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SENSITIVE PERIODS IN THE DEVELOPMENT OF MALE SOCIAL BEHAVIOR: THE ROLE OF GONADAL HORMONES DURING ADOLESCENCE

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SENSITIVE PERIODS IN THE DEVELOPMENT OF MALE SOCIAL BEHAVIOR: THE ROLE OF GONADAL HORMONES DURING ADOLESCENCE

By

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ABSTRACT

SENSITIVE PERIODS IN THE DEVELOPMENT OF MALE SOCIAL BEHAVIOR: THE ROLE OF GONADAL HORMONES DURING ADOLESCENCE

By

Kalynn Marie Schulz

Gonadal hormones exert a powerful influence on male physiology, brain, and behavior during development. The perinatal period is a sensitive period for exposure to testosterone (T) and the differentiation of the genitalia as well as the brain and behavior. The HPG axis is re-activated at puberty, but little is known regarding the role testicular hormones play in shaping the brain and behavior of males during the adolescent period. Using the Syrian hamster as an animal model, this research sought to determine whether adolescence is a second sensitive period for the effects of testicular hormones on adult male social behavior, distinct from the perinatal period. Experiments in Chapter 2 tested the hypothesis that gonadal hormones masculinize and defeminize adult male reproductive behavior. Males deprived of testicular hormones during adolescence displayed much lower levels of masculine reproductive behavior (e.g. mounts and intromissions), and higher levels of feminine reproductive behavior (e.g. lordosis) than did males exposed to testicular hormones during adolescence, suggesting that behavioral neural circuits undergo a second period of masculinization and defeminization by testicular hormones during adolescence. Experiments in Chapter 3 tested whether the influence of testicular hormones extends to other social behaviors. A type of scent marking behavior important for forming male-male dominance hierarchies was impaired in adult

males that were deprived of adolescent testicular hormones, suggesting that in addition to reproductive behavior, testicular hormone exposure during adolescence is also required for the full expression of dominance behavior in adulthood. Although these data clearly suggest that during adolescence testicular hormones exert lasting changes on adult behavioral responses to T, they do not address whether adolescence is a sensitive period distinct from the perinatal period, because hormone levels were not manipulated during the time between the neonatal and adolescent period. To test whether adolescence is a second distinct sensitive period for exposure to T, males were castrated at 10 days (d) of age, and then exposed to 19d of T or blank silastic capsules before, during, or after adolescence. In adulthood, all males received a second implant of T designed to activate reproductive behavior, and behavioral assessment occurred 7d later. Exposure to T before or during adolescence, but not after, significantly increased adult mounting behavior relative to blank-capsule treated controls, suggesting that adolescence is not a distinct period of heightened sensitivity to organizing effects of T separate from the perinatal critical period. Instead, they provide evidence for a protracted postnatal period of nervous system sensitivity that begins perinatally and ends in young adulthood. Thus, the classical view of organizational and activational mechanisms of steroid action should be revised to incorporate an extended window of postnatal sensitivity to the organization of adult social behavior by steroid hormones.

This thesis is dedicated to my mom and dad, Vickie and Everett Schulz, and my grandma, Helen Gunderson. Thank you for always being supportive and proud of me, even though "behavioral neuroendocrinology" is a mouthful!

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CHAPTER 1

Introduction

ADOLESCENT BRAIN DEVELOPMENT

Adolescence is a life transition characterized by dramatic changes in cognition, risk taking and social behavior (Spear, 2000; Steinberg, 2005). Given the extent to which adolescents transform their behavior, it is surprising that brain development was once thought to be complete by age five, an assumption based largely on postnatal changes in brain weight (Dekaban, 1978). Recent neuroimaging work has revolutionized scientific thinking by emphasizing the potential biological underpinnings of human adolescent behavior in addition to the numerous psychosocial explanations of behavioral change (Steinberg, 2007). Many dynamic non-linear changes in cortical gray matter volume occur during adolescence. For example, the gray matter in the frontal cortex increases in early adolescence, and subsequently declines until early adulthood (Giedd et al., 1999). The frontal cortex is an important region for executive control and decision making, and its immaturity in early adolescence may contribute to increased risk taking and sensation seeking behavior (Steinberg, 2007).

Animal research has also revealed that many processes occurring earlier in ontogeny are reinstated during adolescence. These processes include neurogenesis (Ahmed et al., in preparation; Eckenhoff and Rakic, 1988; He and Crews, 2007; Pinos et al., 2001; Rankin et al., 2003), programmed cell death (Nunez et al., 2001; Nunez et al., 2002), elaboration and pruning of dendritic

arborizations and synapses (Andersen et al., 1997; Huttenlocher and Dabholkar, 1997; Lenroot and Giedd, 2006; Sowell et al., 2004; Zehr et al., 2006b), and sexual differentiation (Chung et al., 2002; Davis et al., 1996; Nunez et al., 2001). Thus, adolescence is a time of striking nervous system development, and our understanding of the behavioral consequences of these changes is limited. Furthermore, given the extent of neurobiological change, adolescence may be a time of particular vulnerability to nervous system insult (Andersen, 2003; Spear, 2000)

Although one of the hallmarks of adolescence is pubertal maturation and the resultant rise in gonadal hormone secretions, little is known regarding the role gonadal hormones play in shaping the brain and behavior during adolescence. Indeed, gonadal steroid hormones have been implicated in nearly all the developmental processes listed above during earlier developmental time points, yet our understanding of the intersection between adolescent brain development and gonadal hormones and the behavioral consequences of this interaction remains limited. The overarching goal of this thesis is to determine whether testicular hormone secretions during adolescence cause lasting changes in adult male social behaviors. In addition, experiments in this thesis directly test the hypothesis that adolescence is a particularly sensitive period for organizing actions of testosterone (T) on neural circuits underlying male social behavior. The sections below detail the general evidence required to establish any developmental period as one that is sensitive to a particular experience. Examples of sensitive periods in other behavioral systems are provided to

illustrate the general concept of sensitive periods, how they are regulated, and their lasting influence on an individual's developmental trajectory. The chapter finishes with a description of the perinatal sensitive period for organizing actions of gonadal hormones on male reproductive behavior, and the rationale for designing experiments to determine whether similar effects of gonadal hormones occur during adolescence.

SENSITIVE PERIODS OF DEVELOPMENT

The general theory of sensitive periods states that the organization of a system is most easily modified during periods of rapid change (Scott et al., 1974). Importantly, modifying factors that act during sensitive periods do not have the same impact on the system at other times during the lifespan. Modifying factors can take on any shape or form and be of exogenous or endogenous origin (e.g. sensory experience; steroid hormone exposure), depending on the particular developing system.

Brief history of sensitive periods

Embryologists of the 1920s and 1930s were among the first to describe the features of sensitive periods. Charles Stockard was investigating the consequences of exposing fish embryos to both toxic chemicals and extreme temperatures, and found that disruption of the development of a particular organ (e.g., the retina) could only occur if these factors were applied when the organ was developing. Disturbing the embryo before or after this period would cause

no damage to the particular organ. Thus, he reasoned that the period of rapid cellular growth of the embryonic organ was a "sensitive period" or "critical moment" (reviewed in Bruer, 2001). Other embryologists went on to demonstrate similar effects of early experience on cellular differentiation. For example, Hans Spemann found that embryonic cells transplanted to another site in the embryo were able to differentiate into cells appropriate for their new site only if transplantation occurred prior to a certain developmental time point. If cells were transplanted later than this time they would develop as appropriate for the original site (Bruer, 2001).

The concept of sensitive periods was extended to the development of animal behavior when Konrad Lorenz first described the phenomenon of imprinting in birds. Lorenz found that goslings could be trained to follow humans after within hours of hatching. The likelihood of imprinting to a human (or mother goose) peaked approximately 17 hours after hatching, and would not occur prior to 12 hours or after 24 hours post-hatching (Bruer, 2001). Thus, the phenomenon of behavioral imprinting shares several features with that of cellular induction; environmental influences have the greatest impact during a particular stage of development, and the effects are irreversible.

Terminology

It has become increasingly clear that sensitive periods are seldom as brief and well defined as in the case of cellular induction or imprinting. Instead, the impact of an experience during a sensitive period gradually increases, peaks,

and slowly decreases again. Furthermore, some of the effects occurring during sensitive periods can be modified by certain experiences later in life, and as such are not necessarily permanent and irreversible (Bateson, 1983; Hensch, 2004; Knudsen, 2004). Thus, many researchers have opted to use the term sensitive rather than "critical" period when referring to the increased impact of experience during particular phases of development. This term is also intended to allow for the possibility that effects occurring during sensitive periods can be modified or reversed by later experience (Bruer, 2001; Knudsen, 2004).

Sensitive period regulation

Opening of sensitive periods

Currently, little is known about the specific neurodevelopmental requirements for the opening of sensitive periods, but the best evidence thus far suggests that appropriate excitatory/inhibitory balance may permit experience-dependent plasticity during a sensitive period. The development of inhibitory GABA circuits has been linked to the opening of the sensitive period for the effects of visual experience on cortical development. Neurons in the visual cortex layer IV respond preferentially to light stimuli from the left or right eye. Neurons receiving inputs from the same eye are clustered together in a columnar fashion, and these clusters are termed ocular dominance columns (Figure 1.01). The development of cortical ocular dominance is a competitive process driven by exposure to visual stimuli during a sensitive period (Kandel et al., 2000).

Monocular deprivation during a sensitive period causes a dramatic shift in cortical responses toward the open eye. After the sensitive period has passed, monocular deprivation has little effect on the organization of columns, even after much longer periods of deprivation (reviewed in Horton, 2001).

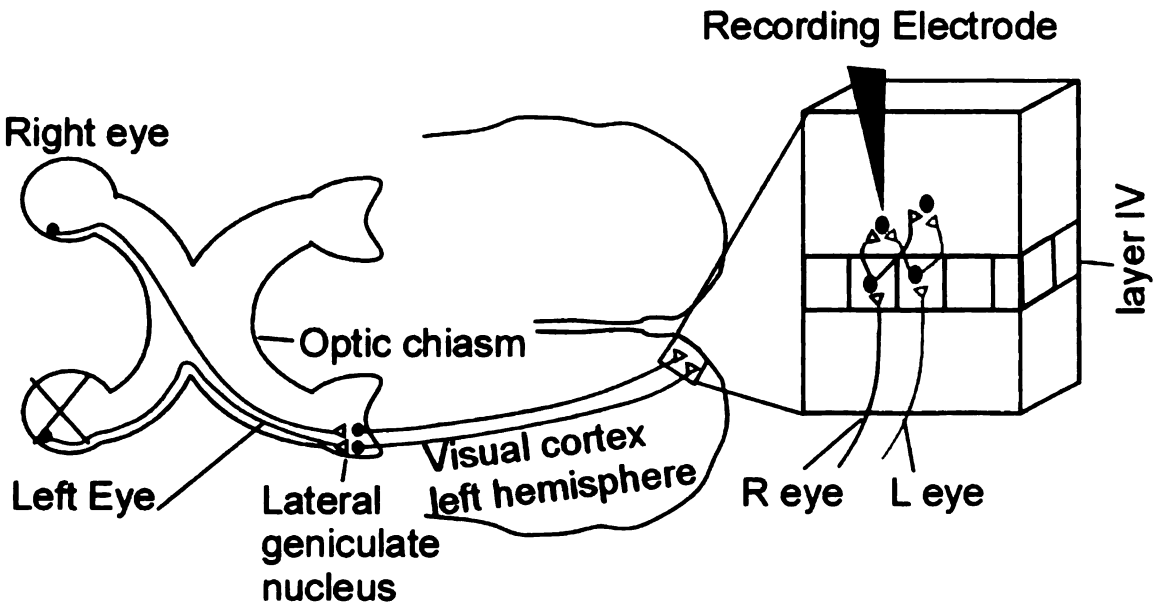


Figure 1.01. Depiction of the pathway by which visual stimuli are relayed to the visual cortex. Light hits the retina and the retinal ganglion cells transduce visual stimuli into electrical signals. The retinal ganglion cells project from the retina of each eye forming the optic nerves, which cross at the midline of the brain (chiasm). After crossing, the ganglion projections from each eye form an optic tract, which is now contralateral to the eye from which information is relayed. The optic tracts terminate at the lateral geniculate nucleus (LGN), which in turn sends segregated projections from the right and left eyes to layer IV of the visual cortex, forming segregated bands termed ocular dominance columns. The monocular neurons within the layer IV dominance columns send converging inputs to neighboring neurons and cortical layers, which forms the basis of binocular vision. Monocular deprivation causes the LGN to retract its axonal projections to the visual cortex (gray axons L eye), which leaves afferents from the open eye to excite cortical neurons and strengthen their synaptic connections. Redrawn and adapted from Kandel, 2000.

Cortical dominance shifts in response to monocular deprivation usually are not possible until 3 weeks of age in mice (Fagiolini and Hensch, 2000). However, benzodiazepine treatment increases intracellular levels of GABA, and prematurely opens the sensitive period for monocular deprivation in wild type mice (Fagiolini et al., 2004; Fagiolini and Hensch, 2000). In addition, mice lacking an isoform for GABA synthetic enzyme (GAD65) display reduced cortical GABA release and are completely insensitive to the effects of monocular deprivation (Hensch et al., 1998). However, enhancing GABA action with benzodiazepine treatment restores normal cortical dominance formation in response to monocular deprivation (Hensch et al., 1998; Iwai et al., 2003). Moreover, benzodiazepine treatment before, during or after the usual sensitive period can induce cortical dominance in GAD65 knock-out mice (Fagiolini and Hensch, 2000). Importantly, early benzodiazepine treatment during monocular deprivation renders cortical neurons insensitive to monocular deprivation in adulthood, suggesting that reaching the appropriate inhibitory threshold can only open the sensitive period *once in life*, similar to what is observed in wildtype mice (Fagiolini and Hensch, 2000). Whether the development of the appropriate inhibitory/excitatory balance allows for the opening of sensitive periods in other neural circuits is not yet known.

Closing of sensitive periods

Some sensitive periods are closed by the very experience to which they are sensitive. For example, during the sensory phase of song learning, male

zebra finches produce immature vocalizations in an effort to mimic a tutor's song, and eventually the correct song is achieved (Konishi, 1985). Accurate replication of a tutor's song is called "crystallization". Learning a different template song is not possible following crystallization in zebra finches, suggesting that the process of song learning itself closes the sensitive period. Similarly, in hatchling birds, once an initial imprinting figure has been selected, imprinting to a second parent figure does not occur (Hess, 1973).

Other sensitive periods close slowly as a byproduct of developmental progress during the sensitive period. Neural circuits are shaped by experience and eventually reach stable structure which is resistant to further modification (Knudsen, 2004). For example, sexual imprinting to a female of the same species results in reduced dendritic spine densities within key forebrain areas. In the absence of sexual imprinting stimuli, the window of sensitivity for imprinting closes at 150 days of age. Interestingly, spine densities also decrease sharply around 150 days of age in these inexperienced males, suggesting that structural modification of spines limits the ability to imprint to stimuli after 150 days of age (Bischof, 2007). Presumably, neural circuits have developed in a particular fashion in the absence of experience and are unable mechanistically or energetically to reestablish normal patterns of connectivity (Knudsen, 2004). Interestingly, the sensitive period for sexual imprinting ends around the time of sexual maturity, a feature common to several sensitive periods including sound localization in barn owls (Knudsen, 1999), and human language learning (Birdsong and Molis, 2001).

Beyond structural consolidation of neural circuits, several individual factors have been associated with the closing of sensitive periods. Increases in the effectiveness of inhibitory circuits (Fagiolini and Hensch, 2000; Hensch, 2004; Zheng and Knudsen, 2001), molecules inhibiting neurite outgrowth (Lee et al., 2003), the stabilization of synapses by glia (Ullian et al., 2004), and myelination (Keirstead et al., 1992; Levay et al., 1978; Sirevaag and Greenough, 1987), all likely play a role in consolidating neural circuits and closing windows of sensitivity.

Sensitive periods for the organizational effects of steroid hormones

Reproductive behavior is also governed by sensitive periods of development. In the case of reproductive behavior, endogenous testicular secretions of T serve as a type of "experience" that masculinizes and defeminizes the brain and peripheral tissues (Phoenix et al., 1959; reviewed in Ward and Ward, 1985). For example, depriving males of testicular hormones during development via neonatal castration decreases masculine reproductive behavior in response to T, and increases feminine responsiveness to estrogen and progesterone in adulthood. Phoenix et al. (1959) hypothesized that the organizational effects of steroid exposure during early development program or "activate" behavioral responses to steroid hormones in adulthood. The criteria primary used to distinguish between organizational and activational effects are defined as follows. First, organizational effects are permanent, and activational effects are transient. Second, organizational effects can only occur early in life

(around the time of birth), and in particular, during a sensitive period. In contrast, activational effects usually occur in adulthood, and steroid hormones cannot activate behaviors until the underlying neural circuits have been organized. Since the time of this classic and important paper, other researchers have pointed out that the above criteria are too restrictive (Arnold and Breedlove, 1985). For example, steroid hormone exposure has been found to have long lasting or permanent effects on the adult nervous system. Androgen treatment of *adult* female zebra finches, which do not normally sing, caused long-lasting increases in the volume of the brain nuclei underlying the production of song, and also induced singing behavior in these females (Gurney and Konishi, 1980). Thus, while sensitive periods always involve organizational change, organizational change does not always require a sensitive period.

Is adolescence a second sensitive period for organizing actions of testosterone on reproductive behavior?

Endocrine and behavioral puberty occurs between 4 and 7 weeks in male hamsters. Puberty begins with increases in testes weight and circulating T (Miller et al., 1977; Sisk and Turek, 1983; Vomachka and Greenwald, 1979), and culminates with adult T levels and maturation of reproductive behavior. The appearance of reproductive behavior during adolescence lags behind the initial hormonal events of puberty because the manifestation of sexual behavior is contingent on elevated levels of gonadal steroids, which via their action in the nervous system facilitate behavioral responses in specific social contexts. The

customary view of the relationship between the elevated hormone levels of puberty and the maturation of reproductive behavior is that hormones activate neural circuits that were sexually differentiated during early neural development. However, if behavioral activation during adolescence is simply the consequence of 7-10 days of T exposure at the onset of puberty, similar exposure immediately prior to adolescence should also elicit adult-like mating responses. Interestingly, this is not the case. Administration of adult levels of T or its metabolites fails to elicit adult levels of sexual behavior in prepubertal male hamsters (Meek et al., 1997; Romeo et al., 2001; Romeo et al., 2002). These data provide preliminary evidence that organizational effects of testicular hormones may be required during adolescence to permit behavioral activation by T in late adolescence and adulthood. Experiments in Chapter 2 specifically tested the hypothesis that exposure to gonadal hormones during adolescence masculinizes and defeminizes behavioral responses to steroid hormones in adulthood. This hypothesis predicts that prepubertal gonadectomy, but not adult gonadectomy, will reduce masculine responses to T and increase feminine responses to estradiol and progesterone in adulthood.

Are other social behaviors organized by adolescent gonadal steroid hormones?

Given that many social behaviors change dramatically across the adolescent period, adolescent exposure to gonadal hormones may induce organizational change in a host of social behaviors. For example, prepubertal and adult males differ markedly in their social behaviors when in novel

environments; adults display reduced social interactions in novel environments whereas prepubertal males do not. Although the masculine response to novel environments is not regulated by T in adulthood, depriving males of T during puberty via prepubertal gonadectomy prevents its development altogether (Primus and Kellogg, 1990).

Organizational effects of gonadal hormones during adolescence have also been found for scent marking and territorial aggression in species as diverse as tree shrews, mice and gerbils. In tree shrews, prepubertal castration prevents testosterone from activating scent marking in adulthood (Eichmann and Holst, 1999). Similarly, mice and gerbils both display testosterone-dependent aggressive behavior in adulthood, and the ability of testosterone to activate adult aggression is substantially reduced in prepubertally castrated males (Lumia et al., 1977; Shrenker et al., 1985). The Syrian hamster also exhibits testosterone-modulated scent marking behavior in adulthood. Syrian hamsters rub specialized sebaceous glands located on their dorsolateral flanks onto objects in their environment. In adults, flank marking is essential for the maintenance of dominance relationships between males, and dominant males flank mark at higher levels than submissive males (Ferris et al., 1987; Johnston, 1970). Testosterone's ability to regulate flank marking behavior changes across adolescence, as prepubertal testosterone-treatment fails to elicit flank marking behavior during social interactions with age- and weight-matched males (Schulz et al., in preparation). The experiment in Chapter 3 was aimed at determining whether adolescent exposure to testosterone programs flank marking responses

to testosterone during adult male social interactions. This hypothesis predicts that males gonadectomized before puberty, but not after puberty, will display reduced flank marking in response to adult testosterone treatment.

Is adolescence a sensitive period distinct from the perinatal period?

The inability of T to activate adult-like reproductive and flank marking behavior in prepubertal males suggests that a second window of sensitivity to gonadal hormones opens at adolescence. However, this possibility has not been empirically tested. It is possible, for example, that organizational effects of steroid hormones occur prior to puberty, but remain latent until requisite steroid-independent development occurs during adolescence. Experiments in Chapters 2 and 3 tested whether the presence or absence of gonadal hormones during adolescence influence adult behavioral responses to T, but were not designed to determine whether organizational effects of testosterone are possible prior to puberty, or whether cumulative life-time exposure to testosterone, regardless of when it occurs, is the key variable in expression of adult male social behaviors. Experiments in Chapter 4 address this question directly by testing the hypothesis that adolescence marks the opening of a second sensitive period for the organizing actions of testosterone on adult male reproductive behavior. This hypothesis predicts that exposure to testosterone during adolescence, but not before or after adolescence, will result in full activational responses to testosterone in adulthood. We also investigated whether regional volumes of brain areas within the forebrain neural circuit underlying reproductive behavior

(Figure 1.02) are altered by exposure to T treatment before, during, and after adolescence.

The experiments within this thesis ask basic questions regarding the role gonadal hormones play in shaping adolescent brain and behavioral development. The findings presented here have implications for normal human adolescent changes in social behavior, and also how aberrant adolescent development may contribute to psychopathologies such as depression, anxiety, and eating disorders.

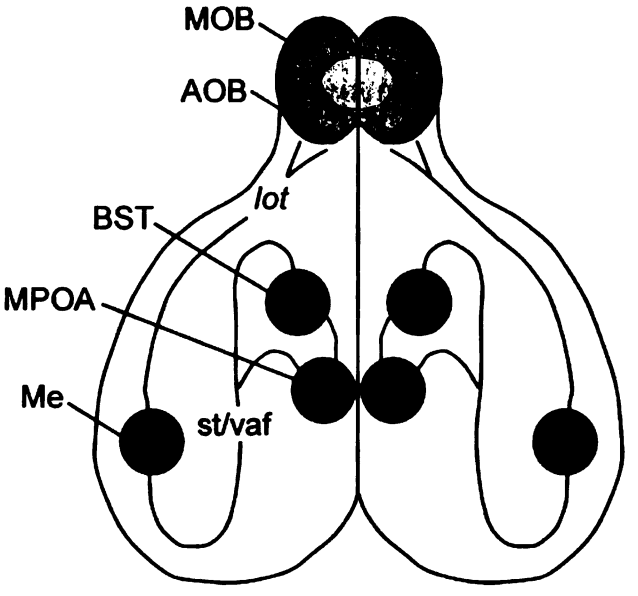


Figure 1.02. Male hamster mating behavior is the result of the integration of chemosensory information from the female and internal steroidal signals within a forebrain neural circuit consisting of the medial amygdala (Me), bed nucleus of the stria terminalis (BNST), and medial preoptic area (MPOA) adapted from Wood, 1998).

CHAPTER 2

Gonadal hormones masculinize and defeminize reproductive behaviors during puberty in the male Syrian hamster

INTRODUCTION

The organizational-activational hypothesis (Phoenix et al., 1959) proposes that exposure to steroid hormones early in development masculinizes and defeminizes neural circuits, programming behavioral responses to hormones in adulthood. Since the organizational-activational hypothesis was first proposed, many studies have demonstrated that disruption of perinatal testicular hormone secretion by manipulations such as prenatal stress or neonatal castration reduces the capacity for masculine behavior and increases the capacity for feminine reproductive behavior in adulthood (Eaton, 1970; Gerall et al., 1967; Grady et al., 1965; for review see Ward and Ward, 1985; Ward and Weisz, 1980; Whalen and Edwards, 1967). Thus, the perinatal period is important for the sexual differentiation of behavior by gonadal steroid hormones.

Neonatal castration followed by assessment of behavioral responses to steroid hormones in adulthood has been a commonly used approach for analyzing the contribution of neonatal hormones to the process of behavioral masculinization and defeminization. However, because neonatal castration also prevents exposure of the nervous system to hormone secretions during puberty, this approach confounds the contribution of neonatal hormones to the process of sexual differentiation of behavior with that of pubertal hormones. Furthermore,

while many studies have employed prepubertal castration as part of their experimental methods, the purpose of these investigations was not necessarily to assess the role of pubertal hormones in the masculinization and defeminization of reproductive behavior. Thus, while the results of some studies employing prepubertal castration suggest that the absence of testosterone (T) during puberty alters adult reproductive behavior (Adkins-Regan et al., 1989; Ford, 1990; Gotz and Dorner, 1976; Larsson, 1967; Sodersten, 1973), the results of other studies do not (D'Occhio and Brooks, 1980; Dixon, 1993; Epple et al., 1990; Larsson et al., 1976; Shrenker et al., 1985), and various methodological considerations make it difficult to draw a firm conclusion. For example, in previous studies, the ability of steroid hormones to activate behavior in males castrated prepubertally was not always directly compared to males castrated as adults (D'Occhio and Brooks, 1980; Epple et al., 1990; Larsson et al., 1976), steroid hormones were not re-administered in adulthood prior to behavior testing (Dixon, 1993), only one measure of reproductive behavior was reported (Larsson, 1967), and sexual behavior may have been influenced by other social experiences such as aggressive encounters (Shrenker et al., 1985).

One indication that further organization and masculinization of behavior occurs during puberty is that hormonal treatments that fully activate masculine reproductive behavior in adult males are less effective in activating behavior in prepubertal males (Baum, 1972; Sisk et al., 1992; Sodersten et al., 1977). For example, one week of testosterone propionate, dihydrotestosterone (DHT), or estradiol benzoate (EB) treatment increases mounts, intromissions, and

ejaculations in adult but not prepubertal male Syrian hamsters (Meek et al., 1997; Romeo et al., 2001; Romeo et al., 2002). Even up to two weeks of T treatment fails to activate reproductive behavior in a 28 day-old male hamster (unpublished data). These data suggest that the prepubertal male brain is not fully organized to mediate masculine reproductive responses to steroid hormones.

We therefore hypothesize that puberty is a second stage of sexual differentiation during which gonadal hormones fine-tune neural circuits to allow full maturation of sex-typical responses to hormones in adulthood. The current study addresses this possibility by testing whether the presence or absence of gonadal hormones during puberty alters masculine responses to T and feminine responses to estradiol benzoate (EB) and progesterone (P) in adulthood. This hypothesis predicts that males gonadectomized (GDX) *before* puberty will display lower levels of masculine reproductive behavior than males GDX *after* puberty when both groups are treated with T in adulthood (Exp. 1). Further, the hypothesis predicts that males GDX before puberty will display higher levels of feminine reproductive behavior than males GDX after puberty when both groups are treated with EB and P in adulthood (Exp. 2). We report here that the absence of gonadal hormones during puberty reduces masculine responsiveness to T in adulthood (Exp. 1), increases feminine responsiveness to EB and P in adulthood (Exp. 2), and that the deficits in masculine behavior are not reversed by prolonged T treatment or sexual experience (Exp. 3).

METHODS

Animals

18-day old male Syrian hamsters (*Mesocricetus auratus*) were obtained from Harlan Sprague-Dawley laboratories (Madison, WI) and arrived with their mothers. Males were housed with mothers and littermates until weaning at 21 days of age. All animals were housed in clear polycarbonate cages (12 x 4 x 8 inches) with ad libitum access to food (Telkad Rodent Diet No. 8640, Harlan, Madison, WI) and water. Animals were exposed to a 14 hr light/10 hr dark schedule (lights off at 1200 hr EST) and the temperature was maintained at 21± 2°C. All animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and all protocols were approved by the Michigan State University All-University Committee for Animal Use and Care.

Experimental Design

Experiment 1A

One group of males (n=6) was GDX before puberty at 21 days of age (noTduringP), and one group of males (n=7) was GDX after puberty at 63 days of age (TduringP). Six weeks following GDX, all males were implanted with a 2.5 mg 3-wk time-release T pellet (Innovative Research, Sarasota FL) in order to clamp circulating T at adult physiological levels. We have previously verified that 7 days of treatment with these pellets results in plasma concentrations of T of between 2-5 ng/ml in both prepubertal and adult males (Meek et al., 1997;

Romeo et al., 2003). Behavior tests with a receptive female were conducted 7 days after the onset of T replacement.

Experiment 1B

Because the noTduringP and TduringP males of Experiment 1A were tested at 70 and 112 days of age, respectively, a parallel experiment was conducted in order to assess whether any behavioral differences between these groups were associated with chronological age. One group of males (n=7) was GDX and implanted with a 2.5 mg T pellet at 63 days of age (same age as noTduringP group at beginning of T replacement), and one group of males (n=8) was GDX and implanted with a 2.5 mg T pellet at 105 days of age (same age as TduringP group at beginning of T replacement). One week following T implantation, males were tested for reproductive behavior with a stimulus female (70 and 112 days of age).

Experiment 2

18 males were GDX before puberty at 22 days of age (noTduringP), and 18 males were GDX after puberty at 63 days of age (TduringP). Seven weeks after GDX, half of the males in each group were injected with EB and P, and half were injected with oil and given one 2-min lordosis test in which behavior was manually induced by the experimenters. Manually stimulated lordosis tests were conducted with the intention of comparing the levels of lordosis behavior induced by manual stimulation with levels induced during interactions with a stud male.

However, the manually stimulated lordosis data are not reported here due to difficulties encountered in reliably inducing lordosis by manual stimulation of the hind flank area in both males and control females. One week after manual stimulation testing, males received the same hormone or oil treatment and were tested for lordosis behavior with a stud male. Hormone primed males received two EB (10 μg in .05 ml sesame oil) injections 72 and 48 hours prior to behavior testing, and one P (500 μg in 0.1 ml sesame oil) injection 6 hours prior to behavior testing. Oil injected males received the same volume of injection as the hormone-injected males and at the same time relative to testing.

Twenty-four females were GDX after puberty between 60-79 days of age. Females had been hormone primed and tested for sexual behavior once in a previous experiment, but had not been exposed to steroid hormones or a copulatory test in the 7 weeks prior to this study. All females received the same 2-wk hormonal priming and behavioral testing schedule as the hormone primed noTduringP and TduringP males. Half of the females were hormone primed and tested for behavior at the same time as the noTduringP males, and half were hormone primed and tested at the same time as the TduringP males.

Experiment 3

Figure 2.01 shows a schematic of the experimental design. Eighteen males were GDX prior to puberty at 21 days of age (noTduringP), and 16 males were castrated as adults at 63 days of age (TduringP). Six weeks following GDX, all males were implanted with a 2.5 mg 3-week time-release T pellet

(Innovative Research, Sarasota FL). The noTduringP and TduringP males were further divided into 2 groups to evaluate the effect of sexual experience on responsiveness to T. One group was tested for behavior 3 times, at 7,12, and 17 days after the onset of T treatment. The other group was tested only once, at 17 days after the onset of T treatment. Although the T capsules were described by the manufacturer as 3-week time release, we discovered in a previous experiment that plasma T concentrations are undetectable 14 days after implantation of these pellets. Therefore, a second pellet was implanted 10 days after the first pellet. This guaranteed that T was present during the last behavior test, which occurred 7 days after the second pellet was implanted.

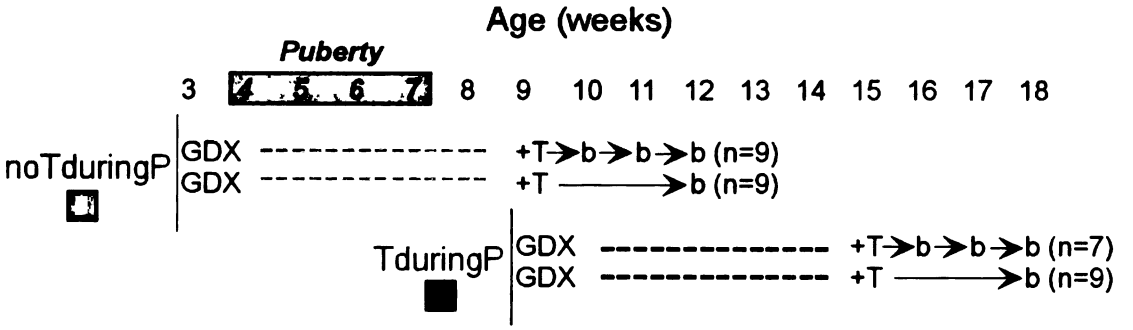


Figure 2.01. A schematic of the experimental design of Experiment 3. GDX, gonadectomy; T, onset of testosterone treatment; b, behavior test. The data from behavior test #2 are not reported in this manuscript.

Testosterone radioimmunoassay

Immediately following the last behavior test in Exp. 3, animals were weighed, administered an overdose of sodium pentobarbital (130 mg/kg ip), and blood sampled via cardiac puncture. All males had experienced 17 days of T treatment at the time of blood sampling. Plasma concentrations of T were measured in duplicate 50 μl samples within a single assay using the Coat-A-

Count Total T Kit (Diagnostic Products, Los Angeles, CA). This assay has been previously validated in our laboratory (Parfitt et al., 1999). The intraassay CV was 9.2%, and the lower limit of detectability was 0.1 ng/ml.

Tests for Reproductive Behavior

Masculine reproductive behavior

All tests for masculine behavior were conducted 1.5 - 4 hours after lights out (Exp 1 & 3). The male was placed in a 10-gal glass aquarium (51 x 26 x 31.5 cm) and allowed to acclimate 5 min before the introduction of a receptive stimulus female. The behavior tests were 15 min. Ovariectomized stimulus females were brought into behavioral estrus with an injection of 10 μg EB (0.2 mg/ml) in sesame oil 48 hours before testing, and an injection of 250 μg progesterone (5μg/ml) in sesame oil 3 hours before testing.

The behavioral tests were videotaped under dim red light illumination. Videotapes were scored to assess the number of vaginally oriented mounts, intromissions, ejaculations, and the latencies to ejaculate. The criteria for these behaviors have been described previously (MPOA-AH, reviewed in Albers et al., 2002). Videotapes were scored blind to experimental condition by a single observer.

Feminine reproductive behavior

All tests for feminine reproductive behavior occurred between 3-6 hours after lights out (Exp. 2). After the experimental male or female experienced a 5

min acclimation period in a 10-gal glass aquarium (51 x 26 x 31.5 cm), a sexually experienced stud male was placed in the aquarium for a 10 min period. To ensure high levels of behavior during testing, stud males were allowed to interact with a sexually receptive female for 1 minute immediately prior to being placed in the test aquarium with the experimental male or female. The behavior tests were videotaped under dim red light illumination. One observer blind to experimental condition later scored the lordosis latencies and the lordosis durations.

Statistical analysis

Experiment 1A and 1B

In experiment 1A, two-tailed t-tests compared the mounts, intromissions, ejaculations and ejaculation latencies of noTduringP and TduringP males. In experiment 1B, two-tailed t-tests compared the behavior of males that differed only by chronological age. These two groups were age matched to the noTduringP and TduringP males from experiment 1A (70 and 112 days old, respectively).

Experiment 2

Two one-way ANOVAS were conducted to compare the lordosis latencies and lordosis durations of three EB and P treated groups; TduringP males, noTduringP males, and females that were ovariectomized as adults. If a one-way ANOVA was significant, Fishers PLSD post hoc tests were conducted to determine specific statistical differences between TduringP males, noTduringP males, and females. Differences were considered significant if $p < 0.05$. The

TduringP and noTduringP males treated with oil were not included in statistical analyses because these groups did not display any lordosis behavior.

Experiment 3

Two 2-way between subject ANOVAs were conducted on the behavioral data. The first 2-way ANOVA examined *time of GDX* (noTduringP, TduringP) and *duration of T treatment* (7 or 17 days) in males that were all sexually naive. The second 2-way ANOVA examined *time of GDX* (noTduringP, TduringP) and *level of experience* (one or three tests) in males that were all exposed to T for 17 days. Because some of the same behavioral data were included in both of these analyses, the Bonferroni correction for multiple comparisons was employed and the significance level was set at $p < 0.025$. Plasma concentrations of T from terminal blood samples were also analyzed by a 2-way ANOVA (*time of GDX* x *level of experience*). Differences in this analysis were considered significant when $p < 0.05$.

RESULTS

Experiment 1A

TduringP males displayed significantly more mounts [$t(1,11) = 11.78$, $p=0.0056$], intromissions [$t(1,11) = 22.13$, $p=0.0006$], and ejaculations [$t(1,11) = 5.38$, $p=0.04$] than noTduringP males (Fig. 2.02). In addition, TduringP males displayed significantly shorter latencies to ejaculate than noTduringP males [$t(1,11) = 5.26$, $p=0.04$; Fig. 2.02].

Experiment 1B

No differences in the number of mounts [t (1,13) = 1.24, p=0.28], intromissions [t (1,13) = 2.37, p=0.15], or ejaculations [t (1,13) = 0.25, p=0.62] were observed between two groups of males that differed only by chronological age (70 vs. 112 days).

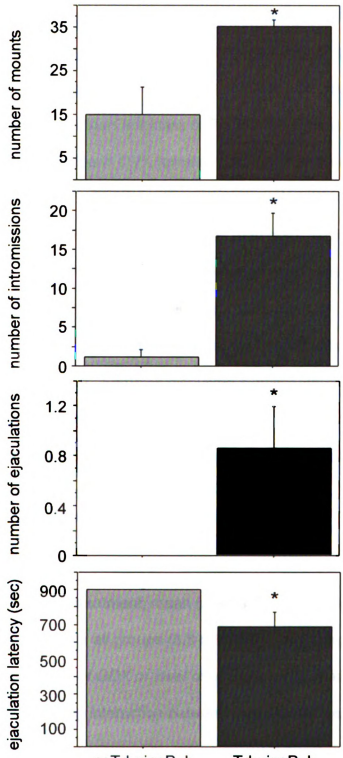


Figure 2.02. Number of mounts, intromissions and ejaculations displayed by sexually inexperienced males that were gonadectomized before puberty (noTduringP, n=6) or after puberty (TduringP, n=7) and tested for reproductive behavior 7 weeks later. All males were administered T for one week prior to behavior tests. All values are expressed as means ± SEM.

Experiment 2

No behavioral differences were found between the two groups of OVX females tested on different days, and so their data were combined. The one-way ANOVA comparing the lordosis latencies of the EB and P treated TduringP males, noTduringP males, and OVX females was significant [t (2,38) = 10.77, p = 0.0002; Fig 2.03]. Fisher's PLSD post-hoc tests revealed that TduringP males displayed significantly longer lordosis latencies than both noTduringP males and OVX females. In contrast, no significant difference in lordosis latency was found between noTduringP males and OVX females.

The overall ANOVA was also significant for lordosis duration [F (2, 38) = 22.34, p < .0001; Fig. 2.03]. Females displayed significantly longer lordosis durations than both TduringP (p < 0.0001) and noTduringP males (p< 0.0004). TduringP males displayed shorter lordosis durations than noTduringP males, but this trend was not statistically significant (p = 0.08).

Experiment 3

After 17 days of T treatment, mean plasma T concentrations were within adult physiological range in all groups (3.8-5.6 ng/ml). A 2-way ANOVA did not reveal any effects of *time of GDX* or *level of experience* on plasma concentrations of T, or any interaction between these variables.

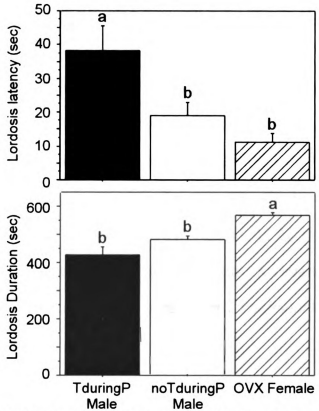
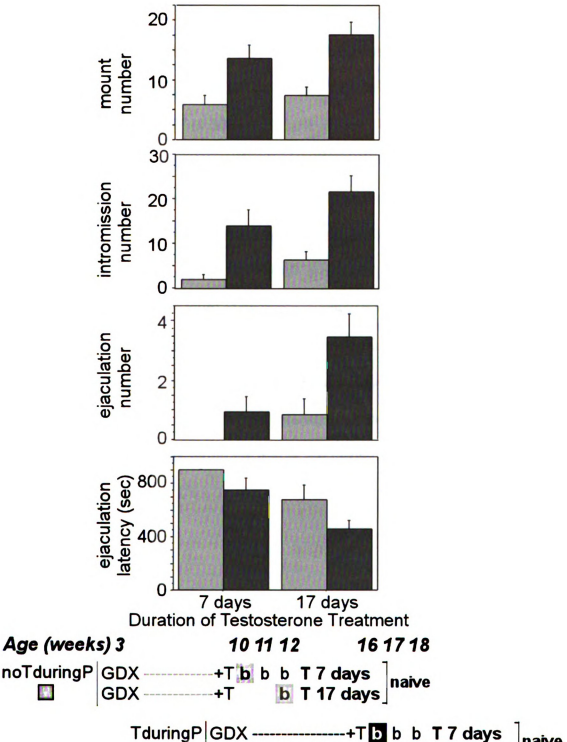


Figure 2.03. Lordosis latencies and total lordosis durations displayed by males castrated before (n=8) or after puberty (n=9), and females ovariectomized after puberty (n=24) when paired with a stud male for 10 minutes. All animals were primed with estradiol benzoate and progesterone prior to behavior tests. All values are expressed as means ± SEM. Bars labeled with different letters represent groups that significantly differ from one another.

The second two-way ANOVA compared sexually naïve TduringP and noTduringP groups that differed only in the duration of T treatment (7 and 17 days; Fig. 2.04). This ANOVA (Time of GDX x duration of T) revealed main effects of *time of GDX* for mounts [$F(1,30) = 23.09$, $p < .0001$], intromissions [$F(1,30) = 25.87$, $p < .0001$], and ejaculations [$F(1,30) = 11.19$, $p = .0022$]. TduringP males displayed significantly higher numbers of mounts, intromissions and ejaculations than noTduringP males. TduringP males also displayed significantly shorter latencies to ejaculate than noTduringP males [F (1,30) =

5.60, p = .0246]. This ANOVA also revealed main effects of *duration of T treatment* (7 or 17 days) for ejaculations [F (1,30) = 10.11, p = .0034], and a trend toward significance for intromissions [F (1,30) = 5.04, p = .0323], such that males exposed to T for 17 days displayed significantly higher numbers of intromissions and ejaculations than males exposed to T for 7 days. Males exposed to T for 17 days also displayed significantly shorter ejaculation latencies than males exposed to T for only 7 days [F (1,30) = 11.02, p = .0023]. Duration of T treatment did not affect the number of mounts. No interactions were observed between *time of GDX* and *duration of T treatment* for any of the behaviors measured.



The third two-way ANOVA compared groups of TduringP and noTduringP males that received the same duration of T treatment (17 days), but differed in

their level of sexual experience (1 or 3 tests; Fig. 2.05). The 2 x 2 ANOVA (time of GDX x level of sexual experience) detected main effects of *time of GDX* for mounts [$F(1,30) = 22.68$, $p < .0001$], intromissions [$F(1,30) = 16.77$, $p = .0003$], and ejaculation latency [$F(1,30) = 6.22$, $p = .0183$]. TduringP males displayed significantly more mounts, intromissions, and shorter ejaculation latencies than noTduringP males. TduringP males also displayed more ejaculations, but this difference was not significant after the Bonferroni adjustment for multiple comparisons was applied [$F(1,30) = 4.67$, $p = .0388$]. The 2 x 2 ANOVA also revealed a main effect of *level of sexual experience* for mounts [$F(1,30) = 6.17$, $p = .0188$], but not intromissions, ejaculations, or ejaculation latencies. Males that experienced three sexual behavior tests displayed significantly more mounts than males that experienced only one. No interaction between *time of GDX* and *level of sexual experience* was found for any of the behaviors quantified.

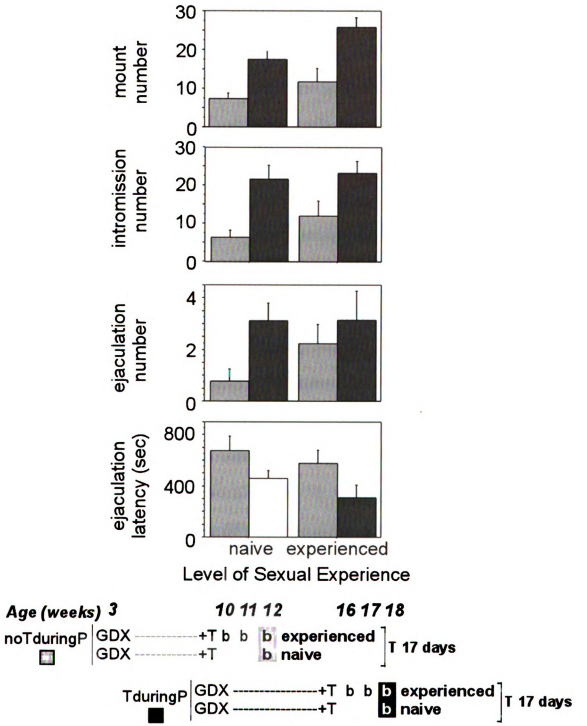


Figure 2.05. Number of mounts, intromissions and ejaculations displayed by sexually experienced or inexperienced males that were GDX before or after puberty. The shaded b's in the schematic indicate the behavior tests being compared in the statistical analysis. All males were treated with testosterone for 17 days prior to testing. All values are expressed as means ± SEM.

DISCUSSION

The current study demonstrates that the absence of testicular hormones during puberty results in reduced masculine behavioral responses to T (Exp. 1&3) and increased feminine behavioral responses to EB and P (Exp. 2) in adulthood. Males that were GDX prior to puberty displayed fewer mounts, intromissions, and ejaculations than males that were GDX after puberty, as well as longer latencies to ejaculate. Males that were GDX prior to puberty also displayed shorter lordosis latencies than males GDX after puberty, and importantly, males GDX before puberty lordosed as quickly as females. Furthermore, increased duration of T treatment and repeated experience with a female did not reverse the deficits in masculine behavior caused by the absence of gonadal hormones during puberty (Exp. 3). The differences in masculine behavior between males GDX before or after puberty are not attributable to chronological age because control males that were age-matched to the TduringP and noTduringP males did not differ in their levels of masculine reproductive behavior (Exp. 1B). Taken together, these results suggest that gonadal hormones secreted during puberty cause long lasting organizational change in the neural circuitry underlying reproductive behavior, thereby altering behavioral Hresponsiveness to gonadal steroid hormones in adulthood.

These findings are the first clear demonstration that the presence of testicular hormones during puberty both masculinizes and defeminizes the capacity for reproductive behavior in males. Neonatal castration is a common method of assessing the contribution of neonatal hormones to the process of

sexual differentiation. Because neonatal castration prevents exposure of the nervous system to gonadal hormones during both the neonatal and pubertal periods of development, previous experimental designs have confounded the contribution of neonatal hormones to the process of sexual differentiation with the contribution of pubertal hormones. The results of this study suggest that puberty is a second period of nervous system and behavioral organization by gonadal hormones. We propose a two-stage model for the full maturation of adult male social behaviors: perinatal sexual differentiation of neural circuits, followed by peripubertal finishing of this process, which results in the sex-specific activation of behavior by steroid hormones in adulthood (Figure 2.06). Thus, the contribution of pubertal hormones to the expression of adult reproductive behavior includes not only an activational component, but also an organizational component, which permits the activation of behavior by steroid hormones.

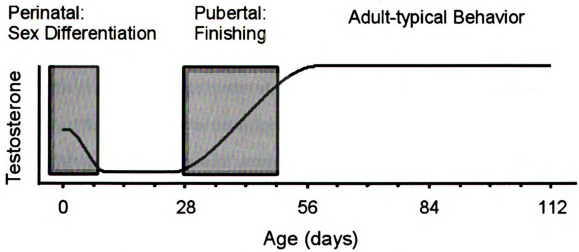


Figure 2.06. Two stage model for the complete maturation of male social behavior.Perinatal steroid hormone secretions sexually differentiate neural circuits underlying behavior, and pubertal steroid hormone secretions fine-tune or finish the process of behavioral masculinization and defeminization.

The 2-stage developmental model may also extend to other male social

behaviors that appear to be organized by T during puberty. For instance, adult male rats reduce social interactions when in unfamiliar environments, and this sexually dimorphic response to novel environments develops during puberty (Primus and Kellogg, 1990). Although the masculine response to novel environments is not regulated by T in adulthood, depriving males of T during puberty via prepubertal GDX prevents its development altogether (Primus and Kellogg, 1990). Furthermore, administering prepubertal castrates with T throughout puberty maintains the normal development of this social response (Primus and Kellogg, 1990). T during puberty may also organize scent-marking behavior. In male hamsters, flank marking is a T-dependent behavior that communicates dominance status to male conspecifics (Albers et al., 1988; Johnston, 1981). Males that are GDX prior to puberty, however, do not respond to T in adulthood with increased flank marking during social interactions (Schulz-Wilson et al., 2002). Similarly, male-typical territorial scent marking in tree shrews requires the presence of T during puberty (Eichmann and Holst, 1999). Thus, the proposed 2-stage model of behavioral development may apply to many sexually differentiated social behaviors.

While increased gonadal secretions of steroid hormones are a hallmark of pubertal development ("gonadal puberty"), substantial alterations in nervous system structure and function occur *independently* of gonadal hormones during puberty ("brain puberty"). For example, the overproduction and subsequent pruning of striatal DA receptors that occurs during puberty in male rats does not depend on gonadal hormones (Andersen et al., 2002). Thus, just as the onset of

gonadal hormone secretions is developmentally timed to occur during puberty, so is steroid-independent brain maturation (Meek et al., 1997). While it is clear that these two developmental processes interact during puberty to organize many adult male social behaviors, an important question for developmental neurobiology and psychobiology is one of timing: does the full maturation of adult male social behaviors depend on the temporal coordination of gonadal puberty and brain puberty? If gonadal puberty and brain puberty must be synchronized, then puberty may be a sensitive period for steroid-dependent organization of behavior. Alternatively, the second wave of organization by gonadal hormones may be possible at any time after the perinatal critical period, but occurs during puberty simply because T secretions normally increase during this time. Distinguishing between these possibilities and others is necessary for a complete understanding of the relationships between brain and gonadal puberty, and the maturation of social behaviors.

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While the current study did not directly test whether puberty fits the criteria for a sensitive period, the results are certainly suggestive. For instance, the behavioral deficits resulting from the absence of testes during puberty persisted even after 17 days of T replacement in adulthood. Since 17 days of adult T treatment is almost as long as the normal time course of the pubertal increase in gonadal hormone secretion and emergence of reproductive behavior, it appears that the effect of T on neural circuits and behavior is different during puberty than after puberty. Furthermore, T treatment prior to puberty does not activate reproductive behavior in 28 day old males (Meek et al., 1997), even after 2 weeks of T treatment (unpublished study), indicating that the effects of T on neural circuits and behavior are different before puberty than after puberty. Taken together, these data suggest that a second window of maximal sensitivity

to steroid-dependent organization may open at the onset and close at the offset of puberty.

Determining whether puberty is a sensitive period for nervous system development is important to our understanding of life situations that cause shifts in the normal timing of exposure to hormones in humans. For instance, eating disorders and extreme exercise can delay gonadal maturation and consequently deprive the nervous system of hormones during puberty. Similarly, the effects of anabolic steroid use in teenagers may be profound and long lasting if puberty is a sensitive period for the organization of the nervous system and behavior. Future work in our laboratory will be aimed at determining whether puberty is a sensitive period for hormone-dependent organization of the brain. These investigations will increase our understanding of the consequences for behavior when the timing of exposure of the adolescent brain to gonadal hormones is disrupted.

ACKNOWLEDGMENTS

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CHAPTER 3

Testicular hormone exposure during adolescence organizes flank marking behavior and vasopressin receptor binding in the lateral septum.

INTRODUCTION

Scent marking is an important form of social communication for many mammalian species (Johnston, 1973). The Syrian hamster exhibits a stereotyped form of scent marking behavior called flank marking (Johnston, 1975). Flank marking occurs when hamsters rub pigmented sebaceous glands located on their dorsal flank region against objects in their environment (Johnston, 1975). This behavior can be stimulated by the odors of conspecifics alone, but is most often displayed during social encounters (Johnston, 1975). Importantly, flank marking behavior serves to communicate dominance status between males and is essential for the maintenance of these dominance relationships (Ferris et al., 1987).

Flank marking behavior is influenced by testosterone (T) in adult male Syrian hamsters (Johnston, 1981). Castration significantly reduces and T replacement restores flank-marking behavior (Johnston, 1981). T modulates flank marking behavior by altering arginine-vasopressin (AVP) neural transmission within a zone that extends from the posterior medial and lateral preoptic area to the posterior medial and lateral anterior hypothalamus (MPOA-AH, reviewed in Albers et al., 2002). Microinjections of AVP within this region cause dose-dependent increases in flank marking behavior (Albers and Ferris,

1986; Ferris et al., 1988), and the presence of T further enhances these effects (Albers et al., 1988). These data suggest that T influences flank marking by altering the sensitivity or response of the MPOA-AH to AVP, possibly by increasing AVP binding. Indeed, castration reduces and T replacement restores V1a binding within the MPOA-AH continuum (Johnson et al., 1995; Young et al., 2000).

The MPOA-AH is reciprocally connected to the lateral septum (LS), bed nucleus of the stria terminalis (BST), and the periaqueductal gray (PAG). AVP microinjection into these areas also induces flank marking behavior (Hennessey et al., 1992; Irvin et al., 1990), suggesting they are part of a flank marking circuit in which AVP is a neurotransmitter at multiple levels. Just as T facilitates the effects of AVP injection into the MPOA-AH on flank marking, T also enhances the effects of AVP injection into the LS-BST and PAG on flank marking behavior, although to a much lesser extent (Albers and Cooper, 1995). Thus, while these brain regions contribute to the display of flank marking behavior, the MPOA-AH may be the primary site mediating the activational effects of T on behavior.

The factors responsible for the development of flank marking behavior are largely unexplored. Preadolescent hamsters are capable of flank marking in response to male odors around day 22 (Ferris et al., 1996). However, levels of flank marking at this age are much lower than what is typically observed in adults (Johnston, 1981), suggesting that this behavior continues to develop during adolescence. Although increased gonadal secretions are a hallmark of adolescent development, what role testicular secretions play in the development

of flank marking behavior is not known. One possibility is that the rise in gonadal hormones during adolescence simply activates adult levels of flank marking behavior. Alternatively, the rise in gonadal hormones during adolescence may permanently *organize* neural circuits to permit activation of behavior by T in adulthood. For example, gonadal hormones during adolescence organize reproductive behavior in male Syrian hamsters (Schulz et al., 2004). Males gonadectomized prior to adolescence, and therefore not exposed to gonadal hormones during this time, show long lasting deficits in adult reproductive behavior that are not reversed by prolonged T treatment and repeated sexual experience. Thus, full hormonal activation of reproductive behavior in this species requires the presence of testicular hormones during adolescence, and the effects of adolescent testicular hormones may generalize to other hormone-modulated social behaviors such as flank marking.

The current study tested the hypothesis that exposure to gonadal hormones during adolescence is necessary for the activation of flank marking behavior by T in adulthood. We further hypothesized that exposure to gonadal hormones during adolescence influences the degree of V1a receptor binding in the brain regions regulating flank-marking behavior.

METHODS

Animals

All animals were housed in a 14 h light 10 h dark schedule (lights off at 1300 h EST), and had ad libitum access to food (Teklad Rodent Diet No. 8640, Harlan) and water. Animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and all protocols were approved by the Michigan State University All-University Committee for Animal Use and Care.

Experimental animals (resident)

Fifty-two 18-day-old male Syrian hamsters (Mesocricetus auratus) were obtained from Harlan Sprague-Dawley (Madison, WI) laboratories and arrived with their mothers and littermates. Experimental males remained with their mothers and littermates until weaning at 21 days of age. At weaning, males were housed individually in 30.5 x 10.2 x 20.3 cm clear polycarbonate cages. Approximately one week prior to behavior testing, animals were transferred to larger home cages measuring 37.5 x 33 x 17 cm, and these cages were not cleaned or disturbed before behavior testing occurred.

Partner animals (intruders)

Fifty two adult male Syrian hamsters were obtained from Harlan Sprague-Dawley approximately one week prior to behavior testing. All intruders were group housed (4-5/cage) in clear polycarbonate cages (30.5 x 10.2 x 20.3 cm).

Experimental Design

Castrate Groups

Two groups of males were gonadectomized (GDX) before adolescence at 21 days of age (n=8-9/group), and therefore were deprived of testicular hormones during adolescence (NoT@P). Two additional groups were GDX immediately after adolescence at 62 days of age (n=8-9/group), and therefore were exposed to testicular hormones throughout adolescent development (T@P). All males were behavior tested seven weeks following GDX in young adulthood (10 and 16 weeks old, respectively). To determine the activational effects of T on flank marking behavior, one of the NoT@P and T@P groups were administered 3.0 mg of T (0.5 mg and 2.5 mg T pellets; Innovative Research of America, Sarasota, FL) one week prior to testing. One animal in each of these four groups died prior to behavior testing, and the data for two animals were not collected due to a brief video camera failure. Therefore, final sample sizes were 6-8/group.

Sham Groups

Two groups of sham-gonadectomized males were included in this study. One group of males received a sham GDX immediately before adolescence (Shm-NoT@P; n = 9), and the other received a sham GDX immediately after adolescence (Shm-T@P; n=8). The sham surgeries and behavior tests were conducted at the same time as their respective castrate group (NoT@P or T@P). The sham groups served two purposes: 1) to assess whether chronological age (10 vs. 16 weeks old) at the time of behavior testing influences V1a receptor binding or flank marking behavior in adulthood, and 2) to assess whether the one

week of adult T replacement experienced by the castrate groups is sufficient to activate adult-typical levels of flank marking behavior. If one week is sufficient, then the T-treated T@P group should display levels of flank marking behavior similar to that of sham males.

Behavior Testing

Testing Paradigm

The resident-intruder paradigm was employed in which an age- (10 or 16 weeks old) and weight-matched (within 10 grams) gonad-intact intruder was placed into the home cage of the resident male for a 10-min test. Prior to testing, the resident's cage lid was removed and clear plexiglass wall extensions were fitted inside the cage (extended to the floor) to prevent animals from escaping during testing (increased total wall height to 32.2 cm). Five minutes following the insertion of cage wall extensions, the intruder was placed into the cage with the resident. Each intruder was only tested once during the experiment. All behavior tests began one hour into the dark phase of the light-dark cycle, and were videotaped under dim red light illumination for later behavioral analysis. A flank mark was recorded by an observer blind to experimental condition each time a resident or intruder rubbed his dorsolateral flank gland against the walls of the test arena. An attack was recorded if the resident or intruder moved quickly toward their partner in an attempt to bite.

Prescreening of Intruders

In order to increase the likelihood of the resident male displaying dominance behavior toward the intruder, and also to minimize individual differences in behavior between intruders, all intruders were screened for behavior one-two days prior to testing. During screening, the gonad-intact intruders were placed into the home cage of another gonad-intact male for five minutes. If the intruder initiated an attack or was excessively submissive (displayed only escape behavior) he was excluded from testing.

Tissue Collection

All hamsters were decapitated immediately following behavior testing. Brains were removed, frozen rapidly on dry ice, and stored at –80°C until sectioning.

V1a Receptor Autoradiography

Brains were cut into 20-μm-thick coronal sections using a cryostat at -20°C and thaw-mounted onto glass slides (Superfrost Plus, Fisher Scientific). Every third section beginning at the anterior medial preoptic area and ending at the posterior anterior hypothalamus [Figures 19-27 of the hamster stereotaxic atlas (Morin and Wood, 2001)] was used for V1a receptor binding using a linear [^{125}I] V1a antagonist (New England Nuclear) as described in previous studies (Caldwell and Albers, 2003; Caldwell and Albers, 2004; Johnson et al., 1995; Young et al., 2000). Sections were thawed at room temperature and fixed with

0.1% paraformaldehyde (pH 7.4) for 2 min. The sections were next pre-incubated in two 10-min rinses of 50 mM Tris buffer (pH 7.4), and then placed in tracer buffer consisting of 50 mM Tris (pH 7.4) with 10 mM $MgCl_2$, 0.1% BSA, 0.05% bacitracin and 50 pM tracer for 1 h at room temperature. The tracer was [^{125}I]phenylacetyl-D-Tyr(me)-Phe-Gln-Asn-Arg-Pro-Arg-Try-NH_2 linear vasopressin V_{1a} receptor antagonist (New England Nuclear, Boston, MA). Previous studies have verified the specificity of this tracer for the V_{1a} receptor (Ferris et al., 1993; Johnson et al., 1995; Young et al., 2000). Sections next underwent two 5 min washes and one 35 min wash in 50 mM Tris with 10 mM $MgCl_2$ at room temperature. Sections were quickly rinsed in cold dH_2O, blown dry with cool air, and placed in an autoradiograph cassette. Kodak Bio-Max MR film was placed on the slides for 3 days and then developed.

Quantification of receptor binding

Standard curves were established for binding density by placing [^{125}I] microscales (Amersham, IL) into X-ray cassettes with the labeled tissue sections. Adjacent cresyl violet stained sections were used to identify brain regions of interest within the sections used for receptor binding. V_{1a} receptor binding was quantified in the brain regions that mediate flank marking behavior: the LS, BST, AH, medial preoptic nucleus (MPN) and medial preoptic area (MPO). Three brain sections were quantified within each anatomical area using a 0.35 mm x 0.35 mm box placed in the center of the brain area using local neuroanatomical landmarks. The box size was not adjusted to accommodate increases or

decreases in the area of a brain region. The background binding was subtracted from density measurements and the optical densities were analyzed using Scion Image software (NIH). Data were converted from optical densities to disintegrating units per minute/milligram tissue equivalents.

Statistical Analysis

Because the resident's flank marking data were non-parametric, Mann-Whitney U tests were used to compare the behavior of the two groups of T@P males that differed only with respect to hormone treatment during behavior tests in adulthood (T vs. noT during testing) and the two NoT@P groups that also only differed by hormone treatment during adult behavior tests (T vs. noT during testing). The flank marking behavior of intruders was analyzed using a two-factor ANOVA treating the resident's adolescent hormone status and T status during testing as independent variables, and the flank marking behavior of the intruder as the dependent variable. Two-factor ANOVAs (adolescent hormone status x T status during testing) were conducted on the resident's V1a receptor data separately for the LS, BST, AH, MPO, and MPN. Two-tailed t-tests were conducted to compare the V1a binding and behavior of resident Shm-T@P and resident Shm-NoT@P groups to each other and also the resident T@P males to the resident Shm-T@P males that were behavior tested at the same time.

RESULTS

Castrate Groups: Behavior

Mann-Whitney U tests were conducted to determine whether T activates flank marking behavior in castrated resident NoT@P and T@P males. T treatment did not alter flank mark number in NoT@P males [Mann-Whitney U = 31.50, $p > 0.05$; Figure 3.01]. In contrast, T significantly increased flank marking behavior in T@P males [Mann-Whitney U = 39.00, $p < 0.05$; Figure 3.01]. As for the intruders, a 2-factor ANOVA found no effects of resident adolescent hormone status [$F(1, 24) = 0.02$, $p > 0.05$] or resident T status during testing [$F(1, 24) = 0.28$, $p > 0.05$] on the flank marking behavior of intruders, nor did these factors interact to influence the intruder's behavior [Figure 3.01; $F(1, 24) = 0.14$, $p > 0.05$]. Too few attacks were displayed by the residents in this experiment to permit statistical analyses.

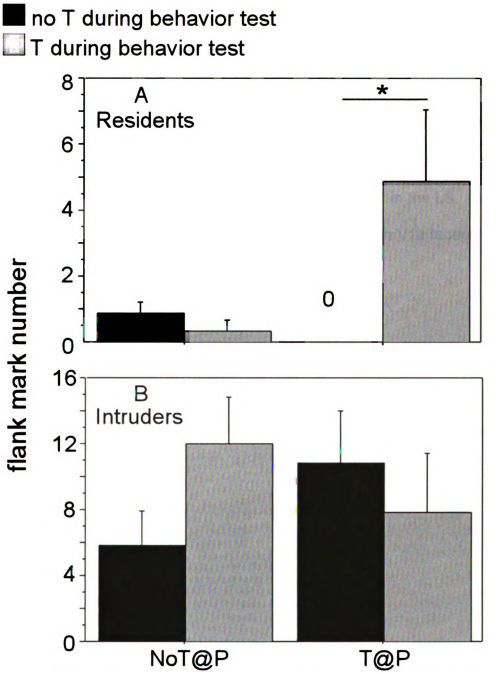


Figure 3.01. A. The mean number of flank marks exhibited by resident adult males exposed to testicular hormones during adolescence (T@P) and males deprived of testicular hormones during adolescence (NoT@P). Testosterone treatment significantly increased flank marking behavior during a resident/intruder test in T@P males but not NoT@P males. B. The mean number of flank marks exhibited by the intruders for each castrate group. Neither resident adolescent hormone status nor resident testosterone status during testing influenced the flank marking behavior of intruders.

Castrate Groups: LS, BST and AH

Adolescent testicular hormone exposure significantly reduced V1a binding in the LS [$F(1, 24) = 4.33$, $p < 0.05$; Figures 3.02 and 3.03], regardless of T status during adult behavior testing. Specifically, T@P males showed less V1a receptor binding in the LS than NoT@P males. No interaction between adolescent hormone status and T status during testing was observed in the LS. No effects of adolescent hormone status or T status during testing on V1a receptor binding were found for the BST or AH (Figure 3.02).

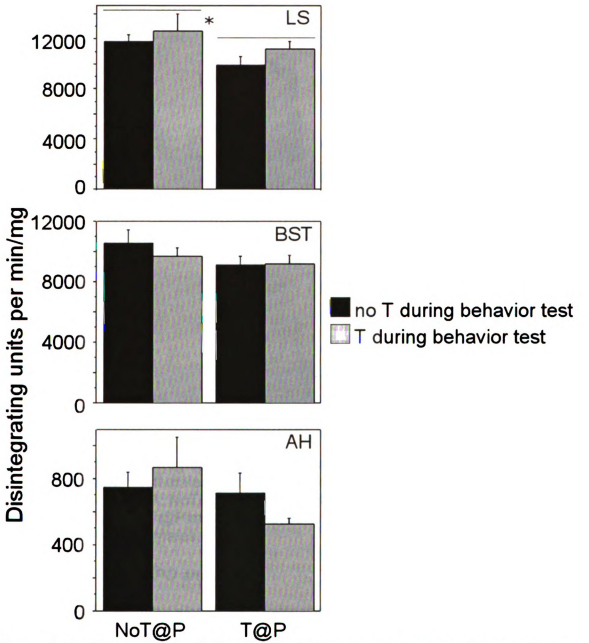
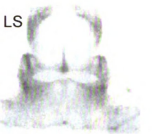


Figure 3.02. The amount of V1a receptor binding (expressed as disintegrating units per min/mg) in the lateral septum (LS), bed nucleus of the stria terminalis (BST) and anterior hypothalamus (AH) exhibited by males exposed to testicular hormones during adolescence (T@P) and males deprived of testicular hormones during adolescence (NoT@P). T@P males displayed significantly less V1a receptor binding in the LS than NoT@P males, regardless of adult testosterone treatment. No significant differences were observed between T@P and NoT@P males in the BST or AH.

A



B

Figure 3.03. Photomicrographs of V1a receptor binding in the LS of two testosterone-treated adult males that were either deprived of gonadal hormones during adolescence (A; NoT@P) or exposed to gonadal hormones during adolescence (B; T@P). T@P males displayed significantly less V1a receptor binding than NoT@P males.

Castrate Groups: MPO and MPN

Adult T treatment significantly increased V1a receptor binding in both the MPN [$F(1, 24) = 97.78$, $p < 0.0001$; Figure 3.04] and MPO [$F(1, 24) = 62.08$, $p < 0.0001$; Figure 3.04], regardless of adolescent hormone status. The T-treated NoT@P and T@P males displayed significantly more V1a receptor binding in these two areas than did untreated NoT@P and T@P males. No interaction between adolescent hormone status and T status during testing was observed.

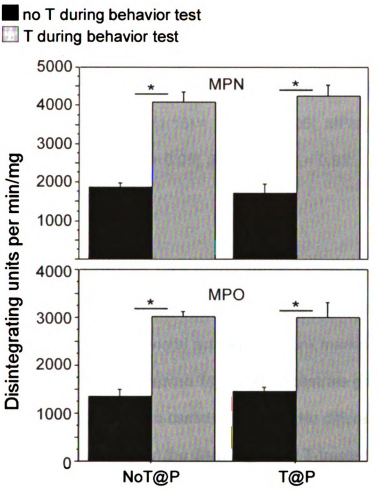


Figure 3.04. The amount of V1a receptor binding (expressed as disintegrating units per min/mg) in the medial preoptic area (MPO) and medial preoptic nucleus (MPN) exhibited by males exposed to testicular hormones during adolescence (T@P) and males deprived of testicular hormones during adolescence (NoT@P). Adult testosterone treatment significantly increased V1a receptor binding regardless of whether gonadal hormones were present during adolescent development.

Comparison of the Sham Groups

The Shm-NoT@P and Shm-T@P males were behavior tested at different ages (10 and 16 weeks, respectively), but at the same time as their respective castrate T@P and NoT@P males. Therefore, the comparison of the two sham groups assessed whether chronological age at the time of testing and brain collection influenced V1a receptor binding or flank marking behavior in this study.

No significant difference in flank mark number was observed between Shm-NoT@P and Shm-T@P groups [Table 3.01; t (1, 15) = -0.66, $p > 0.05$]. Similarly, no significant differences in V1a receptor binding were found between Sham groups in the LS [Table 3.01; t (1,15) = 0.26, $p > 0.05$], MPN [t (1,15) = 0.44, $p > 0.05$], MPO [t (1,15) = 0.17, $p > 0.05$], BST [t (1,15) = 0.83, $p > 0.05$], or AH [t (1,15) = 1.27, $p > 0.05$].

Comparison of the T-treated T@P group and the* Shm-T@P *group

In order to determine whether seven days of adult T treatment was sufficient for adult-typical behavioral activation of flank marking in the castrate groups, the behavior of the T-treated T@P males (castrate group) was compared with the Shm-T@P males (sham castrated group). No differences in flank mark number were observed between the Shm-T@P and T-treated T@P groups [Table 3.01; t (1,14) = 1.045, $p > 0.05$], nor were there any significant differences in V1a receptor binding in the LS [Table 3.01; t (1,14) = 0.72, $p > 0.05$], MPN [t (1,14) = 0.23, $p > 0.05$], MPO [t (1,14) = 0.82, $p > 0.05$], BST [t (1,14) = 0.93, $p > 0.05$], and AH [t (1,14) = 1.78, $p > 0.05$].

Table 3.01. Controlling for the effects of age and long-term castration on flank marking behavior.

	Flank Mark number	Lateral Septum	MPN	MPO	AH	BST
Shm-NoT@P	5.78 ±2.50	10712.0 ± 730.22	4569.76 ± 369.92	3262.88 ±206.70	836.11 ±94.96	9258.77 ±687.90
Shm-T@P	7.88 ±1.89	10429.0 ± 800.20	4349.64 ± 335.178	3314.88 ±232.59	678.50 ±77.601	8550.42 ±467.90
T-treated T@P	4.88 ±2.16	11150.4 ± 609.36	4244.88 ± 296.73	3004.38 ±297.82	524.25 ±38.47	9214.44 ±544.198

Table 3.01. No differences in flank marking behavior or V1a receptor binding (expressed as disintegrating units per min/mg) were observed between Sham castrated groups that controlled for chronological age at the time of behavior testing and brain collection in young adulthood (70 vs 112 days of age). No differences in behavior or V1a receptor binding were observed between the T-treated T@P males (castrated) and the Sham-castrated males, suggesting that the seven days of T replacement prior to behavior testing experienced by the castrated T@P group was sufficient to increase V1a binding and flank marking behavior.

DISCUSSION

This study demonstrates that exposure to gonadal hormones during adolescence is necessary for the activation of flank marking behavior by T in adulthood. Adult T treatment activated flank marking behavior during a social interaction in males exposed to adolescent hormones but not in males deprived of adolescent hormones, suggesting that gonadal hormone exposure during adolescence organizes flank marking neural circuits to allow for activation by T in adulthood.

Although the behavior of the resident animal necessarily depends on the interaction with an intruder, the flank marking behavior of intruders did not appear to influence the general pattern of resident flank marking behavior observed in this experiment. Specifically, the intruders of each castrate group displayed

similar levels of flank marking behavior, and the behavior of the intruders did not vary according to the experimental treatment of the residents.

The behavioral differences between resident T@P and NoT@P males are not likely due to chronological age because the two groups of sham-intact males that were tested at the same ages as the T@P and NoT@P groups did not display different levels of flank marking behavior. This suggests that flank marking behavior in young adulthood does not depend on age at the time of testing. T-treated T@P males also displayed similar levels of flank marking behavior as gonad-intact sham males, suggesting that one week of T treatment was sufficient to fully activate flank marking behavior in the castrated groups. Thus, the activation of flank marking behavior by T in this study depended on the presence of gonadal hormones during adolescence rather than chronological age or the duration of exposure to exogenous or endogenous T.

The LS may be an important neural target for organization by T during pubertal development. In this study, males deprived of testicular hormones during adolescence displayed significantly higher levels of V1a binding in the LS than did males exposed to adolescent hormones, regardless of T treatment in adulthood. Thus, while adult T treatment did not alter LS vasopressin binding, the presence of hormones during pubertal development caused a long-term alteration in vasopressin receptor binding in the LS, suggesting that LS V1a binding is organized by pubertal hormones. Although the same general pattern of result was observed in both the BST and AH, the effect of adolescent hormone status did not reach statistical significance in these regions. In contrast, V1a

binding within the MPN and MPO was significantly affected by the presence of T in adulthood, but not the presence of gonadal hormones during pubertal development, suggesting that V1a binding in these regions is not organized by pubertal hormones. Given that vasopressin microinjections into the LS stimulate flank marking behavior in adult males, and T treatment enhances vasopressin-induced flank marking (Albers and Cooper, 1995), the long-term increase in V1a binding within the LS as a consequence of the absence of pubertal hormones may alter the effects of T on flank marking behavior in adulthood.

It may seem counterintuitive that NoT@P males have more V1a binding in the LS as compared T@P males, since NoT@P males displayed significantly less flank marking behavior. However, the reduced V1a binding in T@P males may be the consequence of synaptic remodeling which normally occurs during pubertal development in both rodents and humans (Andersen et al., 2000; Giedd et al., 1999). While not all synaptic pruning during adolescence depends on the presence of gonadal hormones (Andersen et al., 2002), evidence from our lab suggests that adolescent remodeling of synapses within the mating circuit may rely on gonadal hormones. For example, gonadectomized and T-treated prepubertal males display more androgen receptor immunoreactivity than gonadectomized and T-treated adults in the MPN and BST (Meek et al., 1997), suggesting that androgen receptors decrease across pubertal development. In addition, NoT@P males display greater androgen receptor immunoreactivity than T@P males in both the MPN and BST, suggesting that the pubertal decrease in androgen receptor can be prevented by removing gonadal hormones prior to

adolescence (Romeo et al., 2000). Thus, while the higher levels of LS V1a binding in NoT@P males is dissociated from the low levels of flank marking behavior displayed, the higher levels of V1a binding may indicate incomplete maturation of synaptic organization within the LS, and consequently, flank marking behavior.

One previous study has investigated the effects of prepubertal castration on the development of flank marking behavior. Male hamsters were gonadectomized or sham-gonadectomized prior to adolescence at 21 days of age, and tested for flank marking behavior with the same partner every ten days beginning at 30 days of age in castrate-castrate or intact-intact pairs. Interestingly, flank marking increased during adolescence in both castrate and intact pairs of animals, suggesting that gonadal hormone exposure during adolescence is not necessary for the development of flank marking behavior (Whitsett, 1975). However, experiential factors such as repeated social interactions with another male conspecific may compensate for the detrimental effects of the absence of hormones during adolescence. Indeed, social experience during adolescent development can even overcome the effects of prepubertal medial preoptic area lesions on the development of male sexual behavior (Twiggs et al., 1978). Thus, adolescence is likely a time during which an individual's environment can exert powerful effects on the development of the nervous system and behavior, and therefore provide an opportunity for studying the plasticity of behavioral development. Future work will be aimed at

determining the interactions between hormonal and social experiences during adolescence on the development of social behaviors.

Flank marking is not the only social behavior organized by pubertal hormones in the male hamster. The presence of hormones during adolescence also enhances masculine reproductive responses to T in adulthood such as mounts, intromissions and ejaculations (Schulz et al., 2004). Furthermore, the presence of gonadal hormones during adolescence defeminizes or reduces the male's propensity to display lordosis behavior in response to estradiol and progesterone in adulthood (Schulz et al., 2004). Thus, organizational effects of pubertal hormones generalize to other social behaviors in the male Syrian hamster. In addition, adolescent organizational effects of gonadal hormones on social behavior have been demonstrated in species as diverse as rats (Primus and Kellogg, 1990), tree shrews (Eichmann and Holst, 1999), and gerbils (Lumia et al., 1977). Recent evidence also indicates that the influence of pubertal hormones on brain and behavioral development may generalize to other behavioral systems such as learning and memory (Hebbard et al., 2003). Taken together, work from our lab and others highlights the wide ranging effects of gonadal hormones on the adolescent remodeling of the nervous system and the development of behavior. As research into adolescent brain and behavioral development continues, the adolescent period may prove to be as critical as the perinatal period for establishment of adult behavior patterns.

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CHAPTER 4

Is adolescence a sensitive period for the organizational effects of testosterone on male reproductive behavior?

INTRODUCTION

Adolescence is a life transition characterized by intense physical, emotional, and behavioral maturation. Physical maturation includes the onset of gonadal hormone secretions and resulting changes in bodily appearance, as well as changes in brain structure and function. Indeed, although overall human brain weight increases negligibly after age five (Dekaban, 1978), neuroimaging work has debunked recently held beliefs that human brain development is completed prior to adolescence. This work has revealed that during adolescence, cortical and subcortical regions undergo curvilinear changes in volume, in which they enlarge during early adolescence, reaching peak volumes at different time points in boys and girls, and then subsequently decline in size in late adolescence (Giedd et al., 1999). This biphasic change in gross morphological characteristics reflects a dynamic period of brain development that involves an initial phase of synapse proliferation and a later phase of experience-dependent synapse elimination (Huttenlocher and Dabholkar, 1997). Peak volume of different cortical regions is reached at different times in adolescence. Interestingly, areas important for executive control and decision making, such as the prefrontal cortex, are among latest developing brain regions, which may in part explain some of the behavioral hallmarks of adolescence such as increased impulsivity

and risk taking (Giedd et al., 2006). The growing body of adolescent neuroimaging research has provided an invaluable window into the developing human brain, and even changed popular explanations of adolescent behavior from "raging hormones" to "haywire brain".

Knowledge of the extensive brain remodeling occurring during adolescence provides a richer context in which to interpret adolescent behavioral changes than simply attributing volatile behavior to adolescent hormone changes. However, the brain is a major target of gonadal hormones such as estrogen and testosterone, and many of the changes in cortical and subcortical volume are coincident with the pubertal rise in gonadal hormones during adolescence (Giedd et al., 2006). In addition, numerous animal studies document the powerful effects of gonadal hormones on a variety of behaviors ranging from sexual interest to cognitive abilities. Furthermore, we've known for almost 50 years that exposure to gonadal hormones during early development permanently alters sex-specific behavioral responses to gonadal hormones in adulthood (Phoenix et al., 1959). Despite this knowledge, only a handful of studies have investigated the contribution that pubertal hormones make to adolescent brain development and whether the hormone action in the brain during this developmental period results in enduring changes in adult behavior.

The scarcity of research investigating pubertal hormone influences on adolescent brain development is particularly notable considering that deviations in pubertal timing are associated with psychopathologies in adolescence and adulthood such as depression, anxiety, disordered eating, and conduct disorder

(Ge et al., 2006; Ge et al., 2003; Graber et al., 1997; Kaltiala-Heino et al., 2003; Laitinen-Krispijn et al., 1999; McCabe and Ricciardelli, 2004; Ricciardelli and McCabe, 2004; Zehr et al., 2007). These effects of variation in pubertal timing on psychopathology have largely been attributed to psychosocial factors that come into play with the intense changes in experience that accompany sexual maturation. However, mistimed direct hormonal influences on the brain may also influence risk for psychopathology. Animal models have immense potential for elucidating the mechanisms by which gonadal hormones directly impact adolescent brain and behavioral development. For example, male Syrian hamsters that are deprived of hormones during adolescence display compromised social behavior (Schulz et al., 2004; Schulz and Sisk, 2006), even after hormones are replaced in adulthood, suggesting that exposure of the brain to gonadal hormones during adolescence organizes behavioral circuits and programs long-lasting behavioral responses (Schulz and Sisk, 2006; Sisk and Foster, 2004; Sisk et al., 2003; Sisk and Zehr, 2005). These data further indicate that a window of sensitivity to organizational effects of gonadal steroid hormones may close following adolescence.

The current study sought to determine the temporal parameters spanning the pre- and post-adolescent periods within which neural circuits mediating social behavior are sensitive to organizational effects of gonadal steroid hormones. Although previous work has established that perinatal exposure to gonadal steroid hormones masculinizes and defeminizes behavioral neural circuits, more recent work suggests that a second window of sensitivity may open at

adolescence. For example, steroid hormones fail to elicit maximal expression of male reproductive behavior immediately prior to adolescence (Meek et al., 1997; Romeo et al., 2001; Romeo et al., 2002), but readily activate high levels of reproductive behavior after adolescence, raising the possibility that adolescence is a second sensitive period for the effects of gonadal steroid hormones on male reproductive behavior. However, a direct test of whether adolescence is a second sensitive period distinct from the perinatal period has not been conducted. We tested two competing hypotheses in this study. We tested two competing hypotheses in this study. The adolescent sensitive period hypothesis predicts that T exposure during adolescence, but not before or after, will elicit maximal behavioral expression in adulthood. Alternatively, adolescence may mark the end of a much larger protracted window of sensitivity to the organizing actions of T on adult social behavior. This hypothesis predicts that T treatments before and during adolescence, but not after, will result in high levels of reproductive behavior in adulthood. Given that T does not activate adult levels of reproductive behavior prior to adolescence, this outcome would suggest that while steroid-dependent organization is possible before adolescence, steroid-independent development is still required during adolescence to permit adult behavioral responses to T. We also measured regional volumes of several brain areas essential for social behavior, and predicted that any organizational effects on behavior would be mirrored by changes within the mating neural circuit. Our findings are discussed within the context of sensitive periods for behavioral development, and their relevance to human psychopathology.

METHODS

Animals

Time-pregnant female Syrian hamsters (*Mesocricetus auratus*) were obtained from Harlan Sprague-Dawley laboratories (Madison, WI) and were 4 days pregnant on arrival. Pregnant females housed in clear polycarbonate cages (37.5 x 33 x 17 cm) and provided nesting materials, food (Telkad Rodent Diet No. 8640, Harlan, Madison, WI) and water. Females were exposed to a 14 hr light/10 hr dark schedule (lights off at 1600 hr EST) and the temperature was maintained at 21± 2°C. Nests were checked twice daily for births beginning on gestational day 15 (hamsters have a 16 day gestation). All animals were born on the same day. On postnatal day (PND) 9, litters were sexed and culled to 6-8 mixed-sex pups. Males were housed with mothers and littermates until weaning and single housing occurred (cage dimensions: 30.5 x 10.2 x 20.3) at 19 days of age. Female littermates were also weaned and singly housed at this time, but were used in separate experiment. All animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and all protocols were approved by the Michigan State University All-University Committee for Animal Use and Care.

Experimental Design

<u>Testing the sensitive period hypothesis</u>

Figure 4.01 depicts the experimental design employed to test the hypothesis that adolescence is a sensitive period for the organization of reproductive behavior and its underlying neural circuits by T. Males were randomly assigned to treatment groups and gonadectomized (GDX) or sham castrated at 10d of age (after the perinatal period of sexual differentiation in hamster), and then exposed to 19 days of blank- or T-filled silastic capsules before puberty (10-29d of age; Figure 4.01 row 1), during the normal time of puberty (29-48d of age; Figure 4.01 row 2), or after puberty (64-83d of age; Figure 4.01 row 3). In adulthood, four weeks following pellet removal, all GDX'd males were implanted with T-filled capsules and behavior tested one week later. The 19d and 7d T treatments served two different purposes. Endocrine and behavioral puberty occurs between 4 and 7 weeks of age in the male Syrian hamster (Cherry, 1987; Miller et al., 1977). Within 3 weeks, adult-typical levels of reproductive behavior are displayed and T concentrations have increased from undetectable to approximately 4-5 ng/ml (Sisk and Turek, 1983; Vomachka and Greenwald, 1979). Therefore, 19d T treatment period was intended to organize behavioral neural circuits, whereas the 1 wk treatment was employed to ensure the presence of adult physiological levels of T at the time of behavioral testing and tissue collection. Because our experimental objectives required testing males at three different ages in young adulthood, sham males were included in the design so that any age related differences in behavior could be detected independently of experimental manipulation.

Prepubertal comparison groups

Three groups of prepubertal males were also included in this study to verify previous observations that T fails to elicit adult-like levels of reproductive behavior prior to puberty (Figure 4.01, row 4). Males were gonadectomized on PND 10 and administered 19 days of blank capsules, T capsules, or received a sham gonadectomy. Males were behavior tested on PND 29 (still prepubertal) and brains were collected immediately following testing.

Additional comparison/control groups

The groups treated with T before, during, or after adolescence are without T for 4 weeks before T is re-administered prior to behavior testing. Therefore, we expected their behavior to be lower than sham-castrated animals due to this extended period without T. In order to have a normative behavioral reference point for these castrated animals, we included a group of adult castrated males that experienced endogenous T during perinatal and pubertal development prior to castration (Figure 4.01, row 5). This group was sham operated at 10d of age, and then GDX at 64d of age. Four weeks later, they received silastic T pellets (92d), followed by behavioral testing and tissue collection one week later (99d). Thus, the time between GDX and behavior testing was equivalent to the time the experimental animals waited between T pellet removal and behavior testing, providing an optimal comparison for adult-typical levels of behavior. The last control group consisted of males that were completely unmanipulated prior to

behavior testing (at 104d), and therefore acted as surgical controls for the other sham groups (Figure 4.01, row 6).

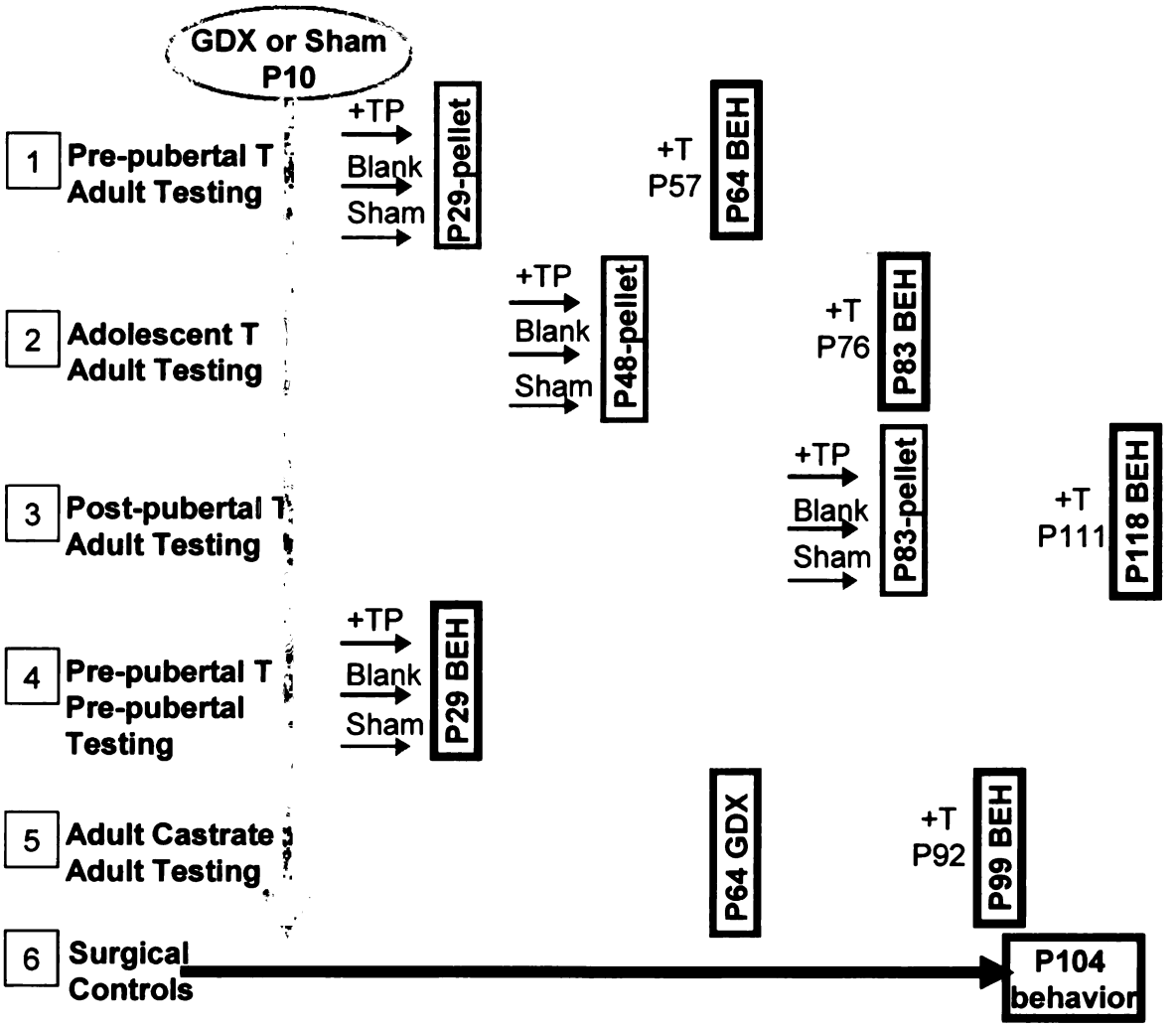

Figure 4.01. Experimental design. Males were gonadecomized or received sham surgeries on P (posnatal) day 10. Testosterone (T) or blank silastic pellets were administered for 19 days before (groups 1 and 4), during (group 2), or after adolescence (group 3). One set of males were behavior tested immediately after the 19 days of prepubertal treatment (group 4). All other groups were behavior tested in adulthood. Castrated males were re-administered T for one week prior to behavior testing, whereas sham GDX males received blank pellets prior to behavior testing. One group of males (group 5) were castrated after puberty, and received T-treatment and behavior testing in adulthood. This group served as a control group for "adult-typical" behavior because they were exposed to gonadal hormones throughout development. In addition, the interval between castration and behavior testing was matched to the interval between pellet removal and behavior testing in groups 1, 2, and 3, and therefore group 5 was also a normative reference for adult-typical behavior following testosterone deprivation. Sham groups served as behavioral controls for age at the time of testing. Surgical controls were also included, and their behavior was compared to sham GDX males.

Surgical Procedures

Gonadectomies and sham surgeries were performed under isofluorane anesthesia and aseptic conditions. Animals were anesthetized with isoflurane and received a subcutaneous injection of the analgesic buprenex (0.02 mg/ml). Next, a bilateral scrotal incision 3-4 mm in length was made, the testes were pulled through the incision, and the testicular veins tied with suture silk (000) before removal of the testes. The incisions were sutured closed. Two sterile silastic T pellets (one 7mm and one 15mm; i.d. =.1.98mm; o.d. = 3.18mm) were inserted subcutaneously through a 3-4 mm incision made on the dorsal midline of the anesthetized animal, and the incision was sutured closed. Animals were returned to their mothers and littermates following surgery, and their health was monitored closely for the following week. Animals also received a post-surgical buprenex injection (0.02 mg/kg) approximately 12 hours after surgery.

Behavior testing

All reproductive behavior tests were conducted 1- 5 hours after lights out. The male was placed in a 10-gal glass aquarium (51 x 26 x 31.5 cm) and allowed to acclimate for 5 min before the introduction of a receptive stimulus female. The behavior tests were 15 min. Ovariectomized stimulus females were brought into behavioral estrus with an injection of 10 μg EB (0.2 μg/ml) in sesame oil 48 hours before testing, and an injection of 250 μg progesterone (5μg/ml) in sesame oil 3 hours before testing. All females were checked for behavioral receptivity prior to behavior tests by pairing them with colony breeder males for approximately one

minute. If females didn't display the lordosis posture within one minute they were not used for experimental behavior tests. Females were only paired with one experimental male per test day.

The behavioral tests were videotaped under dim red light illumination. Videotapes were scored to assess the number of vaginally oriented mounts, intromissions, and the latencies to mount and intromit females. The criteria for these behaviors have been described previously (Meek et al., 1997). Genital and nongenital and nongenital grooming bouts, latencies, and durations were also recorded. In addition, defensive and fear related behaviors were quantified. Stretch-attend behavior is considered a risk-assessment behavior, and it was recorded when the male extended his forepaws stretched his head and nose cautiously toward the female (Albers et al., 2002). Tail-up walking is a defensive behavior that was scored if the male raised his tail above parallel to the floor, arched his back slightly upward (in kyphosis), and moved away from the female (Albers et al., 2002). Finally, escape dashes were recorded when the male darted quickly away from the female after an interaction (Albers et al., 2002). Videotapes were scored blind to experimental condition by a single observer.

Tissue collection and histology

Following behavior tests, male hamsters were euthanized with an overdose of sodium pentobarbital (130mg/kg ip) and perfused intracardially with 100ml of heparinized buffered saline rinse followed by 150ml of 4% paraformaldehyde. Brains were sectioned at 40 μm on a cryostat, and every

fourth section (160 μm interval) was thaw mounted onto glass slides, allowed to dry, and subsequently thionin stained and coverslipped for analysis of regional volumes.

Volume measurements

Regions within the hypothalamus and medial amygdala were traced bilaterally at 40X magnification using Neurolucida (Version number 6 Microbrightfield, Williston, Vermont). Specifically, the hypothalamic regions (Figure 4.02) traced included the medial preoptic nucleus (MPN), sexually dimorphic nucleus (SDN), posterior medial bed nucleus of the stria terminalis (BSTpm), MPN magnocellular (MPN), and ventromedial hypothalamus (VMH; Figure 4.03). Subregions of the medial amygdala (Me; Figure 4.04) included the anterior dorsal (MeAD), anterior ventral (MeAV), posterior dorsal (MePD), and posterior ventral (MePV) nuclei. Regional volumes were calculated by summing the traced cross-sectional areas and multiplying by the distance between sections (160 μm). See Figures 4.02-4.04 for representative coronal sections used to trace these regions. The Morin and Wood (2001) hamster atlas was used as a reference for tracings.

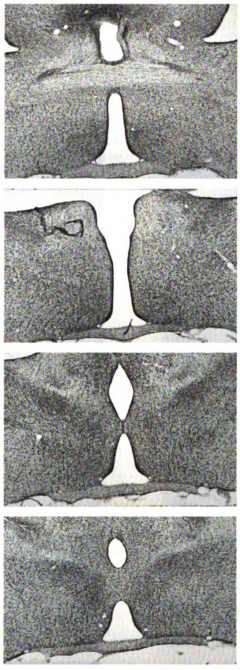

Figure 4.02. Photomicrograph of Nissl-stained coronal sections separated by 160 μm through the hypothalamus. Images were captured using a 4x objective. Regional volumes were estimated for the posterior medial bed nucleus of the stria terminalis (BSTpm), Medial preoptic nucleus (MPN), sexually dimorphic nucleus (SDN), and MPN magnocellular nucleus (MPNmag).

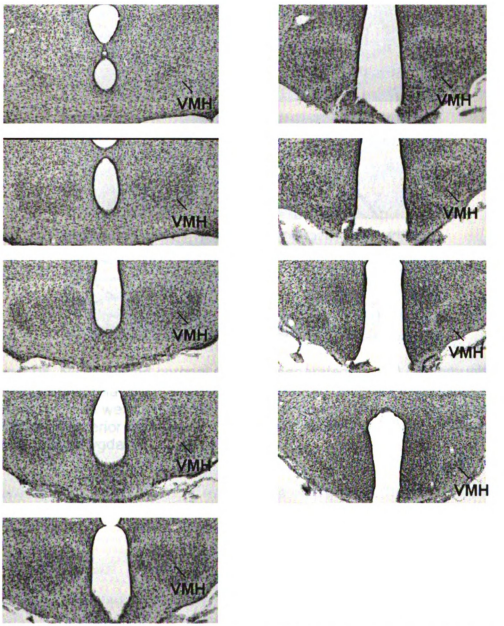

Figure 4.03. Photomicrograph of Nissl-stained coronal sections separated by 160 μm through the ventromedial hypothalamus (VMH). Images were captured using a 4x objective.

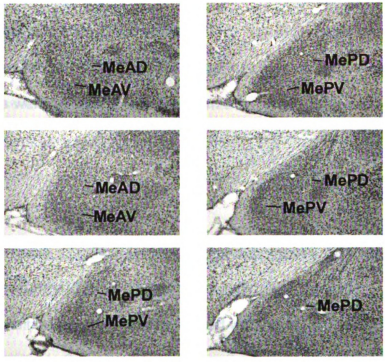

Figure 4.04. Nissl-stained coronal sections separated by 160 μm through the medial amygdala (Me). Images were captured using a 4x objective. Regional volumes were estimated for anterior dorsal (MeAD), anterior ventral (MeAV), posterior dorsal (MePD), and posterior ventral (MePV) nuclei of the medial amygdala.

Sample sizes and experimental attrition

Twelve males were assigned to each group on PND 9, one day prior to the first surgical procedures (gonadectomy). Some animals were lost from each group during this initial surgery and the post-operative period. Most males underwent four surgeries in the course of this long-term experiment, so additional animals were lost during subsequent surgeries. Despite these losses, final sample sizes were adequate, ranging from 8 to 12 per group.

Statistics

Adult comparisons: Effects of testosterone exposure before, during, and after adolescence on reproductive behavior and regional volumes

The adult behaviors of males treated with blank- or T-filled capsules before, during, and after adolescence were analyzed using a 2x3 two-way ANOVA (+/- T treatment x pre-, during-, or post-adolescent age of treatment). A two-factor mixed ANOVA was used to analyze the regional volumes of these groups, treating hemisphere as a repeated measure in all regions but the VMH (tracings were not separated by hemisphere during microscopic analysis).

Adult control groups

The behaviors of sham groups and the surgical control group were analyzed using a one-factor ANOVA. The behaviors of adult castrated and

behavior tested males (normative reference group) were only used comparatively as a yardstick for adult-typical levels of reproductive behavior.

Prepubertal regional volumes

A one-factor mixed ANOVA was used to analyze the regional volumes of prepubertal males that received 19 days of T or blank silastic capsules prior to brain collection at 29 days of age. Brain hemisphere was treated as a repeated measure for all regions but the VMH (tracings were not separated by hemisphere during microscopic analysis).

Prepubertal vs. Adult Behavior and VMH regional volumes

The behaviors of prepubertal blank treated, T-treated, and sham-operated males were compared using one-factor ANOVA followed by Fisher's PLSD tests. Because no differences were found within prepubertal or adult treatment groups, we collapsed across treatment and compared prepubertal and adult VMH volumes using a two-tailed t-test.

RESULTS

Adult comparisons: Effects of testosterone exposure before, during, and after adolescence on reproductive behavior

Mount number and latency

Treatment (T or blank capsule) and time (before, during, or after adolescence) interacted to influence adult mounting behavior [Figure 4.05 top panel; $F(2,53) = 4.90$, $p<0.02$]. T treatment before [$F(2,53) = 27.032$, $p<0.0001$] and during adolescence [$F(2,53) = 9.19$, $p<0.01$] significantly increased mount number, whereas post-adolescent T-treatment did not $F(2,53) = 0.370$, $p>0.05$]. T treatment significantly reduced overall mount latencies [Figure 4.04 bottom panel; $F(2,53) = 16.80$, $p < 0.0001$]. Although T treatment appeared to reduce mount latencies more in the pre- and mid-adolescent treatment groups than in the post-adolescent group, the interaction did not achieve statistical significance $F(1,53) = 2.21$, $p = 0.120$].

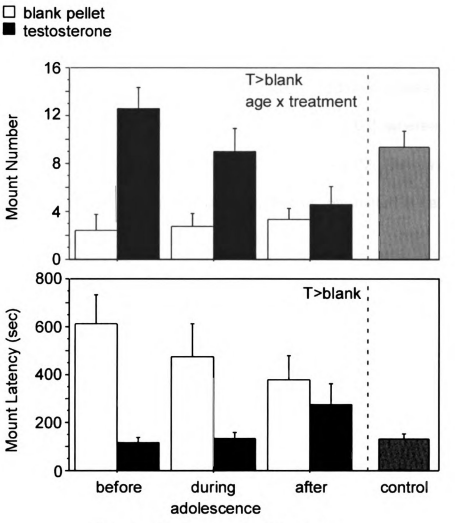

Figure 4.05. Mount number and mount latencies of males gonadectomized on postnatal day 10, and treated with blank- or testosterone-filled silastic capsules before, during, or after adolescence. Behavior tests occurred in adulthood. Testosterone treatment before or during adolescence, but not after, significantly increased mount number, and restored mounting to adult-typical control levels (top right panel). A similar behavioral pattern was observed for mount latencies (bottom panels), but the interaction was not statistically significant.

Intromission number, latency, and proportion of males intromitting

Hormone treatment significantly increased intromission number [Figure 4.06 top panel; $F(1,53) = 8.76$, $p < 0.005$] and decreased intromission latencies [Figure 4.05 bottom panel; $F(1,53) = 5.90$, $p < 0.02$], with no interactions between

treatment and time of treatment. When the proportion of males intromitting was compared separately for groups treated before, during, and after adolescence, pre-adolescent T treatment significantly increased the proportion of males intromitting [blank: 2/11; T: 7/10; $\chi^2 = 5.74$, Fishers exact P = 0.03], whereas mid- [$\chi^2 = 0.69$, Fishers exact P = 0.63] and post- adolescent [$\chi^2 = 0.22$, Fishers exact P > 0.99] treatments did not. The proportion of males that intromitted in each group is noted within the bar graphs of Figure 4.06.

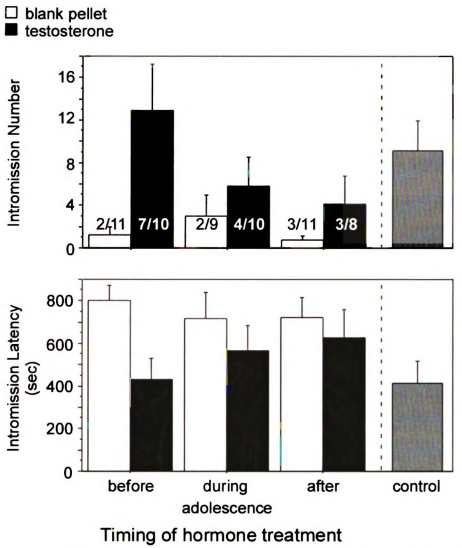

Figure 4.06. Intromission number and intromission latencies of males gonadectomized on postnatal day 10, and treated with blank- or testosterone-filled silastic capsules before, during, or after adolescence. Behavior tests occurred in adulthood. Testosterone treatment increased intromissions and decreased intromission latencies overall, and levels were similar to adult-typical controls (right panels). Prepubertal testosterone treatment significantly increased the proportion of males intromitting the female (proportion noted on each bar graph).

Genital Groom Number, Duration, and Latency

Treatment [$F(1, 53)= 16.79$, $p < 0.0001$] and time of treatment [$F(2, 53)= 3.41$, $p < 0.05$] significantly increased genital grooming (Figure 4.07, top panel), but a trend toward an interaction was also observed [Figure 4.07, top panel; $F(2,$

53)= 3.00, p = 0.058]. T treatment before [F(2,53) = 19.89, p< 0.0001], but not during [F(2,53) = 3.0, p> 0.05] or after adolescence [F(2,53) = 1.01, p> 0.05], significantly increased genital groom number in adulthood. Groom durations [Figure 4.07, middle panel; F(1,53) = 20.140, p<0.0001] and groom latencies [Figure 4.07, bottom panel; F(1,53) = 5.21, p<0.03] were significantly influenced by T treatment, but no interactions between T treatment and time of treatment were observed for these measures.

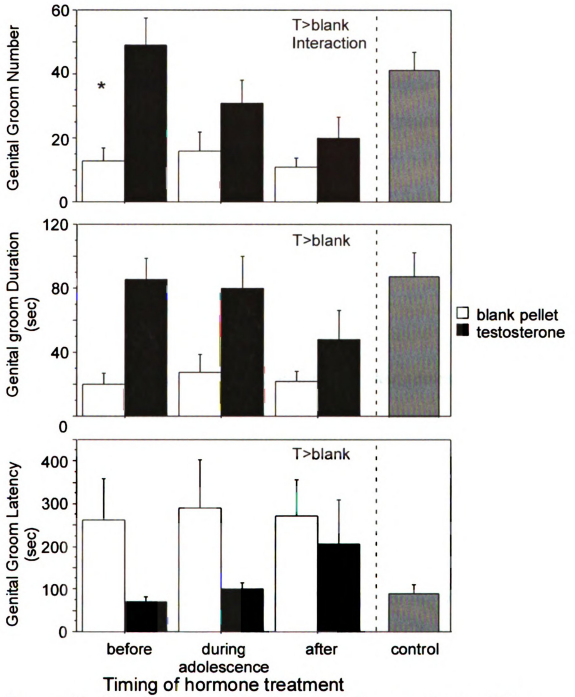

Figure 4.07. Genital groom number, duration, and latency of males GDX on postnatal day 10, and treated with blank- or testosterone-filled silastic capsules before, during, or after adolescence. Behavior tests occurred in adulthood. Prepubertal testosterone-treatment significantly increased genital grooming, whereas testosterone treatment during or after adolescence did not. Grooming levels of prepubertally testosterone-treated males were similar to that of adult-typical behavioral controls (right panels). Although a similar pattern was observed for groom durations and latencies, only a main effect of testosterone treatment was observed.

Behavior of sham-operated control groups and surgical controls

One-way ANOVA was used to compare the behaviors of adult males sham-operated before, during, or after adolescence with a surgical control group that was not exposed to any surgical procedure. These four groups did not significantly differ on any behavioral measure [Mounts: $F(3,38) = 0.890$, $p=0.46$; Mount latency: $F(3,38) = 1.9$, $p=0.15$; Intromissions: $F(3,38) = 1.91$, $p=0.144$; Intromission latency: $F(3,38) = 0.96$, $p=0.423$; Genital groom number: $F(3,38) = 0.61$, $p=0.612$; Genital groom duration: $F(3,38) = 0.10$, $p=0.96$; Genital groom latency: $F(3,38) = 2.45$, $p=0.08$].

Effects of testosterone exposure before, during, and after adolescence on adult brain regional volumes

Adult brain regional volumes were analyzed using a 2-factor (treatment x time of treatment) mixed ANOVA, treating brain hemisphere as a repeated measures variable. Treatment (T or blank) and time of treatment (before, during or after adolescence) did not significantly influence the regional volumes of the MPN, SDN, BSTpm, MPNmag, VMH, MeAD, MeAV, MePD, or MePV and no interactions between treatment or time of treatment were observed for any region. The MePV right hemisphere was significantly larger than the left hemisphere [$F(1,48) = 24.63$, $p < 0.0001$], however.

Prepubertal regional volumes

A repeated measures ANOVA was employed to analyze the regional volumes of prepubertal males that received 19 days of T treatment or blank silastic capsules prior to brain collection at 29 days of age (Figure 4.08). Brain hemisphere was treated as a repeated measure for all regions but the VMH. In the hypothalamus, prepubertal T treatment significantly increased the regional volumes of the SDN [Figure 4.08; $F(1,19) = 5.07$, $p<0.04$], and BST [$F(1,19) = 23.16$, $p<0.0001$], but not the MPN [$F(1,19) = 0.81$, $p=0.78$], MPNmag [$F(1,19) = 0.05$, $p= 0.82$], or VMH [$t(1,19) = 0.23$, $p=0.64$]. In addition, the left hemisphere BST was significantly larger than in the right hemisphere [Figure 4.08; $F(1,19) = 4.98$, $p < 0.04$]. In the medial amygdala, T did not significantly change regional volumes of the MeAD [Figure 4.09, panel A; $F(1,19) = 0.042$, $p=0.84$] or MeAV [$F(1,19) = 1.340$, $p=0.26$], but T significantly increased regional volumes of both the MePD [Figure 4.09, panel B; $F(1,19) = 17.28$, $p<0.0005$] and MePV [$F(1,19) = 4.56$, $p<0.05$]. In the MePD, a marginally significant interaction between T treatment and hemisphere was also observed, suggesting that T increased regional volume to a greater extent in the left hemisphere (Figure 4.09).

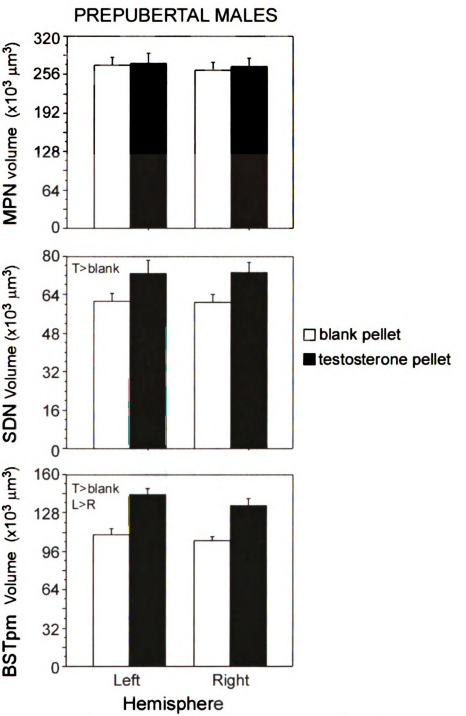

Figure 4.08. Regional volumes of the MPN, SDN, and BST of prepubertal males that received blank- or testosterone-filled silastic capsules for 19 days prior to tissue collection at 29 days of age. Prepubertal testosterone-treatment significantly incresed the volumes of both the SDN and BSTpm, but not the MPN. A left-biased asymmetry was also detected in the BSTpm.

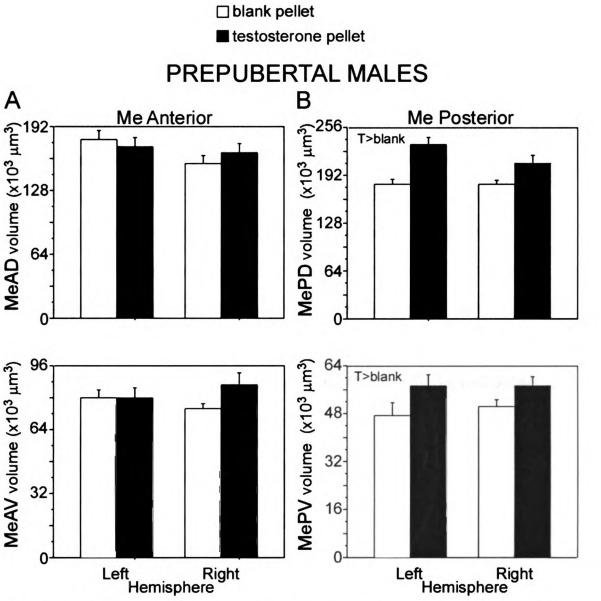

Figure 4.09. Regional volumes of the anterior medial amygdala (A, left panel) and posterior medial amygdala (B, right panel) of prepubertal males that received blank- or testosterone-filled silastic capsules for 19 days prior to tissue collection at 29 days of age. Prepubertal testosterone-treatment significantly incresed the volume of subdivisions within the posterior medial amygdala (MePD and MePV), but not the anterior medial amygdala. A trend toward an interaction was also observed in the MePD, such that testosterone increased regional volume to a greater extent in the left hemisphere.

Prepubertal vs. Adult Behavioral Comparisons

Mount and Intromission Number

Although T-treated prepubertal males displayed more mounts than blank-treated or sham-operated prepubertal males, this difference did not reach statistical significance [Figure 4.10; $F(2,27) = 2.36$, $p=0.11$]. Similarly, prepubertal T-treatment did not significantly increase the proportion of prepubertal males displaying mounts [$\chi^2 = 2.78$, Fishers exact $P=0.16$]. In contrast, prepubertally T-treated males that were behavior tested in adulthood displayed significantly more mounts and intromissions than prepubertally T-treated and behavior tested males [Figure 4.10; $t(1,19) = 18.33$, $p<0.0004$].

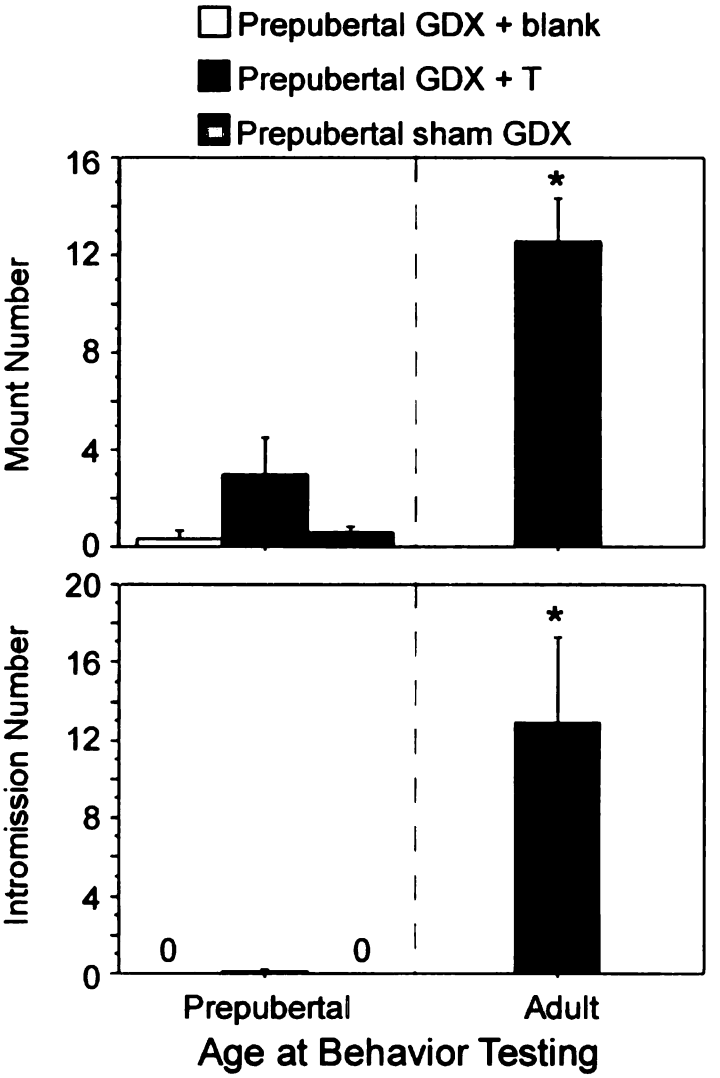

Figure 4.10. Mounts and intromissions of males that received blank pellets, testosterone pellets, or a sham surgery prior to puberty, and were also behavior tested prior to puberty (left panel). The behaviors of males that received testosterone treatment and behavior testing prior to puberty (left panel, black bar) were also compared to the behavior of prepubertally testosterone-treated males that were behavior tested as adults (asterisk indicates a significant difference). No significant differences in mounts or intrommissions were found between prepubertally tested males. Adult tested males displayed significantly more mounts and intromissions than prepubertally tested males.

Genital and non-genital grooming

Genital [$F(2, 27)= 2.30$, $p = 0.12$] and non-genital [$F(2, 27)= 3.00$, $p = 0.06$] grooming durations did not significantly differ between blank-treated, T-

treated, and sham-operated prepubertal males (Figure 4.11). Adult-tested males that received prepubertal T displayed significantly longer genital [$F(1,19) = 29.27$, $p < 0.0001$] and non-genital grooming durations [$F(1,19) = 6.866$, $p < 0.02$] than males that were T-treated and behavior tested prepubertally (Figure 4.11).

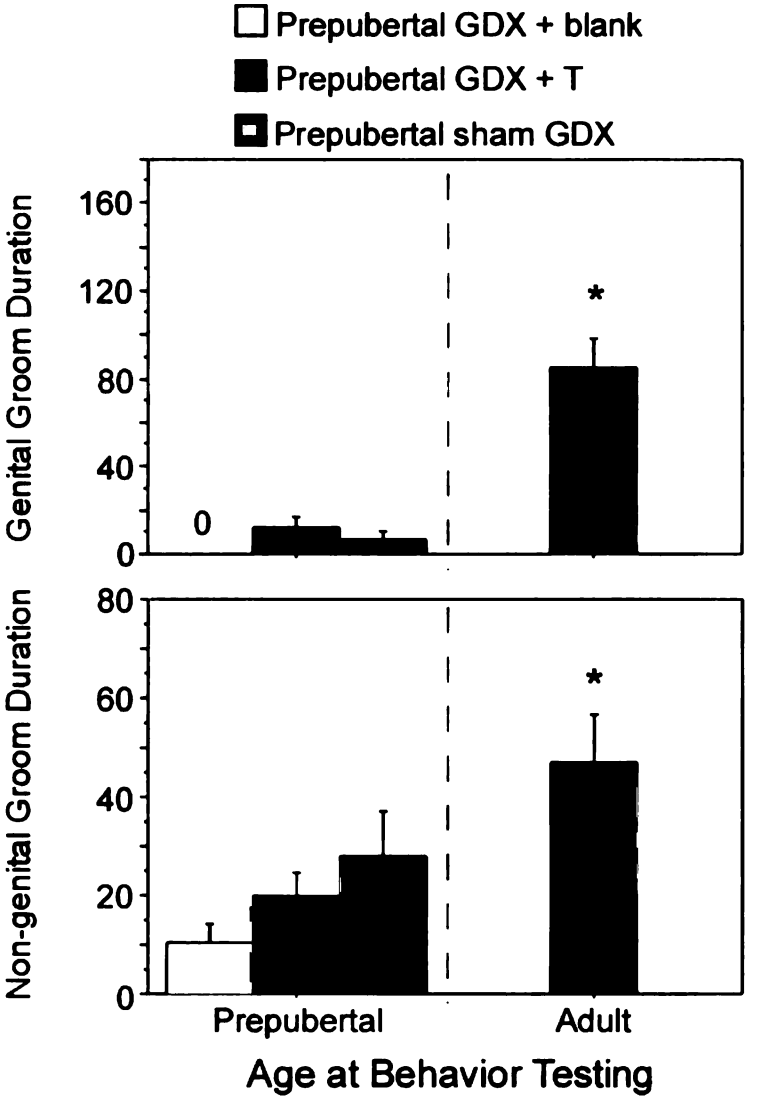

Figure 4.11. Genital and non-genital grooming of males that received blank pellets, testosterone pellets, or a sham surgery prior to puberty, and were also behavior tested prior to puberty (left panel). The behaviors of males that received testosterone treatment and behavior testing prior to puberty (left panel, black bar) were also compared to the behaviors of prepubertally testosterone-treated males that were behavior tested as adults (asterisk indicates a significant difference). No significant differences in genital or non-genital grooming were found between the prepubertally tested males. Adult tested males displayed significantly more grooming behavior than prepubertally tested males.

Stretch-Attend, Tail-up Walking, and Escape Dashes

Prepubertally blank-treated, T-treated, and sham-operated males differed significantly in their number of risk-assessment stretch-attend behaviors [Figure 4.12 panel A; $F(2, 27) = 5.091$, $p < 0.02$]. Fisher's PLSD found that blank-treated prepubertal males displayed significantly more stretch-attend than both T-treated ($p < 0.05$) and sham-operated ($p < 0.004$) prepubertal groups. Adult-tested males that received prepubertal T-treatment displayed little to no stretch-attend behavior (Figure 4.12 panel A). Blank-treated, T-treated, and sham-operated prepubertal males displayed similarly high levels of tail-up walking [Figure 4.12 panel B; $F(2,27)= 1.518$, $p = 0.24$], whereas adult-tested males that received prepubertal T-treatment did not display tail-up walking. Escape dashes significantly differed between the prepubertally tested groups [Figure 4.12 panel C; $F(2,27)= 4.074$, $p < 0.03$], such that blank-treated prepubertal males displayed significantly more escape dashes than both T-treated ($p<0.03$) and sham-operated ($p<0.02$) prepubertally tested males. Escape dash number did not significantly differ between T-treated prepubertally tested and adult behavior tested males, but only one adult-tested male out of ten displayed one escape dash, whereas 5/11 prepubertal males displayed several escape dashes.

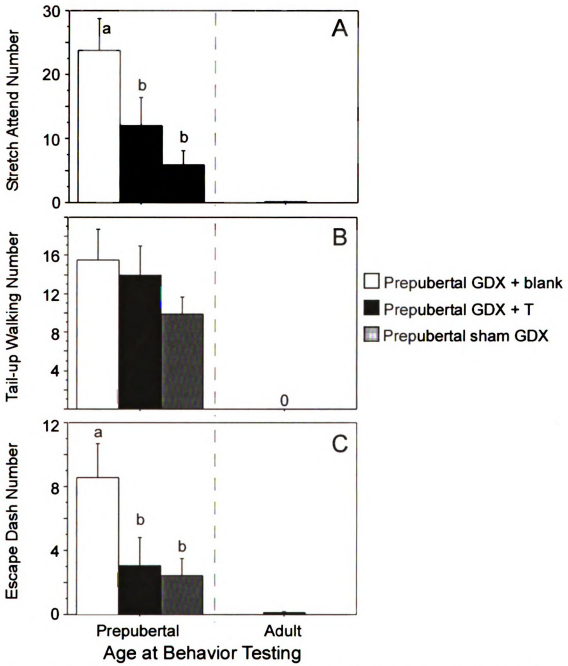

Figure 4.12. Defensive and escape behavior of males that received blank pellets, testosterone pellets, or a sham surgery prior to puberty, and were also behavior tested prior to puberty (left panel). The behaviors of males that received testosterone treatment and behavior testing prior to puberty (left panel, black bar) were also compared to the behaviors of prepubertally testosterone-treated males that were behavior tested as adults. Blank-treated prepubertal males displayed significantly more stretch attend (panel A) and escape dashes (panel C) than testosterone-treated or sham-castrated males. Adult-tested males displayed little to no defensive or escape behavior.

Prepubertal vs. Adult VMH regional volumes

The regional VMH volumes were compared between prepubertal and adult males collapsed across treatments. Adult VMH regional volumes were significantly greater than prepubertal regional volumes [Figure 4.13; t(1,84) = 33.23, p=0.0001].

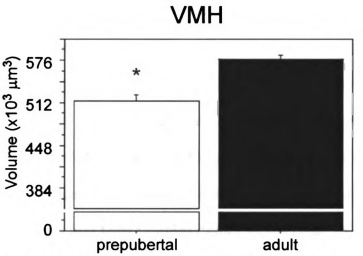

Figure 4.13. Regional VMH volumes of prepubertal and adult males. The volume of the VMH was significantly larger in adults than in prepubertal males.

DISCUSSION

Our data support the hypothesis that adolescence marks the end of a protracted postnatal period of nervous system sensitivity to the organizing actions of T that likely begins perinatally. Both prepubertal and adolescent T-treatment, but not adult T-treatment, facilitated adult reproductive behavior, demonstrating that adolescence is not an isolated sensitive period for the organizing actions of T on adult reproductive behavior. Furthermore, it appears that the potential for T to organize reproductive behavior decreases across postnatal development, as pre-adolescent T-treatment more effectively organized

adult reproductive behavior than mid- or post-adolescent T-treatments. Thus, the classical view of organizational and activational mechanisms of steroid action should be revised to incorporate an extended window of decreasing postnatal sensitivity to the organization of adult social behavior by steroid hormones.

A multitude of studies have demonstrated that perinatal hormone secretions masculinize and defeminize neural circuits, but relatively few have investigated whether similar effects of testosterone are possible prepubertally (Bloch and Mills, 1995a), or during puberty (Adkins-Regan et al., 1989; Costantini et al., 2007; Ford, 1990; Schulz et al., 2004), and until now, no previous studies have assessed whether exposure to testosterone across the pre-, mid-, and post-adolescent periods has differential effects on neural circuits underlying social behavior. Bloch and Mills (1995a) demonstrated that 15 days of prepubertal T-treatment masculinizes and defeminizes adult reproductive behavior in neonatally-gonadectomized males. The same hormone treatment also defeminizes adult lordosis behavior in females (Bloch et al., 1995b). Our data support their conclusion that organizing actions of testosterone are possible well beyond the neonatal period, and extend their findings by demonstrating that the window for organization by testosterone closes in early adulthood. We should note, however, that under normal developmental circumstances, nervous system organization by T is driven by endogenous testicular secretions during two distinct phases of life: the perinatal and adolescent periods.

In addition to organizing adult behavioral responses to T, prepubertal T-treatment increased the regional volumes of several brain areas. Specifically,

prepubertal T treatment from postnatal days 10-29 increased the volume of the SDN, BST, MePD, and MePV at 29 days of age relative to blank-treated prepubertal males. Although we cannot exclude the possibility that the observed volume increases reflect transient activational rather than long-term organizational effects of T on regional volumes, clearly regional volumes of several brain areas important for mating are T-responsive prior to adolescence, even in the absence of behavioral responses to T at this age. Furthermore, although the MePD and SDN are known for their plasticity in response to the presence or absence of gonadal hormones in adulthood (Cooke et al., 2003; Cooke et al., 1999; Davis et al., 1995), the adult the BST and MePV are apparently not as sensitive to adult fluctuations in gonadal hormones (Romeo and Sisk, 2001). Thus, the T-induced increases in regional volumes in prepubertal males likely reflect both long-term organizational and transient activational effects of testosterone, leaving us with the question of why reproductive behavior is not expressed until after adolescence.

We propose that steroid-independent maturation of the VMH during adolescence is necessary before maximal behavioral responses to testosterone are possible. We found that the VMH significantly increases in volume during adolescence, and this volume increase was not influenced by testosterone treatments before, during or after adolescence. Thus, the difference between prepubertal and adult VMH volume most likely reflects a steroid-independent developmental process. Although the VMH is most often associated with female reproductive behavior, recent studies clearly indicate a role for the VMH in male

sexually motivated behavior (Harding and McGinnis, 2003; Harding and McGinnis, 2004; Harding and McGinnis, 2005), and also demonstrate that the VMH is organized by androgens during development (Dugger et al., 2007) which may account for the male-biased sexual dimorphism in volume (Matsumoto and Arai, 1983; Sa and Madeira, 2005). The VMH is laden with androgen receptor (McGinnis et al., 1983; Simerly et al., 1990), and males exhibit more androgen receptor than females (Roselli, 1991). In addition, the VMH sends and receives projections from brain regions traditionally associated with the control of male copulatory behavior (Coolen and Wood, 1998; Wang and Swann, 2006; Wood and Swann, 2005), and therefore may participate in the regulation of male reproductive behavior. Collectively, the data presented here coupled with previous studies suggest that the VMH may play a more central role in male copulatory behavior than previously thought.

Perhaps the role of the VMH in mating behavior has been overlooked because it is particularly important for suppressing sexual behavior in the face of threatening stimuli (Choi et al., 2005). Neuroanatomical tract tracing coupled with FOS labeling reveals that regions of the posterior amygdala that are activated in males by mating (dorsal portion-MePD) or threatening (ventral portion-MePV) stimuli send segregated inhibitory and excitatory projections, respectively, to the VMH. As such, the VMH may gate the relative inhibitory and excitatory tone received from the MePD and MePV, and stimulate or inhibit other brain regions regulating mating and defensive behavior accordingly, resulting in

the adaptive behavioral display in response to the particular social/environmental challenge.

Our behavioral data support the notion that the VMH gates mating or defensive behavioral outputs. Prepubertal males displayed very high levels of defensive behavior during mating tests, even though hormone-primed female Syrian hamsters assume a stationary lordosis posture during much of behavior testing (even in the absence of tactile stimuli), and therefore pose very little threat to males. Furthermore, even prepubertal males that displayed mounting behavior continued to show defensive displays during testing. In contrast, adult males displayed little or no defensive behavior, even when their mating behavior was negligible. Thus, adolescent maturation of the VMH may be necessary before males are capable of assessing an estrous female as non-threatening and suppress defensive behavior. Perhaps the appropriate balance of posterior medial amygdala inhibitory/excitatory input to the VMH develops during adolescence which allows for the suppression of defensive behavior in adulthood. Increased innervation from the posterior amygdala could also account for the VMH volume increase across adolescence observed in this study.

We also investigated whether exposure to T across the pre- mid- and post-adolescent periods programmed adult brain regional responses to T in adulthood. For example, we've previously observed that exposure to gonadal hormones during adolescence programs steroid-induced VMH plasticity in adulthood. Specifically, testosterone treatment in adulthood had little effect on

VMH volumes in males deprived of gonadal hormones during adolescence, but significantly decreased VMH volumes in males exposed to gonadal hormones during adolescence (Schulz et al., 2005). In the current study, pre- mid- and post-adolescent T-treatment did not significantly influence regional volumes when brains were collected in adulthood. However, because all of the adult-analyzed animals were T-treated prior to behavior testing and tissue collection, separating the developmental organizing actions of T from the transient and reversible effects of T-treatment in adulthood was difficult. Future work will investigate whether developmental exposure to T changes the capacity for steroid-dependent regional plasticity in adulthood by varying the timing of T exposure across the pre- mid- and post-adolescent period, and also varying whether males receive blank- or testosterone-treatment in adulthood.

Currently we do not know the mechanism by which the protracted postnatal sensitive period for exposure to T closes with the end of adolescence. However, a common feature among many different sensitive periods is that they are closed by the experience to which the underlying neural circuits are sensitive. For example, once filial imprinting has occurred in birds, subsequent imprinting stimuli have little or no effect on this attachment behavior (Hess, 1973). In the case of the adolescent sensitive period, it is not likely that T closes the window of sensitivity, or at least not as rapidly as in other behavioral systems. If T closed the window of sensitivity, we would not expect cumulative organizational effects of T to occur across multiple developmental periods. For example, T organizes

adult reproductive behavior during both the pre- and post-natal periods (Ward and Ward, 1985), and as demonstrated here, during adolescence.

It is more likely that the sensitive period for exposure to T ends as a consequence of circuit reorganization or consolidatation during adolescence (Bischof, 2007; Hensch, 2003; Hensch, 2004; Knudsen, 2004). In the classic case of primary visual cortex development, monocular deprivation of visual input causes the normally-recipient thalamic axons to withdraw their cortical connections, and competing axons from the open eye take over the vacant cortical space. Thus, visual experience (or the lack thereof) results in enduring structural change that is not easily reversed past a certain developmental stage. However, if both eyes are deprived of visual experience soon after birth, cortical synaptic activity is silenced and the axonal projections from the thalamus remain intact until visual experience is restored. Thus, the sensitive period can be prolonged until the eyes are reopened and activity-dependent synaptic competition ensues. Although plasticity can be prolonged under conditions of complete deprivation, the sensitive period does eventually close in this system and in other brain circuits exhibiting sensitive periods, presumably because neural circuits are unable mechanistically or energetically to reestablish normal patterns of connectivity (Knudsen, 2004). Thus, it seems likely that in the absence of developmental exposure to T, neural circuits consolidate during adolescence in a fashion that is resistant to further modification by T in adulthood.

Similar to many other sensitive periods, patterns of synaptic connectivity also change across adolescence within the hamster MePD, a key region within the neural circuit underlying male reproductive behavior (Wood and Newman, 1995a; Wood and Newman, 1995b). Specifically, dendrites, spine densities, and spinophilin protein all decrease substantially across adolescence, concomitant with dramatic rises in gonadal steroid hormones (Zehr et al., 2006a). These changes in dendritic morphology may reflect organizational changes induced by testosterone during the adolescent sensitive period. Indeed, decreased forebrain spine densities are the physiological manifestation of sexual imprinting in zebra finches (reviewed in Bischof and Rollenhagen, 1999). Furthermore, in the absence of imprinting stimuli, spine densities eventually decrease to the point where subsequent imprinting stimuli can no longer influence spine densities, and the timing is contemporaneous with diminished behavioral sensitivity to imprinting stimuli (Bischof, 2003; Bischof, 2007; Bischof et al., 2002). Whether time-dependent reductions in MePD dendritic branches and spine densities reflect both the organizational influence of testosterone as well as the closing of the sensitive period remains to be determined, but experiments testing these possibilities are underway.

Although it may be very difficult to restore normal patterns of brain connectivity after a sensitive period has passed, restoration of behavior may still be possible via different neural mechanisms, especially in neural circuits regulating complex social behaviors (Knudsen, 2004). As such, studying complex behaviors with known sensitive periods provide the opportunity to

investigate potential mitigators of abnormal sensitive period development. For example, reproductive behavior in the Syrian hamster is regulated by the integration of pheromonal cues from the female and endogenous hormonal signals within a hierarchical neural circuit. Although adult reproductive behavior is markedly reduced in males deprived of adolescent gonadal hormones, exposure to the sight, sound, and smell of an estrous female during adolescence partially ameliorates reproductive behavior deficits in males lacking adolescent exposure to gonadal hormones (Molenda-Figueira et al., 2007). Thus, exposure to a female during adolescence may impact neural circuits via similar mechanisms as testosterone, or have a unique influence on the developing circuit with a similar behavioral outcome.

Human adolescents exhibit substantial individual variability in pubertal onset (Dubas, 1991; Tanner, 1962), and shifts in pubertal timing (early or late) have been associated with a range of human psychopathologies such as depression (Ge et al., 2003; Graber et al., 2004; Michaud et al., 2006), anxiety (Kaltiala-Heino et al., 2003; Zehr et al., 2007), conduct disorder (Burt et al., 2006; Celio et al., 2006) and increased alcohol and tobacco use (Biehl et al., 2007; Bratberg et al., 2007). Although these studies demonstrate that pubertal timing influences adolescent and adult psychopathology, many questions remain regarding the mechanism by which pubertal timing exerts these effects. For example, it is not clear whether the perception of undergoing puberty earlier or later than peers is more important than an individual's actual pubertal status in predicting later psychopathology, or whether mistimed direct hormonal influences

on the brain also influence behavioral psychopathology. To our knowledge, this is the first demonstration in an animal model of how variations in pubertal timing (i.e. hormone exposure) relative to adolescent development result in unique adult behavioral phenotypes. Figure 4.14 illustrates how variations in the timing of puberty alter the instance at which gonadal hormones intersect the developing brain, thus driving individual differences in behavior. Importantly, the effects of gonadal hormones on the developing brain may also be mediated by social and environmental factors that change dramatically across the pre- mid- and post-adolescent periods (Spear, 2000). Additional research employing animal models is needed to elucidate how gonadal hormones and an individual's unique experience impact the developing adolescent brain to drive individual differences in behavior.

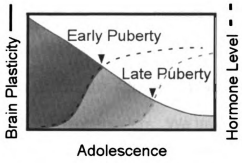

Figure 4.14. Schemating depicting the brains decreasing sensitivity to organizing actions of testosterone across postnatal development (solid sloping line) The dotted lines represent the onset of gonadal hormone secretions in early and late maturing adolescents. Arrows indicate the point at which gonadal hormones intercept the developing brain, which likely contributes to individual differences in adolescent and adult behavior.

CHAPTER 5

Conclusions

The experiments detailed within this dissertation demonstrate that gonadal hormones modify the course of adolescent brain development, resulting in lasting behavioral change. For example, prepubertal gonadectomy reduces masculine and increases feminine responses to gonadal steroid hormones in adulthood (Chapter 2). In contrast, postpubertal gonadectomy for the same length of time does not significantly influence adult behavioral responses to steroid hormones, suggesting that exposure to gonadal hormones during adolescence is required for normal masculine and feminine behavioral responses to steroid hormones in adulthood. Furthermore, these data suggest that a window of sensitivity to gonadal steroid hormones may close following adolescence, because neither prolonged T exposure nor repeated sexual experience with a female were capable of ameliorating the deficits caused by the absence of testicular hormones during adolescence.

We also found that the effects of adolescent testicular hormones generalize to other social behaviors. We investigated flank marking behavior because similar to reproductive behavior, flank-marking is testosterone-modulated in adulthood. Testosterone (T) increased flank marking only in adults exposed to gonadal hormones during adolescence (Chapter 3). Prepubertally gonadectomized males showed little or no flank marking behavior, regardless of adult T status. In addition, AVP binding within the lateral septum was

significantly greater in males deprived of adolescent gonadal hormones. Although it may seem paradoxical that the males displaying the least flank marking behavior display the greatest levels of AVP receptor binding, increased receptor levels may reflect the absence of adolescent synaptic remodeling common to many behavioral neural circuits. For example, if testicular hormones normally cause streamlining of synaptic connections via pruning of dendrites and their associated synapses, AVP receptors would also decrease as a consequence of pruning. Given that decreases in dendritic arbors and spine densities are observed in the amygdala across adolescence (Zehr et al., 2006a), it is possible that similar processes occur in the lateral septum, which would in turn reduce the number of AVP receptors. Another explanation is that testicular hormones cause cell death within the LS during adolescence, which would also reduce absolute levels of receptors. Other systems exhibiting cell loss during adolescence include the visual cortex (Nunez et al., 2002) and the medial prefrontal cortex of rats (Markham et al., 2007), so this is a realistic possibility.

The experiment in Chapter 4 expanded our understanding of adolescent organization of behavior by testing the hypothesis that adolescence is a discrete sensitive period for the organizing actions of T on adult male reproductive behavior. We found that exposure to T before and during adolescence, but not after adolescence, significantly enhanced reproductive responses to T in adulthood. These data suggest that a second window of sensitivity does not open at adolescence, but rather, adolescence marks the end of a protracted postnatal sensitive period for the organizing actions of T on behavioral neural

circuits. Given that gonadectomy and T treatment occurred immediately after the normal time period for sexual differentiation (PND 10), it is likely that this postnatal window of sensitivity extends from birth through adolescence. In addition, prepepubertal hormone treatments more effectively organized reproductive behavior than mid-adolescent or adult treatment, suggesting that sensitivity to the organizing actions of T decreases across the lifespan.

Although experiments in chapters two and three demonstrate that organizational effects of testicular hormones occur during normal adolescence, they did not equate for lifetime exposure to gonadal steroid hormones. Specifically, because these initial studies varied the age at castration, but kept the time between castration and adult T treatment constant, it was not possible to control for cumulative life exposure to T using this design. Thus, at the time of testing, adult castrated males experienced approximately five more weeks of testicular hormones (during adolescence), than prepubertal castrates. The experiment in Chapter 4 controlled for cumulative life exposure to testicular hormones by gonadectomizing all males at 10 days of age, but varying the time of T exposure before, during, or after adolescence. Thus, these data confirmed using a different experimental design that the effects of adolescent hormone exposure on adult behavior are the result of nervous system change, and not cumulative life exposure to T.

WHAT OPENS THE SENSITIVE PERIOD FOR STEROID-DEPENDENT ORGANIZATION?

The neural mechanisms by which sensitive periods are opened and closed have been somewhat elusive, even in well studied systems such as the visual cortex. As discussed in Chapter 1, the development of the relative inhibitory/excitatory tone within neural circuits has been linked with the opening of the sensitive period for visual experience, and interesting links between GABA and the initial perinatal period of sexual differentiation have also been found. During the early development of many brain areas, the action of GABA on postsynaptic cells shifts from excitatory to inhibitory. The inonotropic $GABA_A$ receptor is permeable to Cl^-, which allows for flux in either direction depending on the Cl^- concentration gradient. In mature neurons, intracellular Cl^- levels assure that the reversal potential for $GABA_A$ is set just below the resting membrane potential. Opening of the receptor, therefore, results in membrane hyperpolarization. During late gestation and early postnatal life, however, intracellular Cl^- is greater than extracellular Cl^- (due largely to low levels of the transporter that extrudes Cl^- from mature cells), such that the reversal potential is positive relative to the relative to the membrane potential. Thus, during perinatal development, opening of the $GABA_A$ receptor channel causes Cl^- efflux and membrane depolarization. As early as PND6, however, GABA's actions on hypothalamic neurons are inhibitory, and lead to cell hyperpolarization (Obrietan and Vandenpol, 1995). Notably, estradiol treatment of cultured perinatal hypothalamic neurons doubles intracellular levels of Ca^{2+}, and prolongs the

excitatory actions of GABA during early development (McCarthy et al., 2002; Perrot-Sinal et al., 2001). Given that titers of perinatal testosterone and its estrogenic metabolites are transiently elevated in males but not females, this interaction between the shift in excitatory-inhibitory actions of GABA and circulating steroid hormones may open the sensitive period for hormone action and set divergent developmental trajectories of the male and female nervous system. Additional experimental tests of this hypothesis are warranted, but the initial evidence is tantalizing. For example, if the excitatory-inhibitory shift in GABA action underlies the opening of the sensitive period, pharmacologically inhibiting this shift should render the nervous system insensitive to the organizing actions of steroid hormones. Furthermore, given that our data suggest that this window of sensitivity normally closes in late adolescence, delaying the GABA excitation-inhibition shift may delay the closing of the sensitive period into adulthood.

WHAT CLOSES THE SENSITIVE PERIOD FOR STEROID-DEPENDENT ORGANIZATION?

We do not yet know the mechanism by which the sensitive period for steroid-dependent organization closes. Although the experience to which the nervous system is sensitive closes the sensitive period in other behavioral systems (e.g. filial imprinting), this is not likely the case for reproductive behavior. Specifically, since steroid hormone treatments have apparent cumulative effects on masculinization and defeminization of behavior during both the perinatal and adolescent periods, we must conclude that

experiencing T does not in and of itself close the window of sensitivity. A more plausible explanation is that the sensitive period for exposure to testosterone ends as a consequence of circuit reorganization or consolidatation during adolescence (Bischof, 2007; Hensch, 2004; Knudsen, 2004). For example, patterns of synaptic connectivity change across adolescence within the hamster MePD, a key region within the neural circuit underlying male reproductive behavior (Wood and Newman, 1995a; Wood and Newman, 1995b). Specifically, dendrites, spine densities, and spinophilin protein all decrease substantially across adolescence (Zehr et al., 2006a). These changes in dendritic morphology may reflect organizational changes induced by testosterone during the adolescent sensitive period, and with time, limit the capacity for further steroid-dependent organization. Changes in dendritic morphology have been linked with the closing of the sensitive period for sexual imprinting and song learning in zebra finches (Bischof, 2003; Bischof, 2007; Bischof et al., 2002). Whether time-dependent reductions in MePD dendritic branches and spine densities reflect both the organizational influence of testosterone as well as the closing of the sensitive period remains to be determined. Furthermore, steroid-dependent structural changes in other regions within the mating neural circuit may also limit the capacity for further organizational influence by testosterone.

PREPUBERTAL VS. ADULT BEHAVIORAL RESPONSES TO TESTOSTERONE

It is perhaps surprising that prepubertal T treatments were more effective than adolescent or adult T treatments considering that prepubertal males did not show adult levels of reproductive behavior, as has been demonstrated multiple times now . We are left to conclude that prepubertal T-treatments organize behavioral neural circuits, but requisite steroid-independent developmental processes during adolescence permit the expression of reproductive behavior. We propose that maturation of the VMH may underlie the adolescent transition from defensive behavior to reproductive behavior in the presence of an estrous female. We base this proposal largely on the work of Choi and colleagues demonstrating that the VMH receives inhibitory and excitatory projections from regions of the posterior medial amygdala that are activated by reproductive and defensive behavior, respectively. In their model, the GABAergic and glutamatergic projections first synapse onto a subset of VMH inhibitory neurons, thus exciting or inhibiting their activity (Figure 5.01). Therefore, the presence of a receptive female would cause the MePD to lift the inhibition (inhibit the inhibition) normally placed on the VMH neurons important for male reproductive behavior. Conversely, in the presence of threatening stimuli, the MePV would excite these inhibitory neurons, further inhibiting VMH neurons involved in copulatory behavior (Figure 5.01).

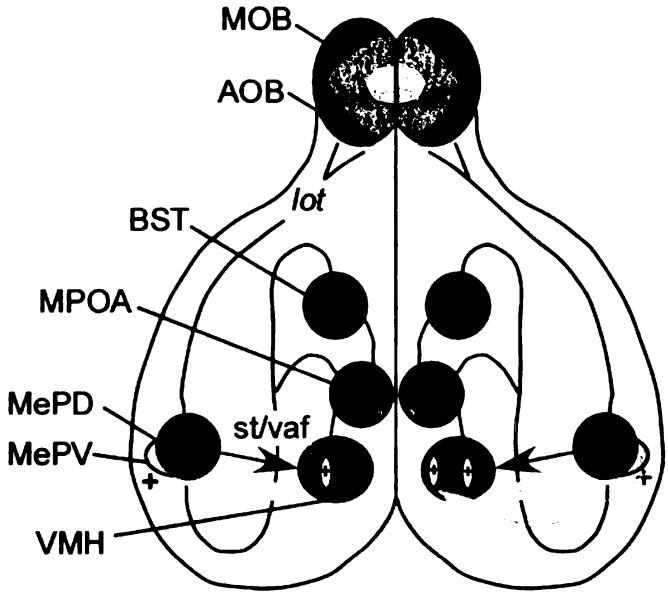

Figure 5.01 depicts the neural circuit underlying male hamster reproductive behavior, and illustrates how inhibition or excitation from the MePD or MePV, respectively, could drive VMH neurons to promote or supress reproductive behavior. The MePD sends an inhibitory projection to the lateral portion of the VMH which is involved in mating behavior. The MePV sends an excitatory projection to the lateral VMH, and also the medial portion of the VMH which is involved in defensive behavior. As depicted, the VMH projects to the medial preoptic area, which may be the critical link between the VMH and the supression of mating behavior in the face of threatening stimuli.

REGIONAL VOLUMES IN ADULT BRAINS

An adequate test of whether T across the pre-, mid-, and post-adolescent periods organizes adult behavioral responses to T required all males to be T treated in adulthood. Given this, we cannot separate long-term effects of T on brain regional volume from transient reversible influences on regions such as the MePD (Cooke et al, 1998, 2003) and SDN (Davis et al., 1995). Indeed, if developmental exposure to T changes the regional responsiveness to T in adulthood, adult T-treatment of all males would likely mask this interaction. For example, T during adolescence may cause cell death in a given region and reduced overall brain regional volume. At the same time, it may also program

the responsiveness of this brain region to adult steroid hormones (e.g. steroid-dependent volume increase due to increased dendritic arborization). If we only analyze the brains of T-treated adult males, detecting a regional volume difference between males deprived of and exposed to T during development would not be possible. Specifically, adult T treatment would mask the effect of adolescent cell loss by increasing regional volume (Fig 5.02), which is most likely why no regional volume differences were found in the adult analyzed groups in Chapter 4. Supporting this notion, prepubertally T-treated males that were behavior tested and sacrificed prior to adolescence showed robust changes in regional volumes as compared to blank-treated controls. Therefore, at the very least, the prepubertally T-treated males that were behavior tested and sacrificed in adulthood should also exhibit steroid-dependent changes in regional volume. Whether the effects are organizational or transient in nature remains to be seen.

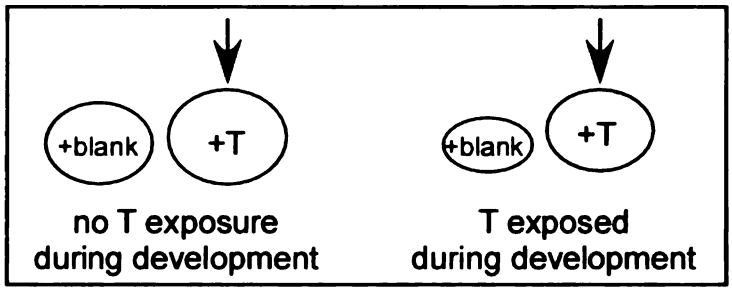

Figure 5.02. Theoretical depiction of how developmental exposure to testosterone could induce irreversable changes in regional volume of a given brain region, and also alter the plasticity of regional volumes in response to adult steroid hormones (e.g. increases in dendritic arborization). Circles represent a regional volume, and the adult hormone treatment is noted within each circle. In this schematic, developmental exposure to testsosterone decreases regional volume overall, but adult testosterone treatment increases regional volume via different mechanisms. The magnitude of volume increase is greater in males exposed to testosterone during development. The arrows highlight that without comparison to blank-treated adults, no difference between males deprived of or exposed to testosterone during development would be detected.

SENSITIVE PERIODS VS. NORMAL BEHAVIORAL DEVELOPMENT

The experiments presented here indicate that the nervous system is sensitive to the organizing actions of T throughout much of postnatal development. However, testicular hormone secretions normally occur at appreciable levels during the perinatal period, and again at adolescence. Thus, we can conceptualize the development of male reproductive behavior as occurring in two stages: perinatal hormone secretions initially differentiate neural circuits, and adolescent hormones further masculinize and defeminize behavioral neural circuits to bring forth full activational effects of steroid hormones in adulthood (Figure 5.03).

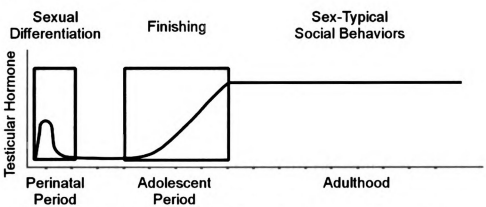

Figure 5.03. Two-stage model of social behavior development. Perinatal hormone secretions sexually differentiate behavioral neural circuits and pubertal hormone secretions refine and "finish" these processes during adolescence to allow for the display of sex-typical social behaviors in adulthood.

Although the process of normal behavioral development may occur in two stages, humans exhibit substantial variability in the timing of pubertal maturation (Dubas, 1991; Tanner, 1962). Furthermore, pubertal onset is steadily advancing

in girls (Ellis, 2004), contributing to the growing "maturational gap" between physical and emotional development and an adolescent's position within society (Waylen and Wolke, 2004). Thus, the developing adolescent brain may be intercepted by pubertal gonadal hormone secretions at different time points, resulting in individual differences in organizational influences of steroid hormones on the adolescent brain and behavior.

Unfortunately, deviations in normal pubertal timing are apparently detrimental to the psychological well-being of both male and female adolescents, and increases their susceptibility to various mental health problems (reviewed by Graber, 2003). Examples range from negative body self-image (McCabe and Ricciardelli, 2004) to increased incidence of depression (Ge et al., 2003; Graber et al., 2004; Michaud et al., 2006), anxiety (Kaltiala-Heino et al., 2003; Zehr et al., 2007), symptoms of disordered eating (Striegel-Moore et al., 2001; Zehr et al., 2007), conduct disorder (Burt et al., 2006; Celio et al., 2006) and increased alcohol and tobacco use (Biehl et al., 2007; Bratberg et al., 2007). Many of these psychological outcomes of pubertal timing have been demonstrated to persist into young adulthood (Graber et al., 2004; Zehr et al., 2007).

The mechanism by which deviations in pubertal timing influence mental health outcomes is likely a complex interplay between the psychosocial impact of physically maturing earlier or later than one's peers, direct hormonal actions on the brain, and the individual's unique environment. For example, the adolescent peer group (Cavanagh, 2004; Ge et al., 2002), parenting styles (Ge et al., 2002), romantic partners (Halpern et al., 2007), and stressful life events (Ge et al., 2001)

have all been found to mediate the effects of pubertal timing on mental health. Experiments modeling off-time pubertal maturation will fill a fundamental gap in our understanding of how interactions between gonadal hormones, adolescent brain maturation, and the environment bring forth adaptive adolescent and adult behaviors.

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