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THE PUBERTAL MATURATION OF MALE SEXUAL BEHAVIOR: THE ROLE OF STEROID HORMONES, THEIR RECEPTORS, AND PHEROMONES

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THE PUBERTAL MATURATION OF MALE SEXUAL BEHAVIOR: THE ROLE OF STEROID HORMONES, THEIR RECEPTORS, AND PHEROMONES

By

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ABSTRACT

THE PUBERTAL MATURATION OF MALE SEXUAL BEHAVIOR: THE ROLE OF STEROID HORMONES, THEIR RECEPTORS, AND PHEROMONES

By

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Prepubertal animals differ vastly from adults in their social behaviors. These behavioral changes during puberty must be mediated by changes in the structure and/or function of the central nervous system. In the present dissertation, the male Syrian hamster (*Mesocricetus auratus*) was used to investigate the neural mechanisms responsible for the pubertal change in reproductive behavior. The full suite of adult mating behaviors in this species is dependent on both steroidal hormones (internal information) and chemosensory cues (external information), thus this model allows us to investigate how puberty affects the ability of an organism to process both endogenous and exogenous information.

A hallmark of pubertal development is the increased production and secretion of the steroid hormones testosterone and progesterone. Since mating behavior emerges after the pubertal rise of these steroids, it was hypothesized that the absence of sexual behavior prior to puberty was due to the lack of stimulation by these hormones. I have found that exogenously adminstered testosterone, and its androgenic and estrogenic metabolites, are capable of activating sexual behavior only in adult males, indicating that not only do

these steroids increase during pubertal development, but the neural responsiveness to these hormones increases as well. Using immunocytochemistry, it was demonstrated that this reduced responsiveness to steroids prior to puberty is not mediated by a lack of androgen or estrogen receptors within the neural circuit that mediates sexual behavior. It was also shown that activation of the progesterone receptor in prepubertal males does not facilitate their ability to engage in copulation.

I found that after pheromonal stimulation, prepubertal and adult males respond with an equivalent level of activity in brain regions that are imperative for chemosensory processing and male sexual behavior. However, adults are dissimilar to juvenile males in that adults experience a rise in testosterone after pheromonal exposure, indicating that adult males are integrating and processing the chemosensory cues differently before and after pubertal development.

Thus, I have found that the lack of mating behavior prior to puberty is not mediated by the availability of steroids, the absence of their receptors, or basic chemosensory processing. However, the lack of a neuroendocrine reflex prior to puberty, suggests that the pubertal maturation of mating behavior is dependent upon the refinement of sensory processing and integration of stimuli, both internal and external.

To my family,

Thank you for your continuous love and support.

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Anatomical Abbreviations

AOB	assessory olfactory bulb
BNST	bed nucleus of the stria terminalis
BNSTpm	posteromedial subdivision of the bed nucleus of the stria terminalis
MeA	anterior subdivision of the medial amygdala
MeAMY	medial amygdala
MeP	posterior subdivision of the medial amygdala
MOB	main olfactory bulb
MPN	medial preoptic nucleus
MPNmag	magnocellular subdivision of the medial preoptic nucleus

General Abbreviations

AGI	anogenital investigation
ANOVA	analysis of variance
AR	androgen receptor
BSA	bovine serum albumin
CV	coefficient of variation
DAB	diaminobenzidine
DHT	dihydrotestosterone
TX	triton X-100
E	estradiol
ER	estrogen receptor
ir	immunoreactive
NGS	normal goat serum
PBS	phosphate buffered saline
PR	progesterone receptor
T	testosterone
TBS	tris buffered saline
TLC	thin layer chromatography
VS	vaginal secretion

INTRODUCTION

The internal and external demands placed on an animal change dramatically throughout its lifespan. To meet these challenges, animals undergo equally drastic changes in their physiology and behavior. One stage of development that typifies a lifespan change is puberty. Prepubertal animals differ vastly from adults in their social behaviors, responses to sensory stimuli, and responses to environmental stressors (Primus and Kellogg, 1990; Primus and Kellogg, 1989; Slob, Huizer, and Van Der Werff Ten Bosch, 1986). These impressive behavioral changes during puberty must be mediated by changes in the structure and/or function of the central nervous system. Therefore, the study of pubertal development of the nervous system addresses the extremely important and interesting question of how the brain mediates profound behavioral changes, and equally as important, how these behavioral changes feed back and impact the brain.

The study of pubertal development in animals is also important for its obvious implications in understanding human adolescence. For example, the morbidity and susceptibility to psychological disorders (e.g., schizophrenia and depression, suicide, and violent behavior/aggression) increase during adolescence (Conger and Petersen, 1984; Hammen and Rudolph, 1996; Masten, 1987). These problems may be mediated by malfunctions in the normal neural and endocrine changes associated with pubertal development

(Buchanan, Eccles, and Becker, 1992; Gooding and Iacono, 1995; Lerner, 1985). Thus, studying puberty through a neurobehavioral perspective may provide important insights into how perturbations of normal pubertal processes may result in inappropriate responses to environmental stimuli in adulthood.

We use the male Syrian hamster (*Mesocricetus auratus*) to investigate the neural mechanisms that are responsible for the pubertal change in reproductive behavior. We have chosen to use this animal model for three reasons. First, the full suite of mating behaviors in the adult male hamster is dependent on both steroidal hormones (internal information) and chemosensory cues (external information; Wood, 1998; Wood and Coolen, 1997; Wood and Newman, 1995c), thus allowing us to investigate how puberty affects the ability of an organism to process both endogenous and exogenous cues. Second, the neural circuit that mediates the mating behavior of adults in this species has been well described and functionally characterized (Coolen and Wood, 1998; Lehman, Powers, and Winans, 1983; Lehman, Winans, and Powers, 1980; Newman, 1999; Wood, 1996b; Wood, 1998; Wood and Newman, 1995b) as well as the endocrinological profile of the male as he progresses through puberty (Miller, Whitsett, Vandenbergh, and Colby, 1977; Vomachka and Greenwald, 1979). Finally, we have demonstrated that behavioral responses to testosterone change as a function of pubertal development in this species (Meek, Romeo, Novak, and Sisk, 1997). Therefore, we are provided

with a large body of information describing the neuroanatomy and reproductive physiology of the adult, and, more importantly, an animal model that processes steroidal information differently before and after pubertal development. Whether Syrian hamsters process chemosensory cues differently before and after puberty is unknown. Therefore, the study of the pubertal maturation of male mating behavior in this species will lead to a deeper understanding of how internal and external cues may interact to affect an individual progressing through pubertal development.

Male Sexual Behavior

Male sexual behavior in most rodent species follows a highly stereotyped pattern of behaviors, at least when observed in a laboratory setting (Meisel and Sachs, 1994). First, the male chemoinvestigates the anogenital region of the female to assess her reproductive status. If the male determines the female is in estrus, as evidenced by particular pheromones and/or behavioral postures emitted by her, the male will attempt to mount the female's hindquarters. At first, mounts are typically not accompanied by thrusting, so the penis does not make contact with the vagina during a mount. When a male thrusts during mounting, and the penis penetrates the vagina, this behavior is termed an intromission, and is the third basic behavior to emerge during copulation. After a series of intromissions (e.g.,

10-15), the male will ejaculate. Following a brief refractory period (i.e., 30 sec to a few minutes depending on the species), the male will return to mounting and intromitting with the female until another ejaculation occurs. This sequence of behaviors will continue until the male reaches sexual satiety. The latency to engage in these behaviors depends upon the experience of the male, and to some degree the female's experience (Bradley and Meisel, 2000). However, the sequence in which these behaviors are displayed remains fixed. Interestingly, the pubertal emergence of reproductive behavior follows the same sequential order as described above. That is, chemoinvestigatory behavior and mounting are the first behaviors to emerge during pubertal development followed by intromissions and ejaculations (Miller et al., 1977).

Hormonal Regulation of Male Sexual Behavior

Physiological and behavioral aspects of male reproduction are typically temporally associated. That is, gametogenesis, steroidogenesis, and sexual behavior are linked, often waxing and waning with each other over the lifespan (Crews, 1984; for interesting exceptions see, Crews, 1984; Mendonça, Chernetsky, Nester, and Gardner, 1996). Therefore, mating behavior typically increases as testosterone (T), the major androgen secreted by the testes, increases. Indeed, when circulating levels of T are manipulated experimentally (i.e., castration, hormonal

injections, or implants), T levels and behavior show a positive correlation (reviewed in, Luttge, 1979; Meisel and Sachs, 1994). However, T is not the only steroid hormone regulating male sexual behavior. It has been shown in a variety of species that its metabolites, such as estradiol (Beyer, Moralí, Naftolin, Larsson, and Pérez-Palacios, 1976; Carroll, Weaver, and Baum, 1988; Christensen and Clemens, 1975; Davidson, 1969; DeBold and Clemens, 1978; Floody and Petropoulos, 1987; Luttge, 1979; Södersten, 1973; Steel and Hutchison, 1988) and dihydrotestosterone (DHT; Butera and Czaja, 1989a; DeBold and Clemens, 1978; Payne and Bennett, 1976; Whalen and DeBold, 1974), are important mediators of male reproductive behavior. In males, these estrogenic and androgenic metabolites of T are formed locally in the brain by the intracellular aromatase or reductase enzymes, respectively (Meisel and Sachs, 1994). Recently, even progesterone has been implicated in the control of male sexual behavior (Crews, Godwin, Hartman, Grammer, Prediger, and Sheppherd, 1996; Lindzey and Crews, 1988; Lindzey and Crews, 1992; Phelps, Lydon, O'Malley, and Crews, 1998; Witt, Young, and Crews, 1994; Witt, Young, and Crews, 1995; Young, Greenberg, and Crews, 1991). The origin of progesterone in males is most likely from both the testes and adrenals, since neither gonadectomy nor adrenalectomy alone significantly influences progesterone titers (Kalra and Kalra, 1977).

Steroid hormones affect behavior directly through the central nervous system. Specific intracellular receptors for

these signals are found in various limbic areas (e.g., hypothalamus and amygdala) in nuclei that form the neural substrate for male sexual behavior (Cottingham and Pfaff, 1986; Pfaff, 1968; Wood, Brabec, Swann, and Newman, 1992; Wood and Newman, 1995a). Steroids bind to their intracellular receptors, which in turn, bind to hormone response elements within promoter regions of DNA to alter gene transcription, protein synthesis, and ultimately cellular function (Tsai and O'Malley, 1994). In a variety of species, intracerebral application of these gonadal steroids into the hypothalamus (Butera and Czaja, 1989a; Butera and Czaja, 1989b; Christensen and Clemens, 1974; Crews et al., 1996; Davidson, 1966; Johnston and Davidson, 1972; Lisk, 1967; Lisk and Bezier, 1980; Moralí, Hernandez, and Beyer, 1986; Rozendaal and Crews, 1989; Wood and Newman, 1995d) and/or amygdala (Baum, Tobet, Starr, and Bradshaw, 1982; Wood, 1996a; Wood and Newman, 1995d) causes an increase in mating behavior in castrated adult males. Taken together, these data indicate that T and progesterone secreted from the testes (and adrenals) by themselves, and in conjunction with metabolites of T, affect steroid-sensitive brain regions in limbic areas, that in turn, activate and facilitate copulation. It should be noted that the efficacy of a particular steroid to activate mating behavior depends on the brain area it is acting on. This issue will be expanded on below when discussing the steroid-sensitive neural circuit that mediates male mating behavior.

Pheromonal Regulation of Male Sexual Behavior

Mammalian reproductive physiology and behavior are largely affected by olfaction (Powers and Winans, 1975). For example, just the odor of an estrous female causes erection and seminal emission in adult male rats (Sachs, 1997; Sachs, Akasofu, Citron, Daniels, and Natoli, 1994). The male hamster exemplifies the importance of olfactory cues in reproduction since adults will not engage in copulation unless olfactory cues from the female are present (Wood, 1998; Wood and Newman, 1995c). In hamsters, the major olfactory cue from the female is a pheromone found in her vaginal secretions around the time of estrus. Males begin to show an interest in these secretions after the onset of pubertal development (e.g., 40 days of age; Johnston and Coplin, 1979), and the interest in these secretions has been reported to be under androgenic regulation (Gregory, Engel, and Pfaff, 1975; Powers and Bergondy, 1983).

Generally, reproductive behavior has two main facets: arousal and performance (reviewed in, Everitt, 1990). The necessity of pheromonal cues for the initiation of male sexual behavior in hamsters illustrates the significance of arousal. That is, if this sensory information is not received and processed in the proper context, then copulation will not ensue. Thus, developmental changes in the animal's ability to interpret these sensory cues as arousing will influence the likelihood that it will engage in the performance aspects of mating behavior.

The olfactory bulbs are the first central brain structures that process olfactory information. The bulbs are composed of the main and assessory olfactory bulbs (MOB and AOB, respectively). The MOB processes volatile odorants, while the AOB processes pheromonal information received from the vomeronasal organ. Interfering with olfactory bulb function (i.e., ablation or inactivating with zinc sulfate) causes a decrease in reproductive behavior (Cain and Paxinos, 1974; Murphy and Schneider, 1970; Powers and Winans, 1975; Rowe and Edwards, 1972). Interestingly, the magnitude of the decrement in behavior one observes after interfering with the bulbs depends on the species of animal being studied. For example, bulbectomy of the rat leads to a partial failure in mating behavior (Rowe and Edwards, 1972), while in the hamster this procedure completely abolishes mating behavior (Murphy and Schneider, 1970). Taken together, these studies indicate that olfactory input to the brain is vital for normal mating to take place, but that the dependence on olfactory information demonstrates some species specificity.

The Forebrain Neural Circuit that Mediates Male Sexual Behavior

In the male hamster, steroidal and pheromonal information is integrated in a forebrain circuit that is composed of nuclei in the hypothalamus and amygdala (Wood, 1996b; Wood, 1997; Wood, 1998), which ultimately project to the spinal cord via the midbrain tegmentum to affect the

motor aspects of mating behavior (Meisel and Sachs, 1994). The correct integration of this internal (steroidal) and external (pheromonal) information is necessary for reproductive behavior to occur. A cartoon of this mating circuit is provided in Figure 1. The function of these various nuclei are discussed below.

Amygdala: The first relay for the olfactory information is the amygdala where both the MOB and AOB send their axons. The MOB projects to the more lateral aspects of the cortical nucleus of the amygdala, while the AOB projects to the more medial aspects (Lehman and Winans, 1982). The medial amygdala (MeAMY) can be subdivided into the anterior medial amygdala (MeA) and posterior medial amygdala (MeP). It has been proposed that these two subdivisions process different types of information (reviewed in, Wood and Newman, 1995b). For instance, selective lesions of the MeA completely abolish chemoinvestigatory behavior, but this component of mating behavior is only modestly affected by lesions of the MeP (Newman, Parfitt, and Kollack-Walker, 1997). These data suggest that the MeA primarily transduces chemosensory information. Conversely, the MeP has a greater number of steroid receptor-containing neurons than the MeA (Wood et al., 1992; Wood and Newman, 1995b), suggesting that the MeP relays mostly steroidal information. Thus, in experiments described later in this dissertation the MeAMY is typically subdivided into the MeA and MeP. However, this is not to say

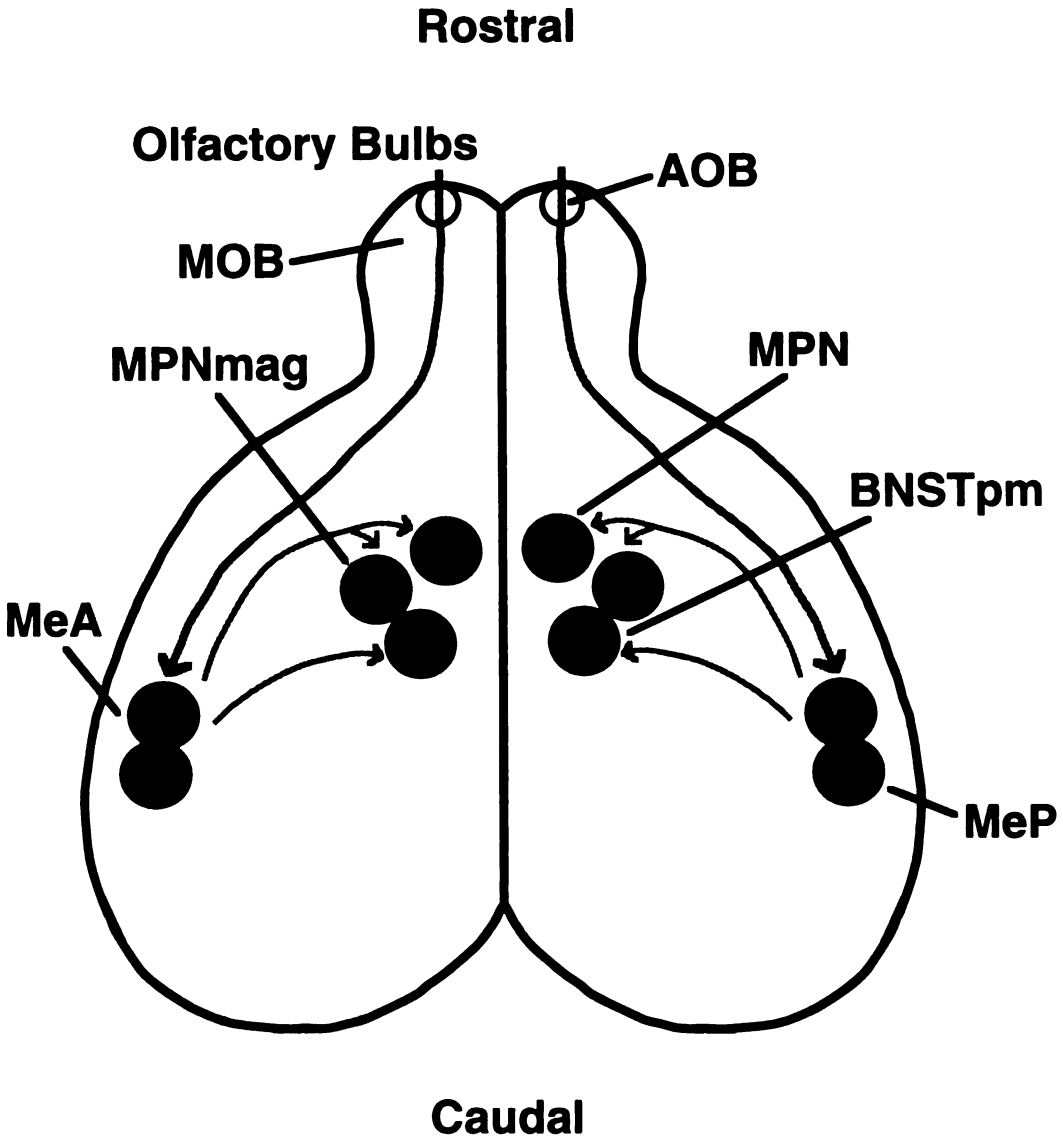

Figure 1. Cartoon of the neural circuit that mediates mating behavior in the male Syrian hamster (horizontal plane). Abbreviations: AOB, assessory olfactory bulb; BNSTpm, posteromedial subdivision of the bed nucleus of the stria terminalis; MeA, anterior subdivision of the medial amygdala; MeP, posterior subdivision of the medial amygdala; MOB, main olfactory bulb; MPN, medial preoptic nucleus; MPNmag, magnocellular subdivision of the medial preoptic nucleus.

that the MeA and MeP do not share some overlap in their function. For instance, both nuclei receive chemosensory information, and both are replete with steroid hormone receptors (Meek et al., 1997; Romeo, Diedrich, and Sisk, 1999; Wood et al., 1992; Wood and Newman, 1995a; Wood and Newman, 1995b). Paradoxically, the MeP shows a greater expression of Fos, a marker of neuronal activity (Morgan and Curran, 1991), than the MeA after the male has been exposed to female pheromones (Fiber, Adames, and Swann, 1993; Fiber and Swann, 1996; Kollack-Walker and Newman, 1997; Swann and Fiber, 1997). There are reciprocal connections between the MeA and MeP (Coolen and Wood, 1998). Thus, whether the more robust increase in activity of the MeP compared to the MeA is due to direct olfactory information from the bulbs or indirect olfactory information from the MeA is unclear. Taken together, these studies indicate that the MeAMY processes both steroidal and chemosensory information, but with some degree of specificity depending on whether the anterior or posterior portion of the nucleus is involved.

Ablation of the MeAMY disrupts the male's ability to engage in copulation (Giantonio, Lund, and Gerall, 1970; Harris and Sachs, 1975; Kostarczyk, 1986; Lehman et al., 1980), but this deficit may be secondary to the inability of the male to sense the pheromonal cues provided by the female (Lehman et al., 1980). Moreover, local steroidal implants in the MeAMY facilitate mating behavior in males (Baum et al., 1982; Wood, 1996a; Wood and Newman, 1995d). However, this

effect appears to be due to the aromatization of T to estrogen, since local application of estradiol, a potent form of estrogen, is more effective than DHT in facilitating male sexual behavior (Wood, 1996a), and intracerebral injections of an androgen receptor blocker aimed at the MeAMY are unable to significantly reduce male mating behavior (McGinnis, Williams, and Lumia, 1996). The effects of an intracerebral implant of progesterone in the MeAMY on male sexual behavior has not been investigated. Thus, the role of progesterone in this area is unknown. It should be noted that these lesion and implant studies look at the MeAMY as a whole since the limited anatomical resolution inherent in these techniques would not allow a finer distinction. Taken together, these data show that the MeAMY is the first brain region in this circuit where both the external (chemosensory cues) and the internal (hormonal stimulation) information start to converge to allow for the full display of male reproductive behavior (Wood, 1998; Wood and Coolen, 1997; Wood and Newman, 1995b; Wood and Newman, 1995c).

Bed Nucleus of the Stria Terminalis: The bed nucleus of the stria terminalis (BNST) receives projections from both the anterior and posterior portions of the MeAMY via the stria terminalis (Wood, 1998), and a sparse projection directly from the AOB (Newman et al., 1997). Similar to the MeAMY, this brain region represents another level where chemosensory cues and hormonal stimulation interact. The

BNST is composed of several subdivisions, one of which is the posteromedial subdivision (BNSTpm). This subnucleus of the BNST shows increases in Fos-immunoreactivity after exposure to female pheromones (Fiber et al., 1993; Fiber and Swann, 1996; Kollack-Walker and Newman, 1997; Swann and Fiber, 1997), and has a high concentration of steroid hormone receptors (Meek et al., 1997; Wood et al., 1992; Wood and Newman, 1995a; Wood and Newman, 1995b), indicating that the BNSTpm is responsive to both steroidal and chemosensory information.

It has been shown in adult male rats and hamsters that when the BNST is ablated, males engage in less sexual behavior than their sham-lesioned counterparts (Claro, Segovia, Guilamón, and del Abril, 1995; Emery and Sachs, 1976; Liu, Salamone, and Sachs, 1997; Powers, Newman, and Bergondy, 1987; Valcourt and Sachs, 1979). Similar to that which is found in a male hamster with lesions of the MeAMY, this deficit in mating behavior may be secondary to the inability of the male to sense the pheromonal cues provided by the female (Powers et al., 1987). It has also been shown that local application of T to the BNST is capable of initiating male sexual behavior (Wood and Newman, 1995c; Wood and Newman, 1995d). The effects of locally administered DHT, estrogen, or progesterone in the BNST on male mating behavior have not been tested. Therefore, whether T is acting directly or indirectly through one if its metabolites, or if progesterone is capable of activating mating behavior when

implanted into the BNST is presently unknown. As noted before, these lesion and implant studies look at the BNST as a whole because of the limited anatomical resolution of these techniques. In summary, these data indicate that the BNST, and in particular the BNSTpm, is similar to the MeAMY in that it is responsive to chemosensory cues and is an important component of the steroid-sensitive neural circuit that mediates male mating behavior.

Hypothalamus: In hamsters, there are two separate subnuclei of the hypothalamic preoptic area that have been implicated in the control of male sexual behavior, the magnocellular portion of the medial preoptic nucleus (MPNmag), and the medial preoptic nucleus (MPN). These nuclei receive projections from the MeAMY via the stria terminalis and ventral amygdalofugal pathway (Wood, 1998). These subregions of the hamster hypothalamus express Fos after exposure to female pheromones (Fiber et al., 1993; Fiber and Swann, 1996; Kollack-Walker and Newman, 1997; Swann and Fiber, 1997), and these areas have a high concentration of steroid hormone receptors (Meek et al., 1997; Romeo et al., 1999; Wood et al., 1992; Wood and Newman, 1995a; Wood and Newman, 1995b). Thus, similar to the MeAMY and BNSTpm, these nuclei integrate chemosensory cues and hormonal stimulation.

It has been shown in numerous species that when the medial preoptic area is removed males engage in less sexual

behavior (Cherry and Baum, 1990; De Jonge, Louwerse, Ooms, Evers, Endert, and Van De Poll, 1989; Floody, 1989; Giantonio et al., 1970; Ginton and Merari, 1977; Hansen, Köhler, Goldstein, and Steinbusch, 1982; Heimer and Larsson, 1966/1967; Larsson and Heimer, 1964; Ryan and Frankel, 1978; Yahr and Gregory, 1993). Moreover, local application of either T, estrogen, or progesterone in the anterior hypothalamus is capable of initiating male sexual behavior (Butera and Czaja, 1989a; Butera and Czaja, 1989b; Christensen and Clemens, 1974; Crews et al., 1996; Davidson, 1966; Johnston and Davidson, 1972; Lisk, 1967; Lisk and Bezier, 1980; Moralí et al., 1986; Rozendaal and Crews, 1989; Tang and Sisk, 1991; Wood and Newman, 1995d). Furthermore, unlike in the MeAMY, the androgenic metabolite DHT is effective in activating male sexual behavior when implanted into the preoptic/anterior hypothalamic area (Butera and Czaja, 1989a; Butera and Czaja, 1989b; Johnston and Davidson, 1972; Rozendaal and Crews, 1989; but see, Lisk and Bezier, 1980). These data indicate that the MPNmag and MPN are responsive to chemosensory cues in the hamster, and are two more important nuclei in the steroid-sensitive neural circuit that mediates male reproductive behavior.

Summary: The network of nuclei discussed above represents the forebrain neural circuit that mediates male reproductive behavior. The steroid-sensitive feature of each component of this circuit demonstrates redundancy in that

hormonal stimulation in any one of these areas is sufficient to facilitate mating behavior in a male with an intact olfactory system (Wood and Newman, 1995d). However, the various nuclei in this circuit do show some differences with respect to which hormone causes the greatest behavioral response and what specific aspect of mating behavior (e.g., mounting, intromission) is affected (Wood, 1996b; Wood, 1997).

The connections between these steroid- and pheromone-sensitive nuclei are reciprocal (Coolen and Wood, 1998). Thus, it is possible that as olfactory information proceeds from the amygdala to the bed nucleus and the hypothalamus these areas can feed back on the amygdala to alter its functioning. Furthermore, depending on the hormonal milieu and the developmental stage of the animal, these areas may be more or less responsive to steroidal and pheromonal information. Hence, the cross-talk between these nuclei may change so that a proper behavioral response may be initiated in the context of a particular set of stimuli, both internal and external.

Pubertal Maturation of Male Sexual Behavior

One benchmark of pubertal development in the male is the marked increase in T secretion by the testes. For instance, in the Syrian hamster serum T levels begin to increase slowly between 21 and 28 days of age and reach adult-like levels at approximately 50 days of age (Meek et al., 1997; Miller et

al., 1977; Sisk and Turek, 1983; Vomachka and Greenwald, 1979), while in the rat, the time course is slightly longer (T titers do not start to increase until around 45 days of age; Ketelslegers, Hetzel, Sherins, and Catt, 1978; Södersten, Damassa, and Smith, 1977). In both hamsters and rats, the pubertal maturation of mating behavior appears to be correlated with these endocrine changes since increases in androgen secretion precede the display of sexual behavior (Miller et al., 1977; Södersten et al., 1977). Similar to an experiment by Miller et al. (1977), we have shown that 28 day old male hamsters engage in little, if any, reproductive behavior, while 49 day old males can be observed engaging in all aspects of reproductive behavior (Meek et al., 1997).

One obvious mediator of this increase in mating behavior is the increase in T secretion by the testes. We conducted a study (Meek et al., 1997) to address this question by castrating and clamping the T levels of prepubertal (21 days of age) and adult (42 days of age) male hamsters by immediately implanting them with a pellet containing either 0, 2.5, or 5 mg of T after castration. It should be noted that the 2.5 mg pellet of T provides back adult-like levels of circulating T. One week after castration and implantation (i.e., at either 28 or 49 days of age), all animals were given a 10-min behavioral test with a receptive female to assess their ability to engage in sexual behavior.

It was found that males treated with T, regardless of age, engaged in significantly greater amounts of anogenital

investigation. In contrast, only adults treated with T showed significant increases in the number of mounts, intromissions, and ejaculations. T did not activate these behaviors in juvenile males. Similar behavioral results were obtained with rats and ferrets in that when prepubertal males are treated with a dose of T that fully activates sexual behavior in adults, prepubertal males still engage in little or no reproductive behavior (Baum, 1972; Larsson, 1967; Sisk, Berglund, Tang, and Venier, 1992; Södersten et al., 1977). Therefore, the lower levels of T experienced by prepubertal males are not solely responsible for the lack of mating behavior exhibited prior to puberty. These data indicate that the nervous system of adults is more responsive than prepubertal males to the activating effects of T on reproductive behavior. The changes that take place in the nervous system during pubertal development that mediate this alteration in responsiveness remain largely unknown, and is the focus of the present dissertation.

Since androgen receptors are the mediators of the intracellular actions of T, we hypothesized that the differential responsiveness to T before and after puberty is mediated by differential expression of the androgen receptor (AR) in prepubertal and adult males. Specifically, this hypothesis predicts that the greater behavioral responsiveness to T exhibited by the adults compared to juveniles is mediated by a greater number of ARs in the adult. Indeed, in support of this hypothesis it has been

demonstrated that pubertal maturation is accompanied by an increase in AR expression in the hypothalamus and amygdala in gonadally intact males (Kashon and Sisk, 1994; Meek et al., 1997). T generally upregulates AR-immunoreactivity in adults (Kashon, Arbogast, and Sisk, 1996; Menard and Harlan, 1993; Prins and Birch, 1993). Therefore, we processed the brain sections from the T-treated adult and prepubertal males to investigate whether T increased AR-immunoreactive (AR-ir) cell number in the mating circuit to a greater extent in adult compared to prepubertal males.

T increased AR to the same degree in prepubertal and adult males in the MPN and MeAMY. Surprisingly, we found that T increased the number of AR-ir cells to a greater degree in prepubertal males compared to adults in the BNSTpm and MPNmag. These data clearly indicate that the lack of behavioral responsiveness to T in prepubertal males is not due to an inability of T to increase AR in the neural circuitry that underlies sexual behavior in the adult male hamster. Furthermore, these data show that the presence of AR in the mating circuit may be necessary, but not sufficient, to mediate the pubertal increase in male reproductive behavior.

The few studies mentioned above are the sum total of our knowledge on the pubertal maturation of male reproductive behavior. Thus, the enormous amount of research that has been done on the steroidal and neural control of adult sexual

behavior stands in stark contrast to the dearth of studies investigating the pubertal development of this behavior in males.

The purpose of the present dissertation is to begin to fill this gap in our understanding by elucidating the role that steroid hormones, their receptors, and pheromones play in the pubertal maturation of male mating behavior. Specifically, the lack of a behavioral response to T prior to puberty could be due to a lack of either the androgenic or estrogenic action, or both, to T's overall action. Moreover, the effects of progesterone on the pubertal maturation of male sexual behavior has not been established. Thus, this dissertation will investigate whether the metabolites of T, namely DHT and estrogen, or progesterone are capable of activating male mating behavior prior to puberty. Furthermore, receptors for these steroids will be assessed in the mating circuit before and after pubertal development to determine whether pubertal changes in responsiveness to these sex steroids is mediated by changes in the number of cells expressing these receptors. Finally, this dissertation will examine whether the mating circuit processes chemosensory cues from the female differently before and after pubertal development.

Section I. Contribution of the androgenic metabolite dihydrotestosterone and the androgen receptor in the pubertal maturation of male sexual behavior.

Rationale

T can be intracellularly reduced to the androgenic metabolite DHT, which facilitates sexual behavior in adult male hamsters (DeBold and Clemens, 1978; Payne and Bennett, 1976; Whalen and DeBold, 1974). Thus, the lack of a behavioral response to T in prepubertal males could arise from a relative lack of androgenic action by DHT. We have previously shown that prepubertal males are behaviorally unresponsive to T (Meek et al., 1997), indicating that cellular responses to activation of the AR by T are different in the prepubertal and adult brain. Whether the androgenic metabolite DHT elicits similar cellular and behavioral effects in pre- and post-pubertal males is not known, and this question was the focus of the present study.

The steroid-sensitive cell groups that comprise the limbic components of this neural circuit express relatively high levels of AR, to which DHT binds (Wood et al., 1992; Wood and Newman, 1995a). DHT increases AR-immunoreactivity in this circuit in adult males (Wood and Newman, 1995a; Wood and Newman, 1999). In the present experiment we compared mating behavior and AR-immunoreactivity in prepubertal and adult castrates treated with DHT to determine whether puberty

is associated with changes in neural and behavioral responses to an androgenic metabolite of T.

Methods

Subjects and Treatment

Male Syrian hamsters used in the present study were bred at Michigan State University (East Lansing, MI). All animals were weaned from their mothers at 21 days of age and singly housed in clear polycarbonate cages (37.5 X 33 X 17 cm) with *ad libitum* access to food (Teklad Rodent Diet No. 8640, Harlan, Madison, WI) and tap water. The animals were maintained on a 14 hr light / 10 hr dark light-dark schedule (lights off at 1200 hr EST) and the temperature was kept at 21±2°C. All animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and the protocols were approved by the Michigan State University All-University Committee for Animal Use and Care.

All animals were castrated under methoxyflurane anesthesia at either 21 (prepubertal) or 42 (adult) days of age. Males received a daily injection (at 1000 hr) of either 500 or 1000 μg of DHT, or the sesame oil vehicle (n=5-6). Hormone treatment began on the day of castration. On the seventh day of treatment, all animals were given a 15-min mating behavior test with a hormone-primed estrous female. Immediately following the behavioral test, animals were weighed, euthanized, and perfused as described below.

Tests for Male Reproductive Behavior

All animals were sexually naive prior to the 15-min behavioral test and were tested 2-5 hr after lights out (4-7 hr after the seventh injection of DHT). 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 the receptive female. Stimulus females were bilaterally ovariectomized under methoxyflurane anesthesia and subsequently made behaviorally receptive by sequential injections of estradiol benzoate (10 μg in 0.05 ml sesame oil, *sc*, 48 hr prior to testing) and progesterone (250 μg in 0.05 ml sesame oil, *sc*, 4 hr prior to testing).

The behavioral tests were videotaped under dim red light illumination with a Panasonic Color Video Camera (WV 3250). Videotapes were scored to assess the amount of time spent in anogenital investigation (AGI) of the female, and the number of mounts, intromissions, and ejaculations achieved by the male. Video tapes were scored by a single experimenter who was blind to the hormonal condition of the animals.

Tissue Collection

Immediately after the 15 min behavioral test, animals were weighed, administered an overdose of sodium pentobarbital (130 mg/kg, *ip*), and perfused. Prior to perfusion, seminal vesicles were removed, seminal fluid was expressed, and the wet weight was recorded. Animals were

intracardially perfused with 100 ml of buffered saline rinse followed by 150 ml of 4% paraformaldehyde. Brains were then removed and postfixed for 1 hr in 4% paraformaldehyde and then transferred to a phosphate buffered saline (PBS) solution containing 20% sucrose. Approximately 48 hr after the brains had been immersed in the sucrose solution, 40μm coronal sections were made on a cryostat and stored in cryoprotectant (Watson, Weigand, Clough, and Hoffman, 1986) at -20°C until the AR immunocytochemistry was performed.

Androgen Receptor Immunocytochemistry

Every fourth brain section from each animal was processed in a single immunocytochemical run. Sections were rinsed 5 times in 0.1 M PBS to remove the cryoprotectant. Sections were then incubated sequentially in 0.1 M glycine in 0.1 M PBS (30 min), 0.3% H_2O_2 in PBS (10 min), 4% normal goat serum (NGS; Vectastain ABC Kit, Burlingame, CA) in 0.3% Triton X-100 in PBS (PBS-TX; 1 hr), 0.25 μg/ml rabbit anti-AR in PBS-TX (PG-21-18a, obtained from G. S. Prins, Michael Reese Hospital, Chicago, IL; 48 hr), secondary antibody (goat-anti-rabbit, Vectastain Elite Kit, 1:200 in PBS-TX; 24 hr), and avidin-biotin-horseradish peroxidase complex (Vectastain ABC Elite Kit, 1:50 in PBS-TX; 2 hr). For the chromogen reaction, sections were incubated for 5 min in 0.05% diaminobenzidine (DAB), 2% 500 nM $NiCl_2$ and 0.075%

H_2O_2. Sections were rinsed 3 times in PBS between incubations in each reagent. All incubations were at room temperature except for the primary antibody, which was at 4°C. Sections were mounted on gelatin-coated slides, dried, dehydrated, cleared in xylenes, and coverslipped. To test for nonspecific binding, sections were processed as described above, but in the absence of either primary or secondary antibody. Absence of either antibody eliminated all detectable immunoreactivity.

Analysis of Androgen Receptor Areal Density

The areal density (cells per unit area) of AR-ir cells, referred to as the number of AR-ir cells, was determined for the MPN, MPNmag, BNSTpm, MeA, and MeP. These areas were chosen for analysis because they are the steroid-containing forebrain nuclei of the neural circuit that mediates mating behavior (Kollack-Walker and Newman, 1997; Newman, 1999; Newman et al., 1997; Wood, 1996b; Wood, 1998; Wood et al., 1992; Wood and Newman, 1995b; Wood and Newman, 1999). These nuclei were located within the respective sections by their relative position to the 3rd ventricle (e.g., MPN), fiber tracts (e.g., BNSTpm, MeA, and MeP), or in relation to each other (e.g., MPNmag is ventral to the BNSTpm). For the MPN, MeA, and MeP, bilateral counts were made in two anatomically matched sections separated by 160μm. For the BNSTpm and MPNmag, a bilateral count was made in a single section

anatomically matched across animals. Using bright-field microscopy, nuclei were centered in the field of view under a 10X objective, and then the magnification was increased using a 40X objective. AR-ir cell profiles that fell within the area of a square ocular grid (62,500μm^2) were counted. Data are expressed as the mean number of AR-ir cells/62,500μm^2. Slides were coded so that the experimenter was blind to the age and treatment of the animal during microscopic analysis.

Statistical Analysis

All peripheral, behavioral, and central variables were analyzed by two-way ANOVAs (age X dose). Fisher's PLSD tests and Tukey HSD tests were used to probe significant main effects and interactions, respectively. Differences were considered significant when $p < 0.05$. Data are presented as means $\pm$ SEM.

Results

Peripheral and Behavioral Measures

There were significant main effects of both age and DHT treatment on the weight of the androgen-sensitive seminal vesicles (Table 1). Posthoc tests indicated that seminal vesicle weight was heavier in adults compared to prepubertal males ($p < 0.05$), and that males treated with either the 500 or 1000 µg dose of DHT had significantly heavier seminal

Table 1. Mean (± SEM) seminal vesicle weight (mg / 100 g body weight) of prepubertal and adult males treated with seven daily injections of either 0, 500, or 1000 µg DHT.

DHT (µg)	Prepubertal	Adult
0	15.99±1.47	62.70±11.37
500	145.28±18.52*	269.48±32.42*
1000	138.69±17.18*	246.75±14.22*

*Significantly different from the 0 µg-treated controls.

vesicles than males of the same age treated with the oil vehicle ($p < 0.05$).

Main effects of both age and DHT treatment were found on the length of time males spent in anogenital investigation of the receptive female (Figure 2A; both $p < 0.05$). Across the hormone treatment groups, adult males engaged in a greater amount of AGI than prepubertal males ($p < 0.05$). Across both age groups, males receiving either the 500 or 1000 µg dose of DHT engaged in more AGI than animals that received the oil vehicle ($p < 0.05$).

Adult males engaged in a greater number of mounts compared to prepubertal males, regardless of DHT treatment (Figure 2B; $p < 0.05$). Figure 2C depicts the significant interaction between age and DHT treatment on the number of intromissions during the behavioral test ($p < 0.05$). Adult males treated with the 1000 µg dose of DHT engaged in a significantly greater number of intromissions than prepubertal males treated with the same dose of DHT ($p < 0.05$). The posthoc tests also revealed that the adult males treated with the 1000 µg dose of DHT engaged in a significantly greater number of intromissions than the adults treated with either the 0 or 500 µg dose of DHT ($p < 0.05$).

There was a significant main effect of age on the number of ejaculations such that adult animals engaged in a significantly greater number of ejaculations than the

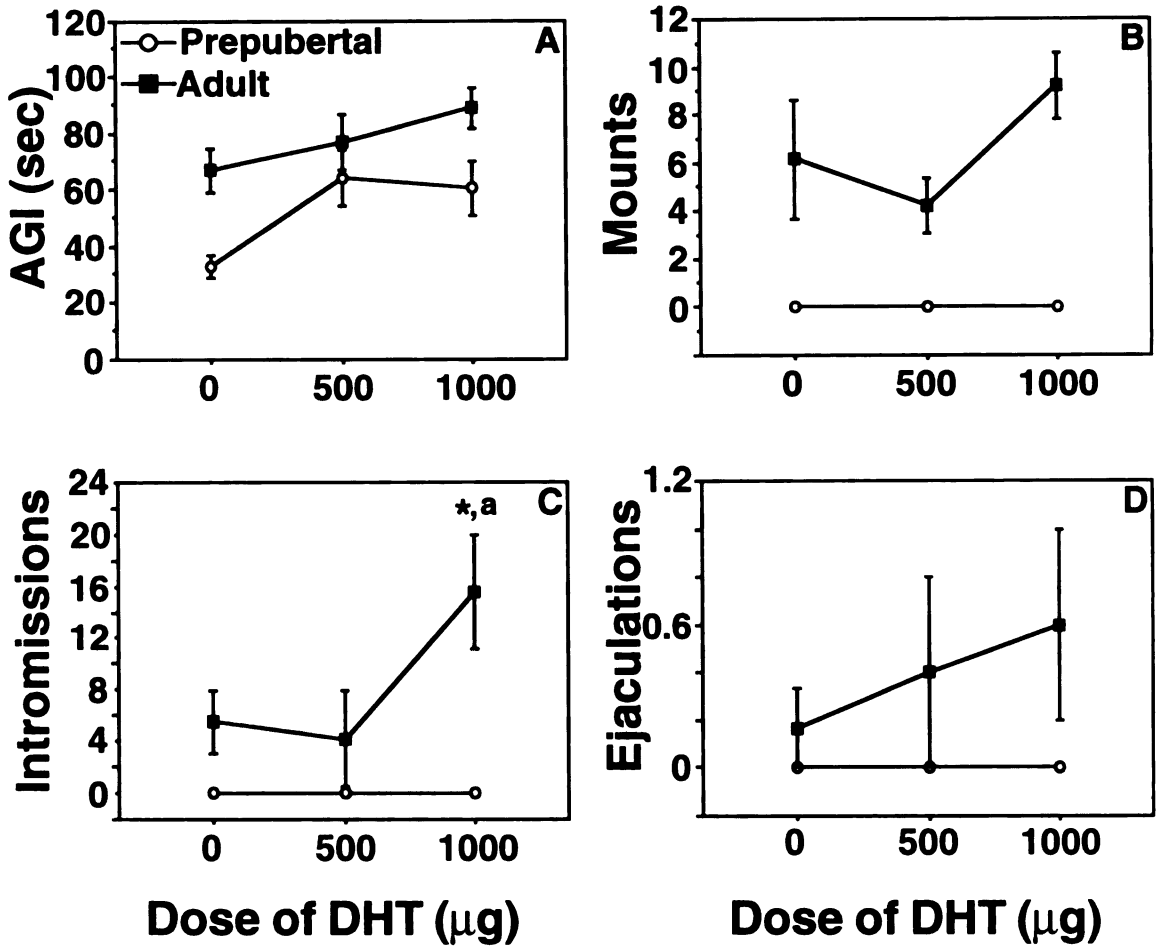

Figure 2. Number of seconds engaged in anogenital investigation (AGI; A) and frequencies of mounts (B), intromissions (C), and ejaculations (D) in a 15 min behavioral test in prepubertal and adult males given daily injections of either 0, 500, or 1000 μg of DHT for seven days. Asterisk indicates a significant difference from prepubertal males. "a" indicates that adults that received 1000 μg of DHT are significantly different from the adults that received either the 0 or 500 μg dose of DHT. All values are means ± SEM.

prepubertal animals regardless of the dose of DHT received (Figure 2D; $p < 0.05$). None of the prepubertal animals treated with DHT were observed to exhibit any mounting, intromissive, or ejaculatory behavior.

AR-Immunoreactivity

Figure 3 shows the number of AR-ir cells within the brain regions examined. Two-way ANOVAs revealed a significant main effect of DHT treatment on the number of AR-ir cells in the MPN, BNSTpm, and MeP ($p < 0.05$). Fisher's PLSD posthoc tests showed that both age groups treated with either the 500 or 1000 µg dose of DHT had a significantly greater number of AR-ir cells than vehicle-treated animals ($p < 0.05$). ANOVAs revealed significant interactions between age and DHT treatment in both the MPNmag and MeA (both $p < 0.05$). The number of AR-ir cells in the MPNmag was significantly greater in prepubertal males treated with the 500 µg dose of DHT than their adult counterparts ($p < 0.05$). In the MeA, the number of AR-ir cells was greater in adult males treated with 1000 µg DHT compared to their prepubertal counterparts ($p < 0.05$). Photomicrographs in Figure 4 show AR-ir in the MPN of a prepubertal and adult male treated with 1000 µg of DHT.

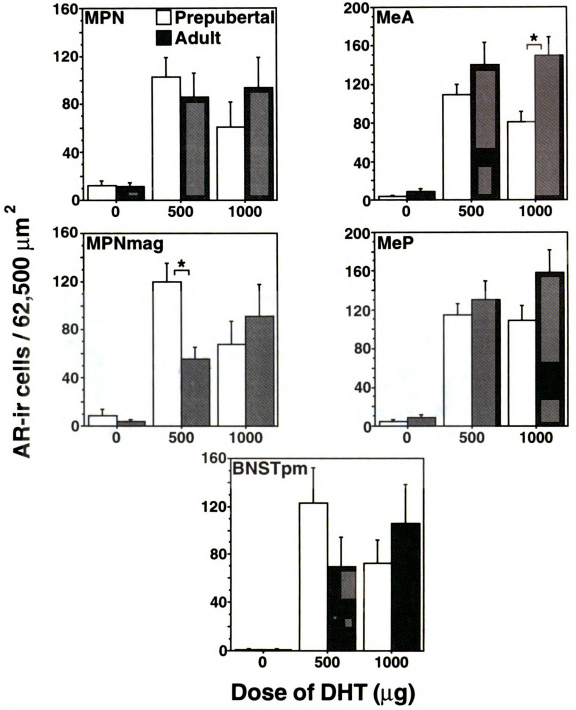

Figure 3. AR-ir cells/62,500 μm^2 in the MPN, MPNmag, BNSTpm, MeA, and MeP of prepubertal and adult males treated daily for seven days with either 0, 500, or 1000 μg of DHT. Asterisks indicate a significant difference between prepubertal and adult males within a dose of DHT. All values are means ± SEM.

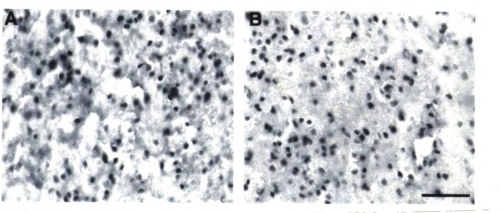

Figure 4. AR-ir in the MPN of a prepubertal (A) and adult (B) male treated with 1000 µg of DHT. Bar, 50µm.

Discussion

These data demonstrate that prepubertal males are less responsive than adults to the activational effects of DHT on male reproductive behavior. These results indicate, therefore, that the lack of a behavioral response to T in prepubertal males includes a specific unresponsiveness to the androgenic component of T's overall action. Furthermore, this experiment shows that the inability of DHT to activate mating behavior in juvenile males is not associated with a relative lack of DHT-induced ARs in most of the nuclei that comprise the neural circuit mediating male mating behavior. We previously observed a similar dissociation between sexual behavior and brain AR in which juvenile and adult males were treated with T (Meek et al., 1997). Specifically, T increased the expression of AR in the steroid-sensitive mating circuit in prepubertal males, but did not activate their sexual behavior. Thus, the present data are in agreement with this earlier study, and indicate that the presence of ARs in this neural circuit may be necessary, but not sufficient, to facilitate the display of male mating behavior.

Adult males treated with 1000 μg of DHT did have a significantly higher number of AR-ir cells in the MeA compared to the prepubertal males treated with the same amount of DHT. Since 1000 μg of DHT was capable of activating intromissive behavior in the adult but not

juvenile males, the greater number of AR-containing cells in the adult MeA is correlated with the adult's greater responsiveness to DHT on this behavioral measure. However, intracerebral implantation of estradiol, but not DHT, in the medial amygdala of castrated adult males activates their mating behavior, suggesting that androgenic stimulation of this area alone is not sufficient to a elicit a behavioral response (Wood, 1996a). Furthermore, T treatment induces equivalent amounts of AR-ir cells in the MeA (and elsewhere) in gonadectomized prepubertal and adult males, yet prepubertal males still do not engage in mating behavior (Meek et al., 1997). Therefore, the greater number of AR-containing cells in the MeA of adult males treated with 1000 µg of DHT is probably not responsible for their greater behavioral responsiveness to DHT. However, the greater number of ARs in the MeA of adult males could mediate neural responsiveness to other motivated behaviors that are influenced by androgens, such as aggression (Payne, 1974).

It should be noted that the prepubertal males treated with the 500 µg dose of DHT had a greater number of AR-containing cells in the MPNmag compared to the adults that were treated with 500 µg of DHT. The functional significance of the higher levels of AR expression in the MPNmag of androgen-treated prepubertal males is unknown. However, this finding further demonstrates the dissociation between AR levels and sexual behavior since these relatively high levels

of AR in the prepubertal MPNmag at this dose did not contribute to any behavioral activation in response to DHT.

Unlike mounting, intromissions, and ejaculations, AGI was activated by DHT in prepubertal males, although to a lesser degree than in adults. The pheromonal stimulation received by the male during chemoinvestigatory behavior is necessary for the subsequent display of mating behavior in this species (Wood, 1998; Wood and Newman, 1995c). These results suggest that the pheromonal cues received by a DHT-treated male may be, like the presence of AR, necessary, but not sufficient, to activate the full suite of reproductive behaviors in juveniles.

DHT was effective in increasing the amount of brain AR-ir and the wet weight of the androgen-responsive seminal vesicles at both ages, indicating that the prepubertal male is responsive to DHT at some level. Importantly, the similar increase in brain AR-ir in most of brain regions examined in the prepubertal and adult males suggests that animals exposed to the same dose of DHT received similar central androgenic stimulation, regardless of age. Thus, the inability of peripherally administered DHT to activate mating behavior prior to puberty cannot be fully explained by unequal exposure of the brain to DHT at the two ages.

In summary, these results demonstrate that prepubertal males are less responsive than adults to the behavior-activating effects of DHT. Furthermore, the inability of DHT to activate mating behavior in juvenile males is not

associated with a relative lack of DHT-induced ARs in most of the nuclei that comprise the neural circuit mediating male mating behavior. Thus, the lack of mating behavior observed in androgen-treated juveniles compared to adults must be mediated, at least in part, by differences in cellular processes downstream of AR induction and activation. This conclusion does not rule out the possibility that prepubertal males are also unresponsive to the estrogenic component of T's actions. This issue is investigated in the next set of experiments.

Section II. Contribution of the aromatase enzyme, estrogen, and estrogen receptors in the pubertal maturation of male sexual behavior.

Experiment I. Androgenic regulation of hypothalamic aromatase activity in prepubertal and adult males.

Rationale

T is converted intracellularly to estradiol in peripheral and central tissues by aromatase. In the brain, estrogenic metabolites of T play a major role in the expression of male sexual behavior (reviewed in, Luttge, 1979; Meisel and Sachs, 1994). For example, systemic injections of estradiol benzoate to castrated male hamsters induce mounting behavior (DeBold and Clemens, 1978), while aromatase inhibitors decrease copulatory behaviors (Floody and Petropoulos, 1987; except see, Cooper, Clancy, Karom, Moore, and Albers, 2000). The aromatase enzyme is present in brain regions that mediate male sexual behavior, such as the amygdala and hypothalamus (Callard, Mak, and Solomon, 1986; Hutchison, Hutchison, Steimer, Steel, Powers, Walker, Herbert, and Hastings, 1991; Roselli, Horton, and Resko, 1985), and appears to be positively regulated by androgens in some regions of the hypothalamus (Abdelgadir, Resko, Ojeda, Lephart, McPhaul, and Roselli, 1994; Hutchison et al., 1991; Negri-Cesi, Celotti, and Martini, 1989; Roselli et al., 1985; Wagner and Morrell, 1996; but see, Callard et al., 1986).

The capacity to engage in steroid-dependent reproductive behavior increases during pubertal maturation. As mentioned in the Introduction, not only is there an increase in circulating levels of T during this time, but responsiveness of the neural circuit to the behavioral actions of T increases as well. We hypothesize that this increased behavioral responsiveness to T in adults is mediated, at least in part, by the efficacy with which T is aromatized to estradiol (E) in the hypothalamus. This hypothesis leads to two related predictions. First, in intact males, aromatase activity within the behavioral neural circuit should be greater in adults than in juveniles. Second, T treatment of castrated males should increase aromatase activity to a greater extent in adults than in juveniles, which would result in higher local concentrations of E to activate male reproductive behavior in adults. As a test of this hypothesis, aromatase activity, as measured by the conversion of 3H-T to 3H-estradiol (3H-E) , was assessed in hypothalamic homogenates obtained from intact and from castrated and T-treated adult and prepubertal male hamsters.

Methods

Subjects and Treatment

Male hamsters were bred at Michigan State University (E. Lansing, MI) from stock obtained from Charles River (Kingston, NY). Animals were housed and cared for as described previously (Subjects and Treatment, Section I).

Four experiments were conducted because the number of samples that can be run in a single assay is limited. Experiment 1 characterized the amount of hypothalamic aromatase activity in untreated, intact prepubertal and adult male hamsters. In this experiment, 63- (adult, n=8) or 28- (prepubertal, n=8) day old male hamsters were weighed and rapidly decapitated. Whole hypothalami, blood samples, and testes were collected as described below. Experiments 2-4 investigated the effects of T on hypothalamic aromatase activity before and after puberty in male hamsters. Experiment 2 assessed the effects of T on aromatase activity in adult males. Adult males (60 days of age) were castrated under methoxyflurane anesthesia and implanted with a 3-week time-released pellet (Innovative Research of America, Sarasota, FL) containing either 0 mg (n=7) or 2.5 mg of T (n=7). One week after castration and implantation, hamsters were weighed, rapidly decapitated, and hypothalami and blood samples were collected as described below. Previous work has shown that aromatase activity is maximally increased within a week of T treatment (Roselli, Horton, and Resko, 1987). Furthermore, the difference in T-stimulated sexual behavior observed in prepubertal and adult male hamsters is observed one week after T treatment (Meek et al., 1997). Thus, animals received one week of T exposure in Experiments 2-4. Experiment 3 assessed the effects of T on aromatase activity in juvenile males. In Experiment 3, prepubertal males (21 days of age) were castrated and implanted with either a 0 mg

(n=7) or 2.5 mg (n=6) pellet of T. One week after treatment, tissues were collected as in Experiment 2. Experiment 4 directly compared the effect of T on aromatase activity in juvenile and adult males. Prepubertal (21 days of age, n=6) and adult (60 days of age, n=8) males were castrated and implanted with a 2.5 mg pellet of T. One week after treatment, tissues were collected as in Experiment 2.

Tissue Collection

Animals were rapidly decapitated by a guillotine. Trunk blood samples were collected and centrifuged. Plasma was removed and stored at -20°C until radioimmunoassays were performed (see below). Brains were quickly removed and the hypothalamus was dissected on a stainless steel surface on wet ice with a razor blade. Coronal cuts were made directly anterior to the optic chiasm and at the posterior end of the hypothalamus, just anterior to the mammillary bodies. Then a horizontal cut was made just ventral to the anterior commissure as it crossed the midline. Finally, the brain was placed on the dorsal surface and the optic chiasm and tissue lateral to the hypothalamus was removed. The dissected hypothalamus was then snap frozen in dry ice and stored at -70°C until the aromatase assays were performed (see below).

Assay for Steroid Metabolizing Enzymes

Individual hypothalami were homogenized in 600 µl of 250 mM sucrose/50 mM potassium phosphate buffer. Assays were

conducted with minor modifications from those used in lizard brain tissue (Wade, 1997). Initially, validation assays that varied the incubation time and substrate concentration were run on adult male hamster hypothalamic homogenates to determine the appropriate assay conditions (details presented with Results). Once the assay was validated, experiments were conducted using duplicate 200 µl aliquots of hypothalamic homogenates incubated for 25 min with 250 nM substrate. The tissue homogenates were added to test tubes in which 3H-T (New England Nuclear, Boston, MA) had been dried. In all cases, substrate was repurified by thin layer chromatography (TLC) before use. Samples were incubated at 37°C with a NADH/NADPH generating system, and the reaction was terminated by freezing the tubes in a methanol/dry ice bath.

Steroids were extracted from homogenates 3 times with diethyl ether. Androgens were then separated from estrogens twice by phenolic partition, and estrogens extracted 3 times with ethyl acetate. Androgenic and estrogenic products were applied to TLC plates following the addition of radioinert carrier steroids (Steraloids, Wilton, N. H.). TLC plates containing estrogens were run twice in ether:hexane (3:1), and the products visualized by exposure to iodine vapors. Plates containing androgens were run twice in chloroform:ethyl acetate (4:1), and the products were visualized under ultraviolet irradiation following a primulin spray. Regions containing the steroids of interest were

scraped from the plates, and after the addition of 400 µl H_2O, steroids were eluted from the silica-gel in 2 ml methanol. A fraction of the eluate was mixed with Bio-safe cocktail II (Research Products International, Santa Cruz, CA) and counted in a Beckman liquid scintillation counter (LS 6500). Each sample was corrected for counter efficiency, volume, and background counts in tubes incubated with buffer and cofactors but no tissue. Results were also corrected for recovery efficiency, which was determined by the addition of a known quantity of 3H-E or 3H-T (approximately 150,000 dpm) to tubes processed in parallel.

Protein content in each assay tube was determined with the method of Bradford (Bradford, 1976; Bio-Rad kit, Hercules, CA) using bovine serum albumin (BSA) as the protein standard. To confirm their authenticity, samples of all steroid products were recrystallized with radioinert steroids (Steraloids, Wilton, N. H.) to constant specific activity using ethanol and water (details presented with Results).

Testosterone Radioimmunoassay

Plasma concentrations of T were measured in two different assays using the Coat-A-Count Total Testosterone Kit (Diagnostic Products, Los Angles, CA). This assay has been validated in our laboratory for the measurement of plasma T concentration in the Syrian hamster. The lower limit of detectability of both assays was 0.1 ng/ml. The

intraassay coefficients of variation (CV) were 5.8% and 4.1%, and the interassay CV was 10%.

Statistical Analysis

The data for each experiment were analyzed using two-tailed *t* tests. Differences were considered significant when $p < 0.05$. All data are reported as mean ± SEM. Values for the K_m and V_{max} were generated from a Lineweaver-Burk plot using a regression line in Statview 4.1 (Abacus Concepts, Inc., Berkeley, Ca).

Results

Validation of aromatase assay

Samples of E were twice verified by recrystallization to constant specific activity in ethanol and water (93.6/85.8, 77.0/77.7; $crystals_{(dpm/mg)}$/$mother\ liquor_{(dpm/mg)}$). In both cases, recovery of the specific activity was 100%. This measure reflects the ratio of the specific activity (dpm/mg) in the crystals following the final recrystallization compared to that prior to the first recrystallization (dpm of assay product used/mg cold steroid used). Although androgens were detectable, the incubation time and substrate concentration for these assays were not optimized to measure the rate of androgen production. Estrone was not detected in any sample. Therefore, quantification of aromatase activity consisted solely of the rate of E production.

Time course and saturation curve

An initial time course study was performed using a 222 nM substrate concentration (specific activity = 45.1 Ci/mmol). Pooled hypothalamic tissue was incubated for 10, 30, 60, or 180 min. E production increased linearly at incubation times up to 60 min, at which point it slowed. Based upon these results, an assay used to generate a saturation curve was incubated for 35 minutes using substrate concentrations ranging from 4 to 2225 nM (specific activity = 45.1 Ci/mmol). The reaction rate increased to the 224 nM concentration at which point E production began to level off. For this saturation curve, the K_m was 27.4 nM and V_{max} was 7.58 fmol / mg protein / min. A second time course experiment was performed using a 250 nM (specific activity = 92.4 Ci/mmol) substrate concentration at 15, 30, 60, and 120 min incubation times. E production increased linearly through 30 min then at a slightly lower rate between the 30 and 120 min time points (Figure 5). Based on the results of these assays, an incubation time of 25 min and a substrate concentration of 250 nM (specific activity = 92.4 Ci/mmol) were used for the four experiments.

Tissue specificity

To confirm tissue specificity, whole cerebellar homogenates from intact prepubertal (25 days of age) and intact adult (65 days of age) males were run in parallel with hypothalamic tissue in Experiment 3. Assay conditions for

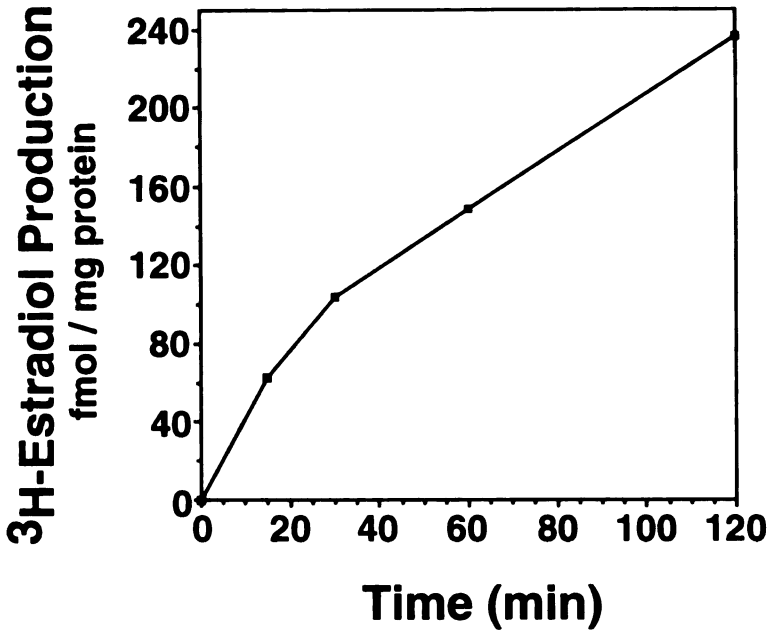

Figure 5. ^{3}H-Estradiol production (fmol / mg protein / min) after incubation of hypothalamic homogenates with 250 nM ^{3}H-testosterone for 15, 30, 60, or 120 min. Estradiol production increased at a lower rate between the 30 and 120 min time points.

the cerebellar homogenates were identical to those used for the hypothalamic homogenates. No aromatase activity was detected in either the prepubertal or adult cerebellar homogenates, consistent with results in other mammalian species (Abdelgadir, Roselli, Choate, and Resko, 1997; MacLusky, Walters, Clark, and Toran-Allerand, 1994; Roselli, Ellinwood, and Resko, 1984).

Experiment 1: Hypothalamic aromatase activity in prepubertal and adult males

Paired testis weight and plasma T were significantly greater in adults compared with prepubertal males ($p < 0.05$, Table 2). Furthermore, adults had significantly higher hypothalamic aromatase activity compared to that of the prepubertal animals ($p < 0.05$, Figure 6). Specifically, adults had a two-fold increase in aromatase activity compared to that of their prepubertal counterparts.

Experiment 2: Androgenic regulation of hypothalamic aromatase activity in adult males

No significant difference was found in paired testis weight on the day of castration between the adults that were treated with either the blank or 2.5 mg pellet of T (Table 2). Plasma T concentrations were significantly higher in castrated adults treated with the 2.5 mg pellet of T compared to castrated adults treated with the blank pellet ($p < 0.05$, Table 2). Furthermore, T-treated adults had significantly

Table 2. Mean (± SEM) plasma testosterone concentrations and paired testis weight.

Age (days)-endocrine status	Plasma testosterone (ng/ml)	Paired Testis weight (g)
	Experiment 1	
28-intact	0.361 ± 0.202	0.557 ± 0.135
63-intact	2.454 ± 1.248*	3.019 ± 0.418*
	Experiment 2	
67-0 mg T	0.143 ± 0.043	3.027 ± 0.099
67-2.5 mg T	3.954 ± 0.585*	2.926 ± 0.120
	Experiment 3	
28-0 mg T	0.100 ± 0.000	0.218 ± 0.019
28-2.5 mg T	4.338 ± 0.564*	0.197 ± 0.015
	Experiment 4	
28-2.5 mg T	4.227 ± 0.367	0.208 ± 0.019
67-2.5 mg T	3.605 ± 0.500	3.401 ± 0.091*

*Significantly different within each experiment. Paired testis weight for Experiments 2-4 was measured on the day of castration (i.e., at either 21 or 60 days of age).

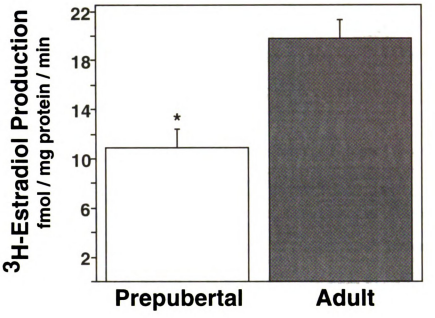

Figure 6. ^{3}H-Estradiol production (fmol / mg protein / min) in hypothalamic homogenates from intact prepubertal and adult males. Asterisk indicates significant difference. Values are means $\pm$ SEM.

higher levels of hypothalamic aromatase activity compared to castrated controls ($p < 0.05$, Figure 7A). Specifically, adults receiving the 2.5 mg dose of T had greater than a two-fold increase in hypothalamic aromatase activity compared to castrated adults treated with a blank pellet.

Experiment 3: Androgenic regulation of hypothalamic aromatase activity in prepubertal males

There was no significant difference in paired testis weight on the day of castration between the juveniles that were treated with either the blank or 2.5 mg pellet of T (Table 2). Plasma T concentrations were significantly greater in prepubertal animals implanted with the 2.5 mg pellet of T compared to those prepubertal animals treated with a blank pellet ($p < 0.05$, Table 2). Moreover, the prepubertal males treated with the 2.5 mg dose of T had significantly higher levels of hypothalamic aromatase activity compared to the prepubertal males treated with the blank pellet ($p < 0.05$, Figure 7B). Similar to the increase in hypothalamic aromatase activity observed in adults, T-treated prepubertal males had over a two-fold increase in hypothalamic aromatase activity compared to their prepubertal placebo-treated counterparts.

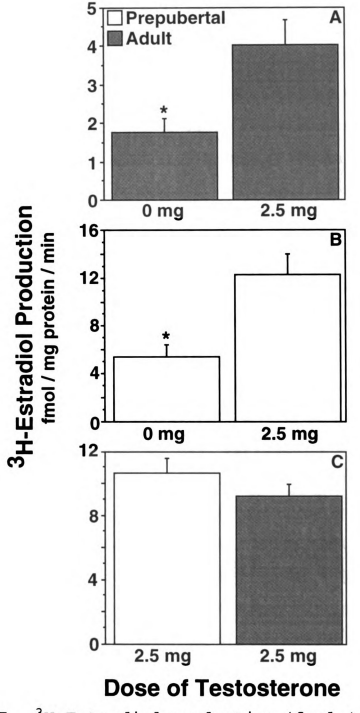

Figure 7. ^{3}H-Estradiol production (fmol / mg protein / min) in hypothalamic homogenates from castrated adult males treated with either 0 or 2.5 mg of testosterone (A), castrated prepubertal males treated with either 0 or 2.5 mg of testosterone (B), and castrated adult and prepubertal males treated with 2.5 mg of testosterone (C). Asterisks indicate significant differences. All values are means $\pm$ SEM.

Experiment 4: Comparison of androgenic regulation of hypothalamic aromatase activity in prepubertal and adult males

Adults had significantly heavier paired testis weight on the day of castration compared to the castrated prepubertal animals ($p < 0.05$, Table 2). Plasma T levels and hypothalamic aromatase activity were equivalent between the castrated adults and the castrated prepubertal animals treated with the 2.5 mg dose of T (Table 2 and Figure 7C, respectively).

Discussion

This series of studies demonstrates that hypothalamic aromatase activity is significantly higher in adult compared to prepubertal males, and that hypothalamic aromatase activity is positively regulated by T in both the adult and prepubertal male hamster. Furthermore, the ability of T to increase hypothalamic aromatase activity appears to be equivalent before and after puberty. The androgenic regulation of hypothalamic aromatase activity most likely accounts for the developmental difference in enzyme activity observed in intact males in Experiment 1, since the prepubertal males in this experiment had significantly lower levels of circulating T compared to the adults.

The present data do not support the hypothesis that increased behavioral responsiveness to T in adults is mediated by the efficacy with which T is aromatized to E in

the hypothalamus. However, we cannot rule out the possibility that aromatase activity is differentially regulated in the hypothalamus of prepubertal and adult males in more discrete hypothalamic nuclei by adult-physiological levels of androgens. Indeed, Hutchison *et al.* (1991) have reported that regulation of hypothalamic aromatase activity by T in the adult male hamster is brain region specific.

This series of experiments has unequivocally demonstrated that aromatase activity in whole-hypothalamic homogenates is positively regulated by T in both adult and prepubertal male hamsters, and this regulation is equivalent at these two developmental stages. Thus, the question remains as to why the same dose of T is unable to activate reproductive behavior in juvenile males. Our experiments suggest that the unresponsiveness of the juvenile male's nervous system to T lies downstream of T's or DHT's action on the AR (Meek et al., 1997 and Section I) and on aromatase activity (present data). One possibility is that the aromatized E may have differential effects on the hypothalami of prepubertal versus adult male hamsters. For instance, there is precedence in the literature suggesting that estrogen receptor expression can be differentially regulated by estrogens depending on the current physiological condition of the animal (Hnatczuk, Lisciotto, DonCarlos, Carter, and Morrell, 1994; Koch and Ehret, 1989; Shughrue, Bushnell, and Dorsa, 1992). Therefore, increased local concentration of estrogen in the hypothalamus may lead to an increase in

steroid receptors in adults, but not in prepubertal males, leading to behavioral activation in the adult males only.

In rats, Lephart and Ojeda (1990) observed a pubertal decrease in aromatase activity and hypothesized that the presumptive decrease in E availability was responsible for the pubertal decline in steroid negative feedback regulation of gonadotropin secretion (Lephart and Ojeda, 1990). However, Roselli and Klosterman (1998) found a pubertal increase in aromatase activity in male and female rats. Variations in experimental methodologies and animal species used could account for the different results in the two experiments mentioned above and the present data. For example, tissue dissection was different in all of these experiments. In the present experiments, a tissue fragment containing both the preoptic area and the hypothalamus was used, while Lephart and Ojeda (1990) and Roselli and Klosterman (1998) assayed more discrete areas of the hypothalamus. It is also possible that the pubertal decrease in responsiveness to steroid negative feedback does involve a change in aromatase activity whereas the pubertal increase in responsiveness to behavioral activation by steroids does not.

In conclusion, the present series of studies has established that adult male hamsters have approximately a two-fold increase in hypothalamic aromatase activity compared to prepubertal males, and that, in both prepubertal and adult male hamsters, hypothalamic aromatase activity is under androgen regulation. Therefore, the failure of T-treated

castrated prepubertal male hamsters to engage in the full suite of male reproductive behaviors is not due to the inability of T to be converted into E in the hypothalamus. Differences in the ability of T to increase aromatase activity in other brain regions, or differences in the action of T and/or E on other cellular processes must account for the inability of T to facilitate male reproductive behavior in juvenile males.

Experiment II. The role of estrogen and the estrogen receptor in the sexual behavior of prepubertal and adult males.

Rationale

The studies we have conducted thus far suggest that the inability of T to stimulate sexual behavior in prepubertal males is due, at least in part, to the unresponsiveness of the prepubertal brain to the androgen metabolite DHT. The lack of a behavioral response to DHT prior to puberty is not associated with an absence of ARs in the mating circuit. Furthermore, these receptors appear to be functional prior to puberty since a T-induced increase in hypothalamic aromatase activity, which is an AR-dependent response (Roselli et al., 1987), is equivalent in prepubertal and adult males.

Similarly, the lack of mating behavior in T-treated prepubertal males cannot be accounted for by an inability of T to upregulate hypothalamic aromatase activity. We cannot,

however, rule out the possibility that aromatase activity is differentially regulated in the hypothalamus of prepubertal and adult males in more discrete hypothalamic nuclei or in areas outside of the hypothalamus. Thus, the distinct possibility remains that T-treated prepubertal males may not be receiving adequate local estrogenic stimulation in brain regions needed to activate male copulatory behavior.

The purpose of the present study was to investigate whether the lack of behavior in T-treated prepubertal males may be the result of inadequate local estrogenic action. The behavioral response to centrally produced estrogen is mediated through the estrogen receptor (ER; reviewed in, Meisel and Sachs, 1994). There are two known forms of the ER, ERα and ERβ. ERα appears to be the predominant ER responsible for the activation of mating behavior, since male mice lacking the ERα gene, but with the ERβ gene intact, engage in relatively little reproductive behavior (Wersinger, Sannen, Villalba, Lubahn, Rissman, and De Vries, 1997). It is unknown whether a prepubertal male would respond behaviorally to estrogen and whether puberty is associated with changes in the number of ERα-containing cells in the mating circuit. Thus, in this study we compared the sexual behavior and ERα expression in the behavioral neural circuit of prepubertal and postpubertal males treated with estrogen.

Methods

Subjects and Treatment

Male hamsters used in this study were bred at Michigan State University (East Lansing, MI), and housed and cared for as described previously (Subjects and Treatment, Section I).

All animals were castrated under methoxyflurane anesthesia at either 21 or 42 days of age and implanted with a 3-week timed-release pellet (Innovative Research of America, Sarasota, FL) containing either 0, 0.05, 0.10, or 0.25 mg of beta-estradiol-3-benzoate (EB; n=6 per age and treatment group). One week after castration and implantation (i.e., at either 28 or 49 days of age), all animals were given a 15-min mating behavior test with a hormone primed estrous female as described in Section I (Tests for Male Reproductive Behavior). All animals were sexually naive prior to the behavioral test.

Tissue Collection

Immediately following the behavioral test, animals were perfused and brains were collected and sectioned as described in Section I (Tissue Collection). Blood samples were obtained via cardiac puncture prior to the perfusion.

Estrogen Receptor-α Immunocytochemistry

Brains from adult and prepubertal gonadectomized males treated with either the blank or 0.05 mg EB pellet were

processed for ERα-immunoreactivity. These treatment groups were chosen for study because they demonstrated the most robust behavioral differences (see below in Results). Every fourth brain section from all animals was processed in a single immunocytochemical run. Specifically, free-floating sections were rinsed 4 times in PBS-TX for 5 min each. Tissue was then incubated in 1% H_2O_2 for 10 min followed by four rinses in PBS-TX. Subsequently, sections were incubated in 0.1% BSA for 1 hr and for 72 hr at 4°C in DAKO (1D5) mouse anti-ERα (1:500; DAKO, Glostrup, Denmark) in PBS-TX and 0.1% BSA. This ERα antibody recognizes ERα in the presence or absence of circulating estrogen (Gréco, Edwards, Michael, and Clancy, 1998). Sections were then brought to room temperature in the primary antibody for 2 hr. After the 2 hr equilibration, sections were incubated for 1 hr in biotinylated donkey anti-mouse IgG (1:500, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; preadsorbed against hamster and other species serum proteins). Sections were then rinsed 4 times in PBS-TX and incubated in avidin-biotin horseradish peroxidase complex for 1 hr (1:100, 1 hr, Vectastain ABC Elite Kit). Sections were again rinsed 4 times in PBS-TX followed by a 5 min rinse in a 0.05M Tris buffer with 1.5% NaCl (1.5T buffer). For the chromagen reaction, sections were incubated in 0.05% DAB with the 1.5T buffer and 0.01% H_2O_2. The DAB reaction was

terminated by 4 rinses in PBS. Sections were mounted on gelatin-coated slides, dried, dehydrated, cleared in xylenes, and coverslipped. To test for nonspecific staining, brain sections were processed as described above following omission of primary antiserum and/or secondary antiserum. Omission of the primary and/or secondary antiserum eliminated all detectable ERα-immunoreactivity.

Analysis of Estrogen Receptor-α Areal Density

The number of immunoreactive cell profiles per unit area (areal density), referred to as number of ERα-ir cells, was determined in an identical manner and for the same brain regions described in Section I (Analysis of Androgen Receptor Areal Density)

Estradiol and Luteinizing Hormone Radioimmunoassays

Plasma concentrations of E were measured using the Coat-A-Count Estradiol-6 Kit (Diagnostic Products). This assay has been validated in our laboratory for the measurement of plasma E concentrations in Syrian hamsters. The lower limit of detectability of the assay was 14.59 pg/ml. The intraassay CV was 13.8%. Plasma luteinizing hormone (LH) concentrations were measured using reagents in the rat LH kit obtained from Dr. A. F. Parlow and the National Hormone and Pituitary Program at the National Institute of Diabetes, Digestive, and Kidney Diseases (NIDDK). Values are reported

as nanogram equivalents of NIDDK-rLH-RP-3. This assay has been validated in our laboratory for the measurement of plasma LH concentrations in Syrian hamsters. The lower limit of detectability was 0.66 ng/ml, and the intraassay CV was 8.6%.

Statistical Analysis

The effects of age and EB treatment on all physiological, neural, and behavioral variables were analyzed by two-way ANOVAs (age X dose). Significant main effects were further analyzed with Fisher's PLSD tests and significant interactions were probed with Tukey HSD tests. Differences were considered significant when $p < 0.05$. Data are presented as means $\pm$ SEM. One adult in the 0 mg EB group was an outlier on the variable of intromissions as determined by the Dixon's outlier test (Sokal and Rohlf, 1981, pg. 413, $p < 0.05$), and was 4 standard deviations above the mean for his group on mounts. Therefore, data from this animal were excluded from all statistical analyses.

Results

Hormonal Measures

Increasing doses of EB resulted in increasing circulating plasma E concentrations (main effect of treatment, $p < 0.05$, Table 3). Castrates treated with the blank pellet or the 0.05 mg pellet of EB had significantly lower circulating levels of E compared to the castrates

Table 3. Mean (+ SEM) plasma estradiol and luteinizing hormone concentrations and paired testis weight.

Age (days)-endocrine status	Plasma estradiol (pg/ml)	Plasma luteinizing hormone (ng/ml)	Paired Testis weight (g)*
28-0 mg	51.40±3.12	3.70±1.09	0.165±0.017
28-0.05 mg	95.51±10.54	0.66±0.00	0.200±0.014
28-0.10 mg	340.90±65.98	0.66±0.00	0.215±0.012
28-0.25 mg	847.84±282.12	0.66±0.00	0.180±0.025
49-0 mg	73.56±2.99	12.36±1.64	2.110±0.258
49-0.05 mg	134.44±15.00	5.37±0.94	1.922±0.208
49-0.10 mg	318.15±24.97	0.66±0.00	2.175±0.148
49-0.25 mg	548.11±60.39	0.66±0.00	2.300±0.150

*Testes were weighed on the day of castration (i.e., at either 21 or 42 days of age).

treated with either the 0.10 or 0.25 mg dose of EB. Furthermore, castrated males treated with the 0.25 mg pellet of EB had significantly greater amounts of circulating E than the castrates treated with the 0.10 mg pellet of EB. Importantly, there was no significant interaction between age and EB treatment on plasma E concentrations. A given dose of EB produced similar levels of circulating E in prepubertal and adult males.

A significant interaction between age and EB treatment on circulating plasma levels of LH was found ($p < 0.05$, Table 3). All doses of EB reduced plasma LH to undetectable levels in prepubertal males, whereas in adults only the 0.10 and 0.25 mg pellet of EB completely inhibited LH secretion.

Behavioral Measures

There were significant main effects of both age and estrogen on AGI, such that adults engaged in significantly more AGI than prepubertal males, and E increased AGI significantly above that of castrated, blank-treated controls ($p < 0.05$, Figure 8A). A significant interaction was found between age and EB treatment on mounts ($p < 0.05$, Figure 8B). Post hoc tests revealed that all the adult groups engaged in significantly greater numbers of mounts than their juvenile counterparts. Furthermore, treatment of adult males with either the 0.05 or 0.10 mg pellet of EB resulted in a significant increase in mounts compared to adults receiving

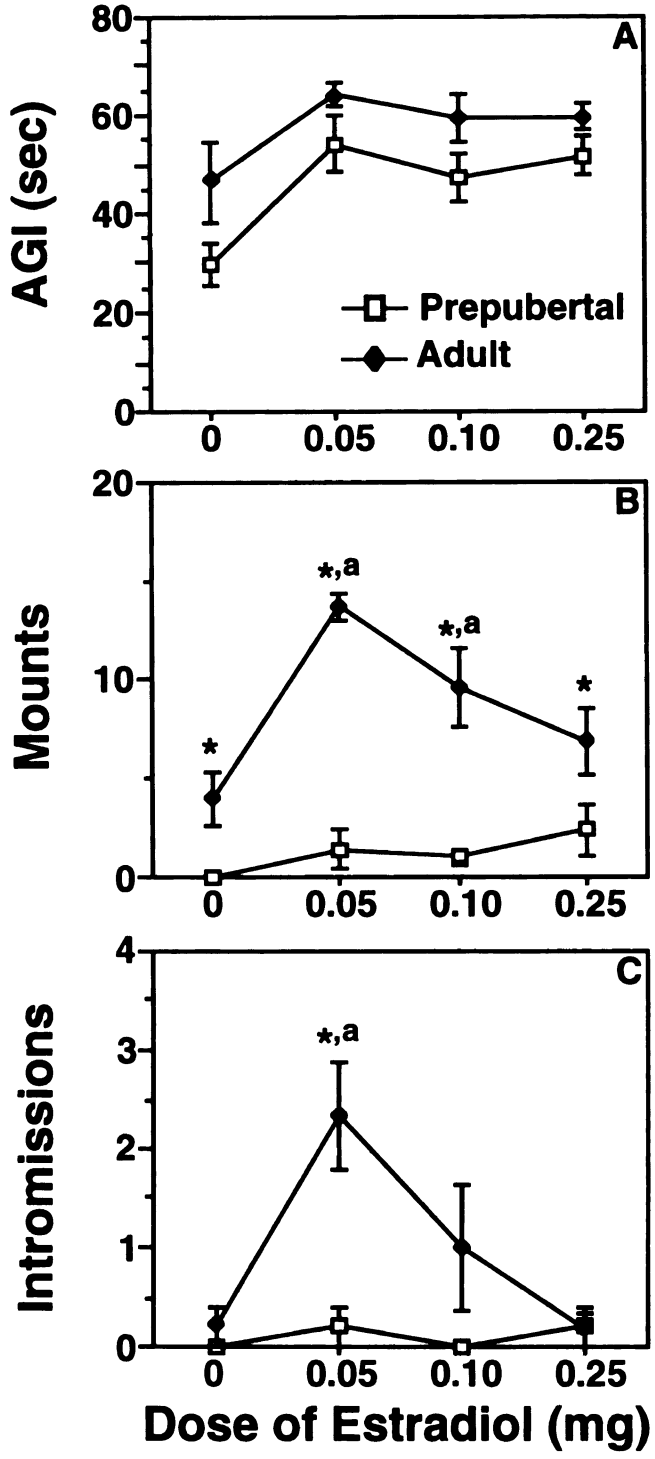

Figure 8. Number of seconds engaged in AGI (A) and the number of mounts (B), and intromissions (C) in prepubertal and adult males implant with a pellet containing either 0, 0.05, 0.10, or 0.25 mg of EB. Asterisks indicate adults are significantly different from their prepubertal counterparts. "a" indicates significantly different from the blank-treated controls within an age. All values are means ± SEM.

the blank control pellet. In contrast, EB treatment had no effect on mounting behavior in prepubertal males.

There was a significant interaction between age and EB treatment on intromissions ($p < 0.05$, Figure 8C). The 0.05 mg dose of EB significantly increased the number of intromissions in adults, but not in prepubertal males. None of the doses of EB significantly increased intromissions in prepubertal males. No ejaculations were observed in any adult or prepubertal group.

ERα-Immunoreactivity

There was a significant interaction between age and estrogen treatment on the number of ERα-ir cells in the MPN. While the number of ERα-ir cells was similar in EB-treated and control adults, prepubertal males treated with the blank pellet of EB had significantly more ERα-ir cells / 62,500μm^2 than EB-treated juveniles ($p < 0.05$, Figure 9). Photomicrographs in Figure 10 show ERα-ir in the MPN of a prepubertal and adult male treated with 0.05 mg of EB. No other main effects or significant interactions on the number of ERα-ir cells were found in any of the other nuclei analyzed.

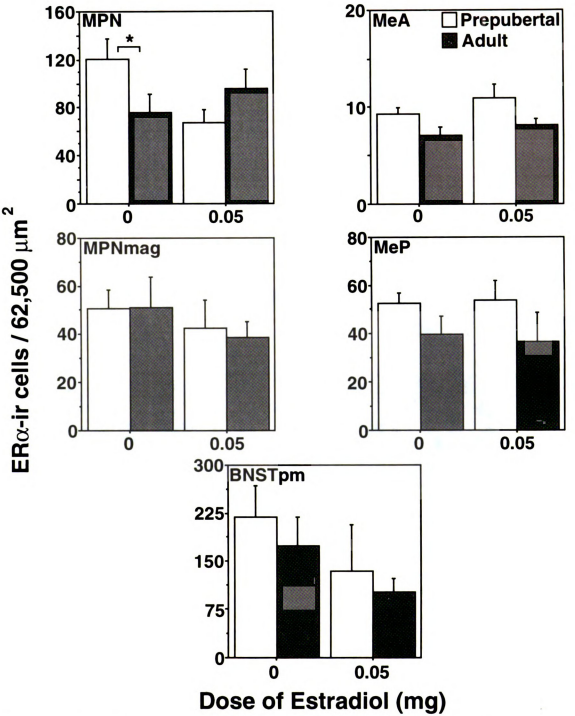

Figure 9. The number of ERα-ir cells/62,500 μm^2 in the MPN, MPNmag, BNSTpm, MeA, and MeP of pubertal and adult males implanted with a pellet containing either 0 or 0.05 mg of EB. Asterisk indicates that castrated prepubertal males have a significantly greater number of ERα-ir cells compared to the castrated adults. All values are means ± SEM.

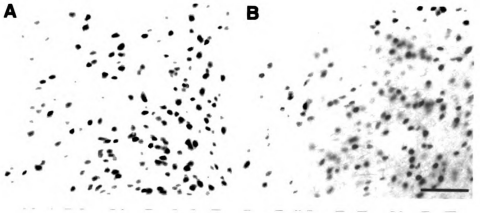

Figure 10. ERα-ir in the MPN of a prepubertal (A) and adult (B) male treated with 0.05 mg of EB. Bar, 50μm.

Discussion

These data demonstrate that puberty is associated with a change in behavioral responsiveness to estrogen. In the adults, the fact that EB activated mounts and intromissions, but not ejaculations, is in agreement with previous reports (DeBold and Clemens, 1978; Floody and Petropoulos, 1987). In stark contrast to the adults, EB was unable to activate either of these behaviors in prepubertal males even though levels of E were similar to those of the EB-treated adults. Thus, these behavioral data indicate that adult males are more responsive than prepubertal males to the activating effects of E on male mating behavior. This reduced behavioral response to EB in prepubertal males is not associated with a lack of ERα, since ERα-ir is similar in EB-treated juvenile and adults within cell groups in the neural circuit that mediate reproductive behavior.

Contrary to mounting and intromissive behavior, EB was able to activate AGI in the prepubertal males, albeit to a lesser degree than adults. Pheromonal stimulation received by the male during AGI of the female is essential for the display of subsequent mating behavior in this species (Wood, 1998; Wood and Newman, 1995c). Therefore, similar to the data obtain with DHT-treated males, pheromonal stimulation received by EB-treated males may be necessary, but not sufficient, to activate the full suite of mating behaviors. Thus, the absence of mating behavior in the EB-treated prepubertal males may be the result of inappropriate

integration of chemosensory cues in the juvenile brain, which in turn does not allow the proper coordination of further reproductive behaviors.

The estrogen-dependent increase in AGI in both prepubertal and adult males was unexpected, since interest in female pheromones has been reported to be under androgenic regulation (Gregory et al., 1975; Powers and Bergondy, 1983). However, a recent study showed that male ERα-knockout mice engage in less AGI than wild-type mice, suggesting that estrogen plays a role in mediating this behavior (Wersinger and Rissman, 2000). Taken together, these data indicate that estrogen has a significant influence on the amount of time a male will spend anogenitally investigating an estrous female and that EB can activate this behavior prior to puberty.

The number of ERα-ir cells in the MPNmag, BNSTpm, MeA, and MeP was not significantly affected by EB treatment or age, indicating that the reduced behavioral responsiveness to EB in prepubertal males is not associated with reduced levels of ER in these brain regions. EB treatment did reduce the number of ERα-ir cells in the MPN of castrated prepubertal males. However, this reduction in ERα-ir cells can not account for the reduced behavioral responsiveness to EB exhibited by the juvenile males since the EB-treated adult males, which do engage in mating behavior, have equivalent numbers of ERα cells in the MPN as the EB-treated prepubertal

males. These ERs appear to be functional in the prepubertal male since EB treatment was capable of suppressing LH secretion. However, the brain regions responsible for steroid negative feedback on the hypothalamic-pituitary-gonadal axis are not known, and the possibility remains that the ERs present specifically in the steroid-sensitive mating circuit may be functioning differently prior to pubertal development.

Interestingly, the lowest dose of EB used in this study was the most effective in facilitating mating behavior in adults. We reported a similar finding with T treatment, in that a high dose was less effective at facilitating adult mating behavior than a low dose (Meek et al., 1997). This low dose produced circulating T levels equivalent to those found in intact adult males. The animals treated with the highest doses of steroid in this and our previous study did not appear ill or agitated. Thus, we do not suspect that high steroid levels reduce behavior indirectly by compromising normal physical capabilities. Instead, high levels of steroid may inhibit neuropeptides that modulate reproductive behavior. For example, LHRH (luteinizing hormone-releasing hormone) facilitates the display of mating behavior in both males and females (Beyer, Gonzalez-Flores, and Gonzalez-Mariscal, 1997; Fernandez-Fewell and Meredith, 1995; Meredith and Howard, 1992; Pfaff, 1982). Since high doses of steroid inhibit the secretion of LHRH, they may result in a reduction in behavior through inhibition of LHRH.

Further experiments will be needed to explore this possibility.

In summary, we have established that prepubertal males are less responsive than adults to the activational effects of EB on reproductive behavior, and that this reduced behavioral response is not associated with a lack of ERs in the neural circuit that mediates male mating behavior.

Summary

Experiments I and II of this section clearly demonstrate that the lack of reproductive behavior exhibited by T-treated prepubertal males is not due to the relative lack of local estrogenic stimulation of the mating circuit. Furthermore, these experiments show that this steroid-sensitive circuit has ample ERs for the locally produced, or exogenously administered, E to act upon. Thus, taken together with the DHT experiment, we can conclude that the prepubertal nervous system does not produce a behavioral response to either the estrogenic or androgenic components of T's effects on sexual behavior.

Section III. Contribution of progesterone and progesterone receptors in the pubertal maturation of male sexual behavior.

Experiment I. Estrogenic regulation of the progesterone receptor in prepubertal and adult males.

Rationale

We have shown that prepubertal males have similar levels of ERα-ir throughout the mating circuit compared to adult males. Although prepubertal males have as many ERα positive cells as adults, the question still remains as to whether the receptors found in the cells of juveniles are functioning in a similar manner to those in adults. To address this question we used progesterone receptor (PR)-immunoreactivity as an index of ERα functionality. One action of an activated ER is to increase transcription of the PR gene, and thus upregulate the PR (Kraus, Montano, and Katzenellenbogen, 1994; MacLusky and McEwen, 1978; Moffatt, Rissman, Shupnik, and Blaustein, 1998; Roy, MacLusky, and McEwen, 1979). Therefore, one would expect that if the ERα present in prepubertal males regulates transcription, then one index of ER activation would be estrogen-induced upregulation of PR. To investigate this possibility, prepubertal and adult males were treated with estrogen and their brains were processed

for PR-immunoreactivity. It was predicted that if the ERα was not functioning prior to puberty, then PR would not be increased in estrogen-treated prepubertal males.

Circulating plasma levels of progesterone increase significantly during pubertal development in intact male hamsters (Vomachka and Greenwald, 1979). Moreover, progesterone facilitates reproductive behavior in adult males (Crews et al., 1996; Lindzey and Crews, 1988; Lindzey and Crews, 1992; Witt et al., 1994; Witt et al., 1995; Young et al., 1991). Thus, we measured plasma progesterone levels in the present study to investigate whether estrogen-treated prepubertal and adult castrates differ on this variable.

Methods

Subjects and Treatment

Male hamsters used in this study were bred at Michigan State University (East Lansing, MI), and housed and cared for as described previously (Subjects and Treatment, Section I).

All animals were castrated under methoxyflurane anesthesia at either 21 or 70 days of age and implanted with a 3-week timed-release pellet (Innovative Research of America, Sarasota, FL) containing either 0, 0.05, 0.10, or 0.25 mg of EB (n=6 per age and treatment group).

Tissue Collection

One week after castration and implantation (i.e., at either 28 or 77 days of age), animals were perfused and

brains were collected and sectioned as described in Section I (Tissue Collection). Blood samples were obtained via cardiac puncture prior to the perfusion.

Progesterone Receptor Immunocytochemistry

Immunocytochemistry was performed on free-floating sections using a rabbit polyclonal antibody (DAKO) directed against the DNA binding domain of the human PR. This antibody detects both the A and B isoforms of PR (Traish and Wotiz, 1990). All incubations were performed at room temperature unless otherwise indicated. Free-floating sections were rinsed in 0.5M Tris buffered saline (TBS) three times to remove the cryoprotectant. Sections were then incubated in 1% sodium borohydride in TBS for 10 min, rinsed in TBS four times for 5 min each and incubated in TBS containing 20% NGS, 1% H_2O_2, and 1% BSA for 20 min. PR antiserum was diluted to a concentration of either 1:1000 or 1:5000 in TBS containing 2% NGS, and 0.3% TX (Tritrigo) for 65 hr at 4°C. These concentrations of primary antibody were chosen based on an earlier pilot study. Specifically, we established that a primary antibody concentration of 1:1000 provided very dark, specific staining, while a concentration of 1:5000 provided lighter staining, such that the number of stained cells and the optical density of those cells fell near the middle of the dilution curve (Figure 11). The more dilute concentration of primary antibody was used to investigate whether reducing the staining intensity would

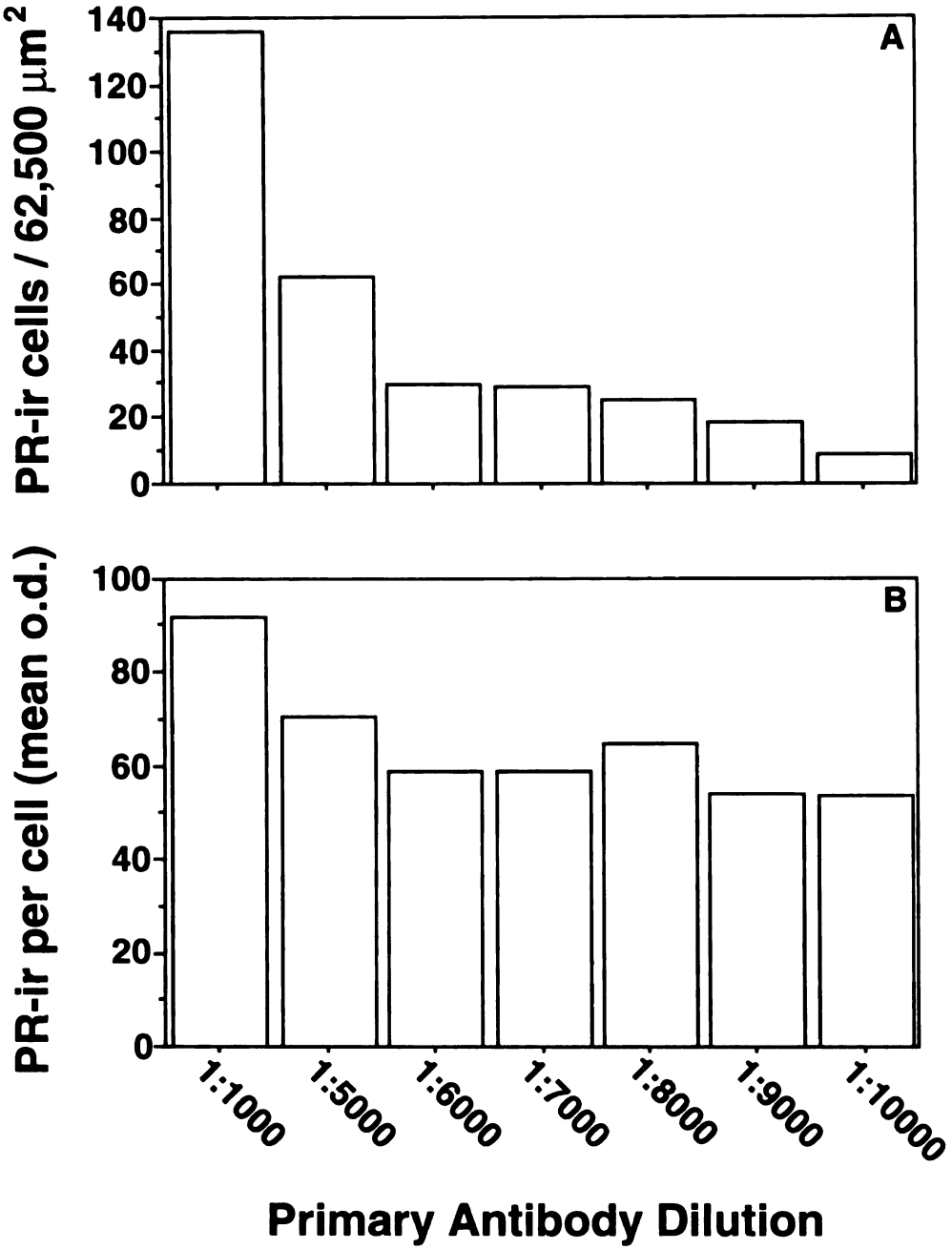

Figure 11. The number of PR-ir cells / 62,500 μm^2 (A) and relative amount of PR-ir per cell (mean o.d.; B) in sections from the MPN exposed to different dilutions of primary antibody. Tissue was from adult castrates treated with a 0.05 mg pellet of EB for one week.

allow us to detect differences between the groups that may have been occluded by the maximally stained tissue in the 1:1000 primary antibody protocol. Following the primary antibody incubation and 3 rinses in Tritrigo, the sections were incubated for 90 min in biotinylated goat anti-rabbit IgG (Vector Laboratories) at a concentration of 5µg/ml in Tritrigo. After 2 rinses in Tritrigo and 2 rinses in TBS, the sections were incubated in avidin-biotin horseradish peroxidase complex for 1 hr (1:100; Vectastain ABC Elite Kit). After 3 rinses in TBS, the sections were incubated in phosphate buffer containing 0.012% DAB, 0.018% $NiCl_2$, and 0.075% H_2O_2 for 10 min. The reaction was terminated by 3 rinses in TBS. Sections were mounted on gelatin-coated slides, dried, dehydrated, cleared in xylenes and coverslipped. To test for nonspecific staining, brain sections were processed as described above following omission of primary antiserum and/or secondary antiserum. Omission of the primary and/or secondary antiserum eliminated all detectable PR-immunoreactivity.

Analysis of PR Immunoreactivity

The number of immunoreactive cell profiles per unit area (areal density), referred to as number of PR-immunoreactive (PR-ir) cells, was determined in an identical manner described in Section I (Analysis of Androgen Receptor Areal Density). However, initial inspection of the sections from

both prepubertal and adult males at both antibody concentrations revealed analyzable PR-ir only in the MPN, so it was the only area quantified.

In addition to the number of PR-ir cells, the relative amount of PR-ir per cell was determined by measuring the optical density of immunoreactivity in individual cell nuclei. One anatomically matched section through the MPN was chosen for each animal. Microscopic images of the PR-ir cells in the MPN were captured on the computer screen using a 100X oil immersion objective. NIH Image software (W. Rasbaud, National Institutes of Health, Bethesda, MD) was used to analyze these captured images. The optical density of individual cell nuclei of all immunoreactive cells within two 110μm X 75μm sampling field within the MPN was measured. In addition, the optical density of five cell-sized areas of neuropil per sampling field was determined for each animal and averaged as a measurement of background optical density. The optical density for each PR-ir cell was then corrected by subtracting the average background value. The mean corrected optical density of all cells within the sampling fields was calculated for each animal and referred to as the relative amount of PR-ir per cell.

Progesterone Radioimmunoassay

Plasma concentrations of progesterone were measured using the Coat-A-Count Progesterone Kit (Diagnostic Products). This assay has been validated in our laboratory

for the measurement of plasma progesterone concentrations in Syrian hamsters. The lower limit of detectability of the assay was 0.05 ng/ml. The intraassay CV was 11.6%.

Statistical Analysis

The effects of age and EB treatment on plasma progesterone levels and PR-ir were analyzed by two-way ANOVAs (age X dose). Significant main effects were further analyzed with Fisher's PLSD tests. Differences were considered significant when $p < 0.05$.

Results

Hormone Measures

There was a significant main effect of age on plasma progesterone levels ($p < 0.05$, Figure 12), such that adult males had significantly higher plasma progesterone levels than prepubertal males. Estrogen treatment had no effect on progesterone levels in either prepubertal or adult males.

PR Immunocytochemistry

There were significant main effects of treatment for both the number of PR-ir cells and the intensity of PR-ir per cell in the MPN for both antibody concentrations (both $p < 0.05$, Figure 13A and B and Figure 14A and B). Specifically, both prepubertal and adult males treated with EB had significantly more PR-ir cells and more PR-ir per cell than prepubertal and adult males treated with the blank pellet.

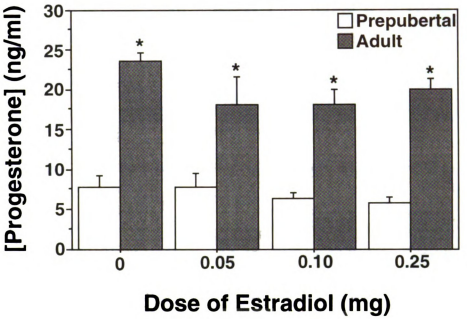

Figure 12. Plasma progesterone concentrations in prepubertal and adult males castrated and implanted with a pellet containing either 0, 0.05, 0.10, or 0.25 mg of estradiol. Asterisks indicate that adults have significantly higher circulating levels of progesterone than prepubertal males. All values are means ± SEM.

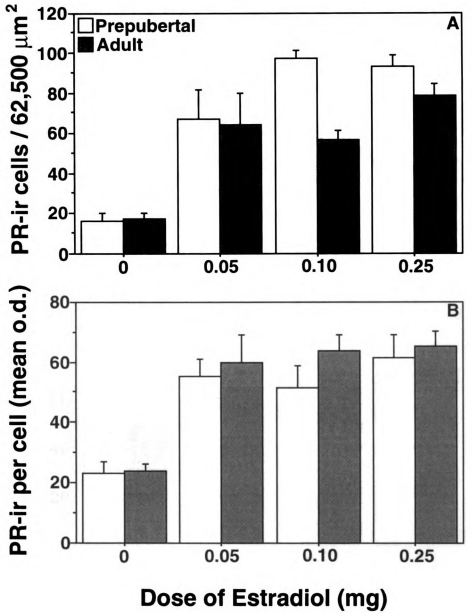

Figure 13. The number of PR-ir cells / 62,500 μm^2 (A) and the relative amount of PR-ir per cell (mean o.d.; B) in the 1:1000 primary antibody concentration in the MPN of prepubertal and adult males that were castrated and implanted with a pellet containing either 0, 0.05, 0.10, or 0.25 mg of estradiol. Males treated with the 0 mg pellet had significantly fewer PR-ir cells and less PR-ir per cell than males treated with either the 0.05, 0.10, or 0.25 mg pellet of estradiol. All values are means $\pm$ SEM.

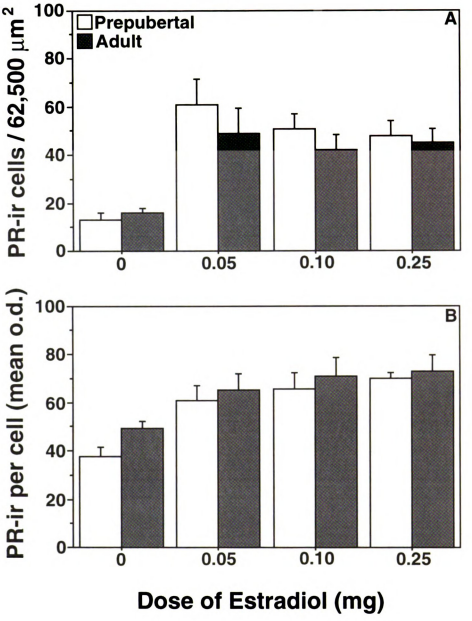

Figure 14. The number of PR-ir cells / 62,500 µm² (A) and the relative amount of PR-ir per cell (mean o.d.; B) in the 1:5000 primary antibody concentration in the MPN of prepubertal and adult males that were castrated and implanted with a pellet containing either 0, 0.05, 0.10, or 0.25 mg of estradiol. Males treated with the 0 mg pellet had significantly fewer PR-ir cells and less PR-ir per cell than males treated with either the 0.05, 0.10, or 0.25 mg pellet of estradiol. All values are means ± SEM.

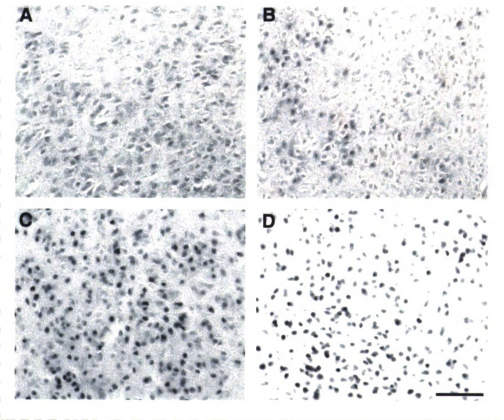

Figure 15. PR-ir in the MPN of a prepubertal (A) and adult (B) male treated with a blank pellet and a prepubertal (C) and adult (D) male treated with a pellet containing 0.05 mg of EB. Sections were from the 1:1000 primary antibody condition. Bar, 50μm.

The photomicrographs in Figure 15 show this similar upregulation of PR-ir in the MPN of EB-treated prepubertal and adult males.

Discussion

These data show that estrogen is capable of upregulating the expression of the PR in the MPN of both prepubertal and adult males, suggesting that the ER is functioning similarly before and after pubertal development. Therefore, the lack of a behavioral response to estrogen prior to puberty cannot be accounted for by estrogen's inability to activate the ER and induce the expression of PR in the MPN of prepubertal males. We cannot, however, rule out the possibly that the ER is functioning differently before and after pubertal development in some capacity other than transcription of the PR gene.

We obtained virtually identical results in the present experiment whether the tissue was incubated in a primary antibody concentration of either 1:1000 or 1:5000. Incubating tissue in a more dilute concentration of primary antibody would result in the reduced ability to detect existing PRs. It appears, therefore, that the lack of a developmental difference found in the 1:1000 condition is not due to a "ceiling effect" caused by the maximal staining of both groups that would not allow any subtle, but significant, effects to be detected. Thus, the second set of data

strengthens the conclusion that estrogen is capable of upregulating PR prior to puberty.

The results from the progesterone assay demonstrate that, regardless of estrogen treatment, prepubertal male castrates have substantially lower plasma progesterone levels than castrated adult males. These data parallel a previous study, which demonstrated a significant pubertal rise in progesterone secretion in intact male hamsters (Vomachka and Greenwald, 1979). Furthermore, these data demonstrate that the source of the pubertal rise in progesterone may be the adrenal glands, because castration did not eliminate the age-related difference in circulating plasma progesterone levels. Since sexual behavior can be facilitated by progesterone in adult males (Crews et al., 1996; Lindzey and Crews, 1988; Lindzey and Crews, 1992; Witt et al., 1994; Witt et al., 1995; Young et al., 1991), it is possible that the low levels of progesterone circulating in the prepubertal male are responsible for the lack of reproductive behavior exhibited by juvenile males.

It is important to note, however, that high progesterone levels alone are insufficient to stimulate mating behavior since castrated adults engage in little reproductive behavior, even though they have relatively high levels of progesterone. Furthermore, high levels of progesterone in the presence of ample PRs (e.g. estrogen-treated males) may be necessary, but not sufficient, to facilitate mating behavior since adult males treated with high doses of EB

engage in significantly less mating behavior than adults treated with lower doses of EB, even though both groups of animals have similar progesterone levels. Taken together, progesterone may facilitate male mating behavior only under certain conditions or hormonal milieus.

Experiment II. The effect of progesterone receptor antagonists on adult male sexual behavior.

Rationale

As mentioned above, activation of the PR is an integral neural event in the facilitation of male reproductive behavior in adult rats, mice and lizards (Crews et al., 1996; Lindzey and Crews, 1988; Lindzey and Crews, 1992; Phelps et al., 1998; Witt et al., 1994; Witt et al., 1995; Young et al., 1991). Furthermore, a pubertal rise in circulating progesterone is temporally associated with the appearance of reproductive behavior (Vomachka and Greenwald, 1979). Therefore, it is possible that progesterone and activation of its receptor are important mediators in the pubertal maturation of reproductive behavior. However, before these questions can be addressed in a pubertal context, a role for the PR in mediating sexual behavior in the adult male hamster needs to be established. Thus, the purpose of the present experiment was to investigate the hypothesis that PR activation is required for full activation of male sexual behavior in the adult hamster, as it is in other species. To

test this hypothesis, two experiments were conducted in which adult males were treated with either the PR antagonist mifepristone (RU486) or onapristone (ZK98299), and tested for their ability to engage in male reproductive behavior.

ZK98299 was used in addition to the more conventional PR antagonist, RU486, because the hamster PR has a cysteine in position 575 of the hormone binding domain, instead of a glycine (Benhamou, Garcia, Lerouge, Vergezac, Gofflo, Bigogne, Chambon, and Gronemeyer, 1992). Remarkably, this single amino acid switch causes RU486 to bind poorly to the PR (Gray and Leavitt, 1987; Okulicz, 1987). ZK98299, however, does not interact with the PR hormone binding domain, but instead inhibits the stable binding of the PR to the DNA (Gass, Leonhardt, Nordeen, and Edwards, 1998; Nath, Bhakta, and Moudgil, 1992), and thus, may be a more effective PR antagonist in the Syrian hamster.

Methods

Subjects and Treatment

Adult male hamsters used in this experiment were obtained from Charles River (Kingston, NY). Animals were housed and cared for as described previously (Subjects and Treatment, Section I).

In Experiment 1, sexually naive adult males were weighed at 1000 h and given a single injection of RU486 (2 mg/kg, *ip*) or the sesame oil vehicle (n=8). Two to four hr after lights out (4-6 hr after the injection), all animals were given a

15-min mating behavior test with a hormone primed estrous female as described in Section I (Tests for Male Reproductive Behavior). This dose and time point were chosen based on previous studies investigating the effects of RU486 on male mating behavior (Witt et al., 1994; Witt et al., 1995; Romeo, unpublished observation).

In Experiment 2, sexually naive adult males were weighed and given a single subcutaneous injection of ZK98299 (6 mg/kg) or the sesame oil vehicle (n=6; injection given at 1000 hr). Two to four hr after lights out (4-6 hr after the injection), all animals were given a 15-min mating behavior test with a hormone primed estrous female as described in Section I (Tests for Male Reproductive Behavior). This dose and time point were chosen based on a previous study in which ZK98299 was reported to block the LH surge in female rats (Chappell and Levine, 2000). Presently, there are no reports in the literature that have used this compound in a behavioral context.

Statistical Analysis

In Experiments 1 and 2, the effects of RU486 or ZK98299 on male sexual behavior were analyzed by *t* tests. Differences were considered significant when $p < 0.05$.

Results

Experiment 1

RU486-treated males engaged in 88.1±12.0 seconds of AGI, while males treated with the oil vehicle engaged in 85.4±9.5 seconds of AGI. Thus, RU486 did not significantly affect the amount of time males spent in anogenital investigation of the female. RU486-treated males did, however, engage in significantly fewer mounts than oil-treated males ($p < 0.05$, Figure 16). No other measures of reproductive behavior differed significantly between the two groups.

Experiment 2

Similar to Experiment 1, the amount of time males engaged in AGI was not significantly affected by treatment. Specifically, ZK98299-treated males spent 118.8±15.4 seconds engaged in AGI, while vehicle-treated controls spent 128.0±12.9 seconds. ZK98299 did significantly reduce the number of mounts exhibited by adult males ($p < 0.05$, Figure 17). Similar to RU486, ZK98299 reduced mounting behavior by roughly 50%. No other behaviors measured were significantly affected by the drug.

Discussion

These data demonstrate that PR activation contributes to the full expression of male sexual behavior in the adult Syrian hamster. Although mounting was the only behavior

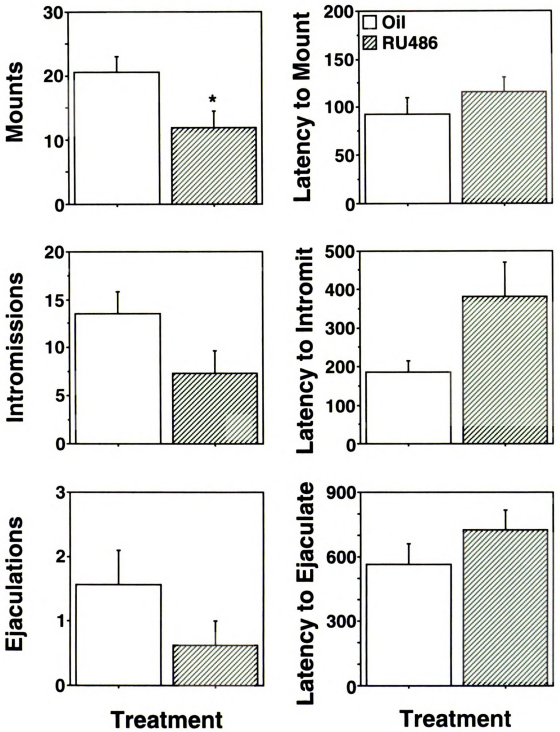

Figure 16. The frequencies and latencies (sec) of mounts, intromissions, and ejaculations in adult males treated with RU486 (2 mg/kg) or the oil vehicle. Asterisk indicates a significant difference. All values are means ± SEM.

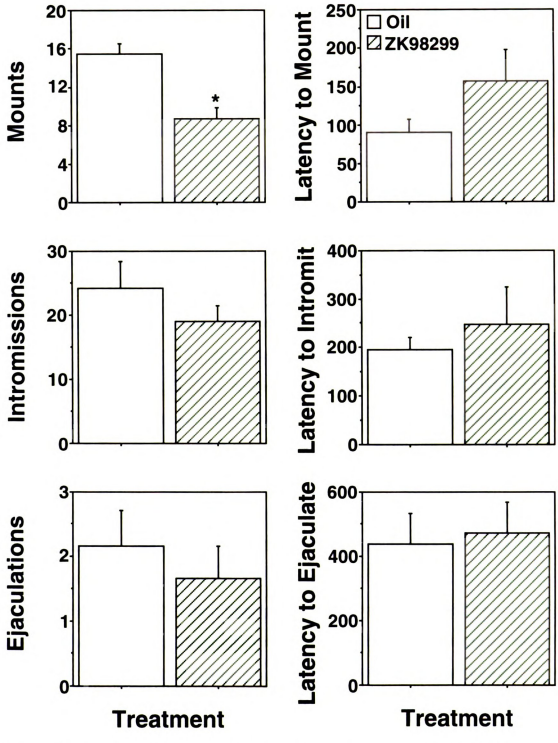

Figure 17. The frequencies and latencies (sec) of mounts, intromissions, and ejaculations in adult males treated with ZK98299 (6 mg/kg) or the oil vehicle. Asterisk indicates a significant difference. All values are means ± SEM.

significantly affected by RU486 or ZK98299, all other behavioral measures showed trends such that RU486- or ZK98299-treated males performed more poorly than their oil-treated controls. These results are in agreement with previous studies that have shown that the PR plays an important role in the reproductive behavior of adult male rats, mice, and lizards (Crews et al., 1996; Lindzey and Crews, 1988; Lindzey and Crews, 1992; Phelps et al., 1998; Witt et al., 1994; Witt et al., 1995; Young et al., 1991). However, the behavioral effects of blocking PR in the hamster are not as robust as those seen in the other species mentioned above. It is possible that higher doses of RU486 or ZK98299 or a different time course would cause a greater deficit in male mating behavior, as seen in other species. In hamsters, however, doubling the dose of RU486 does not decrease their behavior below that observed in the present experiment (Romeo, unpublished observation). Unfortunately, due to limited availability of ZK98299, only one dose of the antagonist could be tested in the present study.

We can not rule out the possibility that ZK98299, although not acting through the hormone binding domain, may still not completely block PR activation in the hamster. However, ZK98299 has been shown to inhibit progesterone-stimulated fibronectin secretion from chicken granulosa cells (Asem and Conkright, 1995; Conkright and Asem, 1995; but see, Moudgil, Nath, Bhakta, and Nakao, 1991). Since chickens, like hamsters, share the single amino acid switch in the

hormone binding domain (Benhamou et al., 1992), these studies together with the present data suggest that this PR antagonist is capable of at least partially blocking PR activation in animals that have the cysteine substitution in the hormone binding domain. Paradoxically, RU486 was able to significantly block mounting behavior in adult male hamsters even though RU486 has been reported to be a poor PR antagonist in hamsters (Gray and Leavitt, 1987; Okulicz, 1987). These studies used cytosolic uterine PR in female hamsters as the substrate for RU486, and the possibility remains that male and female and/or brain and uterine PR are subtly different. It should be noted that RU486 administered to hormonally-primed estrous female hamsters does not block lordosis (Romeo, unpublished observation), a female receptive posture mediated by PR activation (Mani, Blaustein, Allen, Law, O'Malley, and Clark, 1994b; Moguilewsky and Raynaud, 1979; Ogawa, Olazábal, Parhar, and Pfaff, 1994; Parsons, MacLusky, Krey, Pfaff, and McEwen, 1980). RU486 does bind to and antagonize the glucocorticoid receptor (GR; Moguilewsky and Philibert, 1985), albeit with half of the relative binding affinity RU-486 has for the PR. In contrast, ZK98299 binds the GR with orders of magnitude less affinity than RU486 (Koper, Molijn, van Uffelen, Stigter, and Lamberts, 1997). Thus, it is possible that the significant reduction in mounting behavior of RU486-treated males may be through RU486's interactions with the GR, while ZK98299's effects may be more specifically through the PR. Taken together,

although RU486 was effective in reducing mounting behavior in male hamsters, it is not presently understood through what mechanism this effect is mediated.

In summary, these data suggest that PR activation contributes to the display of male mating behavior in adult hamsters. Thus, it is possible that the lack of reproductive behavior exhibited by prepubertal males may be, at least in part, the result of inadequate PR stimulation, as the previous experiment demonstrated significantly lower levels of circulating progesterone in juvenile compared to adult males.

Experiment III. The sexual behavior of prepubertal males treated with estrogen and progesterone.

Rationale

We have shown PR upregulation in the MPN is similar in estrogen-treated prepubertal and adult males, suggesting that the ER is functional prior to puberty. It should be noted that T is also capable of upregulating the expression of PR in the MPN of prepubertal males (Romeo, unpublished observation), most likely through T's conversion to estradiol. It has been demonstrated that estrogen-treated prepubertal males engaged in less mating behavior and have significantly lower circulating levels of progesterone compared to estrogen-treated adults. This developmental difference in plasma progesterone levels was not affected by

estrogen treatment. Since PR activation appears to contribute to the facilitation of male mating behavior in adults, it is possible that the lack of reproductive behavior in prepubertal males is mediated by the relatively low levels of progesterone at this developmental stage, and hence, reduced PR activation.

The present set of experiments had two objectives. First, we needed to established what dose of exogenous progesterone given to juvenile males would provide circulating adult-like levels of progesterone. The second was to tested the hypothesis that adult-like levels of progesterone will facilitate the display of mating behavior in estrogen-treated prepubertal males.

Methods

Subjects and Treatment

Prepubertal male hamsters used in these experiments were obtained from Charles River (Kingston, NY). Animals arrived at 18 days of age with their mothers. At 21 days of age, all animals were weaned from their mothers, weighed, and treated as described below. Animals were housed and cared for as described previously (Subjects and Treatment, Section I).

In Experiment 1, animals were castrated under isoflurane anesthesia and implanted with a pellet containing either 0, 0.25, 0.50, 1.5, 2.5, 5.0, 7.5, 10, 15, 25, or 35 mg of progesterone (n=4-7). One week after castration and implantation (i.e., at 28 days of age), animals were weighed

and trunk blood samples were collected. Plasma samples were stored at -20°C until the progesterone assay was performed (see below). Plasma samples from sexually behaving adult males that had been castrated and treated with a pellet containing 0.05 mg of EB were run in the same progesterone assay as the samples obtained from the progesterone-treated prepubertal males. These samples were included in the assay to establish what dose of progesterone provides a prepubertal male with progesterone levels similar to adult males, which have equivalent levels of PR in the MPN as estrogen-treated juvenile males, but engage in greater amounts of mating behavior than estrogen-treated juvenile males.

In Experiment 2, animals were castrated under isoflurane anesthesia and implanted with a pellet containing 0.05 mg of EB. This dose of EB was chosen since it was the most effective dose at activating reproductive behavior in adult males (Section II, Experiment 2) and has been shown to induce similar levels of PR-ir in the MPN of prepubertal and adult males (Section III, Experiment 1). In addition to the EB pellet, half the animals received a pellet containing either 15 mg of progesterone or a blank pellet (n=6). This dose of progesterone was chosen based on the results obtained in Experiment 1 (see Results below). One week after castration and implantation (i.e., at 28 days of age) all animals were given a 15-min mating behavior test two to four hr into their dark cycle with a hormone primed estrous female as described in Section I (Tests for Male Reproductive Behavior).

Progesterone Radioimmunoassay

Plasma concentrations of progesterone were measured using the Coat-A-Count Progesterone Kit (Diagnostic Products). The lower limit of detectability of the assay was 0.06 ng/ml. The intraassay CV was 10%.

Statistical Analysis

In Experiment 1, plasma levels of progesterone were analyzed by a one-way ANOVA and significant differences were probed with Fisher's PLSD. In Experiment 2, all behavioral measures were analyzed by *t* tests. Differences were considered significant when $p < 0.05$.

Results

Experiment 1: Plasma progesterone levels in progesterone-treated prepubertal males

The one-way ANOVA revealed a significant effect of progesterone treatment on plasma progesterone levels (Figure 18). Posthoc testes revealed that plasma progesterone levels were not significantly elevated above that of blank-treated controls until animals received either the 10, 15, 25, or 35 mg pellet of progesterone. More importantly, these data show that the 15 mg pellet results in plasma progesterone concentrations that most closely resemble the circulating levels of progesterone in sexually behaving adult castrates treated with 0.05 mg of EB (Figure 18).

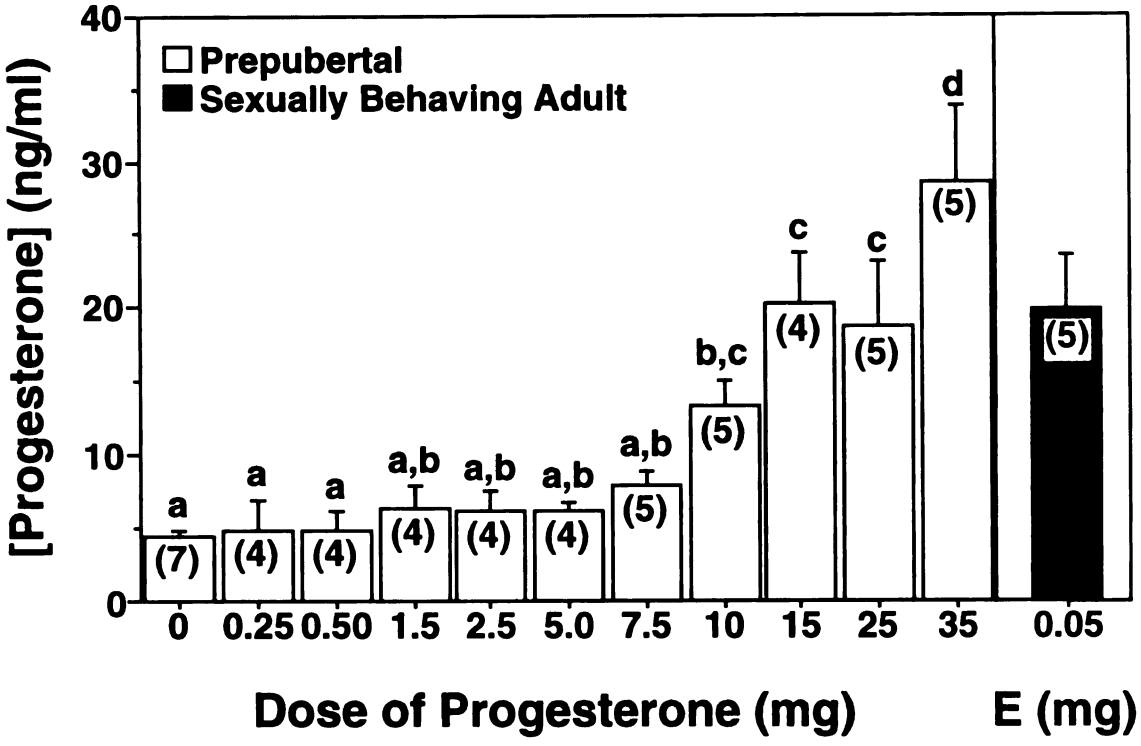

Figure 18. Plasma progesterone (ng/ml) in castrated prepubertal males treated with either a 0, 0.25, 0.50, 1.5, 2.5, 5.0, 7.5, 10, 15, 25, or 35 mg pellet of progesterone for one week. Bars that share a letter are not significantly different from each other. Numbers in the parentheses are the number of animals that comprise that mean. The gray bar represents the average plasma progesterone level in a sexually behaving castrated adult male implanted with a 0.05 mg pellet of estradiol (E). All values are means ± SEM.

Experiment 2: Mating behavior of estrogen- and progesterone-treated prepubertal males

There were no significant differences in any aspect of mating behavior measured. That is, prepubertal males treated with EB and progesterone engaged in equivalent levels of AGI and mounting as prepubertal males treated with EB only (Table 4). Neither group of males engaged in any intromissive or ejaculatory behavior. There was also no significant difference between either group of males in their latency to mount (data not shown).

Discussion

Taken together, these data demonstrated that mating behavior is not activated in prepubertal males treated with EB and supplemented with progesterone. Thus, even when PR and plasma progesterone levels are equated between prepubertal and adult males, prepubertal males still engage in relatively little reproductive behavior. It appears, therefore, that activation of the PR by progesterone is not the rate limiting factor in the pubertal maturation of male reproductive behavior.

It is interesting to note that progesterone is not the only ligand to activate the PR. Dopamine and LHRH have been implicated in the activation of the PR (Mani, Allen, Clark, Blaustein, and O'Malley, 1994a, and Beyer et al., 1997, respectively) and facilitation of sexual behavior in adults (Bitran and Hull, 1987; Mani et al., 1994a; Melis and

TABLE 4. Mean (± SEM) duration of AGI and the frequency of mounts.

Age (days)-treatment	AGI (sec)	Mounts
28-EB+Blank	44.5±7.2	0.3±0.2
28-EB+Progesterone	41.3±4.9	0.7±0.4

Argiolas, 1995, and Beyer et al., 1997; Fernandez-Fewell and Meredith, 1995, respectively). Thus, it is possible that the ligand-independent activation of PR, either by dopamine or LHRH, may contribute to the pubertal maturation of male sexual behavior. Experiments to address these questions are currently in progress.

Summary

Experiment I of this section showed that prepubertal males treated with EB have an equivalent increase in PR expression in the MPN as EB-treated adults. Since one action of an activated ER is to increase transcription of the PR gene, these data suggest that the ER is functional prior to puberty. Also in Experiment I, it was shown that adult males have higher circulating levels of progesterone compared to prepubertal males, regardless of EB treatment. As demonstrated in Experiment II, activation of the PR contributes to the display of sexual behavior in the adult male Syrian hamster. Thus, it was reasoned that although estrogen treatment of prepubertal males upregulates their PR expression in the MPN to a similar level as adults, these PRs in the juvenile's hypothalamus may not be activated because of the relatively low levels of circulating progesterone at this age. However, as was shown in Experiment III, even when PR and plasma progesterone levels are equated between prepubertal and adult males, prepubertal males still do not engage in mating behavior. In conclusion, it appears that

activation of the PR by progesterone is not the rate limiting factor in the pubertal maturation of male reproductive behavior.

Section IV. Contribution of chemosensory cues in the pubertal maturation of male sexual behavior.

In Experiments in Sections I, II, and III, we have demonstrated that prepubertal males are unresponsive to the behaviorally activating effects of both the androgenic and estrogenic components of T, and progesterone. Furthermore, the unresponsiveness to these steroid hormones is not due to a lack of their respective receptors within the neural circuit that mediates sexual behavior. Finally, the ARs and ERαs in the mating circuit appear to be functional. Taken together, it appears that the lack of mating behavior exhibited by prepubertal males must be mediated by processes other than the availability of these steroids, the absence of their receptors, or the receptors' functionality. Thus, this last research chapter will focus on the role that pheromones may play in mediating the pubertal maturation of male mating behavior.

Rationale

As mentioned in the Introduction, full expression of male reproductive behavior in the Syrian hamster is dependent on both pheromonal cues from the female and the presence of gonadal steroids (Meisel and Sachs, 1994; Wood and Newman, 1995b). The pheromones are contained within female hamster vaginal secretions (VS) and stimulate the male vomeronasal system (Fernandez-Fewell and Meredith, 1994), leading to

increased AGI and mounting by the male (Darby, Devor, and Chorover, 1975). The male's interest in VS is increased by the presence of circulating androgens (Powers and Bergondy, 1983). We have demonstrated that chemoinvestigation of the female is stimulated in castrated adult and prepubertal males treated with T, DHT, or E. However, the steroid-treated adults display more mounts and intromissions than the prepubertal males. These results suggest that, despite similar interest in the female, steroid-treated adult and juvenile males may process the chemosensory information from the female differently.

Adult male ferrets (Wersinger and Baum, 1997), hamsters (Fernandez-Fewell and Meredith, 1994; Fiber et al., 1993; Fiber and Swann, 1996; Kollack-Walker and Newman, 1997; Swann and Fiber, 1997), and rats (Bakker, Baum, and Slob, 1996; Bressler and Baum, 1996) respond to chemosensory cues from an estrous female with increased expression of the immediate-early gene product Fos within various forebrain nuclei, which is indicative of increased neuronal activity in these areas (Morgan and Curran, 1991). When adult male hamsters are exposed to VS, cells within subdivisions of the MeAMY, BNST, and MPN express Fos (Fernandez-Fewell and Meredith, 1994; Fiber et al., 1993; Fiber and Swann, 1996; Kollack-Walker and Newman, 1997; Swann and Fiber, 1997). These data suggest that VS causes an increase in neuronal activity in brain regions that mediate chemosensory processing and male sexual behavior in the adult male hamster. In the present

experiment, we investigated the influence of sexual maturity on Fos production in response to VS in the male hamster. We tested the hypothesis that the different amount of reproductive behavior observed in prepubertal and adult males is the result of a pubertal change in the processing of chemosensory information, which leads to a greater degree of VS-induced neuronal activation within the behavioral circuit in adults compared to juveniles. This hypothesis predicted that the Fos response to VS will be greater in adults than in juvenile males.

Methods

Subjects and Treatment

Twelve weanling (21 days of age) and twelve adult (80 days of age) male hamsters were obtained from Charles River (Kingston, NY). All animals were sexually naive, and housed and cared for as described previously (Subjects and Treatment, Section I), except that the vivarium light-dark cycle was 14 hr light/10 hr dark (lights on at 0600 hr EST).

After a one week acclimation period, half of the animals in each age group were given either a clean cotton swab or a cotton swab containing VS. Thus, there were four treatment groups (n=6): (i) 28-day-old prepubertal males exposed to a clean swab, (ii) 28-day-old prepubertal males exposed to a VS swab, (iii) 87-day-old adult males exposed to a clean swab, and (iv) 87-day-old adult males exposed to a VS swab. The VS was collected onto the swab immediately before the test from

naturally cycling females on the day of estrus. The swabs were given to all animals in their home cage in the light phase (between 1300 and 1700 hr EST) of their light-dark schedule. All animals (control and VS) were observed to place the cotton swab in their cheek pouch. Therefore, the only difference between the control and VS-exposed animals is that the animals receiving the swab with VS presumably were able to deliver VS to their vomeronasal organ.

Tissue Collection

One hour after the introduction of the swab into the home cage, animals were perfused and brains were collected and sectioned as described in Section I (Tissue Collection). Blood samples were obtained via cardiac puncture prior to the perfusion.

Fos Immunocytochemistry

Every fourth section from each brain was processed simultaneously during a single immunocytochemical procedure. Free-floating sections were washed 3 times for 5 min in TBS, and incubated in rabbit anti *c*-fos (diluted 1:1000 in TBS with 0.25% TX; a polyclonal antibody raised in rabbit against amino acids 3-16 of *c*-fos p62 of human origin; Santa Cruz, lot #B245, Santa Cruz, CA) for 48 hr at 4°C. Subsequently, sections were incubated in biotinylated goat anti-rabbit IgG (diluted in 1:1000 in TBS with 0.25% TX; Vector) and avidin-biotin horseradish peroxidase complex (Vectastain ABC Kit),

each for 1 hr at room temperature. Horseradish peroxidase was visualized with a 0.0125% DAB solution containing 0.06% hydrogen peroxide with 0.015% nickel chloride in TBS for 5 min. Sections were mounted on gelatin-coated slides, dried, dehydrated, cleared in xylenes and coverslipped. To test for nonspecific staining, brain sections were processed as described above following omission of primary antiserum and/or secondary antiserum. Omission of the primary and/or secondary antiserum eliminated all detectable Fos-immunoreactivity.

Analysis of Fos Areal Density

The number of immunoreactive cell profiles per unit area (areal density), referred to as number of Fos-immunoreactive (Fos-ir) cells, was determined in an identical manner and for the same brain regions described in Section I (Analysis of Androgen Receptor Areal Density). However, in addition to the mating circuit, the lateral septum (LSept) was analyzed as a control nucleus to demonstrate brain region specificity for the Fos response to VS, since this nucleus does not respond to pheromones with an increase in Fos expression (Kollack-Walker and Newman, 1997).

Testosterone Radioimmunoassay

Plasma T concentrations were measured using the Coat-A-Count Total Testosterone Kit (Diagnostic Products). The lower

limit of detectability of the assay was 0.08 ng/ml. The intraassay CV was 7.7%.

Statistical Analysis

Two-way ANOVAs (age X treatment) were used to analyze all data. Significant main effects were probed using Fisher's PLSD tests. Differences were considered significant when $p < 0.05$. All data are presented as mean $\pm$ SEM.

Results

Peripheral Measures

Regardless of treatment condition, adult males had significantly heavier seminal vesicles and testes (both $p < 0.05$, Table 5), and significantly higher plasma T concentrations ($p < 0.05$, Figure 19) compared to the prepubertal animals. An interaction between age and treatment on plasma T concentrations approached significance ($p = 0.06$), such that the adult males exposed to VS tended to show an elevation in T secretion while prepubertal animals exposed to VS did not. Indeed, when *t* tests were conducted within the two ages, VS-exposed adults had significantly higher plasma T concentrations than the adults exposed to a clean cotton swab ($p < 0.05$), while exposure to VS did not significantly alter plasma T concentrations of prepubertal animals (Figure 19).

TABLE 5. Mean (± SEM) seminal vesicle and paired testis weight.

Age (days)-condition	Seminal vesicles (g)	Paired Testes (g)
28-Control	0.078 ± 0.004	1.035 ± 0.066
28-VS	0.058 ± 0.007	0.869 ± 0.061
87-Control	0.239 ± 0.015	2.105 ± 0.214
87-VS	0.284 ± 0.025	2.293 ± 0.141

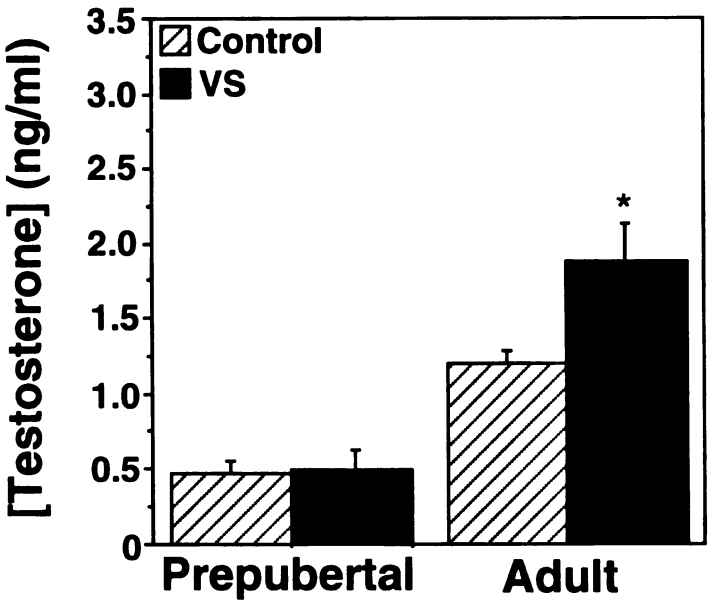

Figure 19. Plasma testosterone concentrations in prepubertal and adult males exposed to VS or a clean cotton swab. Asterisk indicates adult males exposed to VS had significantly higher circulating levels of testosterone than the adults exposed to a clean cotton swab (*t* tests). All values are means ± SEM.

Fos-Immunoreactivity

There was a significant main effect of treatment on the number of Fos-ir cells in the BNSTpm, MPNmag, MPN, and MeP (all $p < 0.05$, Figure 20), but no effect of age and no interaction in these areas. Specifically, there was a greater number of Fos-ir cells in the BNSTpm, MPNmag, MPN, and MeP in animals that were exposed to VS compared with those that were exposed to a clean cotton swab. The greatest increase in Fos-ir cells was in the BNSTpm where VS exposure led to approximately a three-fold increase in the density of Fos-ir cells (Figure 21A-D). In the MPNmag, MPN, and MeP, VS exposure led to approximately a 1.5- to 2-fold increase in Fos-ir cells. There was no effect of age or treatment on the number of Fos-ir cells in either the MeA or the LSept of prepubertal and adult males (Figure 20).

Discussion

Compared to animals exposed to a clean cotton swab, both prepubertal and adult males exposed to VS have a greater amount of Fos-immunoreactivity in the BNSTpm, MPNmag, MPN, and MeP, all of which are essential for chemosensory processing and male sexual behavior (Wood, 1998; Wood and Newman, 1995b; Wood and Newman, 1995c). Furthermore, the Fos response to VS is equivalent in the two age groups, which suggests that VS results in comparable neuronal activation in juvenile and adult males. Importantly, not all nuclei examined showed an increase in Fos-immunoreactivity in

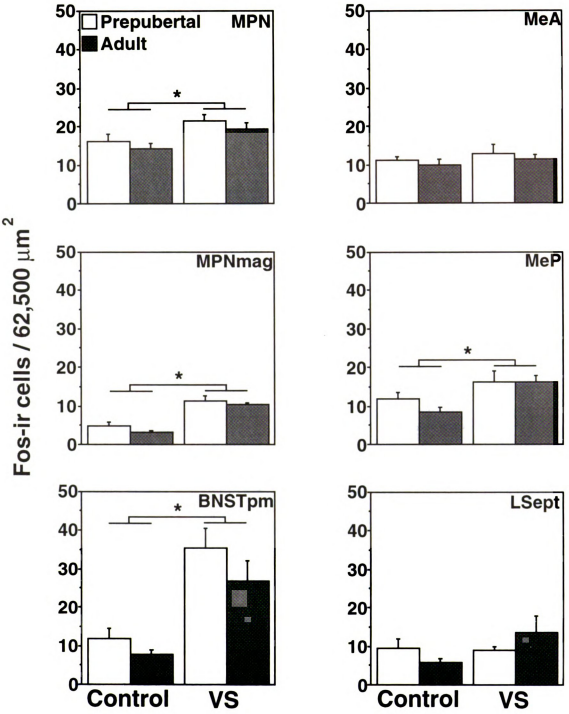

Figure 20. Fos-ir cells / 62,500 μm^2 in the MPN, MPNmag, BNSTpm, MeA, MeP, and LSept of prepubertal and adult males exposed to VS or a clean cotton swab. Asterisks indicate that animals exposed to VS had significantly greater numbers of Fos-ir cells than animals exposed to a clean cotton swab. All values are means $\pm$ SEM.

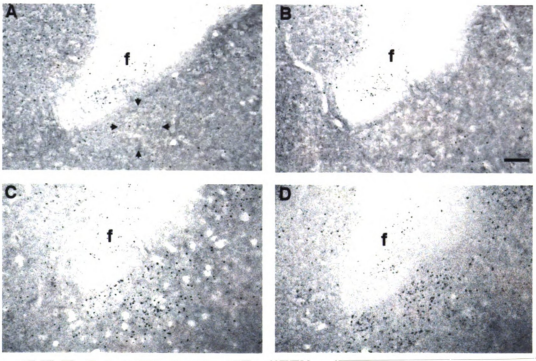

Figure 21. Photomicrographs of Fos-ir cells in the BNSTpm of a prepubertal (A) and adult (B) male exposed to a clean cotton swab and a prepubertal (C) and adult (D) male exposed to VS. Arrowheads are outlining the approximate area of the nucleus that was analyzed. f, fornix; Bar, 100μm.

response to VS exposure (e.g., LSept & MeA), indicating that the stimulation provided by VS is not merely general activation of the brain in response to a novel stimulus.

The induction of Fos-ir by exposure to VS in the BNSTpm, MPNmag, and MeP of adult male hamsters in the present experiment is in general agreement with much of the previously published literature (Fernandez-Fewell and Meredith, 1994; Fiber et al., 1993; Fiber and Swann, 1996; Kollack-Walker and Newman, 1997; Swann and Fiber, 1997). The observed increase in Fos-immunoreactivity in MPN of VS-exposed animals has not been consistently found in the other studies mentioned above, but in each case there was a trend toward greater Fos expression in the MPN after exposure to VS (except in Swann and Fiber (1997) in which the MPN was not analyzed). It is possible that different experimental conditions, such as previous sexual experience or time of day of testing, could account for cross-experiment differences in Fos expression in the MPN. It should be noted that other investigations of the Fos response to chemosensory cues from an estrous female found a lack of a response in castrated males (Bressler and Baum, 1996; Fiber and Swann, 1996; Paredes, Lopez, and Baum, 1998). Thus, the relatively low, but detectable, levels of circulating T observed in the prepubertal male hamster must be sufficient to allow a Fos response to occur.

Although it has been shown that nestling (7-14 days of age) and prepubertal (28-40 days of age) male hamsters are

attracted to VS (Johnston and Coplin, 1979), this is the first report to our knowledge that shows prepubertal males exposed to VS respond with equivalent levels of Fos expression in the same brain regions as adult male hamsters. However, this does not exclude the possibility that the pheromonal cues provided by an estrous female are interpreted differently by adult and juvenile males. For instance, Johnston and Coplin (1979) suggest that VS might act as a stimulus to facilitate nest location by pups. However, once sexual maturation is achieved these cues may become exclusively a sexual stimulus. It would be interesting to investigate the pattern of neuronal activation in response to VS in the brain of male pups and/or weanlings to see if they are similar to the patterns observed in prepubertal and adult males in the present study. Nonetheless, even if these pheromonal cues are being interpreted differently, the brain regions examined in the present study appear to be as responsive in the prepubertal as in the adult male to the pheromonal stimulation provided by VS.

Intact adult male hamsters spend almost twice as much time investigating the anogenital region of a receptive female than prepubertal males (Meek et al., 1997). Therefore, the vomeronasal system of adults may be provided with greater amounts of pheromonal stimulation in a naturally occurring behavioral encounter, resulting in the pubertal increase in mating behavior observed in the adult male. However, Meek et al. (1997) also reported that when T

implants equated the circulating levels of hormone in castrated juvenile and adult males, AGI was activated to the same degree at both ages, presumably providing equal amounts of pheromonal stimulation in both juvenile and adult animals. Furthermore, we have shown that DHT and E can stimulate AGI in prepubertal males above that of age-matched controls. Yet, mounting and intromissions were activated by steroid in the adult group only. The present data suggest that this inability of steroid-treated prepubertal males to engage in the appetitive aspects of mating behavior is not due to an insensitivity of the juvenile brain to chemosensory cues. Therefore, this would suggest that some cellular process downstream of these events is functional in the adult but immature in the prepubertal animal, leading to the differential amounts of reproductive behavior exhibited by the animals at these two developmental stages. For instance, the chemosensory information may not be integrated properly in the prepubertal brain, or other neural events that are required contemporaneously with neuronal activation by pheromones may not be completely developed in the prepubertal animal. It is interesting to note that T-treated female rats (Bressler and Baum, 1996; Paredes et al., 1998) and hamsters (Fiber and Swann, 1996) have a Fos response to estrous female odor cues similar to that observed in the T-treated males. However, the full suite of male sexual behaviors are not exhibited by these females. Hence, the prepubertal male and adult female brain may have similar neural characteristics

that do not permit chemosensory facilitation of a behavioral response.

The significant increases in circulating levels of T in adult animals exposed to VS in the present experiment are also in agreement with the existing literature (Macrides, Bartke, Fernandez, and D'Angelo, 1974; Pfeiffer and Johnston, 1992), and a recent study in our laboratory that described the time-course of the endocrine response to VS in prepubertal and adult males (Parfitt, Thompson, Richardson, Romeo, and Sisk, 1999). Our data suggest (Parfitt et al., 1999 and present study) that adults exposed to VS respond with a greater amount of T secretion compared to the VS-exposed juveniles. The ability to initiate a transient increase in T secretion may facilitate the greater amount of sexual behavior observed in adult male hamsters compared to juvenile males. In female rats, hormonal treatments that result in episodic increases in estrogen prior to a behavioral test lead to a greater amount of lordosis compared with treatments that provide constant levels of estrogen (Kow and Pfaff, 1975; Södersten, 1985). Therefore, the transient increase in T secretion in response to pheromonal stimulation, which in the present experiment was more pronounced in adults, may facilitate sexual behavior in animals experiencing this acute change in steroid hormone secretion.

In summary, we have found that in response to VS stimulation, prepubertal and adult males respond with

equivalent levels of Fos-immunoreactivity in brain regions that are imperative for chemosensory processing and male sexual behavior. Therefore, it appears that the inability of the prepubertal male to perform the full repertoire of male reproductive behaviors is not due to a lack of a neuronal activity in response to the pheromonal cues present in VS.

Section V. Integration and Conclusions

I have demonstrated that prepubertal males are unresponsive to the behaviorally activating effects of both the androgenic and estrogenic components of T. Furthermore, the absence of a behavioral response to these steroids is not due to a lack of ARs or ERαs within the neural circuit that mediates sexual behavior. These receptors appear to be functional prior to puberty, since T and estrogen treatment causes upregulation of hypothalamic aromatase activity and PR expression, respectively, in prepubertal and adult males. I have also shown that blocking PR reduced mating behavior in adult males, but activation of the PR in EB-treated prepubertal males with adult-like levels of progesterone did not facilitate their ability to engage in sexual behavior. Taken together, we have demonstrated that the lack of mating behavior exhibited by prepubertal males must be mediated by processes other than the availability of these steroids, the absence of their receptors, or the receptors' functionality.

It is possible that the greater behavioral response to steroids in adult compared to prepubertal males is related to differences in their hormonal histories. That is, in all the studies we conducted, prepubertal males experienced extremely low levels of gonadal steroids prior to treatment, whereas adults had experienced 2-3 weeks of increasing testosterone prior to treatment. Male reproductive behaviors are more readily evoked in gonadectomized adults when steroid

treatment is begun sooner, rather than later, after castration (Hamburger-Bar and Rigter, 1977; Meisel and Sachs, 1994; Olsen and Whalen, 1984). This difference in responsiveness to hormones is presumably because the proteins, neural connections, and other conditions necessary for mating behavior need only be maintained when steroids are replaced at the time of gonadectomy, whereas they need to be restored when steroids are replaced long after gonadectomy. However, "restoration" versus "maintenance" is unlikely to explain the absence of reproductive behaviors in steroid-treated prepubertal males compared to adults, because even adult males that have not experienced gonadal hormones for several weeks still show some behaviors after 7-14 days of steroid replacement (Hamburger-Bar and Rigter, 1977; McGinnis, Mirth, Zebrowski, and Dreifuss, 1989; Olsen and Whalen, 1984; Valenstein and Young, 1955).

In addition to the studies investigating steroid hormones and their receptors, we have shown that juveniles are able to detect pheromones in an estrous female's VS, and that the mating circuit is similarly activated in prepubertal and adult males in response to these chemosensory cues. Thus, it appears from the data summarized above that the striking differences in mating behavior exhibited by prepubertal and adult males are not reflected by equally impressive differences in AR, ERα, or estrogen-induced PR levels, or basic chemosensory processing.

If these parameters are not responsible for the lack of mating behavior observed prior to puberty, the question still stands as to what mediates the pubertal maturation of male sexual behavior. The distinct possibility remains that other areas of the central and peripheral nervous systems integral for successful reproduction to occur, such as the midbrain tegmentum (Brackett and Edwards, 1984; Eibergen and Caggiula, 1973), nuclei in the lumbar spinal cord (Breedlove, 1984), pelvic ganglion (Keast and Saunders, 1998), and penile musculature (Sachs, 1982), have not yet fully developed in the prepubertal male to allow the proper motor responses to be coordinated and displayed. Interestingly, pubertal development has been implicated in the morphological and phenotypical alteration of neurons in the spinal nucleus of the bulbocavernosus (Goldstein, Kurz, and Sengelaub, 1990)and pelvic ganglion (Keast and Saunders, 1998). Although these areas do not integrate the steroidal and pheromonal information necessary for mating behavior to emerge, the contribution of these structures to the pubertal maturation of male reproductive behavior warrants continued investigation.

In addition to steroids, various neurotransmitters and neuropeptides are capable of activating and modulating mating behavior in adult males (reviewed in, Bitran and Hull, 1987; Meisel and Sachs, 1994). For example, dopamine and LHRH have been implicated in the facilitation of male sexual behavior in adults (Bitran and Hull, 1987; Mani et al., 1994a; Melis

and Argiolas, 1995, and Beyer et al., 1997; Fernandez-Fewell and Meredith, 1995, respectively). Thus, it is possible that factors such as these are not functioning in an effective manner prior to puberty, which in turn may underlie the lack of mating behavior in prepubertal males. Studies investigating these possibilities are currently being pursued in our laboratory.

The assumption that the absence of reproductive behavior in prepubertal males is mediated by the lack of a particular factor(s) may be incorrect. Instead, the possibility exists that too much of some factor(s) is inhibiting the juvenile male from engaging in copulation. For example, serotonin is generally thought to inhibit male sexual behavior (Ahlenius, Larsson, and Svensson, 1980; Baum and Starr, 1980). Hence, the absence of mating behavior observed in juvenile males may be due to a greater serotonergic input in prepubertal compared to adult males in key areas of the brain that mediate reproduction. It would be interesting to test whether a serotonergic antagonist could facilitate reproductive behavior prior to puberty.

In addition to the possibilities mentioned above, the role of chemosensory processing in pubertal males must be considered. Even though the prepubertal and adult mating circuits show similar activation in response to female pheromones, we have found that adults are dissimilar to prepubertal males in that adults experience a rise in T after pheromonal exposure, which is not observed in juvenile males.

These findings have been replicated and extended in a recent experiment that characterized the levels of T and LH at various time points after prepubertal and adult males were exposed to VS (Parfitt et al., 1999). This study demonstrated that 60-min after exposure to VS, adult males had significantly elevated T levels and an increase in LH secretion compared to VS-exposed prepubertal males. Furthermore, this study showed that LH levels do not increase appreciably in juvenile males in response to VS at any time point tested. Taken together, it appears that activation of the hypothalamic-pituitary-gonadal (HPG) axis by pheromones is greater in adult compared to juvenile males. The lack of HPG activation in response to pheromones prior to puberty indicates that prepubertal males are integrating chemosensory cues differently than adult males. Since proper integration of chemosensory cues are an integral neural event for the full suite of mating behaviors to be displayed, the absence of a neuroendocrine reflex in the juvenile may be an important mediator of the lack of reproductive behavior observed at this age.

As alluded to in the Introduction, we hope to obtain a deeper understanding of how internal and external cues interact to affect the physiology and behavior of an individual progressing through puberty. The different endocrine response to chemosensory cues exhibited by prepubertal and adult males provides an interesting possibility of how the interaction of these cues may change

during pubertal development. Specifically, adult male rats and hamsters respond to the scent of an estrous female with a increase in dopamine secretion in the MPN (Hull, Du, Lorrain, and Matuszewich, 1995; Schulz, unpublished observation). It is not known if prepubertal males respond to an estrous female with a central release of dopamine. It is possible that, similar to the lack of HPG activation, prepubertal males may not respond to pheromones with an elevation in dopamine secretion in the MPN. If this were the case, then a potential scenario would be that increased steroid production during pubertal development primes the MPN with an increase in PR expression. Pheromonal stimulation received by males would lead to a significant increase in dopamine secretion in the MPN of adults only. Since dopamine can activate PRs through a ligand-independent mechanism (Mani et al., 1994a), these activated PRs could then facilitate copulatory behaviors necessary for successful reproduction. This possibility illustrates how dopamine secreted from the MPN in response to an estrous female may be an event where the internal (steroids) and external (chemosensory) cues converge and interact to facilitate reproductive behavior during pubertal maturation.

One of our goals in studying the pubertal maturation of mating behavior is to gain a better understanding of how puberty impacts the development of the central nervous system in general. Perinatal development is often viewed as the major window of time for the organization of neural circuits

by steroids (MacLusky and Naftolin, 1981). These early organizational effects of steroids on the nervous system supposedly determine the behavioral potentials of an organism in adulthood (Becú-Villalobos, Iglesias, Díaz-Torga, Hockl, and Libertum, 1997; Phoenix, Goy, Gerall, and Young, 1959). However, the perinatal period is probably not the only period of development during which the sensitivity of the central nervous system to steroid hormones can be determined or influenced. Arnold and Breedlove (1985) suggest that endocrine changes that occur well after neonatal development may have profound effects on the organization of neural circuits. Since a hallmark of pubertal development is the increased production and secretion of gonadal steroid hormones, puberty may be another, perhaps critical, period of steroid-dependent neural development.

Good evidence exists for organizational effects of steroid hormones during puberty on social interaction (SI) in a novel environment and open-field ambulation, two behaviors used as inverse indices of anxiety. Adult male rats show less SI and open-field ambulation than prepubertal males (Primus and Kellogg, 1989; Slob et al., 1986, respectively). If animals are gonadectomized before puberty and tested in adulthood, they display high levels of SI and open-field ambulation, similar to those displayed by juveniles (Brand and Slob, 1988; Primus and Kellogg, 1990). Testosterone replacement at the time of gonadectomy permits the normal pubertal change in these behaviors. In contrast, animals

gonadectomized after puberty engage in the same amount of SI and open-field ambulation as intact adults (Brand and Slob, 1988; Primus and Kellogg, 1990). Thus, the presence of gonadal steroids during puberty results in a behavioral change that does not require the continued presence of steroid, an effect that fits the traditional definition of organizational effects of steroid hormones. Estrogen formed from the aromatization of testosterone mediates the puberty-related decrease in SI (Kellogg and Lundin, 1999). Taken together, these studies demonstrate that behavioral potentials can be recast during puberty by androgenic and estrogenic steroids, and that the critical period for the organization of neural circuits by steroids may be extended into pubertal development.

Direct empirical evidence exists that demonstrates remodeling of the nervous system during pubertal development. For instance, we have demonstrated that the amygdala exhibits morphological plasticity during puberty, such that the MeP is larger in adult compared to prepubertal males, while the MeA is smaller in adult males compared to juveniles (Romeo and Sisk, 2001). These changes may reflect underlying cellular processes (e.g., altered cellular excitability, changes in protein synthesis) related to the pubertal change in motivated and social behaviors (e.g., mating, aggression, affiliation). We have also found that adult males that have been castrated before puberty have a greater number of AR-ir cells in the MPN than adult males that have been castrated

after puberty (Romeo, Diedrich, and Sisk, 2000), indicating that the presence of gonadal steroids during pubertal development decreases the number of AR-containing cells in the MPN. These data suggest that a hormone-dependent change in androgen sensitivity or responsiveness is one outcome of normal pubertal development, and provides further support for the concept that puberty is a developmental stage during which hormones shape steroid-sensitive brain regions.

Expression of adult reproductive behaviors may depend on steroid-independent maturational events that must occur in conjunction with the pubertal rise in testosterone. Thus, prepubertal males may not engage in mating behaviors even when treated with steroids because a steroid-independent neural process or a change in metabolic signals that is permissive for behavior has not yet occurred. However, rats castrated prior to puberty and then treated with hormones in adulthood respond behaviorally to steroids as if they were prepubertal (Larsson, 1967), suggesting steroid-independent maturational events are not the only determinant of the pubertal increase in mating behavior.

Structural changes in the brains of humans during pubertal development have been reported. For instance, a magnetic resonance imaging (MRI) study has shown that the volume of the amygdala increases in boys during adolescence, while hippocampal volume increases during puberty in girls (Giedd, Vaituzis, Hamburger, Lange, Rajapakse, Kaysen, Vauss, and Rapoport, 1996). Furthermore, recent studies have

demonstrated that the ratio of gray to white matter changes in the frontal and parietal lobes as a function of puberty (Giedd, Blumenthal, Jeffries, Castellanos, Liu, Zijdenbos, Paus, Evans, and Rapoport, 1999; Paus, Zijdenbos, Worsley, Collins, Blumenthal, Giedd, Rapoport, and Evans, 1999; Sowell, Thompson, Colin, Jernigan, and Toga, 1999), such that white matter volume increases during adolescence. This raises the possibility that the greater cognitive demand placed on humans as they pass through adolescence is paralleled by greater myelination of fiber tracts or synaptic pruning that facilitate information flow from one area of the brain to another.

Whether these structural changes in the adolescent brain are mediating the changes in behavior and/or cognition, or susceptibility to psychological disorders exhibited during puberty is unknown. Further, it is presently unclear what role the pubertal rise in gonadal and adrenal steroids plays in this process. However, it does provide evidence that in humans, as in experimental animal models, the central nervous system shows changes in structure and function during pubertal development, which may underlie the vast changes in physiology and behavior exhibited by individuals during this period of development.

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