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Repeated Social Stress and the Maturation of Sexual Behavior in Juvenile Male Golden Hamsters

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Repeated Social Stress and the Maturation of Sexual Behavior in Juvenile Male Golden Hamsters

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Dedication

This dissertation is dedicated to anyone who was told they could not. You absolutely can.

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Repeated Social Stress and the Maturation of Sexual Behavior in Juvenile Male Golden Hamsters

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In certain species, puberty is thought to be a period of susceptibility to various stressors, resulting in pathological behavioral and physiological changes subsequent to exposure during this period. However, juvenile male golden hamsters appear to be fairly resilient to pubertal stress, as compared to adult hamsters and many other species. In these experiments, repeatedly stressed juvenile male hamsters were found to be avoidant of aggressive adult male social stimuli, but did not display anxious behavior outside of a social context. In addition, several long-term changes in neural activity were associated with social stress during early puberty. The medial preoptic area and medial preoptic nucleus, and ventral tegmental area showed decreased neural activity in subjugated juveniles than in naïve individuals. Since these brain areas are involved in the expression of motivated behaviors, specifically sexual behavior, and reward pathways, we next investigated sexual behavior in virgin juveniles.

When placed in a confined space with receptive females, consummatory behavior in subjugated juveniles was similar to those observed in naive juveniles. Appetitive aspects of sexual behavior were also tested in a Y-maze to allow subjects to choose whether to approach a social stimulus. When given a choice between a sexually receptive and non-receptive female social stimulus, socially stressed individuals showed anxiety related behaviors and did not show a preference. However, naïve hamsters preferred the non-receptive female. Interestingly, this effect was less significant in naïve animals tested during late puberty and early adulthood, and a preference for sexually receptive females was not observed. In addition, stressed hamsters tested with harnessed females at mid-puberty were slower to approach females, indicating altered motivation to approach adult conspecifics. This research is unique in that it is the first to suggest the disconnect between the development of consummatory and appetitive aspects of sexual behavior. Together, these data examine the effects of stress on the development of pubertal social behaviors.

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Chapter 1: Introduction

Puberty

Definitions of Puberty

Puberty can be defined a number of ways. While the term "puberty" specifically refers to gonadarche (Graber & Brooks-Gunn, 1998), "adolescence" is a more general term referring to a wider spectrum of changes that occur during development and the time period is more loosely defined (Pickles et al., 1998). For the sake of simplicity, I will use the term "puberty" since it is tightly tied with the endocrinological changes that are more easily delineated, particularly in animal models. The timing of puberty, too, can be measured a number of ways. The most common way of measuring the start of puberty is increasing levels of growth hormones, gonadal steroid hormones, and then follicle stimulating hormone (FSH), luteinizing hormone (LH), and plasma testosterone (T). Other measures of the pubertal period can be used in animal models, however. Testes size and weight, seminal vesicle weight, body weight, and reproductive ability all change during puberty and are markers for this period. Motivated and social behaviors also develop during puberty, and it is possible that this is a critical period for emotional regulation. The overarching focus of these studies is how stress affects the development of social behaviors.

Stress Hormones and Puberty

Hormone changes that occur during puberty in certain species include development of the hypothalamo-pituitary-adrenal (HPA) axis. In humans, baseline cortisol levels increases steadily from the beginning of puberty to adulthood in correlation with Tanner stages of development (Elmlinger et al., 2002; Jonetz-Mentzel & Weideman, 1993; Kiess et al., 1995). Several other species show a similar increase during puberty, such as chimpanzees, tree shrews, Wistar rats, and hamsters (Cutler et al., 1978; Kiess et al., 1995; Pignatelli et al., 2006; Van Kampen & Fuchs, 1998; Wommack et al., 2004). In hamsters, both baseline and post-stress levels of cortisol increase gradually in a manner reminiscent of changes in the hypothalamo-pituitary-gonadal axis (HPG) axis (Wommack et al., 2004). In Wistar rats, adrenal weights also increase gradually during puberty (Pignatelli et al., 2006). Consequently, it is possible that pubertal chimpanzees, tree shrews, certain strains of rats and hamsters may be particularly useful in studying the development of stress because of the similarity in how the HPA axis matures. However, not all animal models follow this pattern of development. For example, basal serum corticosterone levels in Sprague Dawley rats remain the same throughout puberty and adulthood (Gomez et al., 2002; Romeo et al., 2004; Vasquez, 1998), and juveniles have a longer duration of recovery post stress, which is likely related to differences in concentration or functionality of glucocorticoid receptors (GR) (Goldman et al., 1973; Meaney et

al., 1985, Vazquez & Akil, 1993). In these studies, we utilized male golden hamsters in order to most closely match the development of human stress reactivity in order to conserve translational value. In hamsters, puberty starts around postnatal day 28 (P-28), and ends by P-70. Around P-45, the gonadal development reaches a midpoint, now referred to as mid-puberty in this dissertation.

Sex Hormones and Puberty

During puberty, the role of gonadal hormones, particularly testosterone (T) in the development of male sexual behavior is of particular interest. In juvenile male hamsters, T levels increase during puberty and are near, but not reaching, adult levels at mid-puberty (P-45) (Vomachka & Greenwald, 1979). Yet pubertal males are capable of copulating at age P-30 (Bond, 1945). This suggests that while T is necessary for the initiation of sexual behavior, adult levels of T may not be necessary for copulation. Indeed, the role of T in sexual behavior has been described as permissive in that activity of any one of several brain regions containing androgen receptors is sufficient for expression of sexual behavior (Sato et al., 2008; Coolen & Wood, 1999). A study on the role of T in juveniles suggests that puberty is a critical period for T to defeminize and masculinize sexual behavior (Schulz et al., 2004). However, because of issues related to the study design, the idea that T in puberty has an organizational role remains an intriguing possibility.

Behavioral Changes during Puberty

Behavioral changes also abound during puberty. The Ernst Model describes the change in approach, avoidance, and decision-making processes (Ernst & Fudge, 2009). The model describes the tendency for adolescents to approach conspecifics. This can be seen as risk-taking or pleasure seeking behavior. The tendency to approach individuals of the same species may be viewed as adaptive in the sense that it would encourage animals in puberty to leave their home nest. However, it may also make them more vulnerable to certain predators and competitors. Nevertheless, a drive to leave the nest and investigate potential competitors or available territories may also be seen as advantageous in a solitary species like hamsters. Pubertal hamsters leave the home nest to establish new territories. This would not be possible without a predilection toward approach of unfamiliar conspecifics and increased risk taking behavior. Indeed, increased risk taking behavior and sensation seeking during puberty are thought to be adaptive in the sense that these transitory changes help pubertal individuals survive challenges during this period as well as prepare for independent living and dispersal away from their family unit (Oppenheim, 1981).

Agonistic behavior also undergoes a transition during puberty. Most mammals undergo a transition from play fighting to adult types of aggression

during this period (Fagen, 1981). Mammalian species display agonistic behavior in the form of play fighting prior to the onset of puberty (Blanchard et al., 2003; Delville et al., 2003; Pellis, 2002). Rats are the most commonly used species in these studies. They begin to show play-fighting behavior around day P-20. Rough play is predominant from P-20 to P-30, then transitions to aggression that is purely playful. Next, around P-40 rats begin a form of rough play fighting that becomes adult aggression (Pellis & Pellis, 1990). This is roughly the pattern of development of aggression during puberty in hamsters, although rats continue to show play-fighting behavior throughout their lives, depending on the social context (Delville et al., 2003; Pellis & Pellis, 1993). In golden hamsters, the pattern of development of this behavior matures as follows. As soon as hamsters are capable of coordinated movement by P-18, they initiate play-fighting interactions (Goldman & Swanson, 1975; Siegel, 1985) and during puberty this behavior undergoes a transition to adult forms of aggression from P-45 to P-70 (Goldman & Swanson, 1975). There are quantitative differences in the attacks performed by juveniles versus adults. Juveniles have a higher frequency of attacks during early puberty around P-35 than adults at P-70 (Pellis & Pellis, 1988; Goldman & Swanson, 1975; Wommack et al., 2003; Taravosh-Lahn & Delville, 2004). In addition, qualitative differences in agonistic behavior occur as part of this transition. For example, in early puberty (P-35) attacks are targeted at the cheeks and face. By late puberty (after P-45), this pattern transitions to the aggressor attacking the underbelly and rump of his opponent

(Wommack et al., 2003). Unlike rats, hamsters stop displaying play-fighting behavior once they have transitioned to adult aggression (Wommack et al., 2003).

Sexual behavior also starts occurring during puberty. When allowed to interact with a sexually receptive female, pubertal males start attempting to mount, intromit, and ejaculate. They start attempting to mount and copulate with females around postnatal day 20 (Bond, 1945). Over time and experience, their behavior becomes better coordinated and they consistently attempt to copulate with females by P-45. Experience plays an important role in this behavior, as experienced copulators are faster and more efficient to consummate sexual behavior (i.e. ejaculate) and, therefore, copulating is considered a type of motor learning (Can et al., 2007; Pfaus et al., 2001; Woodson, 2002). However, copulatory behaviors do not account for the full spectrum of sexual behaviors. Appetitive aspects of sexual behavior are important because in order for reproduction to occur, motivation to approach mates and mate selection must also occur. Male sexual appetitive behavior includes approach components and underlying motivation, as well as mate selection. Besides enhancing the consummatory phase of the behavior, sexual experience has also been reported as critical to appetitive behavior as well (reviewed in Pfaus et al., 2001).

Neural Substrate of Sexual Behavior

In male hamsters, the medial preoptic area (MPOA) plays central role in consummatory sexual behavior (for review, please see Hull et al., 2006). Lesions of the area abolish the behavior (Powers et al., 1987; Floody, 1989). Testicular androgens that are necessary for expression of copulatory behavior (Beach & Pauker, 1949; Campbell et al., 1978) target this area. In hamsters, the MPOA is rich in neurons expressing estrogen receptors (ER) and androgen receptors (AR) (Wood & Swann, 1999). Testosterone administration to the area is also necessary and sufficient for the consummation of sexual behavior. While castration inhibits the behavior, implants specific to the area are sufficient to reinstate male sexual behavior (Wood & Swann, 1999).

However, the MPOA is not the sole neural site controlling the behavior. The extended medial amygdala also participates in the behavior, possibly relaying olfactory inputs to the MPOA (Newman, 1999). Lesions of the medial amygdala or medial division of the bed nucleus of the stria terminalis also abolish or severely the consummation of male sexual behavior (Powers et al., 1987). These areas are also rich in estrogen and androgen receptors (Wood & Swann, 1999). Furthermore, implants restricted to the medial amygdala are as effective on restoring the consummation of male sexual behavior as implants into the MPOA (Wood & Newman, 1995).

While the medial amygdala relays olfactory inputs to the MPOA (Maragos et al., 1989), neurons in the MPOA and medial amygdala form reciprocal connections (Gomez & Newman, 1992; Wood & Swann, 2005). Thus, these two areas have been proposed to be the basis of a neural network modulating sexual behavior in males as well as other social behaviors, the Social Behavior Network (Newman, 1999). This network is constituted primarily of the MPOA, medial amygdala, medial division of the bed nucleus of the stria terminalis, the anterior hypothalamus, the ventolateral hypothalamus and the lateral septum. This network has been identified on the basis of reciprocal neural connections and neural activation in the form of c-*Fos*, an immediate early gene product (IEG). Indeed, sexual behavior is associated with enhanced IEG expression in the MPOA and other areas of this neural network (Baum & Everitt, 1992; Coolen et al., 1996; Fernandez-Fewell & Meredith, 1994; Heeb and Yahr, 1996; Kollack-Walker & Newman, 1995; Robertson et al., 1991). Though different patterns of activation can be observed under different behavioral conditions, such as sexual behavior or aggression (Kollack-Walker & Newman, 1995; Delville et al., 2000). It must be noted, though, that these studies and observations of IEG activation in this network were based mostly on a single behavioral interactions. Immediate early gene (IEG) expression in brain regions is affected by sexual experience. In Japanese quail, sexual experience is associated with a reduction of c-fos expression in various elements of the sexual behavior network that includes the POA, BST and AMY (Can et al., 2007). Indeed, sexually experienced animals no

longer show activation of the immediate early gene zenk in POA and BST with sexual behavior. This effect may be explained by long-term changes in metabolic activity at the level of the synapses mediating the network. This possibility has been tested in lizards. A study of long-term activity of brain regions in lizards showed that experience enhanced activity and modified correlations in areas such as the POA (Sakata et al., 2002).

While a wide variety of neurotransmitters have been associated with the control of sexual behavior within the Social Behavior Network, dopamine (DA) appears to play a key role (see Dominguez & Hull, 2005 for review). Dopamine release in this area is gonadal-hormone regulated (Putnam et al., 2003) and contributes to motivation to perform sexual behavior (Hull et al., 1995). T and sexual behavior alone do not increase DA in the MPOA, however. Indeed, chemosensory input is necessary for this rise in DA (Sato & Wood; Wood & Swann, 1999; Triemstra et al., 2005). Further, it is important to note that while rats with ablated olfactory bulbs continue to display sexual behavior, while olfactory bulbectomized hamsters do not (Beach, 1942; Stone, 1922; Stone, 1923; Wood & Newman, 1995). Testosterone levels rise in adult male hamsters in response to olfactory perception of vaginal discharge of estrous females (Macrides et al., 1974). Similarly, stimulation of the medial amygdala in rats increases MPOA dopamine release (Dominguez & Hull, 2001).

Stress and Behavior

Puberty versus Adulthood

Puberty has been described as both a time of vulnerability and resilience. This period has been discussed as a paradox of increased physical robustness and increased mortality rates during this time (Dahl, 2004). Research in rats indicates that puberty in these animals is a period of enhanced vulnerability to stress. As explained earlier, stress responses last longer in puberty, as corticosterone levels take longer to come back to their baseline after a stressor (Gomez et al., 2002; Romeo et al., 2004; Vasquez, 1998). In addition, stress during puberty is associated with substance abuse and enhanced anxiety (Avital & Richter-Levin, 2005; Avital et al., 2006; Spear, 2002). Thus, it has been proposed that individuals during puberty are particularly vulnerable to stress, as compared to childhood and adulthood (Spear, 2009).

However, not all species show enhanced stress responsiveness during puberty, nor do they have a potential for longer periods of exposure to high levels of cortisol. As explained earlier, the activity of the HPA axis matures slowly during puberty in humans as well as in hamsters. Enhanced vulnerability to substance abuse in puberty could instead be related to the development of reward systems during puberty. The Ernst Triadic Model predicts a higher level of activity either within the ventral tegmental area and its main connections; the nucleus accumbens and prefrontal cortex (Ernst & Fudge, 2009). In particular,

puberty has been associated enhanced dopamine release under specific conditions (Robinson et al., 2011). Thus enhanced vulnerability to stress in drug taking conditions may have more to do with the maturation of reward systems than the HPA axis.

The case for resilience to stress in puberty is best illustrated by studies in hamsters. While exposure to stress in adulthood has widespread inhibitory effects on sexual and aggressive behavior in adult hamsters, different outcome have been observed in juveniles exposed to stress (Cordner et al., 2004; Delville et al., 1998; Potegal et al., 1993; Wommack et al., 2003). For instance, hamsters exposed to repeated social subjugation in early puberty showed enhanced aggression toward smaller intruder in adulthood (Delville et al., 1998, Wommack et al., 2003). In addition, acute effects were observable, such as inhibition of aggression towards same sized-individuals and avoidance of larger adults, though these effects were transient (Delville et al., 1998; Wommack et al., 2004). Furthermore, while stress in adulthood is associated with an inhibition of the HPG axis, observable by strong decline in plasma T levels (Huhman et al., 1991), the effects of stress on T in juveniles were modest and short lasting (Wommack et al., 2004). Taken together, these data point to a relative resilience of these animals to the effects of stress. This, coupled with the similarity of HPA development with the HPA development in humans, prompted these experiments.

Types of Stressors

Stress has been defined in a number of ways, and is at times difficult to elucidate (Levine & Ursin, 1991). Most definitions converge around upsetting a homeostatic balance, be it a psychological or physiological set point. Factors such as maternal care and individual differences in novelty-seeking and short-term memory greatly influence stress responsiveness (Dulcot et al., 2011; Schmidt et al., 2010; Walker, 2010). In addition, the effects of a stressor are dependent on the type of stressor experienced, severity of the stressor, timing of the stressor, frequency, and also the internal state of the animal being tested. Further, effects of stress can be temporary or long-lasting and can range from mild changes in body weight to generalized states of fear or anxiety as seen in animal models of depression, such as learned helplessness in rats (Maier, 1984).

One type of stressor that is particularly relevant to the study of social behaviors is social stress. Social stress is an ecologically relevant stressor that is an animal model for depression. This stressor involves the source of stress being the social context of an animal. In social species, overcrowding, isolation, and the defeat experienced by individuals lowest in the hierarchy are common social stressors used in a laboratory setting. Since most animals in the wild would conceivably experience social stress, the effects that follow are relevant to the animal being studied, as opposed to more artificial means of stressing an animal.

In adults, social stress enhances anxiety in social and non-social contexts. Socially stressed rats display a generalized state of fear, as evidenced by increased anxiety and fear in elevated plus mazes and open field arenas that are non-social settings (Katz et al., 1981; Zelena et al., 1999). Similarly, the aggression of adult hamsters is inhibited, avoidance of threatening and non-threatening conspecifics is increased, androgen levels are decreased, and body weight increases (Foster et al., 2006; Huhman et al., 1991; Huhman et al., 2003; Potegal et al., 1993; Solomon et al., 2007). Sexual behavior can be inhibited by stress, but this is not always the case (Hamilton et al., 2008). Indeed, the effect on sexual behavior is dependent on the type and frequency of the stressor (Tilbrook et al., 2002). Further, the neural substrate specific to the type of stressor and the pathway affected are important to establish in order to make predictions about whether sexual behavior.

Given the generalized effects in adult rodents, we sought to determine whether stress had the same effects during puberty in hamsters. Since the peak of cortisol in golden hamsters lower than that of rats (Delville et al., 2003), it is possible that stress would have less of an effect. Indeed, juvenile hamsters appear to be more resilient to social stress. While acute effects are observable, such as inhibition of aggression towards same sized-individuals and avoidance of larger adults (Wommack et al., 2005), effects during puberty are transient. Decreases in plasma testosterone levels are short-lived and body weight is only modestly affected in subjugated juveniles (Delville et al., 1998; Wommack et al.,

2004). In addition to these effects, further testing is necessary to determine how general the effects are in these animals.

Neural networks of stress

As stress responses are manifested by an activation of the HPA axis, they obviously involve activity of parvocellular CRH neurons in the pavaventricular nucleus of the hypothalamus. But stress responses are not limited to HPA axis activation. Stress responses also include an activation of the sympathetic nervous system. These are controlled by outputs of the paraventricular nucleus and central amygdala acting directly and indirectly on the nucleus of the solitary tract and nucleus ambiguous to inhibit the parasympathetic nervous system and activate sympathetic motor neurons in the vicinity of the nucleus ambiguous. In addition, sensory inputs relevant to stressful situations are processed at the level of the basolateral amygdala which connects to the central amygdala. The central amygdala coordinates behavioral, autonomic and behavioral responses at least partially through its connections with the hypothalamus (for review, see Ledoux, 2007). In adult hamsters, the memory of social defeat is enhanced by overexpression of cyclic AMP response element binding protein (CREB) within the basolateral amygdala (Jasnow et al., 2005). This circuitry is also connected to several parts of the cortex, in particular the prefrontal cortex, insular cortex and entorhinal cortex (for review see Dallman et al., 2002).

Just as IEG expression has be used to study neural networks associated with social behavior, the same procedure has been used to study systems associated with stress. Generally, IEG expression is typically increased during initial exposure to a stimulus, but after repeated exposures to a stimulus IEG expression decreases. Stressful stimuli follow this pattern; acute stress exposure increases IEG expression in many brain areas, while repeated stress causes fewer increases in IEG expression (Girotti et al., 2006; Kollack-Walker et al., 1999; Stamp & Herbert, 1999; Ryabinin et al., 1999). The same is true for social defeat (Martinez et al., 1998). Rats defeated once during adulthood showed increased expression in the lateral septum (LS), bed nucleus of the stria terminalis (BNST), lateral preoptic area (LPOA), lateral hypothalamic area (LHA), paraventricular nucleus (PVN), medial amygdala (MeA), and central amygdala (CeA) an hour after a single defeat. However, increased *c*-fos expression was only observable within the BNST, PVN, and MeA after repeated defeat. Thus, patterns of neural activation in repeatedly socially stressed juveniles may closely resemble the neural activation in repeatedly defeated rats.

Neural activity changes involving immediate early genes utilize genomic action potentials, meaning that the activation of immediate early genes affect the firing of a cell as opposed to the opening and closing of ion channels. This process operates on a much slower time scale and IEG activation sets up the brain to efficiently form memories. Further, genomic action potentials alter the physical remodeling associated with plasticity in neuronal circuitry (Clayton,

2000). Adaptations of social behavior in subjugated individuals indicate there may be some alterations in the activity of regions in the social behavior network. However, IEG expression, or lack thereof, should not be considered an absolute indicator of whether a brain region is activated or not. Rather, IEG expression should be understood in the context of behavioral and neuroendocrine effects of manipulations (Pfaus & Heeb, 1997). This prompted the use of quantitative Cytochrome Oxidase (CO) histochemistry to facilitate the quantification of long-term activity in brain regions involved in social behaviors.

Experimental overview and methods

Golden hamsters

Hamsters are solitary animals as shown by field studies (Gattermann et al., 2001). It could be assumed that juvenile hamsters seek to establish their own territories during puberty. Juvenile hamsters previously exposed to social stress would be likely to avoid burrows occupied by unknown adult males. This would enable them to avoid conflict and injuries. However, these animals would not necessarily be fearful of new contexts, which would enable them to colonize new territories or burrows unoccupied by adult males. Therefore, this species is ideal for the study of the effects of stress on approach and avoidance behavior.

In hamsters, a study by Vomchka and Greenwald (1979) found that gonadotropins, prolactin, and androgens begin their increase at about postnatal day 28 (P-28). In addition, LH and FSH peak around P-40, and subsequently

decline. Prolactin peaks twice: once at P-22 and then again at P-55. Androgens peak at P-50, then decline slightly. These hormones seem to normalize around P-70. Therefore, we define puberty in hamsters as occurring from P-28 (early puberty) to P-70 (early adulthood).

Hamsters in the wild reproduce seasonally. Therefore, light cycle changes affect the reproductive system of these animals (Czyba et al., 1971; Mogler, 1958; Vendrely et al., 1971; Reiter, 1973; Reiter, 1974). In our studies, we use long-day conditions (14h L, 10h D) in order to mimic summer conditions, which is when this species breed in the wild. Therefore aggressive and reproductive behaviors were present in stimulus males and juveniles. In our experiments, our stressed individuals were placed in the homecage of a larger aggressive adult male hamster who would then investigate and attack the subject.

In our experiments, we sought to answer the following questions:

Experiment 1: How are approach, avoidance, and risk-taking behavior affected by stress during puberty and how generalized are these effects?

In this experiment, approach/avoidance and risk-taking behavior were studied with larger aggressive male social stimuli present in a Y-maze. While Y-mazes have traditionally been used as a simple choice task, they have recently been used to assess odor preference in female voles (Johnston et al., 1997), individual recognition in female golden hamsters, and individual recognition of

familiar male hamsters in subjugated and non-subjugated males (Johnston et al., 1997; Lai & Johnston, 2002; Lai et al., 2005; Petrulis & Johnston, 1999).

In addition to the analysis of behaviors with social stimuli present, juveniles were also tested using various procedures to determine whether risk-taking behavior is altered in non-social contexts. Tests in a non-social context were completed in three apparatuses. Subjects were tested in an open field arena, a lat-maze, and an empty Y-maze. In an open field arena, defeated adult rats typically cower in a corner and show reduced locomotion and avoidance of the center of the arena (Meerlo et al., 1996a; Meerlo et al., 1996b; Raab et al., 1986). The lat-maze is a variant of this test that focuses particularly on locomotion in hamsters and rats (Cervantes et al., 2005; Griesbach & Amsel, 1998; Lipp et al., 1987; Wommack & Delville, 2007). Therefore, these apparatuses provided additional opportunities to examine risk-taking behavior in our subjects. Utilizing the Y-maze without a social stimulus present provided a baseline measure for behaviors and allowed comparison between groups in a non-social context.

Experiment 2: Is neural of activity affected by repeated stress?

It is possible that changes in neuronal activity in response to stress are long-term changes in regions involved in the social behavior network and IEG expression may or may not reflect the resultant changes in activity. In addition,

brain regions habituate to long-term activation in IEG activity (Martinez et al., 2001; Melia et al., 1994; Watanabe et al., 1994). Therefore, we opted to use quantitative cytochrome oxidase histochemistry to study neural adaptations in response to repeated stress. This staining method uses CO enzymatic activity as a measure of neuronal functional activity because the two are tightly linked (for review, please see Wong-Riley 1989 and Wong-Riley et al., 1998). Further, neuronal activity uses ATP to maintain transmembrane gradients and action potentials and CO catalyzes the final step in the mitochondrial transport chain (Erecinska & Silver, 1989). The impetus for our using this method is that it makes long-term activity of individual brain regions measureable and comparable, facilitating the study of neural adaptations in response to repeated stress. In addition, this method has been used to analyze plasticity in patterns of activation, particularly in developmental studies (Hevner, 1998).

Experiment 3: Is sexual behavior altered by repeated stress in juveniles and are aspects of sexual behavior desynchronized?

Effects of repeated stress on consummatory and appetitive aspects of juvenile behavior were examined. We placed receptive females in our subjects' homecage to test consummatory aspects of sexual behavior. This protocol is typical in studies of sexual behavior. In order to elucidate appetitive aspects of sexual behavior, we again utilized the Y-maze. In this instance, the Y-maze was

used as a means to give subjects a choice between mates while allowing for the analysis of approach/avoidance and motivated behaviors. Up to this point in our studies when testing subjects in the Y-maze we prevented the direct interaction between animals. However, the mate choice made by naïve animals prompted investigation of the type of interaction sought with the female social stimuli and the pattern of normal development of appetitive sexual behaviors. Therefore, additional tests in the Y-maze allowed naïve individuals access to the female social stimuli in the Y-maze. We also evaluated appetitive behaviors in the Y-maze at mid-puberty and adulthood with female social stimuli present.

Significance

These studies examined the effects of stress on development of social behaviors during puberty. In animal models of chronic stress, prolonged exposure to stress increases activity of the HPA axis, increases cocaine self-administration, induces biochemical changes in the mesolimbic dopamine system, and decreases sexual behavior in adult males (Heinrichs et al., 1992; Johren et al., 1994; Lucas et al., 2004; Miczek and Mutschler, 1996; Ortiz et al., 1996). Development during puberty is of particular interest since the age of onset of several mental disorders in human males is during late puberty. Bipolar disorder, depression, posttraumatic stress disorder (PTSD), and schizophrenia have all been linked with previous exposure to stressors (Kendler et al., 1999; Roth, 1958; Walker et al., 2008; Yehuda & LeDoux, 2007).

In addition, repeated stress during puberty has been associated alterations in HPA reactivity. For example, male victims of bullying during adolescence show altered sympathetic response to stressful situations (Hamilton et al., 2008). However, these effects are mediated by the social context in which the bullying occurs. Boys who feel isolated and are bullied are more damaged than their non-isolated peers (Newman et al., 2005). Indeed, our studies suggest the effects of stress on social development are specific to the social context. Instead of generalized effects in all contexts as is seen in learned helpless models of depression, the alterations in behavior we report in juvenile hamsters are specific to a social context. We suggest that our use of hamsters is most similar to the anxiety associated with social situations humans. Thus, stressed male juvenile hamsters may be best utilized as a model for social anxiety in humans.

Chapter 2: Avoidance of adult males by subjugated juveniles

Introduction

Chronic stress causes a variety of effects in mammals and has been associated with mental disorders in humans, including depression and bipolar disorder (Kim et al., 2007; McEwen, 2003; Nestler et al., 2002; Swaab et al., 2005). In animal models of chronic stress, prolonged exposure to stress increases activity of the HPA axis, increases cocaine self-administration, induces biochemical changes in the mesolimbic dopamine system, and decreases sexual behavior in adult males (Heinrichs et al., 1992; Johren et al., 1994; Lucas et al., 2004; Miczek & Mutschler, 1996; Ortiz et al., 1996). In addition, social stress enhances anxiety and fear responses in both social and non-social contexts. Socially stressed rats display increased anxiety and fear in elevated plus mazes and open field arenas (Katz et al., 1981; Zelena et al., 1999). Thus, social stress results in a generalized state of fear in these animals. Such outcome is not limited to rats, since similar results have been obtained in other species such as golden hamsters. Adult hamsters show long-lasting inhibition of aggression, increased avoidance, decreased androgen levels, increased body weight, and increased adiposity (Foster et al., 2006; Huhman et al., 1991; Huhman et al., 2003; Potegal et al., 1993; Solomon et al., 2007).

However, juvenile hamsters appear to be more resilient to social stress. In previous studies, exposure to social subjugation only results in a transient decrease in plasma testosterone levels (Wommack et al., 2004). Social subjugation has only modest effects on body weights (Delville et al., 1998; Wommack et al., 2004). In addition, there is no inhibition of aggressive responses toward smaller individuals (Delville et al., 1998; Wommack et al., 2003). Nevertheless, subjugated juvenile animals did show inhibition of aggression towards same sized-individuals and avoidance of larger adults, thus showing changes in aspects of fear responses (Delville et al., 1998). However, the extent of these apparent fear responses is unclear. In particular, it is unclear whether subjugated hamsters differ in a non-social context. While basal cortisol levels do not differ between subjugated and non-subjugated animals (Wommack et al., 2004), the way these animals respond to a non-social stressor is unknown.

In this study, juvenile animals were tested using various procedures to determine whether fear is generalized or specific to the presence of a social stimulus. Tests were administered in a Y-maze both with and without a social stimulus to determine the importance of these animals as a cue for the behavioral response of the subjects. Animals were also tested in an open field arena and in a lat-maze to test their responses to a non-social stimulus. In an open field arena, defeated adult rats typically cower in a corner and show reduced locomotion and avoidance of the center of the arena (Meerlo et al., 1996a; Meerlo et al., 1996b; Raab et al., 1986). The lat-maze is a variant of this

test that focuses particularly on locomotion in hamsters and rats (Cervantes et al., 2005; Griesbach and Amsel, 1998; Lipp et al., 1987; Wommack and Delville, 2007).

Materials and methods

Golden hamsters were bred in the laboratory from a colony originally obtained from Harlan Sprague–Dawley (Indianapolis, IN). Each litter was culled to six animals of both males and females a few days after birth, weaned on postnatal day 25 (P-25), and singly housed in Plexiglas cages. Animals were housed in a reversed light-day cycle (14 L: 10 D lights off at 9:00 a.m.) with food and water provided ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin. Animals were kept at the Animal Resource Center, an AALAC-accredited facility. The experimental design is shown in Table 2.1.

On P-27, each hamster was observed in the presence of an adult male for a few seconds to test for inherent fearfulness. Adult males were not allowed to have physical contact with subjects. Those subjects who immediately vocalized and attempted to escape the cage were determined to be inherently fearful and were not used for this study. Approximately 1:12 hamsters fall into this subset of animals and were excluded since they would likely exhibit fearful behavior in most contexts regardless of treatment. The remaining animals were equally distributed into two groups: non-subjugated control and experimental. Control

animals were placed in a clean, empty cage for 20 minutes every day from P-28 to P-42, which correspond to the onset of puberty and mid-puberty respectively. Experimental animals were put into the home cage of a novel adult hamster daily for 20 minutes as previously described (Delville et al., 1998; Wommack and Delville, 2003). Submissive behaviors and the number of attacks/bites inflicted by the unfamiliar aggressive adult were recorded. Animals were subjected to an average of 6 attacks per day and displayed submissive behaviors during the encounters (Wommack and Delville, 2003). Social subjugation and behavioral testing occurred within a 3-hour window during the second half of the dark phase. Groups were counterbalanced during testing. Body weights were monitored regularly during the entire experiment, but no difference was observed between groups. Behavioral observations were made in a Y-maze, an open field arena, and a lat-maze to address various aspects of behavior in the presence of social and non-social stressors.

Tests in a Y-maze may elucidate differences in behavior with more specificity than previous studies by allowing for examination of the motivation of the subjugated animal alone and outside of an immediately dangerous environment. Beyond use as a simple choice task, a Y-maze has been used to assess odor preference in female voles (Johnston et al., 1997), individual recognition in female golden hamsters, and individual recognition of familiar male hamsters in subjugated and non-subjugated males (Johnston et al., 1997; Lai and Johnston, 2002; Lai et al., 2005; Petrulis and Johnston, 1999). By

examining behaviors in sections of the maze, some of the ambiguity of testing during agonistic encounters will be alleviated.

Y-maze sections include the start box, stem, left arm, and right arm. The Y-maze is approximately 170 cm from the base of the maze to the end of the arms, 16 cm wide in each compartment, and 20 cm high, with the arms of the maze at about a 45° angle. The stem and arms are each about 85 cm long. During testing in the presence of a social stimulus, a compartment at the end of one arm contained an unknown experienced adult fighter as a social stimulus, and the other contained no social stimulus. In addition, a group of subjugated (n=11) and control (n=10) hamsters were exposed to a smaller and younger male conspecific with no fighting experience in order to test whether social anxiety generalized to non-threatening social stimuli as well. Previous studies have shown that subjugated juveniles will attack smaller conspecifics at increased frequency, so we do not anticipate defensive or anxious responses (Delville et al., 1998, Wommack and Delville, 2003). The arms of the maze are referred to as the social stimulus (S) arm and no social stimulus (NS) arm of the maze. Lines are marked every 12 cm in each section of the maze to facilitate quantification of locomotor activity. The entire maze is covered with lids and there are 20 cm long compartments at the end of each arm and the stem of the maze (start box). Black perforated barriers separate the compartments from the rest of the maze. A fan outside of the start box facilitates airflow from the arms and out through the start box.

Prior to testing in the Y-maze, animals were habituated to the apparatus through daily 10-minute exposure periods for four days. The maze was cleansed with 70% ethanol between subjects during habituation and testing. Animals were placed in the start box with a solid black barrier separating them from the stem of the maze. The barrier was immediately removed allowing the animal to walk in the maze. During testing in the Y-maze on P-44 and P-45, behaviors were observed and videotaped for 10 minutes. These behaviors were later reviewed and scored through iMovie (Apple Inc., Cupertino, CA). On P-44 animals were tested without a social stimulus present. Animals were tested with a social stimulus in the maze on P-45.

The following measures were recorded in each section of the maze: duration of time spent in the area, latency to reach the end of each arm, and line crossing counts. Frequency of olfactory investigation and a subjective description of the type of walk used by the subject were also recorded for animals exposed to the larger aggressive adult. These measures were meant to address the defensive behavior usually observed in smaller hamsters exposed to larger aggressive individuals. Flank marking was recorded for subjects exposed to smaller conspecifics in order to elucidate whether their behavior toward the stimuli might be aggressive. Olfactory investigation included a stop, neck stretching, and sniffing. Animals observed in the Y-maze exhibited three distinct types of walks: a slow walk, an intermediate walk, and a fast walk. The slow walk was characterized by a low posture, neck stretched forward, and a high

number of starts and stops. This type of walk was is reminiscent of the stretch approach behavior seen in rats in a hostile environment (Ribeiro-Barbosa et al., 2005). In the intermediate walk, hamsters moved at a moderate speed with the body not close to the ground and would occasionally stop and start. In the fast walk, animals moved at a high speed with their bodies somewhat raised from the ground. While olfactory investigation was performed during all walks, it is possible that the slow walk indicates a higher level of vigilance due to increased fear and cautiousness in a particular context.

Behavioral responses to non-social contexts were observed independently in a lat-maze on P-35 and P-46 in animals that were exposed to aggressive adult stimuli in order to test the generalizability of their fear and anxiety. The lat-maze has been used as a method of testing locomotor activity in response to a non-social stimulus (Griesbach & Amsel, 1998; Lipp et al., 1987). A lat-maze consists of a black wooden box (about 63 cm x 63 cm and 21.5 cm tall) with a closed smaller box in the center (39 cm X 39 cm X 17.5cm), leaving a 20 cm wide corridor for hamsters to run through. Lines were marked off every 15 cm in the corridor making it possible to quantify locomotor activity. Each line crossing was scored to quantify locomotor activity of each subject during 10-minute periods. After testing each animal, the lat-maze was cleaned with 70% ETOH. This type of maze is ideal to test locomotor activity in hamsters and they routinely cross between 300 and 500 lines in 10 minutes (Cervantes et al., 2005; Taravosh-Lahn et al., 2006; Wommack and Delville, 2007).

After the second day of Y-maze testing, animals were placed in an open field chamber and their behaviors were recorded. The open field chamber was used as an alternate independent measure of behavioral activity in response to a non-social stimulus. The open field consisted of a box (62 cm x 62 cm) made of 21 cm high clear plastic walls and a white Plexiglas floor. The activity monitor (MED Associates, St. Albans, VT) measured several behaviors. Motion sensors positioned at 2.5 and 10 cm high recorded the distance traveled, rearing, amount of time spent near the walls of the field, and amount of time spent in the center of the field for each subject. The ratio of the amount of time spent near the walls of the open field to the time spent in the center is an index of anxiety in rats (Crawley, 1985).

All data analysis was performed using SPSS 11.5 software. Behavioral observations were compared between the non-subjugated control group and the repeatedly subjugated group through two-tailed Student's t-tests assuming unequal variance. Within groups differences were analyzed using two-tailed Student's t-tests assuming equal variance.

Table 2.1: Experimental design

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Culling	P-5
Weaning	P-25
Subjugation or clean cage	P-28-P-42
Lat-maze testing	P-35,P-46
Open field testing	P-45
Habituation to Y-maze	P-40-P-43
Y-maze NS testing	P-44
Y-maze S testing	P-45

Results

Y-Maze

When the Y-maze contained no conspecific, subjects showed no preference for either arm of the Y-maze. Under these conditions, there were no significant differences between groups in any of the measures studied. Line crossing counts between arms, latency to enter arms, latency to reach the end of the arms, and duration of time spent in areas of the maze showed no differences between groups. Also, there were no significant differences in the frequency of olfactory investigation or in walk type in any area of the Y-maze (Figure 2.1). However, the behavior differed in the presence of an unknown aggressive adult male hamster. The data are explained below for each type of measure.

Durations

The presence of an aggressive adult male in a compartment at the end of the arm of the Y-maze led to completely different observations on P-45. There were significant differences in the duration of time spent in areas of the maze between groups when exposed to an aggressive individual. Subjugated animals spent twice as much time in the start box as control animals [$t(8)=3.79$, $p<.01$]. These animals also spent more time in the stem [$t(12) = 2.74$, $p<.05$]. Subjugated animals spent 4-5 times less in the S arm than non-subjugated control animals [$t(10) = -6.75$, $p<.001$]. Subjugated animals spent more time in

the NS arm than the S arm [t (6) =3.57, p<.05]. Hamsters in the control group spent more time in the S arm than the NS arm [t (6) =-3.17, p<.05].

There was one significant difference in the subjugated animals exposed to a smaller conspecific in the Y-maze. The subjugated animals spent significantly more time in the S arm versus the NS arm [t (11)= -2.60, p<.05].

Latencies

Overall, latencies to enter the NS and S arms of the maze were not significantly different between groups of animals exposed to a larger aggressive adult. Subjugated animals showed no difference in latency to enter the NS versus the S arm and the latency to reach the barrier at the end of the arms did not differ significantly between groups. However, there were differences between arms within the control group. Control hamsters entered the S arm faster than the NS arm [t (6) = 2.51, p<.05]. In addition, control animals showed a difference in the amount of time it took to reach the end of the S arm and the end of the NS arm, with control subjects reaching the end of the NS arm faster [t (6)=3.37, p<0.05].

Subjects placed in the presence of a smaller stimulus animal did not display any significant differences in latency to enter arms of the maze.

Line crossing counts

Subjugated and non-subjugated control animals exposed to larger aggressive adults had similar numbers of line crossing counts in Y-maze overall although there were differences in specific sections of the maze. Compared to control hamsters, subjugated subjects crossed fewer lines in the S arm [$t(7) = -3.28$, $p<.05$]. Within the socially stressed group, the hamsters crossed more lines in the NS arm of the maze than the S arm [$t(6)=4.0$, $p<.01$]. There was no difference in the number of line crossings in the NS and S arms of the maze in control animals.

In animals exposed to smaller conspecifics, there were significant differences between groups. Subjugated animals ambulated less than hamsters in the control group in the stem [$t(20) = -2.46$, $p<.05$], NS arm [$t(20) = -2.27$, $p<.05$], and S arms of the maze [$t(20) =2.36$, $p<.05$]. However, within the socially stressed group, animals crossed fewer lines in the no stimulus arm of the maze than in the stimulus arm of the maze.

Olfactory investigation

With a novel adult in a compartment at the end of an arm of the maze, subjugated animals exhibited a lower frequency of olfactory investigation than control animals in the Y-maze overall [$t(56) =-2.42$, $p<.05$]. Olfactory investigations were significantly less frequent in the S arm in subjugated animals [$t(11) = -5.78$, $p<.001$]. The frequency of olfactory investigation in the NS arm versus the arm containing the aggressive adult did not differ in subjugated

individuals. In contrast, control hamsters performed more olfactory investigation in the arm of the maze containing the novel adult than the NS arm [t (11) = -4.71, p<.01].

Walk types

Repeatedly subjugated animals exhibited a higher number of slow walks in the S arm of the maze as compared to non-subjugated controls [t (11) = 3.1 p<.01]. There was no other significant difference between groups in any types of walks in all part of the maze.

Lat-maze

Hamsters were very active when placed in the lat-maze and crossed between 400 and 500 lines in 10 minutes. There were no differences between groups in the number of lines crossed in the lat-maze on P-35 [t (8) = 0.28, p>0.1] and P-45 [t (5) = 0.22, p>0.1].

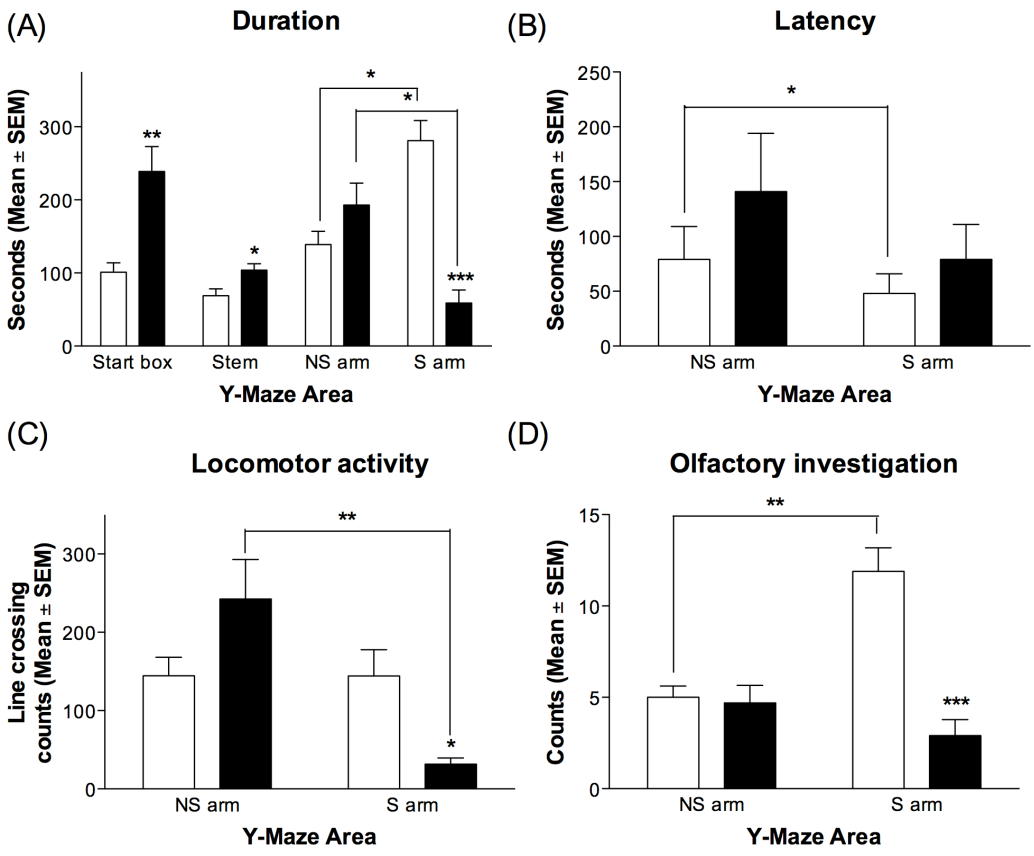
Open Field Arena

Ambulatory time in the center and periphery of the open field were similar between groups and showed no significant differences. Hamsters spent 90% of their ambulatory time in the periphery of the open field arena regardless of group. This was statistically significant in both subjugated [t (4)=5.98, p<.01] and control hamsters [t (4)]=6.55, p<.01].

Ambulatory distance followed the same pattern with no significant differences between groups. Subjugated [$t(4)=7.42$, $p<.01$] and control [$t(4)=7.17$, $p<.01$] animals walked a longer distance in the periphery versus the center of the open field. Most of the time, the animals were very active in all sections of the arena.

To observe the type of activity hamsters perform in the arena, a separate group of untreated hamsters was placed in the arena and videotaped. These videotapes showed that hamsters spend 90% of their time in the periphery of the arena walking along the walls of the box or attempting to climb them. Hamsters in our laboratory exhibit this same behavior regardless of condition.

Figure 2.1: Response to an adult male



Repeatedly subjugated (black bars) and non-subjugated (open bars) juvenile hamsters were tested in a Y-maze with in the presence of a novel stimulus animal (adult male hamster). The Y-maze consisted of a start box, stem, and two arms. The stimulus animal was placed at the end of one of these arms (S arm), while the other was empty (NS arm). Duration of time in each area of the maze *(a),* latency to enter arms of the maze *(b),* line crossing counts in arms of the maze *(c),* and olfactory investigation frequency in arms of the maze *(d)* were compared between groups. *: p<.05, **: p<.01, ***: p<.001, Student's t-test.

Discussion

In this study subjugated juveniles avoided adult males during tests in the Y-maze. In contrast, the behavior of the animals did not differ between groups in the absence of a social stimulus. Thus, it can be concluded that repeated social stress does not result in a generalized state of fear in this species. Instead the behavioral changes are limited to a social context.

In a social context, subjugated individuals displayed fear and anxiety-related behaviors when in the presence of an aggressive adult male. They spent more time in areas of the maze that were distant from the stimulus animal, which indicates anxiety in the presence of a social stimulus. In addition, while both subjugated and control animals spent about the same amount of time in the NS arm, subjugated animals spent much less time in the S arm indicating fear of the stimulus animal. Interestingly, subjugated hamsters avoided the S arm while control animals preferred it. This suggests that repeated subjugation alters the interest hamsters would normally have toward conspecifics at this age. Reduced line crossing counts in the proximity of the social stimulus further supports the conclusion that subjugated animals fear the social stimulus.

In previous studies, social subjugation was associated with an inhibition of risk assessment in the immediate presence of an aggressive adult (Wommack et al., 2004). However, it is possible that these findings resulted from the immediate presence of this individual. In these studies, risk assessment

strategies depended on the perceived level of danger (Blanchard and Blanchard, 1989; Ribeiro-Barbosa et al., 2005). In this study, subjugated hamsters preferentially displayed a slow walk that is reminiscent of risk assessment in rats. Thus it can be argued that risk assessment strategies in hamsters are context dependent as well. Though we noted a reduction of olfactory activity in the proximity of adults, these hamsters also spent less time overall in this part of the maze.

In addition, hamsters exposed to smaller conspecifics show less ambulatory behavior in several areas of the maze, possibly indicating some level of generalization of anxiety in a social context. However, these animals also spend more time near social stimuli, suggesting they are motivated to interact with these animals. Previous research suggests the interaction between a subjugated individual and smaller conspecific would include offensive aggression by the socially stressed animal (Delville et al., 1998; Wommack et al., 2003).

Animals were also tested for behavior in the absence of a social stimulus in the Y-maze, open field, and lat-maze. Open field testing was administered without previous habituation to the arena. While hamsters placed in a new environment show an elevation of plasma cortisol levels within 10-15 minutes (Weinberg and Wong, 1983), there were no behavioral differences between the groups in any test under non-social conditions. Total line crossing counts in the Y-maze and lat-maze also served as an index of behavioral specificity of the effects of social subjugation. Since ambulatory behavior was found to be similar

in both groups, such a finding suggests that chronic social stress did not affect locomotor activity of the animals in the Y-maze and the lat-maze. More importantly, the lack of behavioral differences between groups in non-social contexts supports the absence of a generalized fear in these animals after repeated social subjugation. These behavioral observations are consistent with the absence of differences in baseline plasma cortisol levels in these animals (Wommack et al., 2004).

One aspect of our data in hamsters contrasts with previous studies in rats. In rats, adolescence is considered a period of enhanced vulnerability to stress. For example, stress during puberty is associated with substance abuse and enhanced anxiety (Avital and Richter-Levin, 2005; Avital et al., 2006; Spear, 2002). However, this disparity may be associated with differences in the development of the HPA axis during puberty between rats and hamsters. In rats, basal serum corticosterone levels remain constant throughout puberty and adulthood (Gomez et al., 2002; Romeo et al., 2004; Vasquez, 1998), and juveniles have a longer duration of recovery post stress (Goldman et al., 1973, Vazquez and Akil, 1993). However, hamsters show a gradual increase in cortisol levels throughout puberty (Wommack et al., 2004). The developmental pattern of hamsters may not be unique to this species. Tree shrews and humans also show a steady increase in basal cortisol levels during puberty until adulthood (Kiess et al., 1995; Van Kampen & Fuchs, 1998; Wommack et al., 2004). It is possible that puberty is associated with changes in responsiveness to chronic

stress in humans as well. Indeed, recent data indicate greater vulnerability to social subjugation in late adolescence in humans (Delville et al., 2005).

Hamsters exposed to smaller conspecifics show less ambulatory behavior in several areas of the maze, possibly indicating some level of generalization of anxiety in a social context. However, these animals also spend more time near social stimuli, suggesting they are motivated to interact with these animals. Previous research suggests the interaction between a subjugated individual and smaller conspecific would include offensive aggression by the socially stressed animal (Delville et al., 1998, Wommack & Delville, 2003).

In hamsters, the characteristics of the stimulus animal that are responsible for the behavioral observations remain unclear at this time. In previous studies, subjugated juveniles showed a lack of fear of smaller individuals. Instead, they were more likely to attack them (Delville et al., 1998; Wommack et al., 2003). It is important to note that the hamsters used as stimuli in the Y-maze were unknown individuals. This indicates a generalization of the behavioral response of subjugated animals toward adult male hamsters. The ecological significance of these results could be discussed as follows. Hamsters are solitary animals as shown by field studies (Gattermann et al., 2001). It could be assumed that juvenile hamsters seek to establish their own territories during puberty. Juvenile hamsters previously exposed to social stress would be likely to avoid burrows occupied by unknown adult males. This would enable them to avoid conflict and injuries. However, these animals would not necessarily be fearful of new

contexts, which would enable them to colonize new territories or burrows unoccupied by adult males.

Chapter 3: Neural alterations related to stress during puberty

Introduction

Exposure to stressful events is associated with the development of psychopathology. Bipolar disorder, depression, posttraumatic stress disorder (PTSD), and schizophrenia have all been linked with previous exposure to stressors (Grandin et al., 2007; Kendler et al., 1999; Roth, 1958; Walker et al., 2008; Yehuda & LeDoux, 2007). Social stress is an ecologically relevant model of depression in animals and its effects are relevant to this disorder. For instance, exposure to social stress causes a variety of behavioral changes associated with depression, and thus has been regarded as an ecologically relevant model for the disorder.

Behaviorally, an assortment of effects are observable following repeated exposure to social defeat, including increased anxious behavior, increased submissive behavior, decreased risk assessment, and decreased aggression (Bastida et al., 2009; Blanchard et al., 2002; Potegal et al., 1993; Huhman et al., 1993). In addition to these behavioral manifestations, alterations in the physiology of stressed animals also occur. Immunosuppression, enhanced acute glucocorticoid synthesis, higher baseline cortisol levels, increased adiposity, and decreased plasma testosterone levels have been observed in rodents (Huhman et al., 2003; Potegal et al., 1993; reviewed by Blanchard et al., 2002).

Neural activation following acute stress is different from patterns of activation in repeatedly defeated animals. Martinez et al. (1998) found that rats defeated once during adulthood versus those defeated repeatedly showed different patterns of neural activation. While the LS, BNST, lateral preoptic area (LPOA), lateral hypothalamic area (LHA), paraventricular nucleus (PVN), MeA, and central amygdala (CeA) were activated an hour after a single defeat, only the BNST, PVN, and MeA continued to show increased c-Fos expression after repeated defeat. Thus, patterns of neural activation in repeatedly socially stressed juveniles may closely resemble the neural activation in repeatedly defeated rats.

In addition to the number of exposures to stress, the timing of a stressor during development also influences the associated severity and consequences. Social subjugation during puberty appears to be more transient and less severe than during adulthood in golden hamsters. While socially stressed adult hamsters are submissive to smaller conspecifics, hamsters subjugated during puberty are more aggressive toward them as adults (Delville et al., 1998; Potegal et al., 1993; Wommack et al., 2003). Submissive behaviors toward conspecifics are not increased in these individuals and risk assessment decreases during puberty in stressed juveniles, but recovers in adulthood (Potegal et al., 1993; Huhman et al., 2003, Wommack et al., 2004). Fear associated with exposure to social stress during puberty is only expressed in a social context, as opposed to the more general effects in hamsters stressed as adults (Bastida et al., 2009;

Foster et al., 2006; Huhman et al., 1991; Huhman et al., 2003; Potegal et al., 1993; Solomon et al., 2007). Subjugation during puberty has also been associated with a transient decrease in plasma testosterone levels, and body weight is only mildly increased (Delville et al., 1998; Wommack et al., 2004). Interestingly, despite the transient nature of effects in juveniles, increased tyrosine hydroxylase expression in the extended medial amygdala has been observed in these animals, suggesting possible remodeling of dopaminergic systems (Wommack & Delville, 2002).

Adaptations in social behavior in these animals indicate there may be some long-term differences in activity of regions in the social behavior network. This network is comprised of reciprocally connected, steroid receptor rich brain areas, associated with control of a variety of social behaviors such as sexual, parental, and aggressive behaviors (Newman, 1999). The social behavior network includes the medial extended amygdala (MeA, BNST), medial preoptic area (MPOA), anterior hypothalamus (AH), and the lateral septum (LS). It is hypothesized that activity in the amygdala will be higher in stressed individuals because of the amygala's prominent role in fear responses. Other regions in this circuitry may also be more active in subjugated juveniles since certain social behaviors are more likely to be expressed in socially stressed hamsters. For instance, approach and avoidance behaviors would likely be expressed by stressed individuals, but not naïve animals. However, this may not necessarily be the case, since we would expect to see certain social behaviors, for example

olfactory investigation, to be expressed at a higher frequency in naïve animals. Therefore, we examined neural circuitries involved in fear and anxiety related behaviors, approach and avoidance as well.

Social defeat in adult hamsters has been studied as an ecologically relevant type of social stress. Conditioned defeat has been established as a potent type of stressor (Potegal et al., 1993), and the emotional responses associated with this model are similar to fearful and anxious responses to conditioned fear training. Like the social behavior network, the neural circuitry involved in fear and anxiety also includes the amygdala. The basolateral nucleus of the amygdala (BLA) in particular plays a role the acquisition of conditioned fear (Fanselow & Ledoux, 1999; Akirav et al., 2006; Berlau & McGaugh, 2006). This area integrates input from auditory, somatosensory, and visual areas and sends output to cells that synapse onto the central amygdaloid nucleus (Ghashghaei & Barbas, 2002). The CeA in turn coordinates the fear response and sends out put to the ventromedial hypothalamus (VMH), and the bed nucleus of the stria terminalis (BNST). The BNST is associated with the expression of fear related behaviors, specifically startle response (Lee & Davis, 1997). The infralimbic cortex is capable of inhibiting fearful and anxious responses by acting on the intercalculated amygdaloid cells in the central nucleus (Quirk et al., 2000). We hypothesize that in subjugated individuals the neural activity of the BLA, CeA, VMH, LH, and BNST would be higher than naïve hamsters, and the activity of

these regions would likely be coordinated. The activity of the IL is expected to be lower in the subjugated animals than in naïve juveniles.

The Triadic Model of motivated behaviors in juveniles suggests that there are changes within the circuitry involved in motivated behaviors during puberty. Motivated behaviors are defined as the actions taken to fulfill a goal in response to a stimulus. The regions included in this model are the medial/ventral prefrontal cortex, ventral striatum, and the amygdala. In this model the activity of the amygdala is associated with avoidance, activity of the ventral striatum is associated with approach behavior, and the activity of the prefrontal cortex is associated with higher order processing of the decision whether to approach or avoid a stimulus is appropriate. This model proposes that the ventral striatum would be more active in juveniles, as they tend to approach novel stimuli. Further, activity of the amygdala and prefrontal cortical regions may not be as active in normal juveniles. However, we observed avoidance in our subjugated hamsters in Chaper 2, suggesting the pattern of neural activity in this circuitry may be different in stressed individuals. We therefore hypothesize that we will see less activity in the ventral striatum, more activity in the amgydalar regions, and perhaps less activity in the prefrontal cortex in subjugated animals versus naïve individuals.

In this study, we examined long-term effects of repeated acute stress on activity in brain regions associated with fear response and social behavior in juvenile hamsters. We also examined coordination of the activity of regions

within circuitries by testing the strength of correlations between regions within each group. Cytochrome oxidase activity can be measured using histochemistry, a staining method used to quantify long-term changes in synaptic metabolic capacity in brain regions, will be utilized quantify long-term activity in juveniles exposed to social stress. Changes in CO activity occur in the order of days or weeks, making CO histochemistry ideal for quantifying long lasting changes in metabolic capacity in brain regions (for review, see Sakata et al., 2005).

Materials and methods

Animals

Hamsters were bred in the laboratory from a colony originally obtained from Harlan Sprague–Dawley (Indianapolis, IN). Each litter was culled to six animals (males and females) before postnatal day 7 (P-7). Animals were weaned on P-25, and singly housed in Plexiglas cages. Animals were housed in a reversed light-day cycle (14 L: 10 D, with lights off at 9:00 a.m.). Food and water were provided ad libitum. Body weights were taken twice a week to monitor subjects' development. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin. Animals were kept at the Animal Resource Center, an AALAC-certified facility.

On P-27 hamsters were pre-tested for inherent fearfulness in the presence of an adult male individual. Hamsters found to be inherently fearful (about 1:12) were not used in this study. Remaining animals were distributed into either the

subjugated or control group balanced for litter and weight. On P-28 animals in the experimental group (n=20) were placed into the cage of a larger aggressive adult male for 20 minutes, while those in the control group (n=20) were placed into a clean empty cage for the same amount of time. This was repeated through P-42. On P-45, subjects' behavior in the presence of an aggressive adult hamster was video taped and later scored. These behaviors were used for previous studies.

Cytochrome Oxidase Histochemistry

On P-45, hamsters (n=10 subjugated and n=10 control) were sacrificed by rapid decapitation. Their brains were quickly extracted, flash frozen in isopentane cooled in dry ice, wrapped in Parafilm, and stored at -80°C. Later, the brains were cut into 40µm coronal sections using a Reichert-Jung cryostat set to -20°C. Sections were stored in a -40°C freezer until processed for histochemistry for cytochrome oxidase activity as previously described (Gonzalez-Lima & Garrosa, 1991, Gonzalez-Lima & Cada, 1994). Cytochrome oxidase staining involves placing tissue in several incubation media. First, sections were placed in 0.1 M phosphate buffer (PB) with 10% w/v sucrose and 0.5% v/v glutaraldehyde for 5 min. After three washes in PB with 10% w/v sucrose to remove red blood cells for 5 min each, sections were incubated in Tris buffer (0.05 M Tris buffer solution, pH 7.6, with 275 mg/l cobalt chloride, 10% w/v sucrose, and 0.5% v/v dimethylsulfoxide) for 10 min in order to enhance tissue

staining contrast and reduce the time spent in the diaminobenzidine (DAB) incubation procedure. After another PB wash, sections were then incubated in a solution of 350 mg diaminobenzidine tetrahydrochloride, 52.5 mg cytochrome c, 35 g sucrose, 14 mg catalase, and 1.75 ml dimethylsulfoxide in 700 ml of oxygen-saturated PB for one hour at 37°C followed by a formalin solution (10% w/v sucrose and 4% v/v formalin) to stop the incubation chemical reaction. Finally, slides were dehydrated in a series of ethanol baths (30%, 50%, 75%, 95%, 100%, and 100% v/v ethanol), cleaned with Xylene, and coverslipped with Permount.

Enzymatic activity of separate batches of cytochrome oxidase stained tissue can be standardized by including two sets of tissue homogenate standards with each batch of slides during cytochrome oxidase histochemistry staining (Gonzalez-Lima & Garrosa, 1991; Gonzalez-Lima & Cada, 1994). Brains from 12 adult Sprague Dawley male rats were removed after decapitation, stored at 4°C (in sodium phosphate buffer, pH 7.6), and then homogenized at 4°C.

CO enzymatic activity as assayed by densitometry (Gonzalez-Lima & Cada, 1994). Activity units were defined using pH 7 and 37°C. Standards were cut the week of the cytochrome oxidase histochemistry staining. For each set of standards, two sections of homogenate tissue of thicknesses of 10, 20, 40, 60, and 80 micrometers were cut using the same cryostat and mounted onto a slide, each of which contained two sections of each thickness. Spectrophotometric activity values for standards were correlated with corresponding optical density

measurements of the cytochrome oxidase chromatic indicator (Gonzalez-Lima & Cada, 1994). Optical density readings from sampled regions of interest were converted into units of cytochrome oxidase activity using linear regression equations based on the known spectrophotometric activity values.

Densitometric Analysis of Cytochrome Oxidase Staining

As previously described (Gonzalez-Lima & Garrosa, 1991), optical density was sampled from each region of interest using JAVA version 1.4 (Jandel Scientific Corte Madera, CA) and were later converted to cytochrome oxidase units. The sampling area was adjusted for each region of interest in order to allow for four non-overlapping samples from each region. Three sections for each region were sampled per subject. Data was averaged for each animal and group means were compared. Several brain regions and subregions were sampled, but only those in the social behavior network, anxiety/fear network, and Triadic Model were included in the analysis for this experiment.

Data Analysis

All data analysis was performed using PASW 18 software. Data was normalized using the average of control data points and missing values were replaced to facilitate the use of step-wise discriminant analysis. All data analysis was performed using PASW 18 software. Two-tailed Student's t-tests assuming equal variance (and unequal variance as appropriate) were used to compare the

activity of each region between the subjugated and non-subjugated group. It is also important to note that with a high number of regions sampled for cytochrome oxidase histochemistry it is possible that the three effects we found are significant merely by chance since repeated Student's t-tests have a higher chance for type I error. This issue was addressed through our use of discriminant analysis. The use of this statistic enabled us to determine with greater certainty that the differences in neural activation between groups in the MPOA and VTA were significant effects and not just effects due to error associated with performing repeated Student's t-tests. In addition, Pearson's r correlations were calculated between areas found to have significant differences in activation between groups to determine whether neural activation is coordinated. Differences in coordination between regions in subjugated and control animals were assessed using a Fisher's Z transformation on Fisher's Z scores of regions that showed significant correlations in activity in each group ($p<.05$ for both groups). The equation for the Fisher's Z transformation is as follows:

$$Z = \frac{Z_{ij}(\text{group1}) - Z_{ij}(\text{group2})}{\sqrt{(1/n_{g1} - 2) + (1/n_{g2} - 2)}}$$

where Zij is the Fisher Z transformation value for the correlation coefficient between regions i and j, ng1 is the sample size in group one, and ng2 is the sample size in group two. Those significant at $p<.05$ using a Fisher's Z transformation are discussed in this study.

Results

Cytochrome Oxidase Histochemistry

Cytochrome oxidase histochemistry results in a golden-brown colored staining that is darker in regions of the brain that have higher metabolic capacity. The Hamster Brain Atlas and the Rat Atlas of Cytochrome Oxidase and Cresyl Violet Staining were used to determine where regions should be sampled (Morin & Wood, 2001; Gonzalez-Lima & Cada, 1998). Long-term differences in neural activity between subjugated and control animals were found in the MPOA, MPN, and the VTA. Figure 3.1 shows the regions sampled that were found to have significant differences. Metabolic activity was significantly lower in subjugated animals in the MPOA [$t(18) = -3.10$, $p<.01$], MPN [$t(18) = -2.45$, $p<.05$], and VTA versus controls [$t(18) = -2.11$, $p<.05$] (Table 3.1).

Analysis of data using step-wise discriminant analysis (Wilks' Lambda) suggests that most of the variability between groups was attributable to the MPOA [Wilks' Lambda = .653, $F(1, 18) = 9.58$, $p = .006$], with a canonical discriminant function coefficient of 1.97 (Table 3.2). The VTA is also a significant source of variability between groups, albeit a lesser contributor than

the VTA with a canonical discriminant function coefficient of 1.42 [Wilks' Lambda = .143, F (5,14) = 16.81, p=.0000177]. This suggests that of the regions sampled the differences in metabolic activity found in the MPOA and VTA are likely to be reliable differences between subjugated and control animals, and not significantly different due to Type I errors. The ventroposterior central gray (vpCG), ventrolateral caudate putamen (vlCP), and deep layer of the lateral frontal cortex dLFC, and lateral infralimbic cortex were also found to be discriminating variables in this calculation, but since they were not found to be significantly different between groups in our original Student's t tests, they are not likely to be meaningful differences between groups.

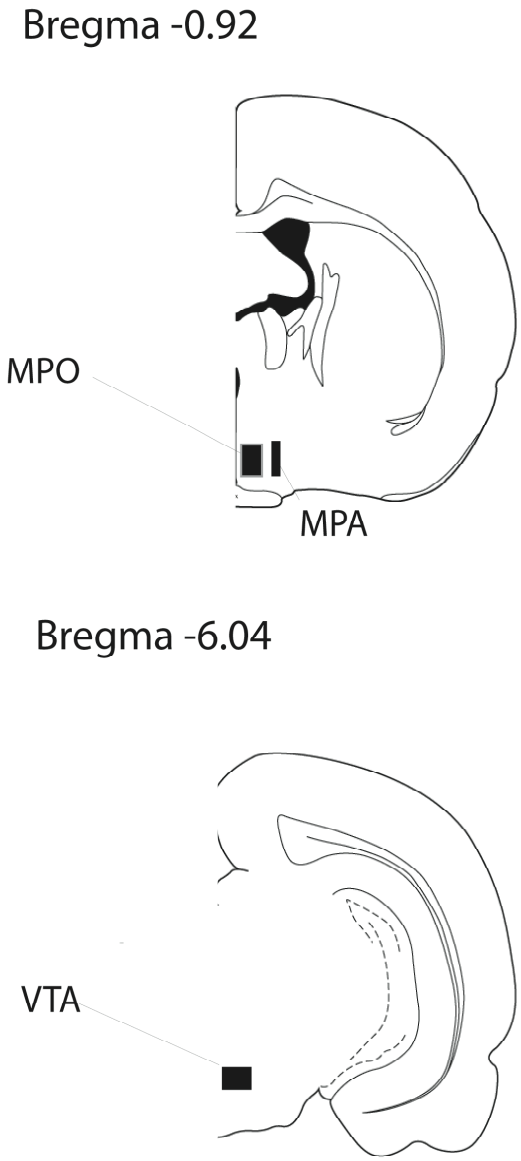
Coordinated activity within networks

Activation of MPOA was significantly correlated with activity in the MPN in controls [r = 0.793, n = 10, p < .01]. The same is true in subjugated individuals [r = 0.959, n = 10, p < .01].

The activity of regions in the social behavior network there were no significant Pearson's r correlations. There were also no significant Fisher's Z transformations. The activity of a few regions were significantly correlated in the fear/anxiety network. The BLA and the CeA were positively correlated in both the subjugated [r= 0.921, n=10, p=0.00015] and naïve groups [r= 0.935, n=10, p=0.000072]. In addition, activity of the CeA and VMH were correlated in the subjugated group [r= 0.885, n=10, p=0.00067]. This correlation was also

significant in the naïve group [r= 0.785, n=10, p=0.0071]. In addition, there were no significant differences in activity of any of the correlations between regions between groups in the fear/anxiety network. There was one significant correlation of activity between regions in the Triadic Model. As stated previously, activity of the BLA and the CeA were correlated in subjugated animals [r= 0.921, n=10, p=0.00015] and naïve groups [r= 0.935, n=10, p=0.000072]. However, again no significant changes in correlations of activity were observed between groups. Tables 3.3, 3.4, and 3.5 show correlation and Fisher's Z transformations for the three networks analyzed.

Figure 3.1 Areas sampled



Altered neural activity in subjugated individuals. The regions that were significantly different in stressed juveniles versus naïve juveniles were the MPOA, MPN, and the VTA.

Table 3.1. Cytochrome oxidase activity values

Region	Control	Subjugated	Sig.
Medial Preoptic Area (MPOA)	199.16 ± 3.7	191.69 ± 6.7	0.006
Medial Preoptic Nucleus (MPN)	199.12 ± 4.1	192.22 ± 7.9	0.024
Ventral Tegmental Area (VTA)	149.07 ± 21.2	132.49 ± 13.0	0.049

Significant differences in cytochrome oxidase activity (mean ± SEM) between naïve and subjugated individuals.

Table 3.2. Discriminant analysis

Step	Region	Statistic
1	MPOA	0.653
2	IL (L)	0.443
3	CG (VP)	0.335
4	LFC (D)	0.247
5	VTA	0.143
6	CP (VL)	0.1

Wilks' Lambda in stepwise discriminant analysis. MPOA=medial preoptic area, IL=infralimbic cortex, CG(VP)=ventroposterior central gray, LFC(D)=deep layer of the lateral frontal cortex, VTA=ventral tegmental area, and CP(VL)=ventrolateral caudate putamen. The activity in the MPOA and VTA were found to be a discriminating factor between groups.

Table 3.3. Social Behavior Network Fisher's Z Transformation

			Pearson's R Correlation		Fisher's Z transformation
Network	Brain region	Brain region	Naïve	Subjugated	
Social behavior	MeA	BNST	.179	.616	1.075
	MeA	MPOA	.478	.564	0.236
	MeA	AH	-.078	.075	0.308
	MeA	LS	-.175	.078	0.509
	BNST	MPOA	.601	.495	-0.303
	BNST	AH	.211	.516	0.714
	BNST	LS	.208	.405	0.436
	AH	LS	.611	.054	-1.313

There were no significant correlations between regions in either group, nor were any changes in correlations between regions between groups significant in the Social Behavior Network. MeA=medial amygdala, BNST=bed nucleus of the stria terminalis, AH=anterior hypothalamus, LS=lateral septum, and MPOA=medial preoptic area.

Table 3.4. Fear/Anxiety Network Fisher's Z transformation

	Brain region	Brain region	Pearson's R Correlation		Fisher's Z transformation
Network			Naïve	Subjugated	
Fear/ Anxiety	BLA	CeA	.935**	.921**	-0.202
	BLA	VMH	.608	.447	-0.449
	BLA	BNST	.562	.580	0.055
	BLA	IL	-.020	-.170	-0.302
	CeA	VMH	.785**	.885**	0.680
	CeA	BNST	-.252	.562	1.785
	CeA	IL	-.065	-.049	0.034
	VMH	BNST	.086	.776**	1.899
	VMH	IL	-.262	-.136	0.263
	BNST	IL	.231	-.182	-0.838

BLA=basolateral amygdala, CeA=central amygdala, VMH=ventromedial hypothalamus, BNST=bed nucleus of the stria terminalis, and IL=infralimbic cortex. * = significant, p<.05, **=significant, p<.01. Within the naïve group, activity between the BLA and CeA, and CeA and VMH was significantly correlated. Within the subjugated group, activity in the BLA and CeA, CeA and VMH, and VMH and BNST was significantly correlated. There were no significant changes in correlations between regions between groups in the Fear/Anxiety Network.

Table 3.5. Triadic Model Fisher's Z Transformation

	Brain region	Brain region	Pearson's R Correlation		Fisher's Z transformation
Network			Naïve	Subjugated	
Triadic Model	IL	NAcc	.362	-.465	-1.765
	IL	BLA	-.020	-.170	-0.302
	IL	CeA	-.065	-.049	0.034
	NAcc	BLA	.165	-.329	-1.016
	NAcc	CeA	-.329	-.242	0.190
	BLA	CeA	.935**	.921**	-0.202

IL=infralimbic cortex, NAcc=nucleus accumbens, BLA=basolateral amygdala, and CeA=central amydgala. . * = significant, p<.05, **=significant, p<.01. Within the naïve group activity between the BLA and the CeA was significantly correlated. Within the subjugated group the activity of the BLA and CeA was significantly correlated. There were no significant changes in correlations between regions between groups significant in the Triadic model.

Discussion

Cytochrome oxidase activity was used as a measure of long-term activity in regions of the brain. The only differences in cytochrome oxidase neural activity between groups were found in the MPOA, MPN, and VTA. Long-term activity in the MPOA, MPN, and VTA was lower in subjugated animals. In addition, activity of the MPOA and MPN was positively correlated in subjugated and naive hamsters. Lower long-term activity of in these regions in subjugated animals may be associated with the behavioral adaptations that occur as a result of with repeated stress. It is also important to note that with a high number of regions sampled for cytochrome oxidase histochemistry, it is possible that the three effects we found are significant merely by chance since repeated Student's t-tests have a higher chance for type I error. This issue was addressed through our use of discriminant analysis. The use of this statistic enabled us to determine with greater certainty that the differences in neural activation between groups in the MPOA and VTA were significant effects and not just effects due to error associated with performing repeated Student's t-tests. In addition we examined changes in correlated activity between regions in the subjugated and naïve groups. While some correlations were found in the activity between regions within groups in the three networks we examined, there were no significant changes in the correlation of activity between groups. This indicates that changes in neural activity associated with social stress in juveniles are limited.

Changes in metabolic capacity in the MPOA, MPN, and VTA in socially stressed animals may correspond with change in social behaviors in these animals. The MPOA and MPN are part of the social behavior network (Newman, 1999), which is congruent with behavioral adaptations to repeated social stress. Despite our prediction that differences in areas more specifically involved in the expression of fear and avoidance, the areas that show differences indicate there may be lasting changes in sexual behavior in these animals. The MPN and MPOA are subdivisions of the same brain area, and are considered major integrative areas for sexual behavior, with lesions ceasing sexual behavior in male rodents (Powers et al., 1987). The MPOA is involved in sexual motivation and behavior as evidenced by cell recordings, electrical stimulation, lesion, hormonal, and pharmacological studies (for review, see Dominguez & Hull, 2005). In addition, these areas are highly subdivisions of the same region, therefore their coordinated activation likely indicates coordinated activity within the MPOA. Unlike the preoptic area, the VTA is not a node in the social behavior network proposed by Newman and elaborated upon by Goodson (Goodson, 2005; Newman, 1999). The VTA is, however, involved in the expression of appetitive, motivated and rewarding behaviors such as drug-seeking and sexual behavior (for review, please see Fields et al., 2007). Numan and Stolzenburg (2009) propose that efferent connections from the MPOA project to the VTA to promote appetitive aspects of maternal and sexual behavior. It also contains cell bodies involved in the reward pathway and sends axons to the amygdala and

several other limbic areas and, most notably, contains reciprocal connections with the MPOA (Simerly & Swanson, 1986; Simerly & Swanson, 1988; Swanson, 1982). Since the MPOA, MPN, and VTA are involved in sexual behavior, lower activity in these regions suggests the development of this social behavior would be altered in subjugated juveniles. In addition, the Triadic model of motivated behaviors in juveniles suggests that normal juveniles would tend to approach conspecifics due to strong reward systems, weak harm avoidance systems, and possibly weak decision-making systems (Ernst & Fudge, 2009). While this model refers to the nucleus accumbens specifically when reward systems are referenced, however the VTA and MPOA are also part of reward systems. Lower activity in these regions in stressed juveniles could indicative of decreased functionality of reward systems, which would fit with the Triadic model in that this would skew approach/avoidance toward avoidance. This is different from what is seen in naïve juveniles, since these animals by default would tend toward approach when in a social context. Thus, rewarding behaviors are of interest in hamsters and the development of sexual behavior in particular will be investigated in future studies.

We expected to observe many differences in long-term neural activation in areas involved in fear responses and defensive behaviors due to the variety of behavioral adaptations animals make after being repeatedly stressed. In previous studies using newborn animals that are congenitally predisposed to learned helplessness show long-term changes in cytochrome oxidase activity in

several brain regions including the PVH, habenula, hippocampus, subiculum, and lateral septum (Shumake et al., 2004). Even more changes in activity have been observed in congenitally helpless juvenile rats (Shumake et al., 2000, Shumake et al., 2001, Shumake et al., 2002, Shumake et al., 2003). This wide range of changes in neural activation did not occur in repeatedly stressed juvenile hamsters. Only 3 of the 60 regions and subregions sampled were differentially activated between groups. However, it is not entirely surprising that long-term changes in neural activation was limited to a few regions since biobehavioral changes associated with exposure to repeated social stress during puberty are transient and lack the breadth of changes seen in defeated adult hamsters. Transient decreases in testosterone, slight increases in body weight, and the highly specific nature of fear response in subjugated juvenile hamsters all suggest effects that are not as generalized and long-lasting as those seen in adult hamsters exposed to the same kind of stressor (Bastida et al., 2009; Delville et al., 1998; Wommack et al., 2004). Because of the nature of changes after repeated social stress indicate that perhaps there is a smaller range of effects on the brain associated with stress during this period. Rather than long-term changes in individual regions studied, behavioral adaptations to repeated stress during puberty may involve short-term differences in expression of some genes.

For example, the expression of the TH gene could be transiently increased in subjugated individuals since tyrosine hydroxylase (TH)

immunoreactvitiy is higher in the MeA and BST in hamsters subjugated during early puberty (Wommack & Delville 2002). This increase in TH immunoreactvitiy is of interest for several reasons. First, this effect is correlated with subjugation and disappears 4 weeks after the end of the subjugation period (Wommack et al., 2004). These transient changes in TH expression in response to stress could be more indicative of what is occurring, as opposed to widespread long-term structural changes. Secondly, the increase in TH immunoreactivity in the MeA has been found in subjugated juveniles, while here we find decreased activity in the VTA. Dopaminergic cell bodies in the VTA have not been examined, so our findings may not necessarily contradict previous work. Also, it is possible that overall activity is decreased in the VTA with most dopaminergic projections sending little input overall, while inputs specifically to the extended medial amygdala increasing dopaminergic release. Thirdly, the increased expression of TH in the MeA is interesting because the same was found in defeated adult golden hamsters. Increased tyrosine hydroxylase expression was found in the MeA of socially stressed adults, indicating a relationship between this stress and dopamine in the MeA (Wommack et al., 2004). This is of importance since hamsters stressed as adults show several generalized effects of stress including increased feeding, body mass, and adiposity (Foster et al., 2006). Defeated adult individuals also show activation of the HPA axis as evidenced by increased plasma ACTH, glucocorticoid, and β-endorphin and decreased plasma testosterone (Huhman et al., 1990, 1991a, 1991b, 1992). In addition, behaviors

associated with conditioned defeat persist over time (Huhman et al., 2003). It has been proposed that certain behavioral effects engender specific metabolic profiles of activation (Sakata et al., 2005). Due to the long-lasting nature of effects in defeated adults, I expect that cytochrome oxidase histochemistry in these animals would show many more differences between groups than animals defeated during early puberty since previous literature and the current study point to resilience in pubertal hamsters.

Interestingly, both the development of stress response and acute stress response of adult rats have been found to differ from those of hamsters. In comparison to golden hamsters, Sprague Dawley rats showed prolonged ACTH release and slower development of corticosterone responses during early puberty. In addition, the baseline and acute corticosteone levels remain unchanged throughout pubertal development, which is in contrast to the gradual increase in both found in hamsters (Gomez et al., 2002; Romeo et al., 2004; Vazquez, 1998; Wommack et al., 2004; Wommack et al., 2005). Interestingly, the development in of HPA activity in Wistar shows similarities to development in golden hamsters (Pignatelli et al., 2006). In addition, in humans the development of HPA axis activation undergoes a gradual increase similar to that seen in golden hamsters suggesting this model for stress and pubertal development may be particularly applicable to human conditions (Jonetz-Mentzel & Weideman, 1993; Kiess et al., 1995; Elmlinger et al., 2002).

In contrast to the few long-term changes in neural activation between groups found in repeated stress, in general, acute stress tends to recruit activation of many more regions than repeated stress. Acute immobilization stress and forced swim test cause similar patterns of neural activation in several IEGs, including c-fos, with peak expression occurring 30 to 60 minutes after stress (Cullinan et al., 1995). Senba et al. (1997) found that 21 brain regions, including the LS, caudate putamen and subregions of the cingulate cortex, amygdala, hypothalamus, and hippocampus were activated 60 minutes after immobilization stress, which is also a processive stressor. While several regions are activated after acute restraint stress, adaptation of c-fos expression has been observed (Chen & Herbert, 1995; Watanabe et al., 1994). Similarly, acute defeat stress induces activity in the lateral preoptic area, LS, lateral hypothalamic area, central amygdala, locus coeruleus, nucleus of the solitary tract, BNST, PVN, MeA, dorsal raphe (DR), median raphe (MR), and central grey (CG) (Martinez et al., 1998). As previously mentioned, of the regions examined, only the BNST, PVN, MeA, DR, MR, and CG were persistently activated after repeated social defeat. Correspondingly, Kollack-Walker et al. (1997) found that subjugated adult hamsters showed activation in a multitude of areas. In so far as acute stress is associated with activation of many regions and repeated stress was associated with activation of fewer brain regions, our observations are accordance with previous studies.

This study is of importance in that it points to the resilience of these animals to repeated exposure to social stress. Long-term neural activation is minimally affected. The changes found in the MPOA, MPN, and VTA in subjugated juveniles point to possible changes in the development and expression of sexual behavior, which will be tested in the next chapter.

Chapter 4: Repeated stress and sexual behavior in juveniles

Introduction

Stress has been shown to have an inhibitory effect on sexual development and behavior. Chronic stress and the associated neuroendocrine changes result in decreased sexual behavior in rodents, while acute stress may increase or decrease sexual behavior depending on the severity of the stressor (Beach et al., 1956; Brotto et al., 1998; D'Aquila et al., 1994; Fernandez-Guasti et al., 1990; Ismail et al., 2011; Retana-Marquez et al., 1996; Sato et al., 1996). For example, the lowest animal in a hierarchy has fewer mating opportunities than higher-ranking animals that live in social groups (Bernstein et al., 1991; Bercovitch, 1992; Clutton-Brock et al., 1982; Kutsukake & Nunn, 2006; Preston et al., 2001; Wickings & Dixon, 1992; Setchell et al., 2010). Albino mice (D'Amato, 1988), deer mice (Dewsbury, 1998), lemurs (Perret, 1992), and rats all display decreased sexual behavior after exposure to social stress. Effects are not limited to behavior, but also include secondary sex characteristics. For example, in subordinate solitary mandrills, the development of secondary sex characteristics is incomplete, testes are smaller, and testosterone levels are lower (Setchell & Dixson, 2002). House sparrows experience testosterone fluctuations that affect male ornamentation that displays hierarchy status in response to stress (Laucht et al., 2010). In the bluebanded goby, the effect of stress on reproductive

success goes beyond secondary sex characteristics. In these fish, the outcome of an aggressive encounter determines whether the individual will remain a female or become a dominant male (Rodgers et al., 2005). Together, these effects are indicative of broad neuroendocrine alterations in subjugated and defeated animals.

The neuroendocrine profiles of socially stressed animals are also affected by social stress. Stress has general effects on the HPA and HPG axis. For example, subordinate rats in a visible burrow system weigh less, have decreased plasma testosterone levels, and increased basal cortisol levels than their high-ranking counterparts (Blanchard et al., 1993). In male African elephants sexually active subordinates, decreased androgen levels and higher basal glucocorticoid levels have been reported, with these deficits being attenuated by the presence of receptive females (Rasmussen et al., 2008). Similar to these solitary subordinate elephants, the golden hamster is a solitary species that is subjected to intraspecies competition for mates (Gatterman et al., 2001; Murphy, 1977). While submissiveness and avoidance in subjugated adult golden hamsters is present during exposure to a non-aggressive intruder and continue to be exhibited 4 weeks after the cessation of the training period, juvenile golden hamsters are more resilient to social stress (Huhman et al., 2003; Bastida et al., 2009). Even so, repeatedly socially stressed juveniles show neuroendocrine changes after subjugation. Stressed juveniles have decreased plasma testosterone, and increased baseline cortisol levels (Wommack et al., 2005). In

addition, low circulating testosterone in males is associated with deficiencies in reproductive behavior (Bartke, 1985). In parallel with the altered endocrine profiles of subjugated juvenile hamsters, there are several associated neural effects. Repeatedly stressed individuals show increased tyrosine hydroxylase immunoreactivity in the extended medial amygdala, and decreased long-term neural activation in the medial preoptic area, medial preoptic nucleus, and ventral tegmental area (Wommack & Delville, 2002; Bastida & Delville, unpublished observations).

Appetitive and consummatory aspects of sexual behavior are of interest, as both could be affected by stress. Aspects of sexual behavior can be categorized as appetitive and consummatory (Hinde, 1970). Appetitive aspects of sexual behavior include behaviors that males exhibit in order to perform sexual behavior. This includes motivated behaviors, mate choice, and mate-seeking behaviors. Consummation of sexual behavior is defined as when copulation occurs. A number of studies have identified key regions in the brain controlling these aspects of male sexual behavior. Lesions to the MPOA inhibit sexual behavior in rodents (first described by Agmo et al., 1977 and reviewed in Hull et al., 2006). Testosterone implants to the MPOA is both sufficient and necessary for the expression of sexual behavior in male hamsters (Coolen & Wood, 1999; Wood & Newman, 1995). In addition, the motivation to perform sexual behavior is associated with dopamine release in this area and precopulatory increases in DA are positively correlated with copulatory behavior (Kleitz-Nelson et al., 2010).

Mate choice in hamsters is highly reliant on the olfactory system, with ablations of this region inhibiting sexual behaviors (Triemstra et al., 2005). Mate choice in hamsters is highly reliant on the olfactory system, with alterations to olfactory bulb and vomeronasal bulb or their connections inhibiting sexual behavior (Ballard & Wood, 2007; Devor & Murphy, 1973, Doty & Anisko, 1973; Lisk et al., 1972). In addition, experienced and inexperienced males prefer the odors of females to those of males (Landauer et al., 1978; Huck et al., 1984). Landauer et al., also found that males are unable to differentiate between receptive and non-receptive females, however their study uses just the vaginal secretions of females. Decreased activity in medial preoptic area (MPOA) of juvenile hamsters is particularly relevant in that this area is a key integrative region for the sexual motivation and expression of male sexual behavior.

In this study, we examined consummatory and appetitive aspects of sexual behavior in subjugated animals. We hypothesized that subjugated juveniles would show deficiencies aspects of their sexual behavior. We anticipated that motivation to seek out female conspecifics would be affected since a generalized avoidance of adult male conspecifics has been observed in these animals and it is possible that this would also generalize to adult females as well (Bastida et al., 2009). It is entirely possible that male hamsters are able to discern the receptivity in females when in the presence of a female and in the context of an interaction, and other studies have found this to be true (Johnston 1974; Lisk et al, 1972). Female hamsters choose dominant males over

subordinate males, with latency to copulate with subordinates much longer (Brown et al., 1988).

Methods

Animals

Golden hamsters (*Mesocricetus auratus*) were bred in the laboratory from a colony founded by animals obtained from Harlan Sprague-Dawley (Indianapolis, IN). Litters were culled to six animals containing both males and females on postnatal day 5 (p-5). On p-25, the animals were weaned and housed individually in polycarbonate cages (20 x 33 x 13 cm). Animals were provided food (rodent diet in pellet form, Harlan Tekland, Madison, WI) and water ad libitum, and housed in a reversed daylight cycle (14:10-hr L:D, lights off at 10:00 am). Body weights were measured regularly through the entire experiment beginning at weaning. All experimental procedures were performed during the early to middle portion of the dark cycle in order to test during the hamsters' active period. All behavioral tests were performed under dim red light. Animals were kept at the Animal Resource Center, an Association for Assessment of Laboratory Animal Care approved facility. All procedures were approved by the Institutional Animal Care and Use Committee of The University of Texas at Austin and were performed according to National Institutes of Health guidelines.

Stimulus Females

Stimulus females were ovariectomized at least one week before experimental use. Receptive females were induced to estrous by two days of daily subcutaneous injections of 5 μg of estradiol benzoate (EB) in 2.5 ml sesame oil, followed by one injection of 500 mg progesterone in 0.1 ml sesame oil approximately three hours before experimental use. Non-receptive stimuli females received vehicle injections. Receptivity was confirmed before use by observing lordosis in response to a non-experimental male. Males were prevented from mounting during this confirmation. All females were at least P-60 at the time of the experiment.

Social Subjugation

At p-27, male hamsters were tested for inherent fearfulness by observing their behavior in the presence of an adult male. Adult exposure lasted a few seconds and adults were not allowed to contact the juveniles. Subjects that immediately fled or vocalized were considered inherently fearful and removed from the experiment. The remaining male hamsters were divided into two groups, balanced by body weight: non-subjugated control and experimental. Experimental males were placed into the home cage of a novel adult male hamster for 20 minutes daily from p-28 through p-42, as previously described (Bastida et al., 2009; Delville et al., 1998; Wommack & Delville, 2003). Naïve males were placed into a clean, empty cage for the same period every day.

Submissive behaviors performed by experimental animals and the number of attacks and bites inflicted by the adult were recorded during each encounter. Any animal found bleeding or showing injuries was immediately removed from study (1 in 20). Typically hamsters do not cause bite marks on the skin (Blanchard et al., 2003).

Experiment 1: Consummatory Sexual Behavior

The consummatory sexual behavior of 12 socially subjugated and 10 control males was assessed at p-45, representing mid-puberty. A sexually receptive female was introduced into the homecage of the male and recorded for twenty minutes. Videos were then coded using iMovie (Apple, Inc. Cupertino, CA) and EventCoder 1.0b(10) software for the male's latency to contact the female, duration of contact, latency to mount, latency to intromit, latency to ejaculate, counts and total duration of mounts, and counts and total duration of intromissions.

Experiment 2: Appetitive Sexual Behavior

The appetitive sexual behavior of 22 socially subjugated and 18 control males was examined by observing their behavior in a Y-maze containing female stimuli. Testing in a Y-maze allows examination of the motivation and stimulus preference of the subject animal. The Y-maze has previously been used to evaluate odor discrimination in female voles, individual recognition in male and

female hamsters, and risk avoidance in hamsters (Bastida et al., 2009; Johnston et al., 1997; Lai & Johnston, 2002; Lai et al., 2005; Petrulis & Johnston, 1999).

The Y-maze is constructed of clear polycarbonate and consists of a stem and two arm sections set 45° from each other (20cm high x 16cm wide each, approximately 170cm from base of stem to end of arms). 20cm long compartments are located at the end of each arm, with a similarly sized start box located at the base of the stem. The arm compartments are separated from the body of the maze by removable black polycarbonate screens with narrow perforations, allowing the transmission of olfactory cues. Lines are marked every 12cm on the maze floor, assisting quantification of locomotor activity. A fan outside the start box facilitates airflow through the maze and draws odors from the arm compartments toward the start box.

All subjects were habituated to an empty y-maze for 10 minutes daily from p-40 through p-44. On p-45, a receptive female (R) was placed into one arm compartment and a non-receptive female (NR) was placed into the other. Stimulus females were allowed to habituate to the compartment for 10 minutes before testing began. Males were initially placed in the start box and then allowed to explore the maze for 10 minutes. The Y-maze was cleaned with 70%-95% ethanol between subjects during both habituation and testing. Subjects' behavior in the Y-maze was recorded on p-44 (no social stimuli) and p-45 (females present). Videotapes were later coded for latency to leave the start box, latency to enter each arm, latency to pass the last arm-line (i.e. enter the

area closest to the compartment screen), durations of time spent in the start box, stem, each arm, and in the area past the last arm-line, and a count of the lines crossed in the start box, stem, and each arm.

Experiment 3: Harnessed Female Preference Test

While we tested male hamsters' appetitive behavior in Experiment 2, it was necessary to study whether the males would attempt to play fight or copulate with the social stimuli. By harnessing females, the males and social stimuli were able to interact, providing tactile and olfactory information not available to the males when the social stimuli were behind a barrier. To test the males' behavior in the presence of the females without forced contact, appetitive and consummatory sexual behavior as well as aggressive behaviors of 10 socially subjugated and 11 control males was examined using harnessed females in the Y-maze. The aggressive behaviors of the females were also recorded. The procedure for this experiment was identical to the Y-maze preference test described above, with exception to the housing of the stimulus females during the p-45 preference test. Instead of being confined to the arm compartments by screens, the females were harnessed to the end of the maze by a commercial rodent harness. The harnesses allowed for full range of movement by the females and access to the females by males, but prevented the females from exiting the arm compartment area in the absence of the polycarbonate screens.

Several behaviors were recorded. Sexual behaviors including ectopic mounts, mounts, intromissions, ejaculations by the male subjects were recorded as in Experiment 1. In addition, aggressive behaviors were also analyzed. Attacks, bites, and pins by males toward each social stimulus were recorded. Attacks were defined as a chase with an attempt to bite. Bite frequencies were recorded when the subject was observed biting the one of the social stimuli. Pins were defined as our subject holding one of the stimulus animals down on her back with his forepaws and/or body.

The behavior of the female social stimuli was also recorded. Females' attacks, bites, pins, and whether or not they lordosed were observed and recorded.

Experiment 4: Development of Appetitive Sexual Behavior

In order to explore the development of appetitive sexual behavior during puberty, the female preference test described earlier (non-harnessed females) was repeated using sexually naïve control males in late puberty (p-55) and adulthood (p-70). 10 males were habituated to the y-maze from p-50 through p-54 and tested on p-55, and another 9 males were habituated to the maze from p-65 through p-69 and tested on p-70. Behavior was recorded on the last day of habituation and during the preference test (p-54 & p-55, and p-69 & p-70 respectively) and coded with iMovie for the same behaviors listed earlier for the p-45 preference test.

Data Analysis

For sexual behavior and preference tests, Student's t tests were used to compare the means between the measures recorded in subjugated and control groups. Data was analyzed separately for NS testing and testing with social stimuli present.

For the developmental study, multivariate ANOVA was used to analyze data, with the postnatal day of animals being the independent variable, and the measures in areas of the maze as the dependent variable.

Results

Experiment 1: Consummatory behavior

Sexual behaviors were compared between subjugated and control animals when a non-artificially sexually receptive female was placed into their homecage. None of these behaviors were significantly different between the subjugated and naïve groups. Table 4.1, Table 4.2, and Figure 4.1 summarize the means and standard deviations of each of the behaviors described in this experiment.

Anogenital investigation and contact

The amount of time subjects spent sniffing the anogenital region of the female stimuli was measured in subjugated and naïve individuals. Both groups

of animals display similar durations of anogenital investigation, with subjugated animals sniffing females an average of 140.06 seconds (SD = 54.02) and naïve animals sniffing 154.75 seconds (*SD* = 36.79). Frequency of anogenital investigation followed the same pattern, with subjugated individuals averaging 19.25 (*SD* = 5.89) counts and controls investigating an average of 22.5 counts (*SD* = 6.63). Investigation of the anogenital region of the female by the males in both groups lasted about 2.5 of the 20-minute test, or about 12% of the time.

A separate measure was taken of the time that subjects spent in direct contact with the females. Subjugated individuals spent an average of 749.6 seconds (*SD* = 119.08) in contact with females and naïve animals spent about 758 seconds (*SD* = 98.68) in contact with the female stimuli. Both groups of males spent about 60 percent of their time in contact with the receptive female. Frequency of contact was similar between groups as well, with subjugated and naïve animals averaging 44.58 (*SD* = 13.27) and 46.50 (*SD* = 6.11) contact counts, respectively.

Mounts

Mounts directed at the anogenital area and other areas on the females were recorded. The frequency of ectopic mounts and mounts directed towards the anogenital area of the female were similar between groups. Naïve animals averaged 37.5 mounts (*SD* = 17.49), and stressed animals averaged 36.67 mounts [*SD* = 18.86].

Intromissions

Intromissions were observed in both groups, usually after about 11 minutes. There were no differences in duration or frequencies of intromissions. Naïve animals spent an average of 184.86 seconds (*SD* = 84.34) and subjugated spent an average of 156.88 seconds (*SD* = 87.28) intromitting. Frequency of intromissions was on average 38 counts (*SD* = 18.03) in naïve animals and 35.17 counts (*SD* = 20.19) in stressed individuals. The frequency of ectopic mounts in control [*M* = 26.33, *SD* = 11.50] and subjugated animals [*M* = 25.25, *SD* = 9.49] did not differ.

Ejaculations

Frequency of ejaculations was also similar between groups, with the frequency of ejaculations ranging from 0 to 9. On average, a little over 3 ejaculations were observed per animal. A small number of ectopic ejaculations were also observed, with one animal per group exhibiting this behavior once. Naive [*M* = 3.25, *SD* = 2.93] and control [*M* = 3.17, *SD* = 2.69] animals did not differ in this measure. Ectopic ejaculations were infrequent and were also similar in naïve [*M* = 0.08, *SD* = 0.29] and stressed juveniles [*M* = 0.08, *SD* = 0.29]. Table 4.2 shows data for intromissions, mounts, and ectopic mounts.

Experiment 2: Appetitive behavior

Preference Test

No differences between groups were observed in any of the measures recorded when no social stimulus was present. However, with a social stimulus present several differences between groups were evident.

Durations

Duration of time spent in the start box was significantly higher in subjugated individuals [$t(35) = 2.70$, $p = .011$]. Stressed animals spent less time in the non-receptive stimulus arm of the maze [$t(38) = -3.34$, $p < .01$]. Control animals also spent more time at the screen near the non-receptive stimulus [$t(38) = -2.98$, $p < .01$]. Durations data is given in Table 4.3.

Latencies

Subjugated hamsters took a longer time to reach the non-receptive stimulus arm of the maze than naïve animals [$t(38) = 2.48$, $p < .05$]. There was no difference between groups in latency to reach the arm of the maze containing a receptive female stimulus.

Line Crossing Counts

The subjugated animals crossed fewer lines in the non-receptive stimulus arm of the maze [$t(29) = -3.78$, $p < .01$]. Within the control group, animals had a trend towards walking more lines in the non-receptive stimulus arm than the receptive stimulus arm [$t(14) = 2.11$, $p = .053$]. There was no difference between groups in line crossing counts in the receptive stimulus arm of the maze. This data is shown in Table 4.4.

Alternations

A subset of animals' alternations were recorded. There was a significant difference between groups, with control animals performing more alternations [$t(6) = -2.6$, $p < .05$].

Figure 4.2 illustrates data for this preference test.

Experiment 3: Harnessed female preference tests

Durations

There were no significant differences between groups in duration of time spent in any area of the maze. Subjugated animals spent an average of 283.91 seconds [*SD* = 287.16] in the start box and naïve animals spent 106.96 seconds on average [*SD* = 189.93]. In the non-receptive stimulus arm, subjugated juveniles spent 139.47 seconds on average [*SD* = 138.77] and naïve individuals

spent 227.2 seconds on average [*SD* = 44.86]. In the receptive stimulus arm of the maze, stressed animals spent 151.85 seconds [*SD* = 144.87] and naïve animals spent 225.34 seconds [*SD* = 134.91].

Latencies

There was a trend toward a significant difference between groups in the latency to reach the non-receptive stimulus arm of the maze, with naïve animals reaching the non-receptive stimulus arm of the maze quicker than the subjugated individuals [t(df) = , p = .051]. In addition there was a between groups effect in latency to reach the receptive stimulus arm of the maze. Naïve animals were quicker to reach the receptive stimulus arm of the maze than subjugated individuals [t(13) = 2.18, p<.05]. Duration and latency data are given in Table 4.5.

Line Crossing Counts

There were no differences between groups in line crossing counts in any area of the Y-maze. Table 4.6 gives the data for line crossing counts when subjects were allowed contact with females.

Male interactions with females

Performance of ectopic mounts, mounts, intromissions, ejaculations, attacks, bites, and pins toward the non-receptive stimuli by subjugated and naïve

animals were similar and low in number. Two animals mounted the non-receptive stimuli; both animals that exhibited this behavior were naïve juveniles. Contact time, too, was similar between groups.

In contrast to this, 31 mounts and 11 ectopic mounts were performed on the receptive social stimuli. Of these, the vast majority of individuals exhibiting this behavior were in the naïve group, with 8 out of the 11 males exhibiting this behavior. Naïve animals performed more ectopic mounts [$t(18) = -2.27$, $p<.05$] and mounts [$t(18) = -2.10$, $p<.05$] to the anogenital region of the receptive females. Only four animals intromitted with receptive females; three from the naïve group and one from the subjugated group showed this behavior. There were no ejaculations, attacks, bites, or pins recorded in any of the subjects toward receptive females. Contact time was similar between subjugated [M = 126.68, SD = 100.26] and naïve [M = 190.99, SD = 111.64] animals.

Female interactions with male subjects

Non-receptive females were not receptive and all but four receptive females lordosed during testing. Non-receptive females exhibited aggressive behavior toward the subjects, with 12 out of the 20 stimuli attacking, 9 of the females biting, 5 of the females pinning the male subjects. 9 attacks were aimed at 4 subjugated individuals, and 24 attacks were aimed at 8 naïve animals. None of these behaviors were observed in the receptive female stimuli.

Figure 4.3 illustrates data for the preference test with contact with social stimuli allowed.

Experiment 4: Development of Sexual Behavior

Line crossing counts

Variability in line crossing counts was significantly attributable to postnatal age in the start box [F (2, 396.87)=3.34, p< .05] and NS arm of the maze [F(2, 5133.36 = 4.57, p < .05], depending on the age of the animal.

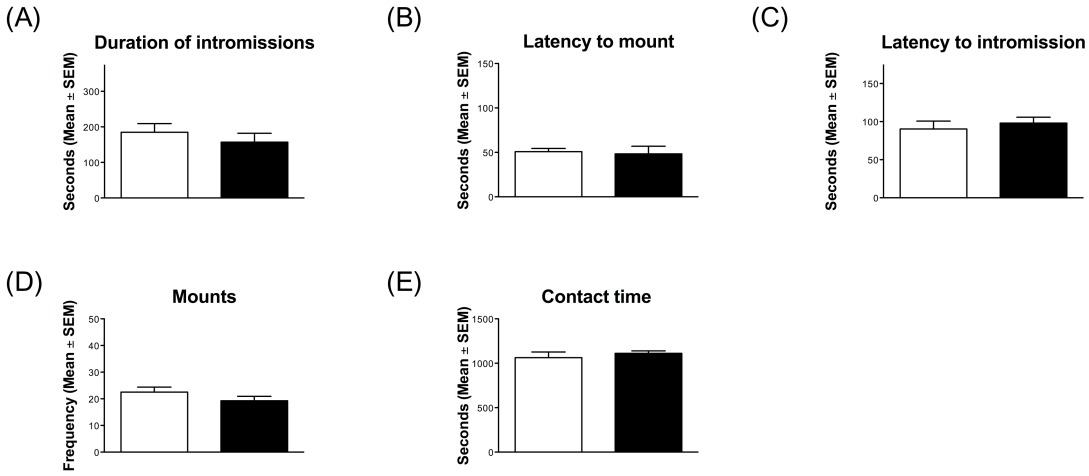
Durations

Variability in screen duration was not attributable to postnatal age. Variability in durations in any other areas of the maze was not attributable to postnatal age.

Latencies

The variability between age groups for how quickly they entered arms of the maze was not attributable to the age of the animals.

Figure 4.1. Consummatory sexual behavior in close proximity



Consummatory sexual behaviors in subjugated and naïve animals while in close contact with a receptive female. Duration of intromissions (A), latency to mount the female (B), latency to intromission (C), frequency of mounts (D), and contact time (E) were similar in subjugated and naïve juveniles.

Table 4.1. Appetitive sexual behaviors while in close proximity

	Anogenital Frequency	Anogenital Duration	Contact Frequency	Contact Duration
Control Mean	22.50	154.75	46.50	758.00
Control Std Dev	6.63	36.79	6.11	98.68
Subj Mean	19.25	140.06	44.58	749.60
Subj Std Dev	5.89	54.02	13.27	119.08

Sexual behaviors were not affected by stress in forced close proximity with a receptive female. Anogenital investigation frequency, anogenital investigation duration, contact frequency, and contact duration were similar in repeatedly subjugated and naïve juvenile hamsters at mid-puberty.

Table 4.2. Consummatory sexual behaviors in close proximity

	Intromiss Frequency	Intromiss Duration	Ect Mount Freq	Mount Freq	Ejac Freq	Ectopic Ejac Freq
Control Mean	38.00	184.86	26.33	37.50	3.25	0.08
Control Std Dev	18.03	84.34	11.50	17.49	2.93	0.29
Subj Mean	35.17	156.88	25.25	36.67	3.17	0.08
Subj Std Dev	20.19	87.28	9.49	18.86	2.69	0.29

Consummatory sexual behaviors were not affected by stress in forced close proximity with a receptive female. Intromission duration, ectopic mount frequency, mount frequency, ejaculation frequency, and ectopic ejaculation frequency were all similar in repeatedly subjugated and naïve juvenile hamsters at mid-puberty.

Table 4.3. Motivated behavior without contact

	Durations						Latencies	
	Start Box	Stem	NR Stim Arm	R Stim Arm	NR Stim Screen	R Stim Screen	NR Stim Arm	R Stim Arm
Subj Mean	243.46	50.34	123.8	147.78	111.51	124.78	261.52	313.12
Subj Std Dev	215.25	36.80	127.6	146.13	123.65	154.11	260.11	235.63
Control Mean	84.86	55.36	257.9	184.46	226.85	141.90	109.03	198.34
Control Std Dev	105.65	55.92	158.1	139.58	166.82	111.11	133.80	190.35

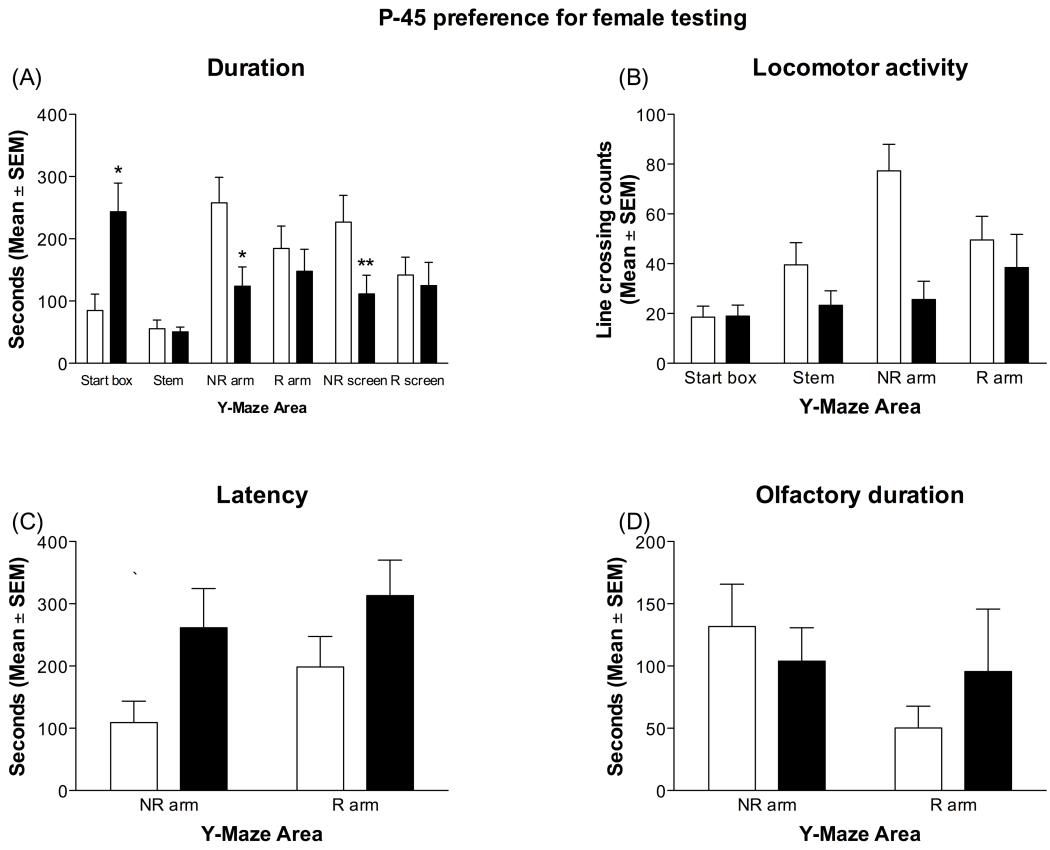
Duration of time in the start box, stem of the maze, non-receptive stimulus arm of the maze, receptive stimulus arm of the maze, near the screen in the non-receptive stimulus arm of the maze, near the screen in the receptive stimulus arm of the maze, and latencies to the non-receptive stimulus arm of the maze, and to the receptive stimulus arm of the maze were recorded.

Table 4.4 Ambulatory behavior without contact

	Start	Stem	NR Female	R Female
Subjugated Mean	18.93	23.29	25.64	38.43
Subjugated Standard Dev	16.51	21.95	27.43	50.00
Control Mean	18.50	39.50	77.25	49.50
Control Standard Dev	15.58	31.01	37.11	32.96

Line crossing counts of males in the Y-maze with receptive (R) and non-receptive (NR) females present, but no physical contact allowed.

Figure 4.2. Preference test at mi-puberty in Y-maze



Preference test with a non-receptive (NR) and receptive (R) social stimulus at mid-puberty. Duration of time spent in areas of the maze (A), line crossing counts (B), latencies to reach arms of the maze (C), and olfactory duration in arms of the maze (D) in subjugated (black bars) and naïve animals (white bars).

Table 4.5. Motivated behaviors in Y-maze with contact

	Durations						Latencies	
	Start	Stem	NR Stim	R Stim	NR Stim Screen	R Stim Screen	NR Stim Arm	R Stim
Subj Mean	283.91	22.48	139.47	151.85	125.44	113.92	290.53	276.34
Subj Standard Dev	287.16	20.14	138.77	144.87	129.71	109.84	290.48	304.54
Control Mean	106.96	28.87	227.20	225.34	197.07	198.94	59.52	37.48
Control Std Dev	189.94	19.97	126.95	134.92	114.55	130.63	86.48	59.55

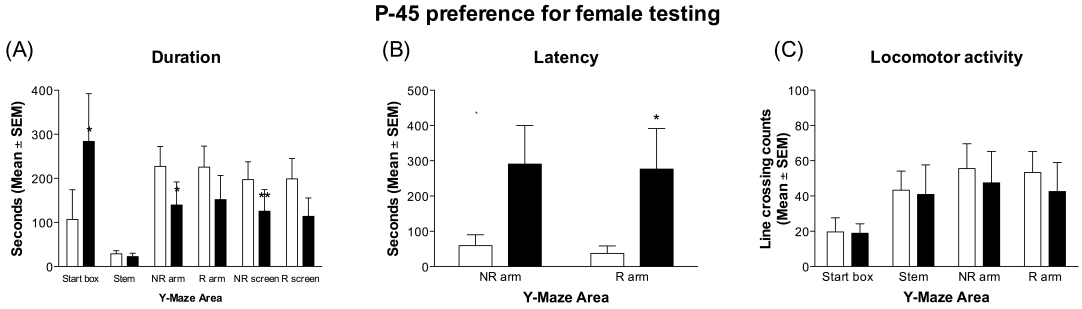
Duration of time spent in areas of the maze and latencies to reach arms of the maze in subjugated and naïve juveniles in the Y-maze with harnessed non-receptive (NR) and receptive (R) female social stimuli.

Table 4.6. Ambulatory behavior in the Y-maze with contact

	Start box	Stem	Nonreceptive	Receptive
Subjugated Mean	18.86	40.86	47.43	42.57
Subjugated Standard Dev	14.09	44.36	47.34	43.40
Control Mean	19.67	43.33	55.56	53.33
Control Standard Dev	23.87	32.31	42.13	35.54

Line crossing counts of males in the Y-maze with harnessed receptive and non-receptive females.

Figure 4.3. Motivated behaviors in the Y-maze with contact



Male preference test with a harnessed non-receptive (NR) and receptive (R) social stimulus at mid-puberty. Duration of time spent in areas of the maze (A), latencies to reach arms of the maze (B), and line crossing counts (C) in subjugated (black bars) and naïve animals (white bars).

Discussion

Consummatory sexual behaviors in subjugated and control animals were similar at mid-puberty. The number of mounts, ectopic mounts, and ejaculations were comparable between the subjugated and naïve groups. Appetitive behaviors, were vastly different between subjugated and naïve animals depending on the context. As opposed to the forced direct contact in the homecage, in the Y-maze animals could pace their contact with social stimuli. In this context, subjugated animals spent most of their time in the start box, rather than near the social stimuli. Interestingly, naïve animals spent most of their time near the social stimuli and showed a preference for the social stimulus that was not sexually receptive. Line crossing counts followed the same pattern; subjugated individuals crossed fewer lines in the arm of the maze containing the non-sexually receptive social stimuli. In addition, alternations were significantly different with the naïve group alternating more than the socially stressed juveniles, suggesting they were attempting to make a choice between the two females. When the females were available for interaction in the Y-maze the only differences between groups was in the latency to reach the arm of the maze with the non-receptive social stimulus. Other measures were interesting but high variability prevented significant findings. For example, more naïve males attempted to mate with females in the Y-maze than subjugated males. Hamsters at mid-puberty, late puberty, and early adulthood did not differ significantly in any

of the measures we examined. Taken together, these data suggest that consummation of sexual behavior is not affected in stressed males, but appetitive behavior is inhibited. However, this is context-dependent. It is also interesting to note that control males generally preferred approaching non-receptive females. Furthermore, by adulthood sexually naïve males still show no preference for receptive females. This could all point to reduced motivation to mate and increased avoidance of adults, whether male or female. These data further elucidate the generalization of avoidance by subjugated males.

Consummatory sexual behaviors were not affected by social stress in juvenile hamsters. This depended on context, however. If approach of a female social stimulus was involved, there was no difference between groups, but when no approach is required, there is no difference between subjugated and naïve animals. This is somewhat different from previous studies reporting decreased reproductive opportunities for animals low in their group's hierarchy in that when given the opportunity these solitary animals choose to not mate. So while stressed juveniles have the ability to copulate, their mating strategy seems to be on par with their naïve counterparts. It would be interesting to study the effects of social subjugation in juvenile hamsters on sperm quality and actual reproductive success in these animals, since copulatory behavior does not necessarily translate into reproduction. Our data confirm previous reports that adolescent hamsters are more resilient to social subjugation than adults. Indeed, it appears that juvenile hamsters are more resilient to stress than adults. One of

these studies reported lower testosterone levels in subjugated adolescents at the time our animals were tested for sexual behavior. It is interesting that our animals copulated despite presumably decreased plasma androgen levels (Wommack et al., 2004). Since copulatory behaviors have been observed in male hamsters as young as P-35, it seems that this behavior develops prior to fully activated testosterone levels (Miller et al., 1977). Yet castration, and resulting decreases in testosterone ceases copulatory behavior (Siegel, 1985). This suggests that while testosterone is required for the expression of these behaviors, copulation is possible in these animals before plasma testosterone reaches adult levels. So, elevated levels of testosterone are not necessary for copulatory behavior during pubertal development. As such, the fact that our subjugated animals copulated with presumptively lower plasma testosterone levels is in accordance with previous literature.

The appetitive behavior of subjugated and naïve males differed. Stressed individuals avoided the female social stimuli, while naïve animals were highly interested in being near conspecifics. This, coupled with our previous observation that subjugated juveniles were avoidant of adult males, suggests a generalized avoidance of older and larger hamsters. Yet these hamsters are capable of consummating when placed in close contact with females. So juveniles develop avoidance and possibly fear of adult social stimuli, but this effect is not strong enough to suppress copulatory behavior. This, too, points to resilience to stress in these animals. It is possible that enhanced fear in these

animals may be due overactive activity in the amygdala. Amydalar activity has been associated with acquisition and expression of fear responses (Campeau et al., 1992; Miserendino et al., 1990; Rodrigues et al., 2001). Indeed, overexpression of cAMP-responsive element binding protein has been found to facilitate behavioral changes associated with conditioned defeat in adult hamsters (Jasnow et al., 2005). In addition, in non-manipulated juveniles, it could be that the balance between approach and avoidance behavior is skewed toward approach in juveniles in order to facilitate their leaving the nest in favor of exploring and establishing a new territory. Thus, exposure to stress during development prevents this normal adolescent pattern. Instead, their behavioral balance is tipped away from approach and toward avoidance.

We observed that in addition to the avoidance of social stimuli by subjugated juveniles, naïve animals chose the non-receptive social stimuli over the receptive stimuli. At P-45 these animals chose non-receptive females when given a choice between receptive and non-receptive social stimuli. This is similar to what has been previously observed in hamsters. Hamsters do not show preference for estrus females, and may not be able to discriminate estrus state when not in contact with the female (Johnston, 1977; Johnston, 1980; Kwan & Johnston, 1980; Landauer & Banks, 1973; Landauer et al., 1978). However, the clear choice made by naïve animals at mid-puberty suggests they are able to discriminate between the social stimuli. It is possible that these juveniles need sexual experience or longer exposure to high levels of testosterone to establish

mate choice that favors sexually receptive females. In this way, it is possible that appetitive aspects of sexual behavior are developed after the consummation of the behavior.

Alternatively, it is possible that the network of brain regions involved in the sensory processing needed are underdeveloped, or require activation by way of sexual experience in order to develop the necessary coordination of activity between brain regions. Mate choice in hamsters involves several nodes, including the olfactory bulb and other regions within the social behavior network (Trimestra et al., 2005). Sexual experience alters sexual behavior in that it becomes more efficient and successful (Domjan, 1992; Pfaus et al., 2001; Woodson, 2002). Therefore, sexual behavior may serve to strengthen the connections and activity between these regions by activating reward pathways. Indeed, experienced males begin sexual behavior and ejaculate faster than inexperienced males (Dewsbury, 1969; Larsson, 1959). In addition, sexual behavior in experienced males is robust in that it is difficult to extinguish this behavior with lesions to the olfactory bulb, lesions to the vomeronasal organ, or castration (Bermant & Taylor, 1969; Costantini et al., 2007; Meredith, 1986; Saito & Moltz, 1986).

In Chapter 3 we found that activity in the medial preoptic area, medial preoptic nucleus, and ventral tegmental area all decreased in subjugated individuals. We proposed that decreases in sexual behaviors might result from these changes in activity of brain regions, and that the avoidance/approach

balance is shifted toward avoidance in subjugated individuals. While consummatory behavior within a restricted area appears to be normal in subjugated individuals, when given a choice subjugated animals are avoidant of adult females. The avoidance by subjugated individuals fits with our finding that they have decreased activity in the MPOA, MPN, and VTA since these regions are involved in reward mechanisms and these animals show, depending on the context, decreased reward-seeking behaviors. However, decreased activity in the MPN and MPOA would be more easily reconciled with decreased consummatory behavior as well. I propose that in a more natural setting with more space and a complex environment, these animals would initially perform copulatory behaviors at a decreased rate, as was observed in the Y-maze. In addition, stressed animals in our previous studies exhibited anxiety and fear only in a social setting, and were otherwise much like their naïve counterparts in other contexts (Bastida et al., 2009). This is perhaps the reason that, despite sampling about 60 regions and subregions, only three brain regions were significantly different between stressed and naïve animals.

While we thought it possible that the control individuals would play fight with the females, and the preference of the control animals for non-receptive social stimuli seemed to indicate this may be the case. However, these animals were more interested in mounting the social stimuli than fighting and when tested with harnessed females, naïve animals were quick to mount females. Interestingly, mounts were mostly exhibited by naïve animals and were directed

toward the receptive female stimuli. Interactions with non-receptive females mostly involved the females attacking the males. This does not appear to have discouraged the males in seeking interaction with these females, as there were no significant differences between behaviors in the arms of the maze within the naïve group. Indeed, males were observed repeatedly approaching females that attacked. This is in accordance with previous findings that indicate that risk-taking behavior increases during puberty and that higher plasma testosterone levels have a protective effect on anxiety or depression-related behaviors and reduce submission in hamsters (Soloman et al., 2009; Steinberg & Belsky, 1996). In addition, Steinberg and Belsky have proposed that risk-taking behavior in juveniles is a mechanism that motivates individuals to separate from their families. This is particularly relevant with male hamsters at mid-puberty, since that is a key time for separation from the home nest. Without risk-taking behavior, it would be impossible for male juvenile hamsters to leave the home nest and establish their own individual territories despite the danger of predators and other hamsters. This of course fits with the Triadic model that indicates that naïve juveniles would tend to approach conspecifics.

Subjugated juveniles had similar sexual behaviors in their homecage and avoided female social stimuli in the Y-maze. However, when females were available for interaction they did mount the receptive female, although to a much lesser extent than the naïve males. This is likely due to learned avoidance or social anxiety in these juveniles. While according to the Triadic model, the

balance of approach/avoidance leans toward avoidance in these animals, and indeed it seems adaptive that an individual that was repeatedly attacked by adults should avoid adult conspecifics, these animals do eventually approach and stay near the female social stimuli. So, the fact that there are fewer mounts and fewer intromissions exhibited may have more to do with the amount of time it takes for these animals to overcome their anxiety. Interaction with the social stimulus provides tactile, olfactory, visual, and auditory interplay that is not available without direct contact. This dialog between the subjects and stimuli appears to be necessary for the anxiety of the socially stressed juveniles to be allayed. So, it is likely that social anxiety in subjugated juveniles, like risk assessment behavior and inhibition of aggression, may also be transient in hamsters subjugated during puberty (Wommack et al., 2003). Further, our research indicates that this fear and anxiety can be diminished very quickly under certain conditions.

Appetitive aspects of social behavior at mid-puberty seemed to require activation by way of sexual experience. This held true for naïve hamsters at mid-puberty, late puberty, and early adulthood as well. At none of the time points tested was there a clear preference for receptive females. This suggests that there is indeed an element lacking in these inexperienced males that is needed to drive their preference toward preference for sexually receptive females. There was a trend toward less avoidance in animals in late puberty and early adulthood, is in accordance with the Triadic model of motivated behavior. While

we expected that naïve juveniles at mid-puberty would be most likely to approach social stimuli, they are relatively more avoidant than their adult counterparts, although not significantly so. However, naïve animals do indeed approach conspecifics more than subjugated juveniles. This may possibly have to do with the aggression profiles and social status of these animals. Had these naïve animals been winners in aggressive encounters, they well may have been more likely to approach conspecifics. Further, I propose that adult male hamsters that win fights would have a balance shifted toward approach, while those who had been defeated would lean toward avoidance as well.

In this study we found that consummation was unaffected by repeated social stress, while appetitive aspects were altered in a context-dependent manner. Social stress induced avoidance in animals that would otherwise be more likely to approach conspecifics. This is only a partial effect, although the balance in socially stressed animals is tipped toward avoidance. It appears that consummatory behaviors develop prior to appetitive aspects because at mid-puberty naïve males, when given a choice between a non-receptive and receptive social stimulus, choose the non-receptive stimulus despite the fact that they are capable of copulating.

Chapter 5: Conclusion

Avoidance and approach

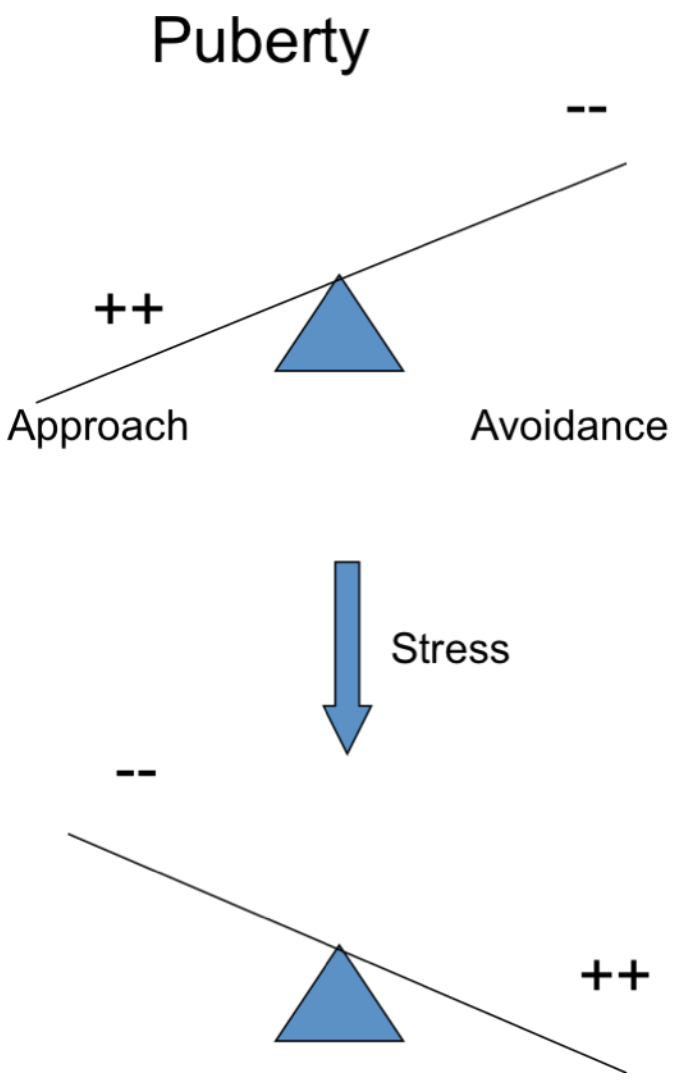
According to the Triadic model of motivated behavior in juveniles, a naïve juvenile individual would tend to approach an unfamiliar conspecific (Ernst & Fudge 2009). This is described as increased risk-taking and reward seeking behavior in normal juveniles. However, after exposure to repeated social stress, we found that these systems were altered insofar as the juveniles tended not to approach conspecifics, but instead avoided them. This is in accordance with a previous study by Wommack et al. (2004) that reported that stressed individuals showed decreased olfactory investigation of adult male social stimuli. Olfactory investigation includes an approach and sniff of the social stimulus, and this behavior was almost completely inhibited in stressed juveniles. Taken together, these findings indicate that approach systems were less active, avoidance systems were more active, and/or regulatory systems were acting divergently. Figure 5.1 shows the effect of stress during puberty on approach/avoidance behavior. In contrast, adult animals have a balance of motivated behaviors (Figure 5.2). Pubertal animals tend to approach conspecifics by default, but after repeated social stress these animals tend to avoid conspecifics in a context-dependent manner. Our experiments investigated this phenomenon, along with the development of other social behaviors during puberty.

In previous studies of stress in juvenile hamsters, subjects and social stimuli were in contact with one another. In our experiments we observed behavior toward conspecifics without allowing contact between our subjects and social stimuli in order to eliminate the confound of our subject's behavior being influenced by the behavior of the other animal. In Chapter 2 we showed that subjugated juveniles exhibit anxiety and avoidance only in a social context. This effect generalized to novel males and was not specific to familiar individuals. This change in motivated behavior could benefit juvenile hamsters in the wild. A juvenile male exploring away from the natal nest would be attempting to establish his own territory. In this setting, it would benefit the juvenile to learn to avoid attacking adults, but not be fearful in general. This would make it possible for them to confidently travel to new territories and approach same-sized and smaller animals to compete for territory if necessary.

Indeed, in the presence of another juvenile, stressed individuals are not avoidant. Previous studies indicated that as adults, repeatedly stressed juveniles were more likely to attack smaller conspecifics than naïve individuals (Delville et al., 1998; Wommack et al., 2003). In Chapter 2 we found that socially stressed juveniles spend more time than naïve individuals near younger, smaller conspecifics. So, subjugated individuals did seek contact with younger individuals, which is similar to approach behavior in naïve juveniles. This is interesting because it further emphasizes that subjugated juveniles do not show generalized fear or avoidance, and do not display inhibition of approach in certain

social contexts. Indeed, it points to the fact that motivated behavior is not black and white, but is instead context-dependent. The size of the social stimulus is a factor, which means the Triadic model of motivated behavior may need further modification to encompass behavior in a wider range of contexts. Ernst and Fudge (2009) proposed a modified version of the Triadic model called the fractal Triadic model. This model partially addresses this issue in that it represents more heteromodal role of each of the nodes in the model. However, the role of motivated could be further clarified with a wider range of contexts discussed.

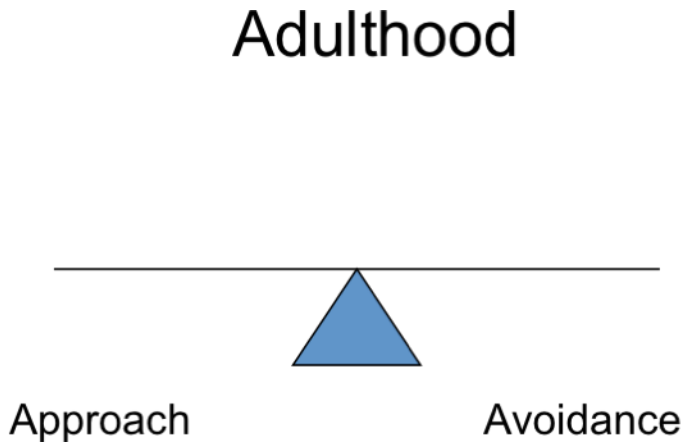
Figure 5.1. The Triadic model in puberty and the effect of social stress



This figure is a heuristic representation of approach and avoidance during puberty. By default, juveniles tend have weaker modulation systems, decreased fear systems with respect to social losses, and increased responsiveness to reward than adults. Thus these pubertal individuals tend to approach

conspecifics. After being socially stressed, however, this inclination is reversed and animals are more likely to avoid conspecifics.

Figure 5.2. The Triadic model in adults



This heuristic representation of the Triadic model of motivated behaviors in adults shows the behavior of mature individuals is indicative of a balance of motivated behaviors.

Appetitive sexual behavior

In addition to avoidance of adult males, repeatedly stressed juveniles show altered motivation to interact with female social stimuli. When given a choice between receptive and non-receptive adult females in the Y-maze, juveniles were avoidant and spent most of their time away from the social stimuli. In addition, subjugated individuals approach conspecifics less than naive juveniles. This may possibly have to do with the aggression profiles and social status of these animals. There are several possible reasons why the subjugated juveniles avoided adult females. It is possible that fear and anxiety are responsible for the avoidance of adult females by stressed individuals. The inhibition of approach behavior observed in these animals when in the presence of an adult male could have generalized not only to all adult males, but also to all adult females as well. This could mean that the juveniles, much like defeated adult male hamsters, are over-generalizing their fear of conspecifics (Huhman et al., 2003). Together with the lack of avoidance of juveniles, these data suggest that these animals may be moving to a more adult-like balance of avoidance, approach, and regulatory systems in that their tendency will not be of approach of conspecifics, but is instead context-dependent.

Another possible explanation for the avoidance of female social stimuli by stressed juveniles is that avoidance of social stimuli by socially stressed juveniles is generalized to all adults, including females. So, not only are these juveniles

avoidant of adult males, but they are also avoidant of adult females. This may be beneficial to hamsters in their natural habitat, considering the females' proclivity to attack. Since male and female adults are likely to attack, it would make sense to avoid any adults that a subject encounters, particularly if the subject has previously been exposed to many attacks. Generalization of avoidance of adults may be an adaptation unique to hamsters, and other solitary territorial species.

Development of Appetitive sexual behavior

While we found that naïve individuals were likely to approach social stimuli, when given a choice between a sexually receptive and non-receptive female, these individuals chose to spend more time near the non-receptive stimulus. This prompted us to study the development of appetitive aspects of social behavior in naïve juveniles. We compared mate choice in naïve animals at P-55 and P-70 with the data we obtained in naïve individuals at mid-puberty. While we found that naïve juveniles at mid-puberty were likely to approach social stimuli, they are relatively more avoidant than their adult counterparts, although not significantly so. Interestingly, even at late puberty and adulthood, the social stimulus chosen by these animals was the non-receptive stimulus. The choice became less obvious, but we expected that by adulthood, naïve subjects would choose the sexually receptive female. This suggests that there is indeed an element lacking in these inexperienced males that is needed to drive their preference toward preference for sexually receptive females. It is possible that

sexual experience is necessary to drive a preference for sexually receptive females. This points toward differential maturation of discrete components of sexual behavior. Admittedly, appetitive and consummatory aspects of sexual behavior are tightly tied to one another and difficult to separate into distinct categories. However, our observations make it clear that appetitive aspects of sexual behavior are underdeveloped in both subjugated and naïve animals at mid-puberty. Further, naïve males continue to show mate choice that does not appear to be conducive to reproductive behavior into adulthood. This is particularly curious since the literature suggests that copulatory behavior is achievable by postnatal day 30.

Consummatory sexual behavior

Since naïve individuals spent a considerable amount of time near non-sexually receptive females, the reason for this preference became of interest. At mid-puberty it was possible that these juveniles were interested in play fighting interactions with the female social stimuli. Thus, we tested animals in the Y-maze again, but removed barriers to allow our subjects to interact with the female social stimuli. Further, since it was possible that subjugated individuals required interaction with social stimuli in order to overcome their avoidance, we again compared stressed and naïve juveniles. We found that all subjects were more interested in mounting the social stimuli than fighting. Indeed, naïve animals were quick to mount females. Mounts were mostly exhibited by naïve juveniles and

were directed toward the receptive female stimuli. Interactions with non-receptive females mostly involved the females attacking the males. This does not appear to have discouraged the males in seeking interaction with these females, as there were no significant differences between behaviors in the arms of the maze within the naïve group. Indeed, males were observed repeatedly approaching females that attacked. This is in accordance with previous findings that indicate that risk-taking behavior increases during puberty and that higher plasma testosterone levels have a protective effect on anxiety or depression-related behaviors (Soloman et al., 2009; Steinberg and Belsky, 1996).

In addition, we found that when female social stimuli were available for interaction in the Y-maze the only significant difference between groups was in the latency to reach the arm of the maze with the non-receptive social stimulus. This again suggests altered motivation to seek out conspecifics in subjugated individuals. Stressed juveniles did copulate, however, albeit at a lower frequency than their naïve counterparts. This is likely caused by a longer latency to reach conspecifics. Thus, altered motivation to seek out conspecifics affected their approaching females, but copulatory behavior itself did not appear to be changed in subjugated individuals. These data suggest that consummation of sexual behavior is not affected in stressed males, but appetitive behavior is inhibited.

A typical way of testing consummatory sexual behavior is to place a female into the homecage of the subject, or to place both into a small arena. This type of setting forces contact between the animals, somewhat removing

appetitive aspects of sexual behavior. We tested subjugated and naïve juveniles in this context to discover whether this change in context would eliminate the differences between groups in their interactions with females in the Y-maze. Indeed, when a female was placed into the homecage of stressed and naïve animals, it appears copulatory behavior was not affected by repeated social stress. All of the analyzed measures of consummatory behavior were similar between the subjugated and naïve individuals. Further, this confirmed that when forced into contact with a female, males at mid-puberty are very well able to perform sexual behaviors. Indeed, it has been found that hamsters at P-30 are capable of performing copulatory behaviors (Bond, 1945). This again points to the fact that effects of repeated social stress during puberty are context-specific.

Further, these data indicate the resilience of juvenile golden hamsters to the effects of repeated social stress. Despite the fact that these animals clearly show avoidance of adult conspecifics, including females, the performance of sexual behavior is unaffected. What is of significance in the particular case of sexual behavior in stressed juveniles is that consummatory behavior is conserved despite presumably lower testosterone levels in these animals. Castration, and resulting decreases in testosterone ceases copulatory behavior (Siegel, 1985). So, testosterone is required for the expression of these behaviors, but copulation is possible in these animals before plasma testosterone reaches adult levels. It appears that elevated levels of testosterone are not necessary for copulatory behavior during pubertal development. Further,

previous literature suggests that sexual behavior in juveniles is reduced, but is indeed present when testosterone levels are increased to adult levels (Baum, 1972; Larsson, 1967; Sisk et al., 1992; Sodersten et al., 1977). However, the question then becomes what purpose elevated levels of testosterone during adulthood serves. Higher testosterone levels may be required for the maintenance of consummatory behavior, motivational aspects of sexual behavior, or for other social behaviors during late puberty and adulthood.

Neural effects

We found that the behavior of juvenile hamsters was in accordance with the Triadic model of motivated behaviors, although the model could be expanded to address a wider variety of social behaviors. In our addendum to the model, we add that social stress results in avoidant behavior. In order to test the neural correlates of motivated behavior in juveniles delineated in our version of the Triadic model, we studied neural activity in stressed and naïve individuals. Cytochrome oxidase histochemistry was used to examine changes in long-term neural activity in regions of the brain after repeated social stress during puberty.

The avoidance of conspecifics by subjugated individuals in our version of the Triadic model fits with the finding that they have decreased activity in the MPOA, MPN, and VTA. Since these regions are involved in reward mechanisms and these animals show, depending on the context, decreased reward-seeking behaviors, this is in line with the Triadic model in Figure 1. In addition, this data

fits with the Triadic model in that these areas are related to motivated behaviors and it appears they may have reduced motivation for sexual behavior or delayed maturation of appetitive sexual behavior. Further, both appetitive and consummatory sexual behaviors have been associated with increased extracellular dopamine in the medial preoptic area, with dopamine levels during the appetitive aspect of sexual behavior predicting the amount of copulation that follows (Kleitz-Nelson et al., 2010). So, reduced activity in this region could indicate that decreased appetitive sexual behavior by stressed individuals would be followed by decreased copulation. This is in accordance with what we found in tests in the Y-maze. Since we found that decreased appetitive sexual behavior in subjugated individuals was dependent on the testing apparatus, it would be interesting to measure extracellular dopamine levels in the MPOA in these individuals during appetitive aspects of sexual behavior in a small arena versus in a large or more complex environment. Presumably, in animals in a complex environment we might see decreased extracellular dopamine since they are less motivated to seek out females under these conditions.

While our version of the Triadic model suggests that the basolateral amygdala would have increased activity, we did not find a difference between subjugated and naïve juveniles. However, stressed animals in our studies exhibited inhibited approach only in a social setting, and were otherwise much like their naïve counterparts in other contexts (Bastida et al., 2009). This is perhaps the reason that there were no differences in activity in the amygdala and

other structures related to stress. Further, the context-specific nature of altered motivated behaviors could explain why the activity of so few brain regions were significantly different between stressed and naïve animals.

Predictions can be made for the long-term consequences in stressed juveniles. Stressed juveniles have accelerated development of aggression and HPA axis alterations similar to highly aggressive hamsters, which are used as a model for reactive aggression (Cervantes, 2010; Wommack & Delville, 2003; Wommack et al., 2003; Wommack et al., 2005). Thus it is possible that stressed juveniles would also display impulsivity and could be useful as a model of reactive aggression as well. Further, besides accelerated maturation of aggressive behavior and impulsivity, another long-term consequence could be a rebounding of approach behavior in adulthood (Figure 5.3).

Significance

Puberty is of particular importance as it is a critical period for the emergence of mental disorders in humans. The onset of disorders such as schizophrenia, major depression, and anxiety disorders typically start during late puberty in males, suggesting that events that occur during puberty may either serve as protective or aggravating factors in the development of these disorders. Aggravating factors include exposure to a stressor. For example, bullied juveniles are at risk for depression and anxiety disorders. Puberty is also a critical period for the development of anger and antisocial behaviors. Again,

social stress is a critical factor in the onset of these behaviors. In contrast, puberty is also a period of exposure or a period of development of protective factors in some individuals. As social stress is preponderant throughout life, the development of coping skills are critical. For instance, social support helps mitigating the effect of bullying. Our studies on stress during puberty are particularly relevant to these issues.

The applicability of the rodent model we used lies in the similarity between the development of the HPA axis in hamsters and humans. In hamsters and humans, the activity of the HPA axis matures during puberty in correlation with Tanner stages (Elmlinger et al., 2002; Jonetz-Mentzel et al., 1993; Kiess et al., 1995; Van Kampen & Fuchs, 1998; Wommack et al., 2005). Individuals exposed to stress in early puberty likely experience lower cortisol levels than in late puberty, thus the potential for damaging effects of stress from cortisol are greater in late puberty than in early puberty. In hamsters, as in humans, baseline stress responsiveness is lower during early puberty than late puberty; therefore peak responsiveness during early puberty is not as high as peak responsiveness in late puberty and adulthood (Wommack et al., 2005). This similarity in HPA axis development is central to the relevance of hamsters as a model for stress in puberty and allows us to look at different phases of puberty. The relevance of hamsters was further tested in our laboratory through studies in human subjects based on predictions derived from our hamster model. In a self-report study the consequences of bullying durinng late puberty were far more severe and more

likely to be associated with depression and attitudes toward violence than stress in early puberty (Delville et al., 2005).

In these studies, exposure to stress in early puberty has limited consequences, as animals showed some resilience. Stressed hamsters became avoidant and showed inhibited risk assessment, but these effects are limited to specific social situations and appear to be short lasting. Similarly, in sexual contexts, stressed individuals were able to copulate, and only showed a short lasting avoidance of females. This lack of effect contrasts greatly with observations in adults. Perhaps in future studies it would be interesting to compare stress in early versus late puberty, predicting greater vulnerability in late puberty. Nevertheless, this lack of behavioral consequences is interesting and may make sense for these animals at a time they are developing social memories. The causes of this resilience in early puberty are interesting too, and may be related to the developing HPA and HPG axes. So far the only long lasting consequence of stress in early puberty in hamsters consists of enhanced aggression later in adulthood. The nature of this aggression is reminiscent of impulsivity; as such this may be related to conduct disorders apparent in individuals with a history of abuse.

In humans, coping with stress can take various forms. Coping has been defined as a conscious or unconscious effort to diminish the intensity of a stressor and/or endure a stressful situation with the minimum amount of pain (Matheny et al., 1986). In human studies on bullying social support mitigates

effects on depression scales. Coping behaviors have been observed in subjugated juvenile hamsters as well. Certain individuals are more likely to display submissive postures during subjugation. These animals were least affected by these repeated attacks. Though this aspect was not covered in our study, it would be interesting to include correlations with individual differences in responses to the attacks.

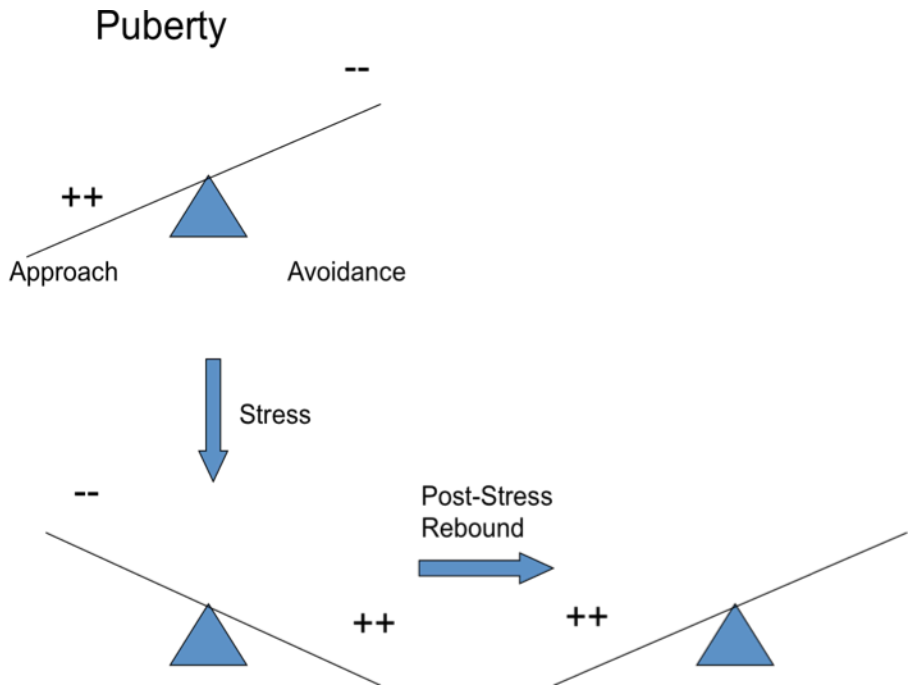
However, not all forms of coping are necessarily helpful. In bullying studies, avoidance coping has been correlated with negative outcomes. The avoidance performed by our subjugated hamsters may be applicable to these correlations. It could be argued that avoidance by our hamsters is a form of coping, and may be the most primitive form of coping in evolution. This avoidance may help individuals avoid injuries, or even encourage them to find new territories. But these new territories may be more risky and contain fewer resources. In humans, avoidance coping does not help individuals in difficult social settings. Similarly, avoidance may not help individuals remain in resource-rich territories.

As explained above our hamster model allows us to dissect different period of puberty. Our studies point to early puberty as a period of relative resilience to stress. Most studies in humans do not show this separation and include all of adolescence. It would be interesting to expose our animals to stress throughout the entire period of puberty. It is likely that the effects would be

far more severe, and would be traceable to enhanced vulnerability in late puberty.

Together, these studies of bullying in adolescents, which was first examined in our rodent model, show the translational value of our previous studies. Further, our human studies were used to make recommendations for increasing social support in school systems to attenuate the effects of bullying. With respect to the applicability of this animal model to the human psychopathology, it can be postulated that this model would best fit the profile of social anxiety. Unlike in models of depression, the behavior of repeatedly stressed juvenile hamsters most closely resembles social anxiety. This is because as opposed to the behaviors observed in animal models of depression such as learned helplessness, socially stressed juvenile hamsters were not more anxious or fearful in other, non-social, contexts. The specificity of this effect, and the resilience of these animals suggest juvenile hamsters would not be a good model for other mental disorders such as schizophrenia and generalized anxiety disorder. However, studying resilience to stress in these animals would give clues as to how the affects of stress may be attenuated. This could perhaps be used to prevent development of these and other mental disorders whose onset are associated with experiencing severe stress.

Figure 5.3. The stress and motivated behaviors



This heuristic representation of motivated behaviors shows the tendency of these individuals to approach conspecifics, and later avoid conspecifics after social stress. Subsequent acceleration of the development of aggressive behavior could lead to a proclivity to approach in adulthood.

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