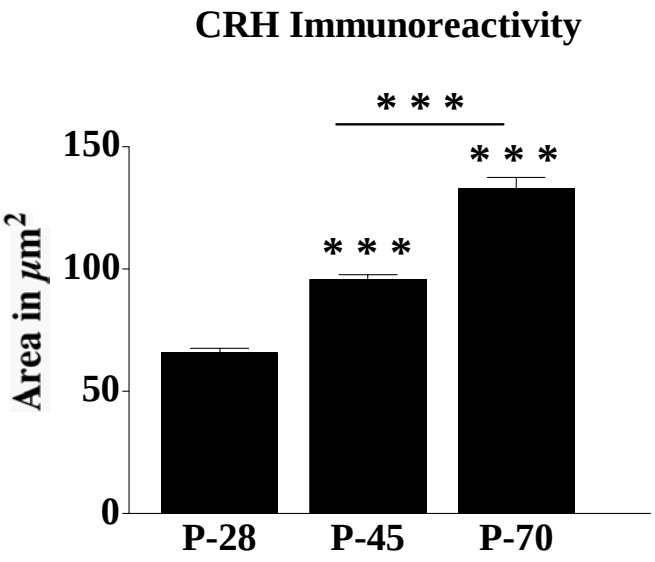
CRH innervation of the median eminence increased during puberty. The density of CRH fibers projecting into the median eminence increased by two-fold from P-28 to P-70 [$F(2,21)=106.5$, $p<0.001$] (Fig. 3.6). Compared to P-28, the density of CRH fibers projecting to the ME was higher on P-45 and P-70 ($P<0.001$, for both). The density of fibers also increased from P-45 to P-70 ($p<0.001$).

Figure 3.5: Post-Restraint Cortisol Levels



Plasma cortisol levels were assayed immediately following 20 minutes of restraint stress on postnatal day 28 (P-28), P-45, and P-70. ANOVA followed by Fisher's PLSD, * $p<0.05$, *** $p<0.001$.

Figure 3.6: CRH Immunoreactivity at the Median Eminence



The density of CRH immunoreactive fibers projecting into the median eminence of male golden hamsters sacrificed on postnatal day 28 (P-28), P-45, or P-70.

Discussion

The current studies examined the endocrine consequences of repeated social subjugation during early puberty and the development of the HPA axis. First, social subjugation is a potent activator of the HPA axis, as subjugated animals showed increases from baseline in cortisol levels even following two weeks of daily defeat. When compared to naïve controls, however, subjugated animals showed blunted stress responses following defeat, suggesting a partial habituation to repeated social stress. Nevertheless, both subjugated and naïve animals showed increases in basal and post-stress cortisol levels across puberty, suggesting a developmental increase in HPA

activity. Additional studies that used restraint as a standardized stressor confirmed that cortisol responses increased across puberty. Moreover, increases in CRH innervation of the median eminence suggest that neural components of the HPA axis also change during puberty. Importantly, subjugated animals showed decreased testosterone levels following two weeks of daily stress, indicating that subjugation negatively affects the HPG axis. In all, the current studies show that the development of offensive responses is likely mediated through stress hormones and not testosterone.

Data from the first studies confirmed that social defeat during puberty causes a daily increase in plasma cortisol levels. The initial exposure to either a social or non-social challenge was a stressful experience for juvenile hamsters. After the first exposure (P-28) to either a novel cage or an aggressive adult, juveniles had elevated plasma cortisol levels. The cortisol elevations observed in juveniles following a single exposure to a novel environment or social stress is consistent with previous findings in adult hamsters (Weinberg and Wong, 1986; Huhman et al., 1991). After two weeks, though, individuals exposed to a clean and empty cage no longer had increased plasma cortisol levels. Only subjugated individuals maintained the three-fold increase from baseline as observed after the first exposure. Thus, the primary difference between the stressors is the ability of juvenile hamsters to habituate to repeated isolation but not repeated social subjugation. Additionally, no differences in baseline cortisol levels were observed between groups following two weeks of subjugation. These findings contrast with the cortisol elevations observed in chronically subjugated adult hamsters (Huhman et al., 1992). The cause of this difference is unclear at this time. Overall, the phase of the study

showed that social defeat causes a daily increase in plasma cortisol levels. Data from the second phase of the study, however, showed that juvenile hamsters are capable of adapting to repeated social defeat. As compared to naïve animals, subjugated juveniles had blunted cortisol responses to defeat on P-45. It is important to note, that while this change suggests habituation, the post-defeat cortisol levels of subjugated juveniles were still approximately three-fold higher than baseline.

Data from the current study suggest a developmental increase in activity of the HPA axis. Both baseline and post-defeat cortisol levels increased during puberty in control animals, with post-defeat levels peaking at around P-45 and remaining steady until P-70. For both subjugated and naïve animals, plasma cortisol levels, both before and after stress, increased by three-fold from P-28 to P-70. The data on baseline cortisol levels are consistent with reports in tree shrews (Van Kampen and Fuchs, 1998) and human adolescents (Elmlinger et al., 2002; Jonetz-Mentzel et al., 1993; Kiess, et al., 1995). The development of the HPA was not permanently altered by repeated defeat. Regardless of the blunted stress responses observed in subjugated animals on P-45, their post-defeat cortisol levels were similar to naïve animals on P-70 (early adulthood). Subjugated and naïve animals showed similar basal and post-defeat cortisol levels after this four-week recover period, on P-70. As such, the observed increases in stress hormones suggest that puberty in male golden hamsters is not just limited to an activation of the HPG axis, but also includes an activation of the HPA axis. Additionally, as HPA activity increases coincide with the transition from play fighting to adult aggression, it is

possible that this neuroendocrine system controls the development of offensive responses.

HPA activity seems to change most dramatically during mid puberty. The present data showed enhanced cortisol response to defeat on P-45 in naive animals, as compared to the other days. On P-45, naïve animals showed increases over baseline much higher than on any other day. These observations suggest that the major developmental changes of the HPA axis occur around mid puberty. The physiological mechanisms mediating enhanced stress responsiveness are still unclear. In rats, mid puberty is also associated with enhanced ACTH responsiveness and prolonged corticosterone release (Gomez et al., 2002; Romeo et al., 2004; Vazquez, 1998). The timing of these changes in stress responsiveness corresponds with the central point of the transition from play fighting to adult aggression. During mid puberty, neither play-fighting responses nor adult attacks are the predominant form of aggression (Wommack et al., 2003). This relationship further emphasizes the possibility that the development of the HPA axis influences the development of offensive responses.

An additional experiment confirmed that changes in post-defeat cortisol levels were likely due to developmental differences in HPA activity and not increasing intensity of social defeat. From early puberty to mid puberty and adulthood, cortisol levels increased following restraint, a standardized stressor that does not change in intensity during development. The previous experiment in this chapter showed that post-defeat cortisol levels also increase during puberty, but it was unclear whether increasing stress responsiveness was due to the increased number of attacks that males received across

puberty (data from chapter 2). The new data show that increases in post-stress cortisol levels are not specific to social defeat.

The pubertal increase in stress responsiveness may explain the age-dependent consequences of social stress in male golden hamsters. Social defeat during early puberty results in an accelerated development of agonistic behaviors (Wommack et al., 2003). In contrast, socially defeated adults show a long lasting inhibition of offensive aggression known as conditioned defeat (Huhman et al., 2003; Potegal et al., 1993). These differing consequences may be explained by increasing cortisol levels. If true, the relationship between stress hormones and the outcomes of stressful experiences could be extended to other species showing developmental changes in HPA activity such as humans (Elmlinger et al. 2002; Jonetz-Mentzel et al., 1993; Kiess et al., 1995). Indeed, preliminary studies in adolescent humans show that the timing of social stress during puberty dramatically influences the consequences.

Pubertal changes in HPA activity in golden hamsters are not limited to cortisol levels. Analysis of CRH immunocytochemistry showed that the density of CRH immunoreactive fibers projecting into the ME also increased by two-fold from early puberty to adulthood. It is possible that enhanced CRH immunoreactivity is a result of increased CRH mRNA. It is also likely that increased CRH immunoreactivity is related to increased ACTH release from the anterior pituitary golden hamsters. These issues will have to be addressed in future studies. Nonetheless, these data suggest that multiple elements of the HPA axis increase during puberty in male golden hamsters.

In contrast to the activation of the HPA axis, social defeat decreased testosterone levels. Socially subjugated animals showed a transient decrease in plasma testosterone levels, while their testes weights did not differ from naïve animals. As expected, testosterone levels increased from P-28 to P-70 in subjugated and naive animals. These developmental increases correspond with previous studies on puberty in male golden hamsters (Vomachka and Greenwald, 1979). However, repeatedly subjugated juveniles showed lower testosterone levels than controls on P-45, but this difference did not last until P-70. While the reduction is only transient, it is important to note that similar decreases in plasma testosterone have been reported in adult males exposed to stress (Huhman et al., 1991). These data are also consistent with social stress in other species, including rodents (Blanchard et al., 2002) and cichlid fish (Francis et al., 1993). Additionally, adult human males exposed to chronic social stress display a phenomenon known as psychological castration.

Interestingly, post-defeat testosterone levels did not differ between groups on P-45. Testosterone levels remained unchanged in the subjugated group, but were lower in naïve animals. While the post-stress testosterone was only slightly reduced in subjugated animals on P-45, the decreased levels are consistent with the effects of social stress in adults (Blanchard et al., 2002; Huhman et al., 1992). The difference potentially results from the dramatic increase in cortisol levels in naïve animals. These age-related differences suggest that the sensitivity to stress differs between juveniles and adults.

The decreased testosterone levels in subjugated animals are consistent with previous studies that androgens do not control the transition from play fighting to adult

aggression (Beatty et al., 1981; Pellis, 2002; Romeo et al., 2003; Smith et al., 1996). Subjugated animals show decreased play fighting and increased adult attacks, yet they have decreased testosterone levels (Wommack et al., 2003). These findings clarify the results from the Romeo study that observed the offensive responses of castrated juvenile hamsters. The main concerns with this study were based on the methods of behavioral analysis and the duration between gonadectomy and aggression testing. Specifically, the Romeo study measured bite location instead of attack target. As bite locations can be altered by the defense of the intruder, this behavioral measure is flawed. Additionally, castrated hamsters were tested in this experiment only a week after castration. However, studies in castrated adults show that the downstream effects of testosterone may still be present weeks after surgery (DeVries et al., 1985; Miller et al., 1992). This latter concern may not be as important, as circulating testosterone levels are very low during the time when the animals were castrated. Regardless of methodological concerns, the current study confirmed the main findings of the Romeo paper: testosterone is unlikely to control the transition from play fighting to adult aggression. This does not mean that testosterone does not affect aggression at all during this time, nor does it rule out a developmental role for testosterone. More careful studies could be performed to determine the role of testosterone during puberty on other aspects of aggression, such as motivation to attack.

In summary, HPA activity increases during puberty and social stress causes daily increase in plasma cortisol levels. Regardless of prior experience, social defeat increased cortisol levels. The daily elevation from baseline cortisol levels suggests that social

defeat is an experience that likely results in daily activations of the HPA axis. Importantly, the current study showed HPA activity increased over the course of puberty, as both basal and post-defeat cortisol levels increased during puberty. While testosterone levels increased in both groups over puberty, subjugated animals showed lower testosterone levels around mid puberty. This decrease shows that social defeat inhibits HPG activity. Moreover, as both increases HPG and HPA activity coincides with the transition from play fighting to adult aggression, the effects of social stress on the development of agonistic behaviors are likely mediated through stress hormones rather than gonadal steroids. This possibility will be tested in chapter 4.

Chapter 4: Glucocorticoids and the Pubertal Development of Offensive Responses in Male Golden Hamsters

Introduction

In most mammalian species, the transition from play fighting to adult aggression occurs during puberty (Fagen, 1981). In male golden hamsters, for example, play fighting attacks gradually decline during puberty while adult attacks increase during this time (Pellis and Pellis, 1988; Taravosh-Lahn and Delville, 2004; Wommack et al., 2003). Play-fighting attacks can be differentiated from adult attacks by the area of an opponent first targeted by the aggressor. For example, play-fighting attacks are directed towards the face and cheeks of an opponent, while adult attacks are focused on the belly/rear (Wommack et al., 2003). The neuroendocrine mechanisms guiding this behavioral transition are not clearly understood.

Contrary to what one would expect, the pubertal development of male aggression is not controlled by increasing gonadal steroid levels. While testosterone facilitates aggression in adult male rodents, the relationship between pubertal changes in androgen levels and agonistic behavior is not as clear (Albers et al., 2002; Simon et al., 2002). For example, hamsters and rats engage in play fighting prior to the pubertal increases of testosterone levels, suggesting that the onset of agonistic behavior is independent of gonadal steroids (Goldman and Swanson, 1975; Pellis et al., 1993; Schoenfield and Leonard, 1985). Additionally, increasing testosterone levels do not appear to be associated with the transition from play fighting to adult aggression. While significant increases in plasma testosterone levels coincide with the transition from play fighting to adult aggression in male golden hamsters, preweaning castration does not affect this

behavioral shift (Pellis and Pellis, 1988a; Romeo et al., 2003; Vomachka and Greenwald, 1979; Wommack et al., 2003). Additionally, data from chapter 3 show that subjugated animals have lower testosterone level than naïve animals. As subjugation accelerates the transition from play fighting to adult aggression, these data further indicate that testosterone is not involved in the pubertal maturation of offensive responses. The failure to establish a link between the HPG axis and the maturation of offensive responses suggests that this behavioral transition is controlled by a separate neuroendocrine axis.

Indeed, the HPG axis is not the only neuroendocrine system that undergoes significant development during puberty. There is mounting evidence that hypothalamic-pituitary-adrenal (HPA) axis changes considerably during puberty, although the nature of these changes varies between species. For example, pre-pubertal male rats show prolonged adrenocorticotropin releasing hormone (ACTH) release and delayed corticosterone responses (Gomez et al., 2002; Romeo et al., 2002; Vazquez, 1998). In this species, baseline HPA activity does not change during puberty (Gomez et al., 2002; Romeo et al., 2002; Vazquez, 1998). In contrast, baseline glucocorticoid levels increase gradually throughout puberty in other species including tree shrews (Van Kampen and Fuchs, 1998) and humans (Elmilinger et al., 2002; Jonetz-Mentzel et al., 1993; Kiess et al., 1995). As chapter 3 showed, both baseline and post-stress cortisol levels increase during puberty in male golden hamsters.

Could changes in stress hormone levels influence the development of agonistic behavior? Studies on social stress in male golden hamsters suggest that glucocorticoids do indeed influence behavior development. Social subjugation is a potent stressor that

produces numerous behavioral and neuroendocrine effects (Blanchard et al., 2002). In male golden hamsters, repeated social stress produces unique and context-specific enhancement in the development of agonistic behavior (Delville et al., 1998; Wommack et al., 2003). Male hamsters socially subjugated during early puberty show an accelerated transition of offensive responses from play fighting to adult aggression when animals are paired with a smaller and younger intruder (Wommack et al., 2003). Dat for chapter 3 suggest that social subjugation causes a daily increase in plasma cortisol levels and is a stressor to which juveniles are incapable of habituating. As puberty is a time of increasing stress responsiveness in hamsters, it is possible that the daily activation of corticosteroid receptors is a mechanism that drives the development of offensive aggression.

The goals of the current study were to investigate the effects of glucocorticoids on the development of offensive responses.

Methods

Animals and Treatment

The animals (male golden hamsters) were obtained from a breeding colony housed within the laboratory derived from Harlan Sprague Dawley (Indianapolis, IN). Approximately a week after birth, all litters were culled to 6 pups including males and females. The males were weaned on postnatal day 25 (P-25) and individually housed in Plexiglas cages (20x33x13 cm). Within 2 days, each animal was briefly (a few seconds) observed in the presence of an adult intruder. Individuals that immediately fled from the adult were considered to be inherently fearful (approximately 1 in 12) and were removed

from the experiment. All animals received food and water *ad libitum* and were housed under a reversed light/dark cycle (14L-10 D, lights off at 9:00am).

Experimental Design

The hypothesis that cortisol positively influences the development of offensive responses was tested through a series of experiments. First, a pilot study using repeated cortisol injections was performed to determine whether this hormone was capable of accelerating the transition from play fighting to adult aggression. Once I found a sufficient dose of cortisol for accelerating behavioral development, I designed additional studies to determine the role of type II corticosteroid receptors. In the first of these studies, animals received repeated injections of the dose of cortisol sufficient to accelerate behavioral development combinded with RU-486, a type II receptor antagonist. In an additional experiment, animals were repeatedly injected with Dexamethasone (Dex), a type II receptor agonist. In this experiment, it was hypothesized that type II activation would affect behavior similarly to cortisol. To confirm that Dex activated type II receptors within the brain, immunocytochemistry was performed on animals sacrificed 4 hours after an injection of Dex, cortisol, or vehicle.

Cortisol and Offensive Responses

To test the effects of cortisol on the development of offensive responses, male golden hamsters were repeatedly injected with cortisol during early puberty. On P-31 male golden hamsters were weighed and screened for offensive responses using a resident-intruder paradigm (Miczek, 1979). Smaller (10-20%) and younger animals were

placed in the home cage of experimental animals for 10 minutes. The size difference between the resident and intruder favors offensive responses (Delville et al., 2003). After confirmation that each resident readily performed offensive aggression, animals were separated into experimental groups balanced for litter and body weight. Animals received daily injections of cortisol in propylene glycol (0, 10, or 40 μg/100g; n=6-7 per dose) from P-31 to P-36. On P-37, the offensive responses of experimental animals were observed in the presence of an unfamiliar and smaller intruder. Offensive responses such as attacks and pins (See below for Detailed Description) were video recorded for later review. This time corresponds to the first week of puberty in golden hamsters (Vomachka and Greenwald, 1979). Body weights were recorded prior to testing.

RU-486/Cortisol and Offensive Responses

To test the involvement of type II corticosteroid receptors in the development of offensive responses, a second experiment was performed using a combination of cortisol and RU-486. In golden hamsters, RU-486 is a corticosteroid type II receptor antagonist and not a progestin receptor antagonist (Gray and Leavitt, 1987). To test the possible effects of each substance on offensive responses, a total of four groups were included in this study: Vehicle/Vehicle, Vehicle/Cortisol, RU-486/Vehicle, and RU-486/Cortisol. A similar paradigm as the previous cortisol experiment was used. On P-31 male golden hamsters were weighed and screened for offensive responses using a resident-intruder paradigm (Miczek, 1979). Smaller (10-20%) younger animals were placed in the home cage of experimental animals for 10 minutes. The size difference between the resident

and intruder favors offensive responses (Delville et al., 2003). After confirmation that each resident readily performed offensive aggression, animals were separated into experimental groups balanced for litter and body weight. From P-31 to P-41, animal received an injection of 0 or 1.0 mg RU-486 in 0.3ml sesame oil. Similar dose of RU-486 have been used to block corticosteroid type II receptor activation in prairie voles (Curtis and Wang, 2005). One hour later, animals received daily injections of cortisol in propylene glycol (0, 10μg/100g; n=9-10 per dose). Body weights were recorded prior to testing. As stated above, animals were tested for offensive responses in the presence of an unfamiliar and smaller intruder for 10 minutes on P-37 and P-42. Offensive responses such as attacks and pins (See below for Detailed Description) were video recorded for later review. Body weights were recorded prior to behavioral testing. Additionally, locomotor activity was recorded the days following tests for offensive responses, on P-38 and P-43 (See below for Detailed Description).

Dexamethasone and Offensive Responses

The effects of daily treatment with dexamethasone (Dex) during early puberty on the development of agonistic behavior were examined using a similar paradigm as the cortisol experiments. On P-31, male golden hamsters were weighed and screened for offensive responses using a resident-intruder paradigm (Miczek, 1979). Smaller (10-20%) and younger animals were placed in the home cage of experimental animals for 10 minutes. The size difference between the resident and intruder favors offensive responses (Delville et al., 2003). After confirmation that each resident readily performed

offensive aggression, animals were separated into experimental groups balanced for litter and body weight. Animals received daily injections of Dex in propylene glycol (0, 10, or 40 μg/100g; n=9-10 per dose) from P-31 to P-37. These doses of Dex are within the range used in rats in learned helplessness studies (O'Connor et al., 2003). Dex was chosen as a corticosteroid receptor agonist due to its long half-life and its ability to selectively activate the low affinity, type II corticosteroid receptors in hamsters (Sutanto and De Kloet, 1987). On P-37, 24 hours following the final Dex injection, the offensive responses of all animals were observed in the presence of an unfamiliar and smaller intruder for ten minutes. Offensive responses such as attacks and pins (See below for Detailed Description) were video recorded for later review. Body weights were recorded prior to testing.

Offensive Responses

On P-37 (and on P-42 for the RU-486/cortisol experiment), 24 hours following drug treatment, animals were tested for offensive aggression using a resident intruder paradigm (Miczek, 1979). Smaller (10-20%) and younger intruders were placed into the home cages of experimental animals for 10 minutes. Behaviors were recorded with a Sony video camera and later analyzed using iMovie software by an observer blind to treatment. Several behavioral responses were recorded as previously described (Wommack et al., 2003). Attacks were recorded when the resident approached and attempted to bite the intruder. Attack latency was recorded as the amount of time that transpired between the initial pairing of the resident and the intruder and the first attack.

Attacks were separated into four distinct categories based on the region of the intruder initially targeted by the resident during an attack. In play fighting attacks (PF Att), the resident first targeted the face and/or cheeks of the intruder. Side attacks were recorded when the resident targeted the flanks and/or the dorsal midsection of the intruder, and adult attacks (Ad Att) were recorded when the resident target the belly and/or rear of the intruder. A fourth attack type, classified as a walk-in attack, was recorded when the resident attacked the intruder while it was lying on it's back or righting itself from a previous attack. The percentage of attacks that were PF Att or Ad Att was calculated for each individual by dividing the number each subcategory by the total number of attacks. Due to the ambiguous nature of the region of the intruder targeted during walk-ins, this attack type was excluded from calculations of attack percentages. Pins were recorded when the resident forced the intruder into the supine position during an attack. Contact time was recorded as the number of seconds the resident initiated and maintained contact with the intruder.

Locomotor Activity

To assess whether the effects of RU-486/cortisol were specific to offensive responses, animals from each of the four groups in the RU-486/cortisol experiment were tested for locomotor activity. On P-38 and P-43 (the day after tests for offensive aggression), animals from each group were observed in a lat-maze for a 10-minute period. The lat-maze is commonly used to assay general activity (Greisbach and Amsel, 1998; Lipp et al., 1987). The name for the lat-maze originates from a sacred stone cube

worshiped by pre-Islamic Arabs (Al-Lat). The apparatus consists of an open square box (63x63x21.5 cm). In the center is placed a smaller, closed end box (39x39x17.5 cm). The space between the smaller box and the larger box forms a corridor, with lines marked every 12 cm. Animals were placed in a corner of the maze and their behaviors were recorded. Specifically, the number of lines crossed and corners turned were measured to assess general locomotor activity. Additionally, the number of times an animal climbed the wall in an attempt to escape was recorded. This behavioral measure in the lat-maze has been previously analyzed as a behavioral index of anxiety (Cervantes et al., 2004).

The Effects of Cortisol and Dex Treatment on GR Immunocytochemistry

To test if Dex activated corticosteroid receptors within the brain, female hamsters were injected in their home cage with either Dex (40 μg/100g), cortisol (40 μg/100g) or vehicle (propylene glycol) on P-26 or P-27. At this age, female hamsters have very low circulating cortisol levels, around 1 ng/ml (Taravosh-Lahn and Delville, 2004) and, therefore, should have low basal levels of corticosteroid type II receptor activation. Four hours after injection, animals were anesthetized with nembutal (35 mg/kg) then with 4% paraformaldehyde/KPBS. The brains were then sectioned into 40 μm-thick coronal sections with a freezing rotatory microtome and were stored in a cryoprotectant (Watson et al., 1986) at -20°C until labeled by immunocytochemistry. Briefly, free-floating sections were pretreated in 1% sodium borohydrite (to remove residual aldehydes) followed by a preincubation in a solution containing 20% normal goat serum, 1% hydrogen peroxide, and 0.3% Triton X-100 (respectively, to block nonspecific labeling,

eliminate endogenous peroxidase activity, and permeabilize the tissue). Sections were then incubated with a rabbit polyclonal antibody against the carboxy terminus of human corticosteroid type II-α receptor (1.5µg/ml Cat # SC-1002, Santa Cruz Biotechnology, Santa Cruz, CA), in a solution containing 2% normal goat serum and 0.3% Triton X-100 for 48 hours at 4°C. After washing, the sections were incubated with a secondary goat antirabbit IgG (7.5µg/ml, Jackson Immunoresearch Laboratories) followed by a tertiary incubation (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA). Between incubations, sections were washed in 0.1 M PBS (pH 7.4). Finally, the sections were reacted with with diaminobenzidine (DAB, 0.5mg/ml) and 0.05% hydrogen peroxide. Labeled sections were mounted on gel-coated slides, dehydrated in a series of alcohols, and coverslipped with permount. Neurons containing corticosteroid type II receptor immunoreactivity were counted within the paraventricular hypothalamic nucleus (PVN), specifically the anterior parvicellular subdivision of the PVN (PaPA) (Morin and Wood, 2001). In the same brains, receptor immunoreactivity was also quantified within the rostral portion of the dentate gyrus (DG). Four brains were used for the cortisol and vehicle groups, while three brains were used for the Dex group. The analysis of immumocytochemical labeling was performed by an observer blind to treatment groups. Cells were counted using a 200 µm x 200 µm grid using a camera lucida attachment under 40X magnification. Once counted, neurons were subdivided into two groups: cells with clear nuclear and cytoplasmic immunoreactivity and cells with only cytoplasmic immunoreactivity. A cell was defined as nuclear labeled if there was a visibly dense and round compartment within the center of the cell. Cells without nuclear labeling often had

clearly visible processes cytoplasmic labeling, with an absence of an identifiable nuclear compartment. Similar immunocytochemical approaches have been used for determining the intracellular location of corticosteroid (Ahima and Harlan, 1991) and other steroid receptors (Blaustein, 1993; Wood and Newman, 1993). For each brain, the total number of cells, total number of nuclei and the percentage of cells without nuclear labeling were calculated.

Data Analysis

For the cortiol and Dex experiments, parametric data (PF Att and Ad Att percentages, contact time and body weights) were compared between treatment groups on P-37 by ANOVA followed by Fisher's post hoc tests. For the cortisol/RU-486 experiment, the data (attack percentages, contact time, and body weights) were compared between treatment groups by repeated measures ANOVA. Group differences on specific days were analyzed by ANOVA followed by Fisher's PLSD post hoc tests. Nonparametric data (attack frequency, lines crossed, corners turned, and escape attempts in the activity test) were analyzed by separate Kruskall-Wallis (*H*) tests followed by Mann-Whitney (*U*) tests (two-tailed) for each test day and by Friedman (X^2) tests followed by Wilcoxon (Z) tests (two-tailed) for each group. Additionally, corticosteroid receptor immunoreactivity was compared within the brains of Dex-, cortisol-, and vehicle-treated animals by ANOVA followed by Fisher's post hoc tests.

Results

Cortisol and Offensive Responses

Repeated cortisol injections during early puberty accelerated the transition from play fighting to adult aggression (Fig. 4.1). Cortisol treatment decreased the percentage

of PF Att [$F(2,17)=5.8$, $p<0.05$, ANOVA]. Both doses of cortisol were sufficient to decrease PF Att ($p<0.01$ for both). Cortisol-treated animals also showed increases in the percentages of side attacks [$F(2,17)=5.9$, $p<0.05$]. For side attacks both the 10- and 40 µg/100g doses of cortisol increased the percentage of side attacks ($p<0.05$, $p<0.01$ respectively). Cortisol treatment caused an apparent increase in Ad Att, yet this effect was not statistically significant [$F(2,17)=2.9$, $p=0.0843$]. No group differences were observed for any other behavioral measure (attack frequency and contact time) or body weight.

RU-486/Cortisol and Offensive Responses

RU-486 blocked the acceleratory effects of cortisol on the transition from play fighting aggression (Fig. 4.2). PF Att was decreased by cortisol treatment [$F(3,35)=4.7$, $p<0.01$, repeated measures ANOVA]. No group differences were observed for PF Att on P-37, but on P-42, vehicle/cortisol animals showed decreased PF Att percentages as compared to the vehicle/vehicle group ($p<0.001$). This effect not observed in RU-486 treatment as Vehicle/RU-486 and cortisol/RU-486 animals did not differ from the vehicle/vehicle group in PF Att percentages on either test day. RU-486 treatment also blocked the cortisol-induced increase in Ad Att [$F,3,35)=6.4$, $p<0.01$]. Vehicle/cortisol animals showed increased Ad Att percentages on P-37 and P-42 ($p<0.05$, $p<0.01$, respectively). RU-486/vehicle and RU-486/cortisol animals did not differ from vehicle/vehicle animals in Ad Att percentages on either test day.

While no group effect was observed for body weight [$F(3,35)=0.5$, $p>0.1$, repeated measures ANOVA] group x day interaction was observed for this

measure[$F(2,6)=3.8$, $p<0.01$]. Both RU-486 groups were slightly smaller (circa 5% body weight) than vehicle/vehicle and vehicle/cortisol animals on P-42. However, no significant group differences were observed on this day [$F(3,35)=1.3$, $p>0.1$].

Locomotor activity was not affected by any combination of RU-486 or cortisol treatment. In the lat-maze tests, the number of line crosses ranged from 408 to 445. The number of corners turned ranged from 67-75, and the number of escape attempts ranged from 44 to 49 between groups. These data did not statistically differ between treatment groups.

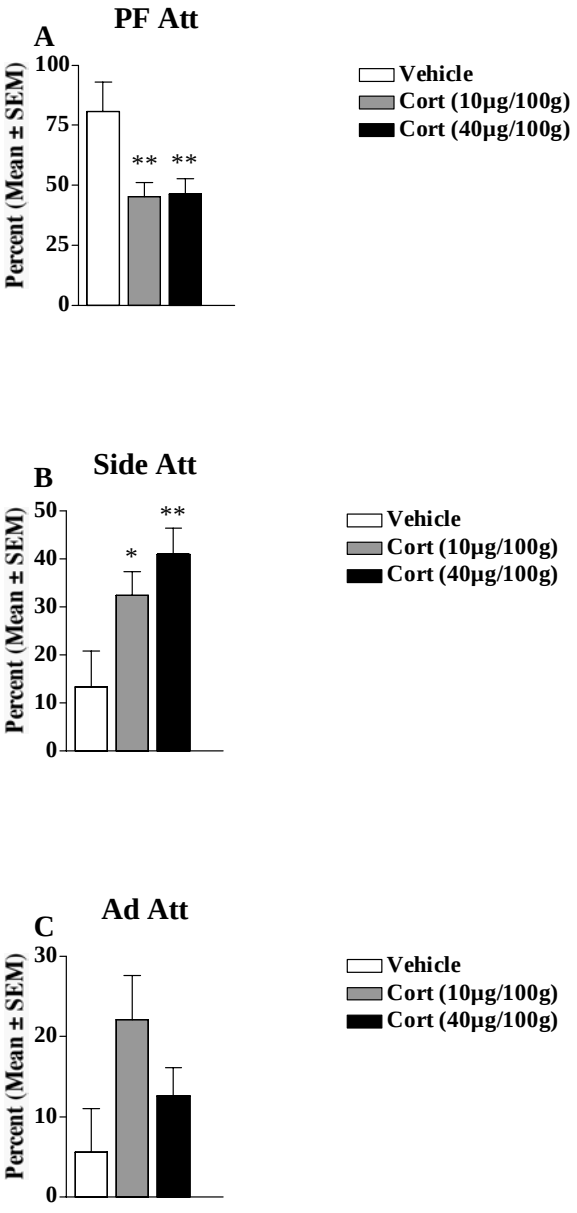
Dex and Offensive Responses

Repeated Dex treatment during early puberty accelerated the transition from play fighting to adult aggression (Fig. 4.3). Dex caused a dose-dependent decrease in the percentage of PF Att [$F(2,26)=4.9$,$p<0.01$]. As compared to vehicle treated animals, individuals exposed to low dose and high dose Dex showed a significant decrease in the percentage of PF Att ($p<0.05$ and $p<0.01$, respectively). Dex also caused a dose-dependent increase in the percentage of Ad Att [$F(2,26)=6.8$, $p<0.01$]. Animals that received the highest dose of Dex also displayed the highest percentage of Ad Att (as compared to vehicle treated animals, $p<0.001$). The effects of Dex were specific to the targets of attack as neither attack frequency nor latency differed significantly between groups. No group differences were observed for pins, flank marks, and contact time. Importantly, group differences in body weight were not observed, indicating that growth was not affected by repeated Dex treatment.

Dex Treatment and GR Immunocytochemistry

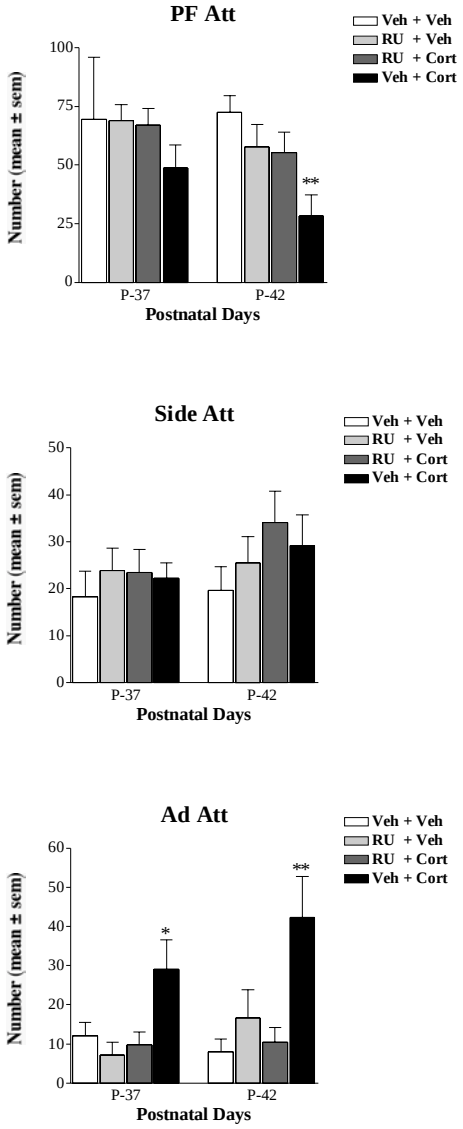
Dex injections are capable of activating type II corticosteroid receptors with the brains of golden hamsters (Table 4.1). Animals treated with Dex or cortisol had lower percentages of cells without clear nuclear labeling than vehicle treated animals within the PVN. The overall number of cells and nuclei analyzed did not differ between treatment groups. It is important to note that no group differences in corticosteroid receptor immunoreactivity were observed between treatment groups within the DG.

Figure 4.1: Cortisol and Offensive Responses



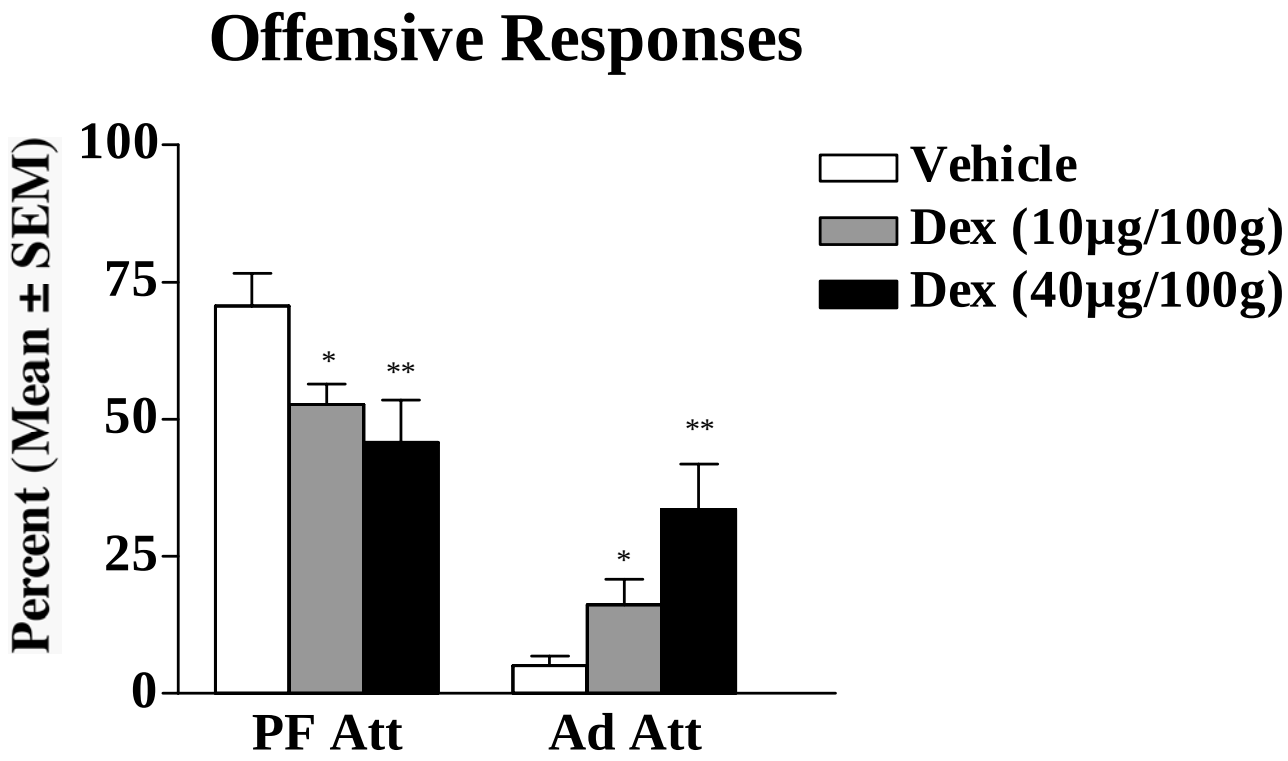
Animals were injected with cortisol (10- or 40µg/100g) or vehicle (propylene glycol) from postnatal day 31 (P-31) to P-36. On P-37, all animals were tested for offensive aggression in the presence of smaller, younger intruder. The percentage of attacks that were directed at the face and ckeeks (PF Att), the side (Side Att), or the belly/rear (Ad Att) were compared between treatment groups. ANOVA followed by Fisher's PLSD, *$p<0.05$, **$p<0.01$.

Figure 4.2: RU-486, Cortisol and Offensive Responses



From P-31 to P-41, animal received an injection of 0 or 1.0 mg RU-486 in 0.3ml sesame oil. One hour later, animals received daily injections of cortisol in propylene glycol (0, 10µg/100g. On P-37 and P-42, all animals were tested for offensive aggression in the presence of smaller, younger intruder. The percentage of attacks that were directed at the face and ckeeks (PF Att), the side (Side Att), or the belly/rear (Ad Att) were compared between treatment groups. ANOVA followed by Fisher's PLSD, *$p<0.05$, **$p<0.01$.

Figure 4.3: Dex and Offensive Responses



Animals were injected with Dexamethasone (Dex) (10- or 40µg/100g) or vehicle (propylene glycol) from postnatal day 31 (P-31) to P-36. On P-37, all animals were tested for offensive aggression in the presence of smaller, younger intruder. The percentages of attacks that were directed at the face and ckeeks (PF Att), the side (Side Att), or the belly/rear (Ad Att) were compared between treatment groups. ANOVA followed by Fisher's PLSD, *$p<0.05$, **$p<0.01$.

Table 4.1 Dex and GR Immunocytochemistry.

	PVN			DG		
	Cells	**Nuclei**	**Pecent**	**Cells**	**Nuclei**	**Pecent**
Vehicle	362.8 + 24.0	189.3 ± 30.4	48.75 ± 7.5	106.3 ± 10.4	99.2 ± 10.1	6.7 ± 4.6
Dex	365.3 ± 13.9	246.0 ± 12.3	32.6 ± 1.1	104.5 ± 23.7	101.2 ± 25.3	3.5 ± 2.2
Cortisol	301.5 ± 54.5	235.8 ± 44.3	23.9 ± 4.3	113.1 ± 11.1	108.3 ± 8.9	4.1 ± 1.9
ANOVA	$p>0.1$	$p>0.1$	$p<0.001$	$p>0.1$	$p>0.1$	$p>0.1$

Female golden hamsters were treated with Dexamethasone (Dex 40 µg/100g, cortisol (40 µg/100g) or vehicle (propylene glycol) on postnatal day 26 (P-26) or P-27. Presented are the number of cells, nuclei, and percentage of cells without nuclear labeling (mean ± sem) for each treatment group. Receptor immunoreactivity was quantified within the paraventricular hypothalamic nucleus and the dentate gyrus. ANOVA followed by Fisher's PLSD, $^{**}p<0.01$, $^{***}p<0.001$, compared to vehicle.

Discussion:

The current study investigated the influence of glucocorticoids on the development of agonistic behavior. In the first experiment, daily injections of cortisol during early puberty accelerated the transition from play fighting to adult aggression. In the second phase of the study, daily injections of RU-486, a corticosteroid type II receptor antagonist, were found to block the behavioral effects of cortisol. To further emphasize the role of type II corticosteroid receptors, animals receiving daily injections of Dex also showed a decrease in play-fighting attacks and an increase in adult attacks. In summary, the current studies showed that the transition from play fighting to adult aggression is controlled by glucocorticoid via activation of type II corticosteroid receptors.

Daily treatment with cortisol accelerated the development of agonistic behavior. Cortisol treated animals showed lower percentages of play-fighting attacks. Additionally, both doses of cortisol increased the percentage side attacks, indicating that daily injections of this hormone accelerated behavioral development. Interestingly, the lower cortisol dose was the most effective at altering offensive responses, suggesting that the effects of this hormone on behavioral development follows an inverted-U dose response. No other behavioral measures differed between cortisol- and vehicle-treated animals, indicating that the effects of this hormone are specific to attack target.

In a second experiment, the effects of cortisol on attack target were blocked by co-treatment with the corticosteroid type II antagonist, RU-486. Animals that received a combination of RU-486 and cortisol showed similar levels of play fighting and adult

aggression as animals received vehicle only or vehicle and RU-486. In contrast, vehicle/cortisol, compared to all other treatment groups, showed a decrease in play-fighting attacks and an increase in adult attacks. These data show that type II corticosteroid receptors are required for cortisol to accelerate the development of offensive responses.

While widely known as an anti-progestin, RU-486 is a type II corticosteroid receptor antagonist in golden hamsters (Gray and Leavitt, 1987). This drug has also been used to block corticosteroid receptors in prairie voles, another rodent species (Curtis and Wang, 2005). Additional investigations of this substance have shown that RU-486 also produces anti-androgenic effects by enhancing activity of androgen receptor corepressors (Hodgson et al., 2005; Song et al., 2004). As these studies were conducted *in vitro* it remains unclear whether or not RU-486 had any effect on androgen receptors in this study. Nevertheless, the potential interaction between RU-486 and androgen receptors deserves consideration when interpreting the experimental results.

Confirming the role of type II corticosteroid receptors, daily treatment with Dex also accelerated the development of offensive responses. Animals that received daily injections of Dex during the first week of puberty performed a lower percentage of play-fighting attacks and a higher percentage of adult attacks than vehicle-treated animals. These effects were dose dependent, as animals receiving the highest dose of Dex showed the largest decreases in play-fighting attacks and increases in adult attacks. Animals from all treatment groups showed similar levels of attack frequency and latency, contact time,

and pins. The lack of difference in these other variables indicates that the effects of Dex are specific to attack target.

Previous studies focusing on the role of the HPG axis in the development of aggressive behavior have produced surprising results. In both rats and golden hamsters, castration during puberty does not alter the development of agonistic behavior (Beatty et al., 1981; Romeo et al., 2003; Smith et al., 1996). Data from chapter 3 showed that repeated social stress accelerated the transition from play fighting to adult aggression, yet subjugated juveniles have lower testosterone levels than non-stressed controls. These findings are of interest because puberty is a period commonly characterized by dramatic increases in androgen levels, and a large number of studies have shown testosterone to be critical for male aggression during adulthood (Albers et al., 2002; Simon, 2002). The lack of involvement of the gonadal steroids in pubertal changes in behavior has led some to hypothesize that these maturational processes are regulated by non-steroidal mechanisms (Sisk and Foster, 2004). Other reports show that progesterone levels increase during puberty in male golden hamsters, but the relationship between progesterone and the development of agonistic behavior is unknown (Vomachka and Greenwald, 1979). Data from the current studies show that the development of offensive responses is influenced by another type of hormone: glucocorticoids.

The effects of cortisol treatment on offensive responses potentially explain how social subjugation during early puberty influences the development of agonistic behaviors. Male golden hamsters that are repeatedly subjugated during puberty show an accelerated transition from play fighting to adult aggression (Delville et al., 1998;

Wommack et al., 2002). Data from chapter 3 suggest that social subjugation is a stressor to which juveniles are incapable of fully habituating. As such, social defeat during early puberty likely causes a daily increase in plasma cortisol levels and subsequent activation of corticosteroid receptors. As the behavioral consequences of social subjugation and repeated cortisol or Dex treatment during puberty are similar, it is likely that the maturation of agonistic behavior is controlled by corticosteroid receptors. Moreover, as type II receptors have lower affinities than type I corticosteroid receptors, they are more likely to remain inactive except during times of stress (Sutanto and De Kloet, 1987). As such, the effects of social subjugation on offensive behavior are likely mediated by type II receptors. Future studies are required to investigate this topic in more detail.

The analysis of the immunoreactivity for corticosteroid receptors type II showed differences between brain areas. Compared to the vehicle, Dex and cortisol injections resulted in significantly higher proportion of nuclear immunolabeling within the parvicellular subdivision of the PVN. This activation by treatment with Dex and cortisol was not observed throughout the brain. For instance, no group differences in nuclear labeling were observed within the DG. Consequently, our data suggest that cortisol and Dex were capable of activating corticosteroid type II receptors in some parts of the brain. However, it is important to note that the animals used for this study were not adrenalectomized, rather they were immature female hamsters. These animals were used for this study because they have very low plasma cortisol levels (Taravosh-Lahn and Delville, 2004), though high enough to activate corticosteroid type II receptors in the DG.

Possibly, corticosteroid type II receptors in this area are more responsive to low plasma levels of cortisol.

In rats and mice, there are data to suggest that Dex does not cross the blood brain barrier (Cole et al., 2000; De Kloet et al., 1975; Meijer et al., 1998). Rats treated with Dex show no increase in nuclear corticosteroid receptor binding within the hippocampus (De Kloet et al., 1975). On the other hand, Dex treatment causes a significant increase in corticosteroid binding in the anterior pituitary gland of rats (Cole et al., 2000). Dex penetration through blood brain barrier is enhanced in mice with a knockout for the mrd1A p-glycoprotein gene (Meijer et al., 1998). It is important to note that the primary glucocorticoid in rats and mice is corticosterone. In contrast, cortisol is the primary glucocorticoid in hamsters. Cortisol has three hydroxyl groups as compared to two hydroxyl groups on the carbon rings of corticosterone. This indicates that the two molecules have slightly different chemical properties. In fact, cortisol poorly crosses the blood brain barrier in mice (Karssen et al., 2001). Besides hamsters, cortisol is the primary glucocorticoid in a number of other species such as guinea pigs (Sachser, 1986), pigs (Hao et al., 2003), new and old world primates (Abbot et al., 2003) and fish (Overli et al., 2004; Pepels et al., 2004). However, there are no existing studies on the blood brain barrier in these species. Dex is similar to cortisol in that it also has three hydroxy groups on its main carbon chain. As such, Dex may more readily cross the blood brain barrier in species where cortisol is the predominant glucocorticoid. These data show that Dex is indeed capable of activating corticosteroid receptors within the brains of golden hamsters suggesting, species differences in the blood brain barrier

In conclusion, the current studies show that glucocorticoids accelerate the development of offensive responses via the type II corticosteroid receptor. These results are important as, for the first time, they show that glucocorticoids are important for the maturation of agonistic behavior. The implications of these data are broad, as the HPA axis undergoes pubertal changes in a number or species. As such, it is unlikely that the effects of glucocorticoids on the maturation of social behaviors are limited to golden hamsters.

Chapter 5: Neural Correlates of Behavioral Development

Introduction

Puberty is a characterized by numerous changes in agonistic behavior. In male golden hamsters, offensive response undergoes a transition from play fighting to adult aggression during this time (Wommack et al., 2003). The specific neural mechanisms guiding this behavioral shift have been largely unexplored.

While it is unclear whether play fighting and adult aggression are controlled by the same neural network, the brain areas that involved in offensive aggression in adult male golden hamsters have been identified. Studies using either electrical stimulation or lesions have shown that a number of limbic areas, such as the septum, hypothalamus, amygdala, and preoptic area, regulate offensive responses (Bunnell et al., 1970; Hammond and Rowe, 1976; Potegal et al., 1981; 1996; Shipley and Kolb, 1977; Sodetz and Bunnell, 1970). Retrograde labeling and c-fos immunocytochemistry has also been used to identify the neural pathways controlling offensive aggression (Delville et al., 2000). Areas such as the anterior hypothalamus (AH), ventrolateral hyhpothalamus (VLH), medial amygdala, bed nucleus of the stria terminals, and the dorsolateral part of the midbrain central gray show increase in c-fos immunolabelling specific to offensive aggression (Delville et al., 2000). Moreover, each of these nuclei is reciprocally connected to the AH, suggesting that this area is the center of a neural network of offensive aggression in male golden hamsters.

Neurochemical studies have shown that offensive aggression in male golden hamsters is facilitated by arginine vasopressin (AVP). Microinjections of vasopressin

into the AH increase offensive responses in adult male hamsters (Delville et al., 1996a; Ferris et al., 1999). Moreover, microinjections of a selective V1A antagonist in the AH cause a dose-dependent inhibition of offensive aggression (Ferris and Potegal, 1988). AVP also stimulates aggressive behavior when injected into the VLH (Delville et al., 1996a). Interestingly, AVP receptors in the VLH are testosterone-dependent, as receptor binding significantly decreases after castration (Delville, et al., 1996b).

Serotonin (5HT) has been identified as a neurotransmitter that inhibits offensive aggression. Animals that receive either peripheral treatment of Fluoxetine, a 5HT reputake inhibitor, or central injections of selective 5HT 1A receptor agonists show decreased aggression (Delville et al., 1996a, Ferris et al., 1997; 1999; Joppa et al., 1997). The effects of 5HT are likely due to an interaction with AVP in the AH (Ferris et al., 1997). Treatment with Fluoxetine blocks AVP-induced aggression (Ferris et al., 1997). 5HT also blocks the effects of AVP at the level of the AH via the 5HT 1A receptor (Ferris et al., 1999). Additional evidence suggests that offensive aggression is influenced by a similar 5HT/AVP interactions within the VLH (Delville et al., 1996b; Ferris and Delville, 1994).

External factors such as social stress during early puberty accelerate the transition from play fighting to adult aggression when tested with smaller and younger intruders (Wommack et al., 2003). Subjugated juveniles show increased 5HT innervation and decreased AVP content within the anterior hypothalamus (Delville et al., 1998). These findings are inconsistent with the enhanced aggression subjugated juveniles display in the presence of smaller and younger intruders and are more likely related to the decreased

aggression they display in the presence of an equally matched intruder (Delville et al., 1998). Other studies focusing on receptors have also failed to show a link between 5HT and AVP and aggression towards smaller opponents (Ferris and Delville, unpublished data). However, recent data has suggested that 5HT innervation of the AH increases during puberty (Taravosh-Lahn et al., 2004). In light of these new data, the subjugation-induced changes in 5HT innervation represent a potential developmental acceleration. Moreover, studies on 5HT 3 receptors show that this receptor subtype facilitates offensive aggression in male golden hamsters (Ricci et al., 2004; 2005). As such, it is possible that subjugation alters behavioral development via 5HT within the AH. It is also possible the development of offensive responses and the acceleration caused by subjugation are controlled through alternate neural mechanisms.

Indeed, other neurotransmitter systems have recently been shown to influence offensive aggression in male golden hamsters. For example, recent reports have implicated dopamine as a mediator of offensive responses during puberty. Repeated treatment with low doses of cocaine enhances aggression in juvenile hamsters (DeLeon et al., 2002b; Harrison et al., 2000). Also, social subjugation, an external factor that accelerates the transition from play fighting to adult aggression, results in enhanced tyrosine hydroxylase immunoreactivity (TH-IR) within the posterodorsal portion of the medial amygdaloid nucleus (MePD) and the posterior portion of the medial division of the bed nucleus of the stria terminalis (BSTPM) (Wommack and Delville, 2002). These brain regions are interesting for a few reasons. First, the BSTPM and MePD show increased FOS immunoreactivity following the consummation of offensive aggression

(Delville et al., 2000). These areas are also reciprocally connected to each other and the anterior hypothalamus, an area critical for the control of offensive aggression (Coolen and Wood, 1998; Delville et al., 2000). Additionally, these species-specific populations of TH neurons are testosterone dependent and present in areas likely influenced by the physiological changes associated with puberty (Asmus and Newman, 1993; Asmus et al., 1992; Romeo and Sisk, 2001). Considering all these factors, it is quite possible that TH neurons within the MePD and BSTPM are involved in the pubertal maturation of agonistic behavior. A more detailed investigation on the pubertal changes in TH-IR within these neurons would be helpful in determining their role in the development of offensive responses.

The transition from play fighting to adult aggression may also be controlled by yet another neurotransmitter: Corticotropin releasing hormone (CRH). CRH innervation is widely observed throughout the brain of male golden hamsters, including areas involved in offensive aggression (Delville et al., 1992). Additionally, oral administration of a CRH type-1 receptor antagonist inhibits aggression in adult male hamsters (Farrokhi et al., 2004). Moreover, data from chapter 3 showed that CRH innervation of the median eminence increases during puberty. As such it is possible that CRH innervation of other brain regions may also change during puberty. Additionally, CRH is a neurotransmitter associated with stress and may also play a role in the alterations of behavioral development observed in subjugated male golden hamsters.

The goals of this study are two-fold. First, dopamine neurons within the medial and extended amygdala and CRH innervation of the limbic system will be investigated

across puberty to determine whether they are related to the transition from play fighting to adult aggression. Second, the effects of social subjugation on TH-IR and CRH innervation will also be investigated to understand if these neurotransmitters are involved in the behavioral consequences of social stress. In an additional experiment, TH-IR was analyzed within the BSTPM and MePD of subjugated adults to determine whether or not the effects of social stress on TH are specific to juveniles.

Methods

Animals and Treatment

The animals (male golden hamsters) were bred in the laboratory from a colony that originated from Harlan Sprague Dawley (Indianapolis, IN). Approximately five days after birth, all litters were culled to 6 pups including males and females. On postnatal day 25 (P-25), all animals were weaned and individually housed in plexiglass cages (20x33x13 cm). Within 2 days of weaning, each animal was briefly (a few seconds) observed in the presence of an adult intruder. Individuals that immediately fled from the adult were considered to be inherently fearful (approximately 1 in 12) and were removed from the experiment. All animals received food and water *ad libitum* and were housed under a reversed light/dark cycle (14L-10 D, lights off at 9:00am).

Experiment 1: TH-IR in the BSTPM and MePD

Experimental Design

On P-28, animals were separated into two groups (Naive and Subjugated). Social subjugation was performed according to a previously described protocol (Delville et al.,

1998; Wommack and Delville, 2002). Daily subjugation started on P-28, which coincides with the onset of puberty (Vomachka and Greenwald, 1979), and ended on P-42, near mid puberty. This period was roughly equivalent to the first half of puberty (Delville et al., 2003). Animals in the subjugated group were placed in the home cage of an aggressive adult for 20 minutes daily while naive animals were placed in a clean and empty cage for the same period. Subjugated juveniles were cycled through a group of aggressive adults (n=8) for each subjugation day. Prior to the experiment, adults were tested for offensive aggression by the resident-intruder paradigm (Miczek, 1979). Smaller and younger intruders were placed in the home cage of adult hamsters. The adults used in this experiment were trained fighters selected for their aggressiveness. During repeated subjugation, animals were observed daily while in the home cage of an adult to ensure they were chased and attacked. Daily subjugation was performed during the second half of the dark phase.

At the onset of puberty, on P-28, a group of animals that had not been assigned to either the naive or subjugated group were sacrificed under basal conditions or immediately after twenty minutes in the home cage of an aggressive adult. These animals were tested on this day to establish a developmental trajectory. On P-35 (early puberty), P-45 (mid puberty), or P-70 (early adulthood), both naive and subjugated groups were sacrificed under basal conditions (n=6 per group per day). These test dates were chosen to establish a developmental trajectory and to understand the time course of experimental effects. The animals were sacrificed by rapid decapitation.

An additional experiment was designed to determine if the effects of social stress on TH-IR were specific to puberty. In this experiment, adult male hamsters (born and raised in the laboratory) were separated into naive and subjugated groups (n=6 for each group) on P-70. This period corresponds to early adulthood (Vomacka and Greenwald, 1979; Delville et al., 2003). Experimental animals were subjugated (as previously described) daily between P-70 and P-84. On P-85, both subjugated and naive animals were sacrificed by rapid decapitation under basal conditions. For each individual, testes weights were recorded, and brains were extracted.

Tyrosine Hydroxylase Immunocytochemistry

During sacrifice, brains were collected and fixed by overnight immersion in 10% acrolein in 0.1M KPBS buffer (pH 7.2) at 4°C and later saved in 20% sucrose/KPBS. The brains were then sectioned into 40 µm-thick coronal sections with a freezing rotatory microtome and were stored in a cryoprotectant (Watson et al., 1986) at -20°C until labeled by immunocytochemistry. Brain sections from animals sacrificed prior to subjugation were used for this procedure. Immunocytochemistry for TH was performed as previously described (Wommack and Delville, 2002). Briefly, free-floating sections were pretreated in 1% sodium borohydrite (to remove residual aldehydes) followed by a preincubation in a solution containing 20% normal goat serum, 1% hydrogen peroxide, and 0.3% Triton X-100 (respectively, to block nonspecific labeling, eliminate endogenous peroxidase activity, and permeabilize the tissue). Sections were then incubated in a mouse monoclonal antibody to TH (1:20,000; Sigma Chemical Co., St. Louis, MO), containing 2% normal goat serum and 0.3% Triton X-100 for 48 hours at

4°C. After washing, the sections were incubated in the secondary antibody (biotinylated goat anti-mouse IgG; 7.5µg/ml; Jackson Immuno Research Laboratories, West Grove, PA) followed by a tertiary incubation (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA). Between incubations, sections were washed in 0.05M TBS (pH 7.6). Finally, the sections were reacted with with diaminobenzidine (DAB, 0.5mg/ml) and 0.05% hydrogen peroxide. This procedure is optimized for labeling of TH-immunoreactive (TH-ir) neurons, dendrites and axons throughout the brain (Wommack and Delville, 2002). We have previously labeled alternative sections by immunocytochemistry for dopamine beta-hydroxylase, and found no cell bodies within the BST or MePD (Wommack and Delville, 2002). Therefore, the neurons located within these nuclei are most likely dopaminergic, not noradrenergic. Labeled sections were mounted on gel-coated slides, dehydrated in a series of alcohols, and coverslipped with permount. Later, TH-IR was observed with a Nikon Eclipse E600 microscope. TH-ir neurons were counted with a camera lucida attachment.

The areas selected for cell counts were BSTPM, MePD, and the periventricular hypothalamic nucleus (Pe) at the level of the optic chiasma (Morin and Wood, 2001) (Fig 5.1). These areas are interesting for a number or reasons. First, the BSTPM and MePD are both involved in a number of social behaviors, including aggression (Bunnell et al., 1970; Delville et al., 2000; Lehman et al., 1980; Potegal et al., 1996). Second, these specific neurons are testosterone-dependent (Asmus et al., 1992; Asmus and Newman, 1993) and may be sensitive to the physiological changes associated with puberty (Romeo and Sisk, 2001). These areas are also highly responsive to stress (Kollack-Walker et al.,

1999; Martinez et al., 1998; Pawlak et al., 2003; Roozendaal et al., 1997). The Pe, an area rich in gonadal steroid receptors (Li et al., 1993; Wood et al., 1992; Wood and Winnans-Newman, 1998) was an additional area analyzed to determine whether the changes in TH-IR were specific to the BSTPM and MePD. Recent reports in mice show that TH-IR within the Pe is increased in subordinate males (Fiore et al., 2005). Six to twelve cell counts were taken bilaterally from consecutive sections for each area analyzed and each individual. Immunoreactive cells were counted only when their nucleus was clearly visible. The average number of TH-ir neurons per count and area was calculated for each individual.

Data Analysis

Parametric data (e.g. average number of TH-ir neurons) were compared between groups over time by 2-way ANOVAs (independent variables: treatment groups and age). For comparison between naïve and subjugated groups on specific test days and for adult subjugation, the numbers of TH-ir neurons was compared between groups by a student's t-test (two-tailed).

TH Results

In the BSTPM, the number of TH-ir cells changed significantly during puberty [$F(3,40)=8.2$, $p<0.001$] decreasing from P-28 to P-70. The analysis of TH-ir neurons also showed increased number of labeled cells within specific areas in experimental animals starting one week after the onset of social subjugation (Fig. 5.2A). The difference

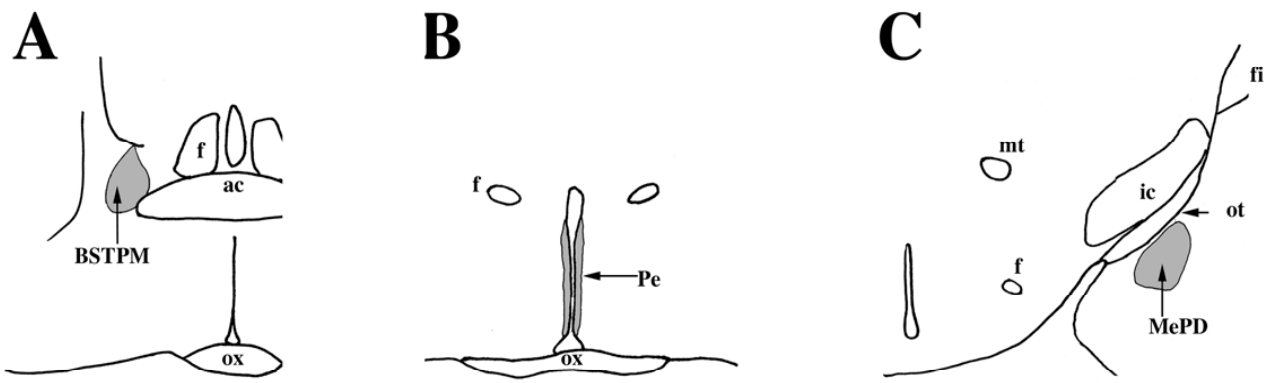
between groups was also statistically significant [$F(1,40)=30.5$, $p<0.001$], so was the interaction between groups and age [$F(3,40)=6.7$, $p<0.001$]. Repeatedly subjugated animals had more TH-ir neurons than naive animals on P-35 and P-45 [respectively, $t(10)=5.29$, $p<0.001$; $t(10)$-3.39, $p<0.01$]. No group difference was observed on P-70.

The number of TH-ir neurons within MePD also decreased during puberty from P-28 to P-70 [$F(3,40)=45.4$, $p<0.001$] (Fig. 5.2 B). Additionally, a statistically significant group difference [$F(1,40)=24.1$, $p<0.001$] and a group x age interaction [$F(3,40)=7.6$, $p<0.001$] were also observed in this area. On P-35 and P-45, subjugated animals had higher numbers of TH-ir neurons [respectively, $t(10)=-3.37$, $p<0.01$; $t(10)=-5.76$, $p<0.001$]. However, subjugated and naive animals had similar numbers of TH-ir neurons by P-70.

Within the Pe, TH-IR did not change during puberty nor was it affected by repeated subjugation (Fig 5.2 C).

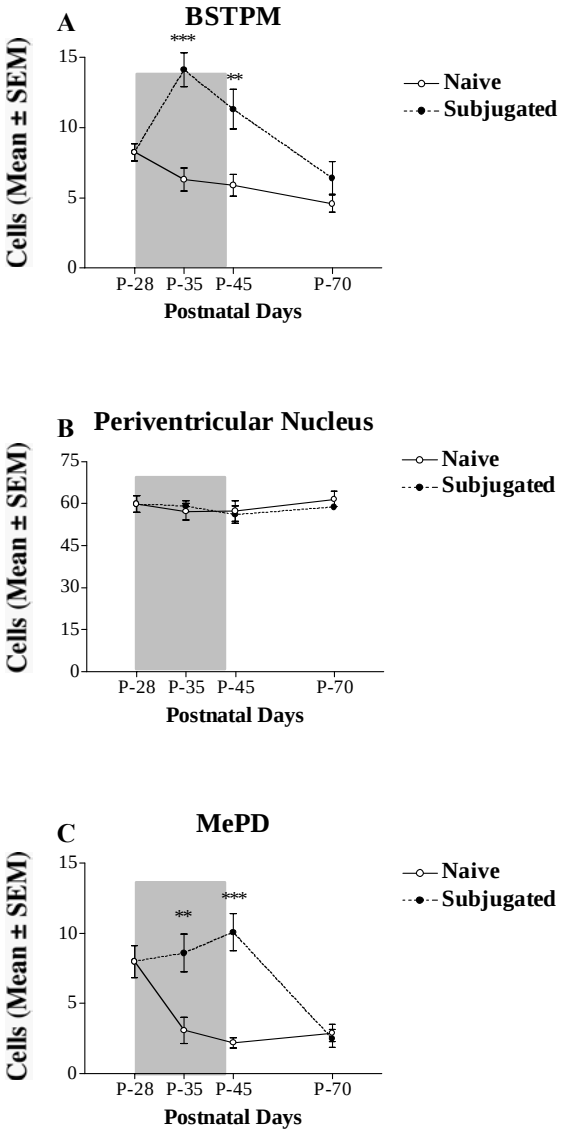
Subjugated adults showed 2-fold increases in TH-ir neurons within the BSTPM [$t(10)3.8$, $p<0.01$] and in the MePD [$t(10)4.1$, $p<0.01$] (Fig. 5.3). Importantly, subjugation during adulthood, did not affect body weights or testes weights.

Figure 5.1: Brain Areas for TH-IR Quantification.



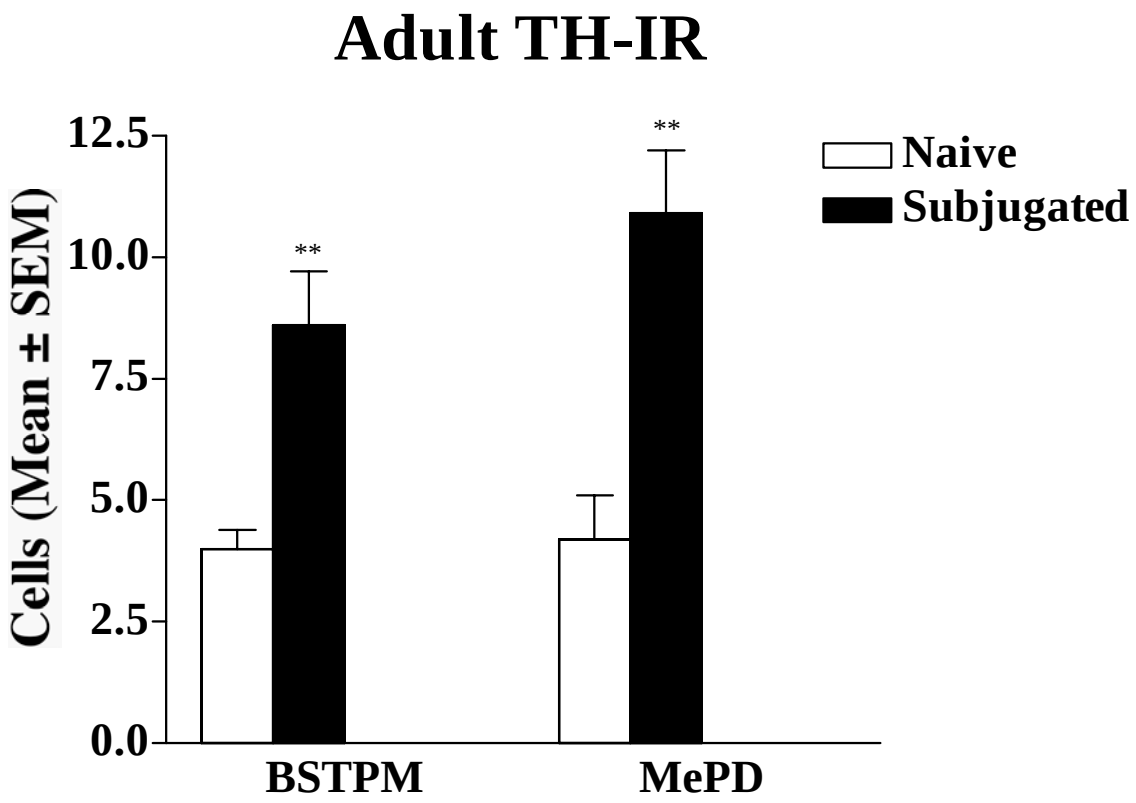
Camera lucida drawings showing the location of areas quantified (shaded areas) for TH-IR. These areas included (A) the posterior portion of the medial division of the bed nucleus of the stria terminalis (BSTPM), (B) the periventricular hypothalamic nucleus (Pe), and (C) the posterodorsal portion of the medial amygdaloid nucleus (MePD). ac, anterior commisure; ic, internal capsule; f, fornix; fi, fimbria fornix of the hippocampus; mt, mammillothalamic tract; ot, optic tract; ox optic chiasm.

Figure 5.2: Development of TH-IR in Male Golden Hamsters.



Comparison tyrosine hydroxylase immunoreactive neurons between subjugated and naïve animals. Cell counts are expressed as average cell number per count per area. The shaded area represents the period of daily subjugation. BSTPM, Posterior portion of the medial division of the bed nucleus of the stria terminalis; MePD, Posterodorsal portion of the medial amygdaloid nucleus. Student's t-test (two-tailed), **$p<0.01$, ***$p<0.001$.

Figure 5.3: TH-IR in subjugated and naïve adult hamsters.



Comparison of tyrosine hydroxylase immunoreactive neurons between subjugated and naïve adults. Cell counts are expressed as average cell number per count per area. Student's t-test (two-tailed) **$p<0.01$.

TH Discussion

The current study investigated whether subjugation-induced increases in TH-IR within the BSTPM and MePD were related to the development of offensive responses. To test this hypothesis, TH-IR was measured within the BSTPM and MePD of naïve and subjugated animals at various points across puberty. In both groups, TH-IR decreased during puberty. However, subjugated animals showed higher numbers of TH-ir neurons during the period of social subjugation. Following a four-week recovery period, the differences in TH-IR between subjugated and naïve animals were no longer significant. An investigation of TH-IR within the subjugated and naïve adult hamsters showed that TH-IR within the BSTPM and MePD was also increased by social stress during adulthood. These latter findings indicate that the effects of social stress on TH-IR are not specific to juvenile hamsters.

In view of the present data, this hypothesis that TH-IR within the BSTPM and MePD is involved in the development of offensive responses has to be rejected. TH-IR within the BSTPM and MePD decreased during puberty in naïve animals. Therefore, the transient increase in TH-IR within the BSTPM and MePD of subjugated animals does not correspond o a developmental acceleration. TH-IR was also similar between groups in early adulthood, although subjugated animals are more aggressive than naïve animals at that time (Wommack et al., 2003). Moreover, social stress during adulthood also increased TH-IR within the BSTPM and MePD. Opposite to the effects of social subjugation during puberty, the social stress during adulthood inhibits offensive aggression during this time (Delville et al., 1998; Huhman et al., 2003; Potegal et al.,

1993; Wommack et al., 2003). Therefore, stress-induced increases in TH-IR within the BSTPM and MePD are likely unrelated to offensive aggression. Moreover, these data fail to support the hypothesis that TH-IR within the BSTPM and MePD is related to the accelerated transition from play fighting to adult aggression observed in subjugated juvenile hamster (Wommack et al., 2003).

My previous findings that repeated social subjugation during puberty causes site-specific increases in TH-IR within the BSTPM and the MePD were confirmed in this study (Wommack and Delville, 2002). Repeatedly subjugated animals showed an increased number of TH-ir neurons within the BSTPM and MePD on P-35 and P-45. While TH was increase within both the BSTPM and MePD of subjugated animals, there are subtle differences between these nuclei. The point-to-point data indicate that TH-IR increased from pubertal levels within the BSTPM. In contrast, the increased TH-IR within the MePD was reflective of a maintenance of prepubertal TH levels. Nevertheless, these effects were site-specific; no group or age differences were observed for TH within the Pe. As subordinate male mice show increased TH-IR in the Pe, the lack of difference between subjugated and naïve hamsters presents and interesting species difference (Fiore et al., 2005).

Although these differences could be a result of increased cell numbers, they are more likely reflective of increased TH expression and neuronal activity in stressed individuals. This speculation is supported by the fact that social subjugation during adulthood does not alter the labeling of a cell birth marker, bromo-deoxyuridine, within the MePD (Eby and Delville, unpublished observations). Moreover, chronic stress is

known to increase TH mRNA expression (Angulo et al., 1991; Ortiz et al., 1996; Serova et al., 1998; Jordan et al., 1994) and enhance dopamine release in the brain (Jordan et al., 1994). However, in this study, stress-induced TH-IR was site-specific. Within the Pe, an area rich in gonadal steroid receptors, TH-IR was not affected by repeated subjugation or puberty (Li et al., 1993; Wommack and Delville, 2002; Wood et al., 1992; Wood and Winnans-Newman, 1998). The enhancement of TH-IR within the BSTPM and MePD in subjugated animals is reversible. By P-70, naive and subjugated animals had equal numbers of TH-ir neurons within both areas.

In addition, the observation of enhanced TH-IR within the same areas in subjugated adults shows this effect is not age-specific, but it has yet to be determined if TH-IR in subjugated adults and juveniles undergo similar decreases during the recovery period. Nevertheless, these data show that increased TH-IR within the BSTPM and MePD is a reliable neurobiological marker of repeated exposure to social stress. This possibility is supported by previous observations. These areas show enhanced neuronal activity after both acute and chronic exposure to social stress in hamsters and rats (Kollack-Walker et al., 1999; Martinez et al., 1998) and undergo neurobiological changes following fear conditioning (Roozendaal, et al., 1997). In addition, acute restraint stress enhances neuronal remodeling within the MePD in association with anxiety-induced behaviors (Pawlak et al., 2003). As such, these neurons are more likely involved in the behavioral consequences of social stress rather than the development of offensive responses.

Indeed, increases in TH-IR within the MePD and BSTPM correspond with the inhibition of risk assessment behavior observed in chapter 2. During the time when olfactory investigation is inhibited, subjugated juveniles show higher numbers of TH-ir neurons than naïve animals. Morevover, TH-ir neurons do not differ between groups on P-70, a point where both subjugated and naïve animals show similar levels of olfactory investigation. As such TH within the BSTPM and MePD is likely related to this behavioral effect. Future studies will be required to investigate this issue.

Experiment 2: CRH Innervation of the Limbic system

Experimental Design

Data from chapter 3 showed that CRH innervation of the median eminence increases during puberty. This finding highlights the possibility that CRH innervation changes during puberty in other brain areas. To determine if CRH innervation is involved in the development of offensive responses, CRH fiber density was analyzed in several limbic regions over the course of puberty. Additionally, social subjugation accelerates the transition from play fighting to adult aggression (Wommack et al., 2003). Therefore, CRH fiber density was also compared between subjugated and naïve animals, to determine if this neuropeptide is involved in the effects of social subjugation on the development of offensive responses.

First, to understand the pubertal development of CRH innervation within the limbic system, male golden hamsters (n=8 per group) were sacrificed on P-28 (early puberty), P-45 (mid puberty), and P-70 (late puberty). An additional experiment was conducted to test the effects of social subjugation on CRH fiber density. On P-28, male golden hamsters were divided into subjugated and control groups (n=6-7 per group). Subjugation was performed as described in Experiment 1 and the animals were sacrificed on P-43.

CRH Immunocytochemistry

Male golden hamsters were sacrificed by rapid decapitation. Following sacrifice, brains were collected and fixed by overnight immersion in 10% acrolein in 0.1M KPBS buffer (pH 7.2) at 4°C and later saved in 20% sucrose/KPBS. The brains were then

sectioned into 40 µm-thick coronal sections with a freezing rotatory microtome and were stored in a cryoprotectant (Watson et al., 1986) at -20°C until labeled by immunocytochemistry using a previously described protocol (Delville et al., 1992). Briefly, free-floating sections were pretreated in 1% sodium borohydrite (to remove residual aldehydes) followed by a preincubation in a solution containing 20% normal goat serum, 1% hydrogen peroxide, and 0.3% Triton X-100 (respectively, to block nonspecific labeling, eliminate endogenous peroxidase activity, and permeabilize the tissue). Sections were then incubated in a rabbit polyclonal antibody to Human/Rat CRH (1:6,000; Peninsula Laboratories, Inc., San Carlos, CA), containing 2% normal goat serum and 0.3% Triton X-100 for 48 hours at 4°C. After washing, the sections were incubated in the secondary antibody (biotinylated goat anti-rabbit IgG; 7.5µg/ml; Jackson Immunoresearch Laboratories, West Grove, PA) followed by a tertiary incubation (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA). Between incubations, sections were washed in 0.05M TBS (pH 7.6). Finally, the sections were labeled with with diaminobenzidine (DAB, 0.5mg/ml) and 0.05% hydrogen peroxide. Labeled sections were mounted on gel-coated slides, dehydrated in a series of alcohols, and coverslipped with permount.

CRH fiber density in a number of areas was quantified using NIH Image Software (v. 1.62, NIH, Bethesda, MD). Brain sections containing the lateral septum (LS), the anterior hypothalamus (AH), the posterior dorsal part of the medial amygdaloid nucleus (MePD), and the ventrolateral hypothalamus (VLH) were selected due to their involvement in social behaviors, including offensive aggression, in male golden hamsters

(Delville et al., 2000; Newman, 1999). CRH fiber density was also analyzed within the dorsomedial hypothalamic nucleus (DM) due to its involvement in circadian fluctuations in glucorticoid release (Kalsbeek, et al., 1996a; b). All areas were observed through a 40X objective as digitized images recorded by a Cohu CCD camera mounted on a Nikon Microscope and imported to a Macintosh computer using a frame grabber (LG3, Scion Corporation, Frederick, MD). The density of CRH-ir fibers was expressed as thesize of the area covered by immunoreactive fibers within a standard sample placed over matching zones or each respective nucleus. For each brain area, fiber density was sampled using a 150 µm diameter circle. Measurements were taken after normalizing for background and foreground differences (Delville et al., 1998). Several measurements (n=6-10) were taken from each sides of the brain in consecutive sections and averaged for each individual.

Data Analysis

CRH fiber densities were compared over time by ANOVA). For comparison between naïve and subjugated groups on, CRH fiber densities were compared by a student's t-test (two-tailed).

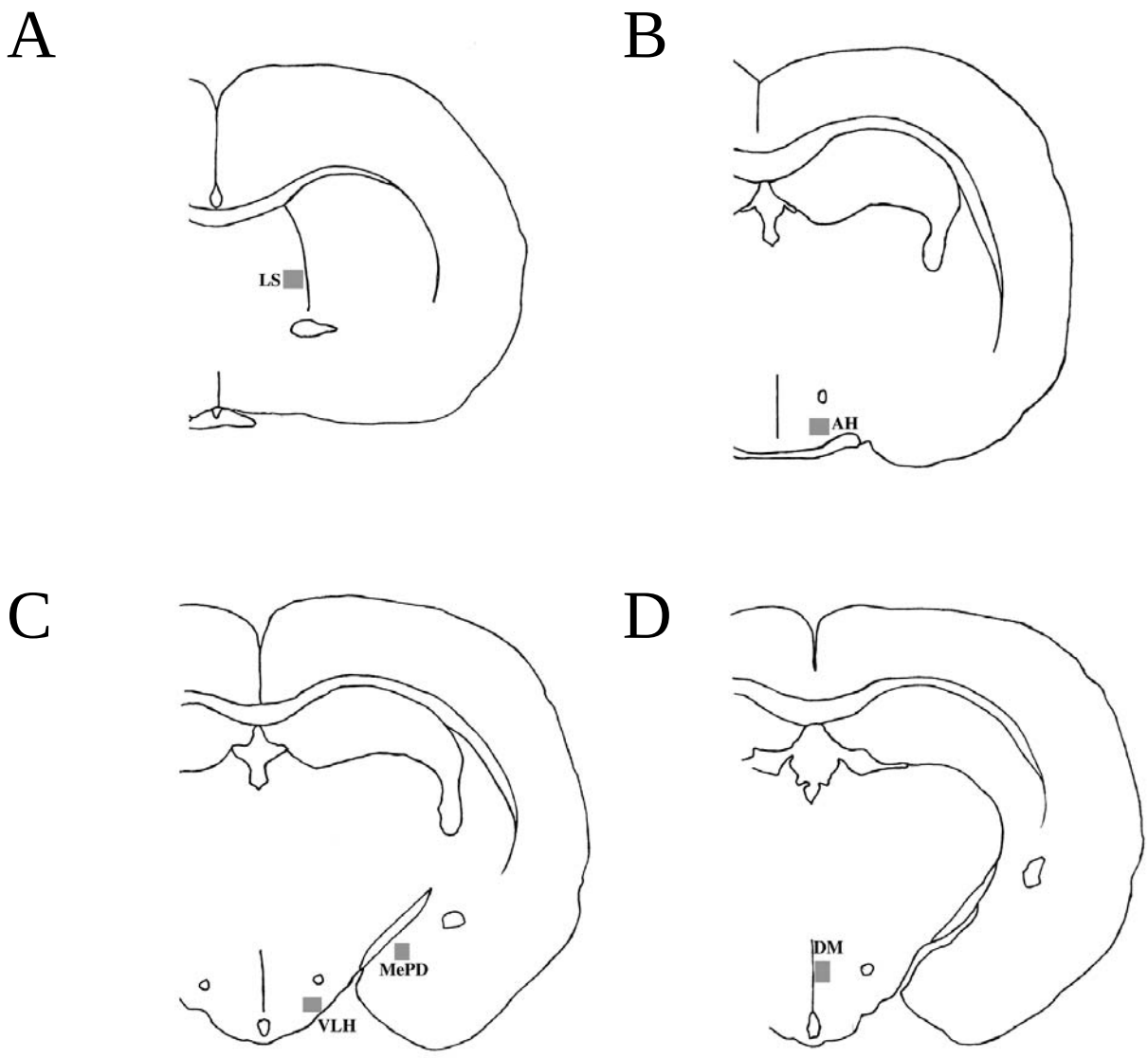
CRH Results

Developmental increases in CRH fiber density were observed in 3 of the 5 brain regions analyzed. During puberty CRH immunoreactive fiber density doubled within the AH [$F(2, 21)=11.8$, $p<0.001$, ANOVA]. Animals sacrificed on P-45 and P-70 showed a greater density of CRH immunoreactive fibers than animals sacrificed on P-28 ($p<0.01$;

$p<0.001$ respectively). A two-fold increase in CRH fiber density was also observed within the VLH [$F(2,21)=36.8$, $p<0.001$]. Animals sacrificed on P-45 had a higher fiber density than animals sacrificed on P-28 ($p<0.001$). Additionally, animals sacrificed on P-70 showed higher fiber density than animals sacrificed on P-28 and P-45 ($p<0.001$ for both). Across puberty, CRH fiber density also incrementally increased within the DM [$F(2,21)=22.8$, $p<0.001$]. For this area, animals sacrificed on P-45 and P-70 the density of fibers was higher than in animals sacrificed on P-28 ($p<0.01$; $p<0.001$, respectively). The density of CRH fibers was also higher on P-70 than on P-45 ($p<0.01$). The effects of puberty on CRH fiber density were region specific, as no age-related differences were observed within the LS or MePD.

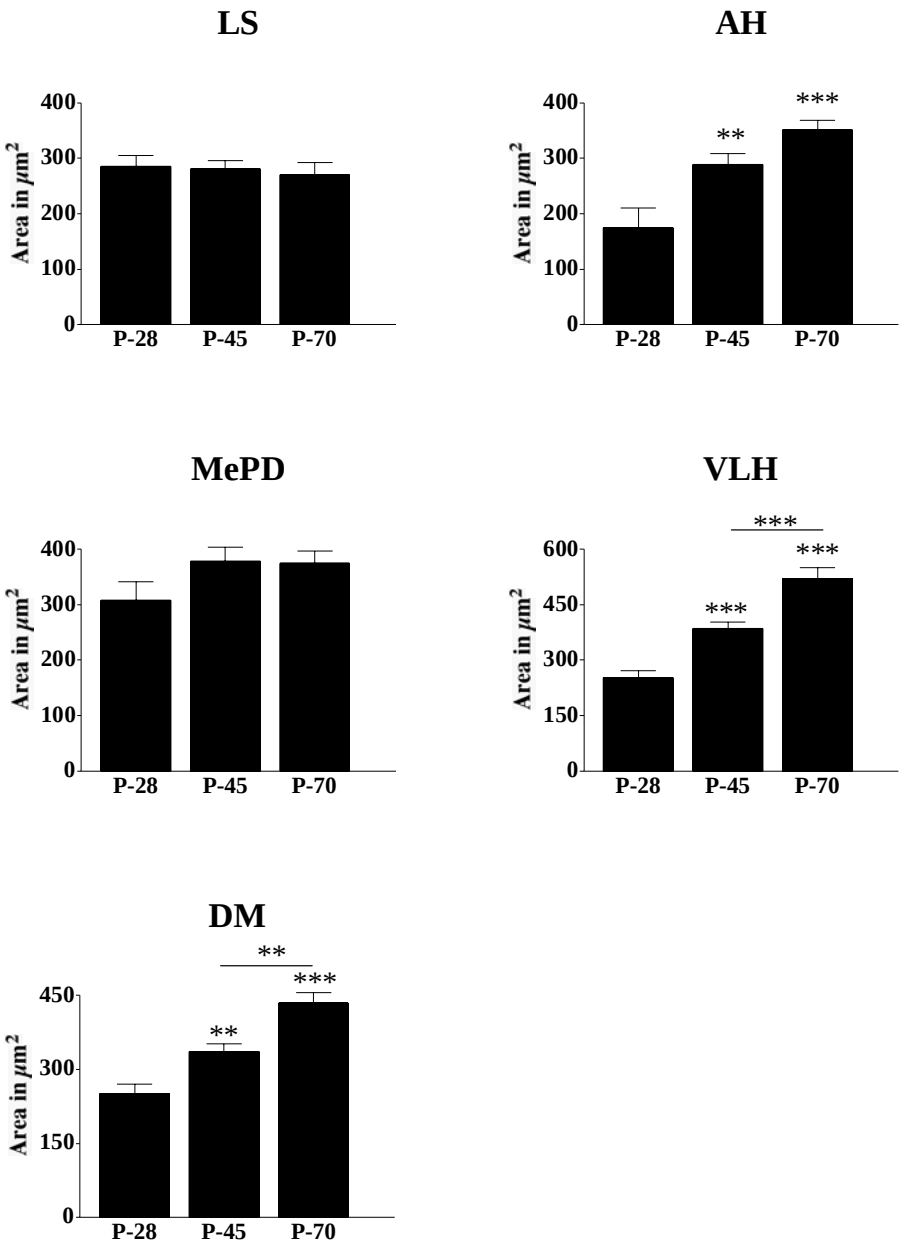
Repeated social stress also caused increased CRH fiber density within the VLH, trending towards significance [$t(11)=2.1$, $p<0.06$, Student's t-test]. In contrast, CRH innervation of the LS was decreased in subjugated animals [$T(11)=2.9$, $P<0.05$]. The effects of subjugation were site specific as group differences in fiber density were not observed for the AH, MePD, or the DM.

Figure 5.4: Brain areas of CRH innervation.



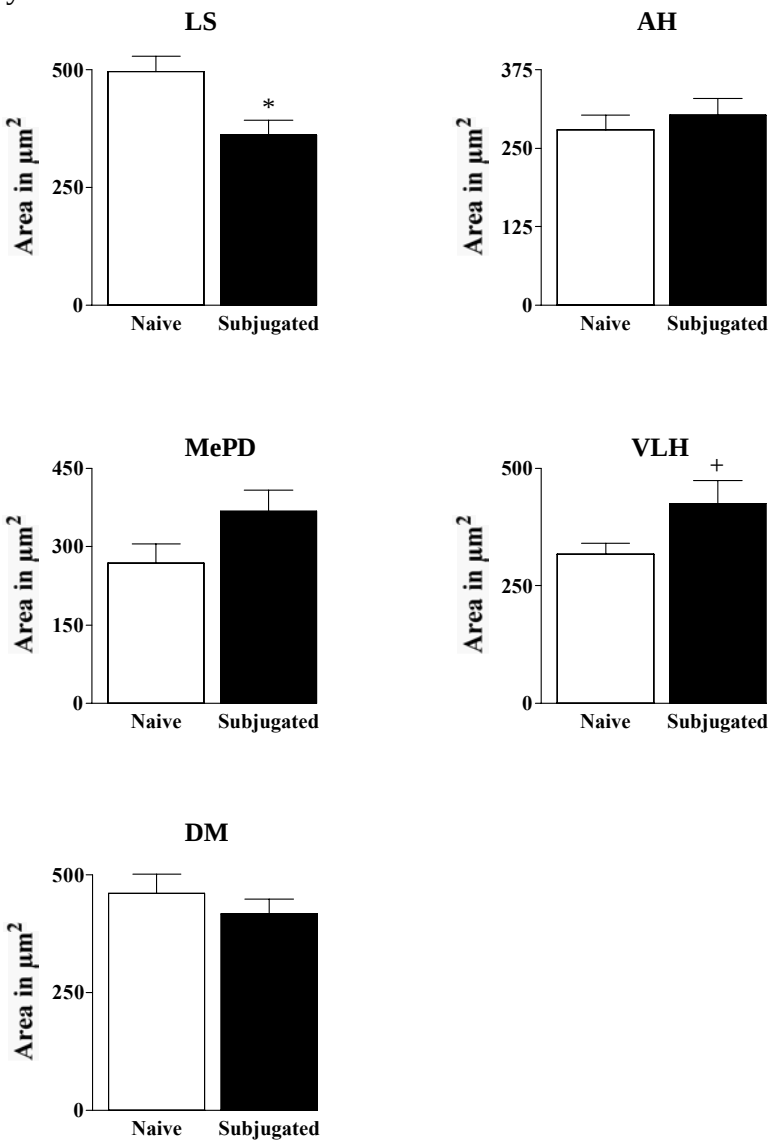
CRH innervation was quantified within the (A) the lateral septum (LS), (B) anterior hypothalamus (AH), (C) ventrolateral hypothalamus, posterodorsal portion of the medial amygdaloid nuclues, and (D) the dorsomedial hypothalamic nucleus.

Figure 5.5: The development of CRH innervation within the limbic system.



CRH fiber densities were compared between animals sacrificed during early puberty on postnatal day 28 (P-28), mid puberty (P-45), and early adulthood (P-70). ANOVA followed by Fisher's PLSD, **p<0.01, ***p<0.001. AH, anterior hypothalamus; DM, dorsomedial hypothalamic nucleus; LS, lateral septum; MePD, posterodorsal portion of the medial amygdaloid nucleus; VLH, ventrolateral hypothalamus.

Figure 5.6: The effects of social stress during pubety on CRH innervation of the limbic system.



CRH fiber densities were compared between subjugated and naive animals on postnatal day 43. Student's t-test, +$p<0.06$, *$p<0.05$. AH, anterior hypothalamus; DM, dorsomedial hypothalamic nucleus; LS, lateral septum; MePD, posterodorsal portion of the medial amygdaloid nucleus; VLH, ventrolateral hypothalamus.

CRH Discussion

This study reports several main findings. First, CRH innervation of several brain regions increases during puberty. Across puberty, CRH fiber density significantly increased within the AH, VLH, and DM. These developmental changes were region-specific, as CRH fiber density remained unchanged across puberty within the LS and MePD. Second, social subjugation caused a site-specific enhancement of CRH innervation within the VLH. In contrast, CRH fiber density within the LS decreased following two weeks of subjugation. Since these brain areas are involved in the control of social behavior, these observations implicate CRH as a potential mechanism for behavioral development and may also explain how social subjugation accelerates the development of offensive responses.

During puberty, CRH fiber density increased within limbic areas involved in the control of offensive aggression. In both the AH and VLH, CRH fiber density was twice as high on P-70 than on P-28. In male golden hamsters, both the AH and VLH show increased Fos immunoreactivity following the consummation of offensive aggression (Delville et al., 2000). In contrast, other areas involved in offensive aggression such as the LS and MePD showed no developmental changes in CRH fiber density (Delville et al., 2000; Potegal et al., 1981; 1996). Interestingly, the region-specific increases in fiber density within the AH and VLH parallel the transition from play fighting to adult aggression (Wommack et al., 2004). As such, pubertal increases in CRH innervation of these areas may mediate the behavioral transition from play fighting to adult aggression. Future studies will be required to address this topic.

Pubertal increases in CRH innervation were also observed in a brain region controlling cortisol release. The density of CHR fibers within the DM rose by approximately two-fold over the course of puberty. The DM is involved in the circadian timing of glucocorticoid release (Kalsbeek et al., 1996a; b). As such changes in CRH fiber density within the DM are potentially related to pubertal changes in the circadian release of cortisol. Data from chapter 3 showed that CRH innervation of the median eminence also increase during puberty. As such, it appears that CRH innervation of multiple areas controlling cortisol release change during puberty. Future studies would be required to test this hypothesis.

Social subjugation appeared to enhance CRH fiber density within the VLH that corresponded with developmental changes. CRH innervation was increased in the VLH of subjugated animals; however, the difference between groups was not quite statistically significant ($p<0.06$). It is possible that larger group numbers would yield statistically significant results. Nevertheless, this increase was site-specific and parallels the developmental changes observed in the VLH during development. Changes in CRH innervation of the VLH can be viewed as an accelerated development of the CRH system and may be related to the accelerated transition from play fighting to adult aggression observed in subjugated juveniles. Urocortin III innervation of the VLH is also increased in subjugated animals (Wommack, et al., unpublished observations). Urocortin III is a stress related neuropeptide that selectively binds to CRH type 2 receptors (Lewis et al., 2001). As such, the increase in urocortin III innervation suggests that CRH type 2 receptors are important for the behavioral effects of social subjugation during puberty.

Additional data in hamsters suggest that CRH type 1 receptors are also involved in the control of offensive aggression (Farrokhi et al., 2004), suggesting that this receptor subtype may also be involved in the development of offensive response. Currently, the distribution of CRH type 1 and 2 receptors within the VLH is not understood. Therefore, a number of future studies will be required to properly address this issue.

Recently, CRH has been implicated in the control of offensive aggression in hamsters (Farrokhi et al., 2004). Although the effects of CRH on play fighting behavior are unknown, it is possible that changes in the innervation of the VLH are related to some aspect of offensive aggression. Interestingly, subjugation did not alter CRH innervation of the AH, an area that showed pubertal increases in fiber density. Although I cannot rule out the possibility that CRH within the AH is involved in behavioral development, the current data present the possibility that the accelerated development behavioral development of juvenile hamsters is mediated by CRH within the VLH. Futures studies will be designed to address this possibility.

Chapter 6: Conclusions

The primary goals of this dissertation were to investigate the neuroendocrine factors modulating the pubertal development of agonistic behavior by using golden hamsters (*Mesocricetus auratus*) as an animal model. Although there is a great deal of literature on neuroendocrine regulation of offensive aggression, the mechanisms responsible for pubertal changes in agonistic behavior are poorly understood (Sisk and Foster, 2004). Previous studies focusing on gonadal steroids have suggested that the HPG axis is not involved in the pubertal maturation of offensive response in male golden hamsters (Romeo et al., 2003). Alternative studies have shown that repeated social stress during early puberty is a factor capable of accelerating the development of offensive responses in male golden hamsters (Wommack et al., 2003). The latter findings led to the main hypothesis of this dissertation: the HPA axis controls the development of agonistic behavior during puberty in male golden hamsters. This hypothesis was explored through experiments designed to answer the questions enumerated below.

1) How do defensive forms of agonistic responses change over the course of puberty, and is this aspect of behavioral development influenced by repeated social defeat?

While the pubertal development of offensive responses in male golden hamsters has received detailed attention (Pellis and Pellis, 1988; Wommack et al., 2003), the development of other forms of agonistic responses, such as submission and defense, have been less explored in this species. Therefore, the first goal of this dissertation was to investigate the development of agonistic behaviors in a defensive behavioral context. As

subjugation accelerates the development of offensive responses (Wommack et al., 2003), this experiment also tested whether social stress could alter the potential development of other forms of agonistic behavior.

To understand the development of defense, submission, avoidance, and risk assessment, a behavioral test for defensive responses was developed. The behavioral responses of male golden hamsters were observed in the home cage of an aggressive adult for 20 minutes at the onset of puberty and other key points until early adulthood.

Unlike offensive responses, other forms of agonistic behavior displayed in a defensive context underwent no apparent changes during puberty. Male golden hamsters showed similar levels of defense, submission, and risk assessment regardless of the age at which they were tested. A developmental increase in avoidance behavior was observed, as the number of retreats performed during the 20-minute test increased across puberty. However, the number of attacks received by experimental animals also increased during puberty, suggesting that the increased avoidance was a result of increased aggression from the adult stimulus animals.

As no apparent developmental changes were observed in these behavioral measures, subjugation obviously did not alter the maturation of defensive responses. However, repeatedly defeated juveniles, as compared to socially naïve controls, showed a complete inhibition of risk assessment behavior during the period of daily subjugation. As the inhibition of risk assessment was only transient, this effect can be viewed as an adaptation to repeated defeat rather than an altered developmental trajectory.

It is unclear whether the lack of pubertal changes in defensive responses was indicative of a true pattern of behavioral development or arose from methods of behavioral analysis. For example, experiments on defensive responses in hamsters that used more qualitative behavioral analyses could potentially uncover developmental changes not observed in the current study. Additionally, studies on defensive responses of male golden hamsters using a different testing paradigm have suggested that these behavioral changes during puberty (Pellis and Pellis, 1988a). This issue will have to be addressed in future studies. As the data stand, these findings in hamsters contrast with rats, as the latter species shows significant changes in defense across puberty (Pellis, 2002; Pellis et al., 1992; Pellis and Pellis, 1997). This disparity would not be the first species difference observed in the development of defensive responses, as the pubertal changes in offensive responses also differ between hamsters and rats (Pellis, 2002; Pellis and Pellis, 1988a; Wommack et al., 2003). These apparently separate developmental patterns of defensive responses emphasize the importance of recognizing species differences in the maturation of agonistic behaviors.

2) Does activity of the HPA axis of male golden hamsters change during puberty?

I hypothesized that the HPA axis controls the development of offensive responses in male golden hamsters. This possibility was recognized based on findings from my earlier experiments and a number of studies in golden hamsters and other species. In male golden hamsters, offensive responses undergo a transition from play fighting to adult aggression during puberty (Pellis and Pellis, 1988a; Wommack et al., 2003).

Previous studies have shown that this aspect of behavioral development is not controlled by testosterone (Romeo et al., 2003). While testosterone has yet to be shown to affect behavioral development, social subjugation, a potent activator of the HPA axis in adult hamsters, results in an accelerated transition from play fighting to adult aggression (Huhman et al., 1991; 1992). A handful of experiments have reported pubertal changes in glucocorticoid levels in other species, suggesting that HPA activity could also change during puberty in male golden hamsters (Gomez et al., 2002; Elmlinger et al., 2002; Jonetz-Mentzel et al., 1993; Kiess et al., 1995; Romeo et al., 2004; Van Kampen and Fuchs, 1998; Vazquez, 1998). Therefore, I hypothesized that activity increases during puberty in male golden hamsters. To test this hypothesis, I designed several studies to investigate the pubertal development of the HPA axis and the effects of social defeat during puberty on plasma cortisol levels.

In the first experiment, I sought to determine whether social defeat during puberty increased cortisol levels in juvenile hamsters. To test this, I measured cortisol levels just before or after social defeat or exposure to a novel cage on the on a single day, P-28. Both of these treatments caused increases in cortisol on P-28, and these data confirmed reports in adult hamsters that exposure to a novel environment and social defeat activate the HPA axis (Huhman et al., 1991; 1992; Weinberg and Wong, 1986). Therefore, to determine if juveniles were capable of habituating to each of these stressors, I additionally assayed cortisol levels following defeat or 20 minutes in a novel cage after two weeks of daily exposure to each respective treatment, on P-42. On this test day, only social defeat increased cortisol levels from baseline. These data suggest that social defeat

results in daily increases in cortisol throughout the period of subjugation. Therefore, subjugation can be viewed as a stressor to which juveniles do not habitutate.

In a second experiment, I sought to determine whether baseline and post-stress cortisol levels increased during puberty and whether or not HPA development could be affected by social subjugation. In this study, cortisol levels were assayed in samples collected in naïve and subjugated animals under basal conditions or immediately following social defeat on various days across puberty. Both baseline and post-defeat cortisol levels increased across puberty in subjugated and naïve animals. Importantly, the most dramatic developmental increases in cortisol levels were observed around mid puberty (P-45). As offensive responses also dramatically change during this time, it is possible that this development of offensive responses is related to changes in cortisol levels.

These data confirmed my earlier hypothesis that HPA activity increases during puberty in male golden hamsters. However, data from chapter 2 showed that the number of attacks received during social defeat increases during puberty. As such, it was not clear whether the age-related increases in post-defeat cortisol levels resulted from developmental changes or enhanced aggression from the larger animal. It was also unclear whether or not any other aspects of the HPA axis changed during puberty. To address these issues, I conducted a third experiment using restraint as a standardized stressor that would not change in intensity over the course of puberty. I also measured CRH innervation of the median eminence to determine whether or not neural components of the HPA axis also changed during puberty. Both post-restraint cortisol levels and

CRH innervation of the median eminence increased during puberty. These data confirm that HPA activity increases during puberty in male golden hamsters and show that the developmental changes in this system included alterations in neural control of stress glucocorticoid release.

These findings suggest that, similar to humans and tree shrews, HPA activity increases during puberty in male golden hamsters (Elmlinger et al., 2002; Jonetz-Mentzel et al., 1993; Kiess et al., 1995; Van Kampen and Fuchs, 1998). However, these findings contrast with reports in pubertal rats that show that this time is characterized by prolonged glucocorticoid release without developmental changes in baseline or peak corticosterone levels (Gomez et al., 2002; Romeo et al., 2004; Vazquez, 1998). This latter comparison emphasizes the idea that the development of the HPA axis varies widely between species, no matter how closely they are related.

In contrast to the effects on the HPA axis, social subjugation during puberty decreased testosterone levels. Basal testosterone levels were decreased in subjugated animals on P-45. Moreover, naïve animals showed an acute decrease in plasma testosterone levels following social defeat on P-45. Although the decrease in naïve animals was not statistically significant ($p<0.06$), these data indicate that social defeat suppresses HPG activity. These studies are also consistent with previous reports that testosterone is not involved in the transition from play fighting to adult aggression (Romeo et al., 2003). Socially subjugated animals had lower testosterone levels than naïve animals on P-45. However, earlier studies have shown that subjugated animals are more likely than naïve animals to perform adult-like offensive responses on this day

(Wommack et al., 2003). In view of the accelerated behavioral development caused by social subjugation, these data also show that testosterone is not likely involved in the maturation of offensive responses. Instead, the increasing cortisol levels are likely responsible for the transition from play fighting to adult aggression. This hypothesis was more directly tested in chapter 4.

3) Could increasing cortisol levels affect the pubertal development of offensive responses?

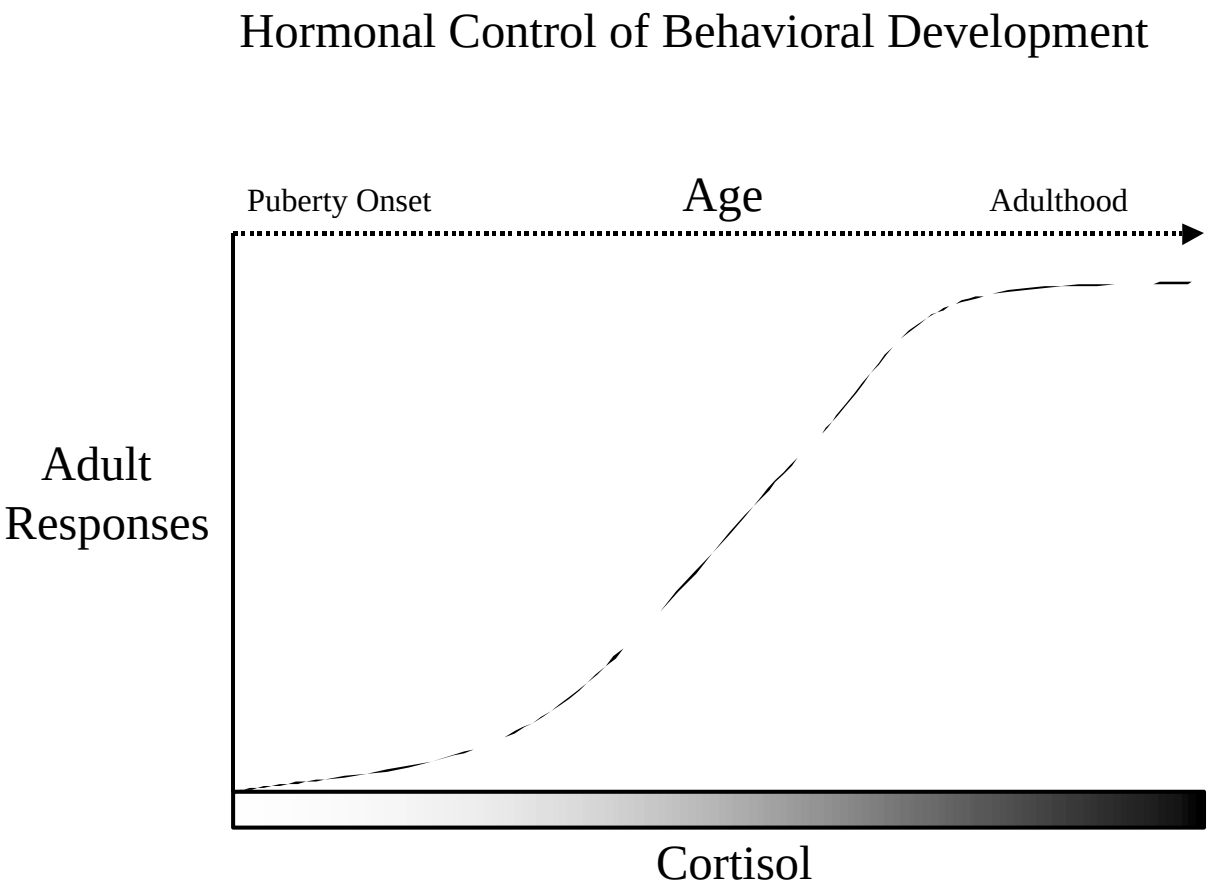
Findings from chapter 3 showed that pubertal increases in cortisol levels coincide with the transition from play fighting to adult aggression. Additionally, experiments from the previous chapter suggested that social subjugation, a factor that accelerates the transition from play fighting to adult aggression, causes a daily increase in cortisol levels. These finding were consistent with my overall hypothesis that the cortisol controls the development of offensive responses. Therefore, a series of experiments was designed to test whether daily increases in cortisol are sufficient to accelerate the development of agonistic behavior. Subsequent experiments were conducted to determine the role of type II corticosteroid receptors in the development of offensive responses

As hypothesized, animals that were repeatedly injected with cortisol showed a more advanced behavioral repertoire than vehicle-treated animals. This outcome was reflected in the increased percentage of adult attacks and decreased percentage of play fighting attacks observed in cortisol-treated animals. Moreover, the subsequent experiment showed that the effects of cortisol are likely mediated via type II corticosteroid receptors, as co-administration of RU-486, a type II receptor antagonist,

blocked the behavioral effects of cortisol. An additional experiment in which repeated injections of dexamethasone, a type II receptor agonist, could mimic the effects of cortisol treatment and social subjugation, confirmed the hypothesis that corticosteroids influence the development of agonistic behaviors through a specific receptor pathway.

Together with the data from chapter 3, the current findings can be used to construct a theoretical model for the interaction between cortisol and the development of offensive responses (Fig. 6.1). As HPA activity increases coincide with behavioral development, cortisol can be considered the driving force behind the transition from play fighting to adult aggression. Data from the current studies confirm this hypothesis and suggest that this hormone-behavior relationship is mediated by increasing activation of type II corticosteroid receptors. As such, any factor (e.g. social defeat or possibly daily restraint) or manipulation (e.g. cortisol injections) that increases the activation of type II corticosteroid receptors also accelerates the ongoing transition from the juvenile to the adult behavioral phenotype.

Figure 6.1: Schematic model of interaction between cortisol and the development of offensive respones.



The proposed interaction between cortisol and offensive responses provides an ecologically relevant model of behavioral development. As golden hamsters are a solitary species, puberty is a period of significant changes in an individual's social structure (Gatterman et al., 2001; Murphy, 1977). Prior to the onset of puberty, juvenile hamsters maintain a consistent level of social interaction with male and female

littermates. The onset of puberty corresponds with weaning from the natal group and dispersal of individuals into a solitary existence. In turn, the demands placed on the organism are much different due to the fact that individuals are no longer nourished or protected as they were within their natal group and become likely to receive attacks from conspecifics. During the pubertal phase, individuals must become capable of establishing and maintaining their own individual territories. Developmental increases in stress hormones during this transitional period could potentially enable the organism to cope with the increasing environmental demands by altering energy utilization and shaping appropriate behavioral responses such as aggression.

4) What is the neural mechanisms that controls the development of offensive responses?

In addition to the endocrine control of behavioral development, the neural mechanisms responsible for the maturation of agonistic behaviors are also poorly understood. Based on previous studies, I first set out to investigate the influence of dopamine neurons within the BSTPM and MePD on the development offensive responses. Additionally, data from chapter 3 suggested that CRH innervation was altered during puberty, and a recent study showed that CRH influences offensive aggression in male golden hamsters (Farrokhi et al., 2003). Therefore, a second set of studies was designed to analyze CRH innervation of brain areas involved in aggression.

TH

Previously, I reported that social subjugation, a factor that influences behavioral development, also results in increased TH-IR within dopamine neurons in the posterior portion of the medial division of the bed nucleus of the stria terminalis (BSTPM) and the posterodorsal portion of the medial amygdaloid nucleus (MePD). Data from the Melloni group have demonstrated that increased dopamine activity, via repeated cocaine treatment, increases aggression in male golden hamsters (Harrision et al., 2000; DeLeon et al., 2002b). As such, the increased TH expression within the BSTPM and MePD were hypothesized to be the neural mechanism by which subjugation altered the development of offensive responses. For this hypothesis to be correct, TH expression within these brain areas should increase in naïve animals during puberty. Moreover, to correlate with the accelerated transition from play fighting to adult aggression caused by repeated defeat, subjugated animals should show an early increase in TH-IR. My earlier study only measured TH-IR on a single developmental day during mid puberty, and therefore, did not address this possibility. As a result, I designed a study to investigate changes of TH expression within these neural populations across multiple time points during puberty to better understand their relationship to behavioral development. Results from this study confirmed my earlier findings that subjugation increases dopamine neurons within the BSTPM and MePD. However, longitudinal changes in these cell populations led me to reject the hypothesis that these neural populations were in the development of offensive responses. In socially naïve animals, the TH-IR decreased during puberty. In contrast, TH-IR remained high in subjugated animals during the period of daily stress. If these

neurons were to be involved in an acceleration of behavioral development, one would expect a more rapid increase in TH expression, not a slower decrease.

While not associated with changes in offensive responses, the TH expression within the MePD and BSTPM correlated with the effects of stress on risk assessment. Specifically, TH-IR was highest in subjugated juveniles during the period of daily defeat, a time when risk assessment was completely inhibited in these animals. Moreover, TH-IR did not differ between naïve and subjugated animals following a 4-week period of recovery, on P-70. On this day, naïve and subjugated animals also engaged in similar amounts of risk assessment behavior. Data from an additional experiment showed that social subjugation during adulthood also increased TH-IR within the BSTPM and MePD. Other reports in adult hamsters have shown that social defeat during this time also decreases risk assessment behavior. As such, it is likely that increases in TH-IR within the BSTPM and MePD are related to the transient behavioral adaptation to repeated social defeat. Further studies are required to properly address this possibility.

The precise mechanisms responsible for the stress-induced increases in TH-IR were not specifically addressed within these experiments. However, the differences between subjugated and naïve animals likely resulted from changes in TH mRNA expression. Previous studies on dopamine neurons within the BSTPM and MePD of adult hamsters showed that these cells contain androgen receptors and are testosterone-dependent (Asmus and Newman, 1993). Therefore, gonadal hormones could also play a role in TH expression within these areas. Indeed, studies in rats have shown that castration reduces TH mRNA expression (Kumai et al., 1995). However, data from

chapter 3 showed that testosterone levels increased during puberty in naïve animals while TH-IR decreased. Also in chapter 3, subjugated animals had higher levels of TH-IR, yet lower testosterone levels than naïve animals. Together, these results show that differences in TH expression between subjugated and naïve animals are unrelated to signaling through the androgen receptor pathway. Studies in rats have shown that stress increases TH mRNA within dopaminergic neurons (Angulo et al., 1991). The effects of stress on TH expression could result from either mechanisms of neural transmission or steroid action. For example, *in vitro* studies have shown that TH expression is regulated by both cAMP and glucocorticoid responses elements in the upstream promoter region of the TH gene (Hagerty et al., 2001a; 2001b; Piech-Dumas and Tank, 1999). As such, changes in TH expression could result from neural activation, increases in glucocorticoid levels, or a combination of both. Future studies would be required to determine the specific roles each of these regulatory pathways.

CRH

Additional experiments focused on CRH as another neural factor capable of influencing behavioral development. Studies in adult hamsters have shown that CRH can activate or inhibit offensive aggression (Jasnow et al. 1999; Farrokhi et al., 2004). Central injections of antagonists can block the inhibition of offensive observed in socially defeated adult hamsters (Jasnow et al., 1999). Additionally, oral administration of a CRH type I receptor antagonist inhibits offensive aggression in adult male hamsters (Farrokhi et al., 2004). These reports, coupled with the pubertal increase in CRH innervation of the

median eminence reported in chapter 3, led me to ask the question of whether CRH innervation of various other brain regions also underwent developmental alterations. If CRH is involved in the transition from play fighting to adult aggression, patterns of CRH innervation should change within brain areas controlling offensive aggression. Additionally, as social stress accelerates behavioral development, social subjugated juveniles should show corresponding changes in CRH innervation within these areas.

To investigate this possibility, CRH innervation was analyzed across puberty in a number of brain areas. Interestingly, CRH innervation increased in two brain areas involved in the control of offensive aggression, the anterior hypothalamus AH and the ventrolateral hypothalamus (VLH) (Delville et al., 2000). These data suggest that CRH not only affects aggression but also is involved in the development of offensive responses.

To further investigate the role of CRH on the development of offensive responses, CRH innervation of the previously analyzed brain regions was also compared between subjugated and naïve animals. Similar to the original hypothesis regarding TH in the BSTPM and MePD, if CRH innervation was to be involved in the development of offensive responses, one would expect the changes in fiber density that corresponded to an accelerated behavioral development. Indeed, CRH innervation of the VLH was increased in subjugated animals. As this area is involved in the control of offensive aggression (Delville et al. 2000), it is quite possible that the increased CRH innervation of this brain region is involved in the acceleratory effects of social stress on the

development of offensive responses. This idea is currently being developed, and further experiments will certainly be required to test this hypothesis.

While CRH innervation and corticosteroid receptors are both associated with the transition from play fighting to adult aggression, it is unclear whether these factors are mechanistically related. As type II corticosteroid receptor activation is critical for the development of offensive responses, it is possible that increased CRH innervation of the VLH was driven by this mechanism. But at first look, this possibility seems unlikely. A larger number of studies have shown that glucocorticoids negatively affect CRH synthesis, storage, release, and expression (Watts, 2005). However, these experiments have largely focused on the negative feedback of glucocorticoids on neurosecretory neurons within the paraventricular hypothalamus. Studies focusing on other populations of CRH neurons have shown that the relationship between glucocorticoids and CRH expression varies from region to region (Reviewed in Watts, 2005). For example, glucocorticoids enhance CRH expression within the central amygdala (Watts and Sanchez-Watts, 1995). As the source of CRH innervation of the VLH is undetermined, the increase in CRH immunoreactivity observed during puberty and following stress, could result from region-specific expression patterns in areas other than the paraventricular hypothalamus. Moreover, the CRH gene lacks a consensus glucocoricoid response element, and glucocorticoid-mediated CRH expression often involves secondary pathways (Beato et al., 1995; Brann et al., 1995; Vamvakopoulous and Chrousos, 1993). It is also possible that the mechanisms mediating glucocorticoid/CRH interactions are altered during puberty, and allow for cortisol to enhance CRH expression during this

time. As the role of CRH and glucocorticoids in the pubertal development of offensive aggression has only recently been explored, future studies will be required to properly address these issues.

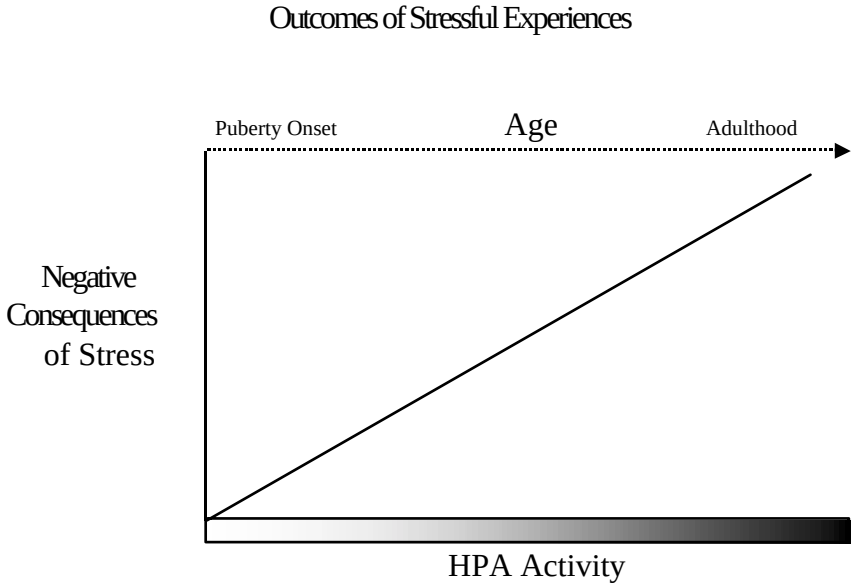
Juvenile Resiliency to Social Stress?

Another main finding that emerged from these studies was the possibility that juveniles are resilient to the effects of social stress. In adult hamsters, social defeat increases submissive behavior and completely inhibits offensive aggression, a phenomenon known as conditioned defeat (Potegal et al., 1993; Huhman et al., 2003). Moreover, these behavioral effects can persist for up to a month following the last defeat. The effects of social stress during adolescence are markedly different in male golden hamsters. Previous studies have shown that social subjugation enhanced aggression in juveniles (Delville et al., 1998; Wommack et al., 2003). The current study showed that, while social subjugation resulted in decreased risk assessment, decreased testosterone, habituated stress responses, and increased TH within the BSTPM and MePD, these effects were only observed during the period of daily subjugation. Following a four-week recovery period, none of these variables differed between subjugated and naïve animals. Their lack of persistence indicates that the effects of social stress during early puberty are transient and represent adaptations to repeated defeat rather than long-term changes in the phenotype of the animal. In other words, repeated defeat during puberty does not cause the male hamsters to become lifelong subordinates like subjugated adults. Instead, socially subjugated juveniles are more capable of adapting to defeat and retain

their ability to become dominant adults. The concept of juvenile resiliency has potential ecological significance. In areas of high population density, juveniles would likely be defeated frequently as they encountered adult males. However, the ability of these juveniles to adapt to social defeat would enable the younger generations to attain dominance once they reach adulthood.

The age-related consequences of social stress are potentially explained by the development of the HPA axis (Fig. 6.2). During early puberty, when activity of HPA axis is low, the consequences of stress are not as severe or long lasting as the consequences of social defeat during adulthood (Huhman et al., 2003; Potegal et al., 1993; Wommack et al., 2003). These findings allow for the construction of a model predicting that the negative outcomes of stressful experiences become more likely as the HPA axis matures.

Figure 6.2: Schematic showing the proposed relationship between the development of the HPA axis and the likelihood that stress will produce negative consequences.



Closing Remarks

This dissertation investigated the relationship between stress and the development of social behaviors. The central finding of these studies is that pubertal changes in the HPA axis control the development of social behaviors. These studies also contributed to an emerging picture of puberty as a period when the consequences of stressful experiences are unique. None of these findings would have been found if the HPA axis did not undergo pubertal development. While pubertal changes in the glucocorticoid levels of male golden hamsters contrast with reports in rats, they also correspond with reports from other species such as tree shrews and humans. This difference emphasizes

the possibility that the nature of developmental changes in HPA activity is species dependent. Moreover, as rising cortisol levels were shown to accelerate the development of offensive aggression in male golden hamsters, it appears that stress hormones affect behavioral development in other species. Similar to the development of the HPA axis, the development of social behaviors widely varies, even between closely related species. Therefore, future studies should be carefully designed to investigate the relationship between stress hormones development of social behaviors in other species.

Existing data from other species show similar relationships between stress hormones and the development of social behaviors as reported in this dissertation. One example of how stress can accelerate behavioral maturation comes from eusocial wasps, a nonvertebrate species. In these wasps, biting is a social cue that causes individuals to show an accelerated transition from the role of nest repairer to forager (O'Donnell, 2003). Although the physiological mechanisms responsible for the accelerated transition are not understood, it is possible that this effect is caused by increases in stress-related neurotransmitters. Due to the similarity of effects of stress in such largely separate species, it is possible that similar interactions between stress hormones and behavioral development exist in a diverse array of other animal species.

The age-related effects of social stress can also be extended from hamsters to other species. Data from hamsters prompted us to predict that negative consequences of social stress become more likely as HPA activity increases during puberty. Applications of this model in human adolescents have produced promising results. Similar to hamsters, basal cortisol levels increase during puberty in humans (Elmlinger et al., 2002;

Jonetz-Mentzel et al., 1993; Kiess et al., 1995). As such, the model for determining the outcome of stressful experiences predicts that social stress would more profoundly affect individuals as puberty progressed. Indeed, this prediction was confirmed. In self-report studies, individuals that reported bullying during late puberty were more likely to experience feelings of depression than individuals that reported bullying during early puberty (Newman et al., 2004). Moreover, individuals that reported bullying during late puberty were also more likely to choose alcohol consumption as a coping strategy than individuals that were only bullied during early adolescence (Newman et al., 2004). While this model has only been tested in an undergraduate population, historical and anecdotal data suggest that this relationship is quite real. For example, compare the differences in aggressive behavior and mental health status between children raised in peaceful environments versus individuals born into war-torn or impoverished countries. Without much more thought, it becomes apparent that the implications of the current studies have valid, serious, and even frightening implications regarding the behavioral development in humans.

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Vita

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