Pheromones Elicit Equivalent Levels of Fos-Immunoreactivity in Prepubertal and Adult Male Syrian Hamsters

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Male reproductive behavior in the Syrian hamster is dependent on both pheromones from the female and the presence of gonadal steroid hormones. The pheromones are contained within female hamster vaginal secretions (FHVS) and stimulate anogenital investigation and mounting by the male. Administration of testosterone to castrated male hamsters facilitates anogenital investigation, mounts, and intromissions in adults, but elicits only anogenital investigation in prepubertal males. One hypothesis for why the full complement of reproductive behaviors is not activated by testosterone in prepubertal males is that the neural processing of pheromonal cues encountered during anogenital investigation is different in juveniles and adults. In the present experiment, we investigated the influence of sexual maturity on Fos expression in response to FHVS in the male Syrian hamster. We predicted a greater increase in Fos immunoreactivity after exposure to FHVS within the neural circuit mediating male reproductive behaviors in adult compared to prepubertal males. Intact adult and prepubertal males were exposed to either a clean cotton swab or a swab containing FHVS. We found that, compared to animals exposed to a clean cotton swab, both prepubertal and adult males exposed to FHVS have a greater amount of Fos immunoreactivity within several brain nuclei comprising the neural circuit mediating male reproductive behavior. Furthermore, this Fos response was equivalent in the two age groups. These results suggest that the inability of the prepubertal male hamster to perform the full repertoire of male reproductive behaviors is not due to a lack of a neuronal activation in response to the pheromonal cues present in FHVS.

Key Words: pheromones; puberty; reproductive behavior; Fos; testosterone.

Full expression of male reproductive behavior in the Syrian hamster is dependent on both pheromonal cues from the female and the presence of gonadal steroid hormones (Meisel and Sachs, 1994; Wood and Newman, 1995b). The pheromones are contained within female hamster vaginal secretions (FHVS) and stimulate the male vomeronasal system (Fernandez-Fewell and Meredith, 1994), leading to increased anogenital investigation and mounting by the male (Darby, Devor, and Chorover, 1975). The male’s interest in FHVS is increased by the presence of circulating androgens (Powers and Bergondy, 1983). We have demonstrated that anogenital investigation of the female is stimulated to a similar extent in castrated adult and prepubertal male hamsters treated with equivalent does of testosterone (Meek, Romeo, Novak, and Sisk, 1997). However, the testosterone-treated adults display more mounts and intromissions than the prepubertal males. These results suggest that, despite apparently equal interest in the female, testosterone-treated adults and juvenile males process the chemosensory information from the female differently.

In the adult male hamster, pheromonal and hormonal signals are integrated within a central neural circuit that is essential for both chemosensory processing and the expression of male sexual behavior. The circuit includes the medial nucleus of the amygdala (Me), the bed nucleus of the stria terminalis (BNST), and nuclei within the medial preoptic area (MPOA) (Wood and Newman, 1995c). Androgen receptor-containing cells are abundant within each component of...
the circuit (Wood and Newman, 1995a). If the pheromonal cues, steroid hormones, and/or the neural substrates on which they act are disrupted, male copulatory behavior is greatly compromised (Meisel and Sachs, 1994; Wood and Newman, 1995b).

Adult male ferrets (Wersinger and Baum, 1997), hamsters (Fernandez-Fewell and Meredith, 1994; Fiber, Adames, and Swann, 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 FHVS, cells within subdivisions of the Me, BNST, and MPOA 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 indicate that FHVS 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 FHVS in the male golden hamster. We tested the hypothesis that the different amount of reproductive behavior observed in prepubertal and adult male hamsters is the result of a pubertal change in the processing of chemosensory information, which leads to a greater degree of FHVS-induced neuronal activation within the behavioral circuit in adults compared to juveniles. This hypothesis predicted that the Fos response to FHVS will be greater in adults than in juvenile males.

**METHODS**

**Animals and Housing**

Twelve weanling (21 days of age) and 12 adult (80 days of age) male golden hamsters (*Mesocricetus auratus*) were obtained from Charles River Laboratories (Kingston, NY). All animals were sexually naive, were singly housed in clear polycarbonate cages (37.5 × 33 × 17 cm) with wood chips (Aspen Chip Laboratory Bedding, Warrensburg, NY), and had *ad libitum* access to food (Teklad Rodent Diet No.8640, Harlan, Madison, WI) and water. In the vivarium, room temperature was maintained at 21 ± 2°C and the light–dark cycle was 14 h light/10 h dark (lights on at 0600 h EST). All animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and protocols were approved by the Michigan State University All-University Committee for Animal Use and Care.

**Procedure**

After a 1-week acclimation period, half of the animals in each age group were given either a clean cotton swab or a cotton swab containing FHVS. Thus, there were four treatment groups (all n = 6): (i) 28-day-old prepubertal males exposed to a clean swab, (ii) 28-day-old prepubertal males exposed to an FHVS swab, (iii) 87-day-old adult males exposed to a clean swab, and (iv) 87-day-old adult males exposed to an FHVS swab. The FHVS 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 h EST) of their light–dark schedule. All animals (control and FHVS) were observed to place the cotton swab in their cheek pouch. Therefore, the only difference between the control and FHVS-exposed animals is that the animals receiving the swab with FHVS presumably were able to deliver FHVS to their vomeronasal organ.

One hour after the introduction of the swab into the home cage, animals were weighed, administered an overdose of sodium pentobarbital (130 mg/kg ip), brought to a separate room, and perfused. Prior to perfusion, the testes and seminal vesicles were removed, and a cardiac blood sample was taken. Animals were perfused intracardially with 100 ml of buffered saline rinse followed by 200 ml of 4% paraformaldehyde. All brains were postfixied for 1 h in 4% paraformaldehyde and then transferred to a 20% buffered sucrose solution for approximately 48 h until sectioning. Four sets of 40-μm sections were cut on a cryostat and stored in cryoprotectant at −20°C until the immunocytochemistry was performed (see below). Data from one prepubertal (FHVS exposed) animal and one adult (Control) animal were dropped from all analyses due to poor tissue quality resulting from inadequate fixation during perfusion.

**Fos Immunocytochemistry**

Every fourth section from each brain was processed simultaneously during a single immunocytochemical procedure. Free-floating sections were washed three times for 5 min in Tris-buffered saline (TBS) and in-
cubated in rabbit anti-c-fos (diluted 1:1000 in TBS with 0.25% Triton; a polyclonal antibody raised in rabbit against amino acids 3–16 of c-fos p62 of human origin; Santa Cruz, Biotechnology, Lot B245, Santa Cruz, CA) for 48 h at 4°C. Subsequently, sections were incubated in biotinylated goat anti-rabbit IgG (diluted in 1:1000 in TBS with 0.25% Triton; Vector, Burlingame, CA) and avidin–biotin horseradish peroxidase complex (Vectastain ABC Kit, Vector), each for 1 h at room temperature. Horseradish peroxidase was visualized with a 0.0125% diaminobenzidine (DAB) solution containing 0.06% hydrogen peroxide with 0.015% nickel chloride in TBS for 5 min. 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 Immunocytochemistry

The areal density (cells per unit area) of Fos-immunoreactive (Fos-ir) cells was determined for the lateral septum (LSept, Fig. 1A), the medial preoptic nucleus (MPN, Fig. 1A), the posteromedial subdivision of the bed nucleus of stria terminalis (BNSTpm, Fig. 1B), the magnocellular region of the medial preoptic nucleus (MPNmag, Fig. 1B), the anterior subdivision of the medial amygdala (MeA, Fig. 1C), and the posterior subdivision of the medial amygdala (MeP, Fig. 1D). These nuclei were located within the respective sections by their relative position to ventricles (e.g., LSept and MPN) or fiber tracts (e.g., BNSTpm, MeA, and MeP) or in relation to each other (e.g., MPNmag is ventral to the BNSTpm). These areas were chosen for examination because they have been demonstrated to be important parts of the neural circuit mediating chemosensory information and reproductive behavior in the male golden hamster (Fiber et al., 1993; Kollack-Walker and Newman, 1997). Sections were analyzed under bright-field microscopy using an Olympus BX60 microscope (Melville, NY). Each brain region was initially centered under 10X magnification and the magnification was then increased to 40X. A cell was considered immunopositive when dark blue reaction product was observed in the nucleus. Two bilateral counts were made for each nucleus, except for the MPN, for which three bilateral counts were made. The two (or three) brain sections used for analysis were separated by 120 μm and were anatomically matched across animals, using the landmarks described above. Within a nucleus the number of immunopositive cells/secion did not vary significantly. All data are expressed as mean number of Fos-ir cells/62,500 μm². One experimenter blind to the condition of the animals was responsible for all Fos-ir cell counts.

Testosterone Radioimmunoassay

Plasma testosterone concentrations were measured using the Coat-A-Count Total Testosterone Kit (Diagnostic Products, Los Angeles, CA). This assay has been validated in our laboratory for the
measurement of plasma testosterone concentration in Syrian hamsters. The lower limit of detectability of the assay was 0.08 ng/ml. The intraassay coefficient of variation was 7.7%.

**Statistical Analysis**

Two-way ANOVAs (age × 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 means ± SEM.

**RESULTS**

**Peripheral Measures**

Regardless of treatment condition, adult males had significantly heavier seminal vesicles \( (F = 145.280, P < 0.05, \text{Table 1}) \) and testes \( (F = 88.816, P < 0.05, \text{Table 1}) \) and significantly higher plasma testosterone concentrations \( (F = 43.541, P < 0.05, \text{Fig. 2}) \) than the prepubertal animals. There was a significant main effect of treatment on testosterone concentration, so that circulating levels of testosterone were higher in the FHVS condition \( (F = 4.835, P < 0.05) \). However, an interaction between age and treatment on plasma testosterone concentrations approached significance \( (P = 0.06) \), such that the adult males exposed to FHVS tended to show an elevation in testosterone secretion while prepubertal animals exposed to FHVS did not. Indeed, when \( t \) tests were conducted within the two ages, FHVS-exposed adults had significantly higher plasma testosterone concentrations than the adults exposed to a clean cotton swab \( (t = -2.255, P < 0.05) \), while exposure to FHVS did not significantly alter plasma testosterone concentrations of prepubertal animals (Fig. 2).

**Fos-Immunoreactivity**

There was a significant main effect of treatment on the number of Fos-ir cells in the BNSTpm, MPNmag, MPN, and MeP \( (F = 28.574, F = 103.850, F = 8.795, \text{and } F = 9.409, \text{respectively, all } P < 0.05, \text{Fig. 3}) \), but no effect of age and no interaction in these areas. Specifically, there were a greater number of Fos-ir cells per unit area in the BNSTpm, MPNmag, MPN, and MeP in animals that were exposed to FHVS than in those that were exposed to a clean cotton swab. The greatest increase in Fos-ir cells per unit area was in the BNSTpm where FHVS exposure led to an approximately 3-fold increase in the density of Fos-ir cells (Figs. 4A–4D). In the MPNmag, MPN, and MeP, FHVS exposure led to approximately a 1.5- to 2-fold increase in Fos-ir cells per unit area. There was no effect of age or treatment on the number of Fos-ir cells per unit area in either the MeA or the L.Sep of prepubertal and adult males (Fig. 3).

**DISCUSSION**

Compared to animals exposed to a clean cotton swab, both prepubertal and adult males exposed to FHVS 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 (Meisel and Sachs, 1994; Wood and Newman, 1995b). Furthermore, the Fos response

**TABLE 1**

<table>
<thead>
<tr>
<th>Age (days)-condition</th>
<th>Seminal vesicles (g)</th>
<th>Paired testes (g)</th>
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<tbody>
<tr>
<td>28–Control</td>
<td>0.078 ± 0.004</td>
<td>1.035 ± 0.066</td>
</tr>
<tr>
<td>28–FHVS</td>
<td>0.058 ± 0.007</td>
<td>0.869 ± 0.061</td>
</tr>
<tr>
<td>87–Control</td>
<td>0.239 ± 0.015</td>
<td>2.105 ± 0.214</td>
</tr>
<tr>
<td>87–FHVS</td>
<td>0.284 ± 0.025</td>
<td>2.293 ± 0.141</td>
</tr>
</tbody>
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**FIG. 2.** Plasma testosterone concentrations in prepubertal and adult males exposed to FHVS or a clean cotton swab. Asterisk indicates that adult males exposed to FHVS had significantly higher circulating levels of testosterone than the adults exposed to a clean cotton swab \( (t \) tests). All values are means ± SEM.
to FHVS is equivalent in the two age groups, which suggests that FHVS results in comparable neuronal activation in juvenile and adult males. Importantly, not all nuclei examined showed an increase in Fos-immunoreactivity in response to FHVS exposure (e.g., LSept and MeA), indicating that the stimulation provided by FHVS is not merely general activation of the brain in response to a novel stimulus.

The induction of Fos-ir by exposure to FHVS 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 (Fernan-
dez-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 FHVS-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 FHVS [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 testosterone observed in the prepubertal male hamster (Meek et al., 1997, present experiment) 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 FHVS (Johnston and Coplin, 1979), this is the first report to our knowledge that shows that prepubertal males exposed to FHVS 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 FHVS 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 FHVS in the brain of male pups and/or weanlings to see

FIG. 4. Photomicrographs of Fos-ir cells in the BNSTpm of prepubertal and adult male hamsters and arrowheads outlining the approximate area of the nucleus that was analyzed. Prepubertal (A) and adult (B) males exposed to a clean cotton swab. Prepubertal (C) and adult (D) males exposed to FHVS. f, fornix. Bar, 100 μm.
whether 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 sensitive in the prepubertal male as in the adult male to the pheromonal stimulation provided by FHVS.

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 testosterone implants equated the circulating levels of hormone in castrated juvenile and adult males, anogenital investigation was activated to the same degree at both ages, presumably providing equal amounts of pheromonal stimulation in both juvenile and adult animals. Yet, mounting and intromissions were activated by testosterone in the adult group only. The present data suggest that this inability of testosterone-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 testosterone-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 testosterone-treated males. However, the full suite of male sexual behaviors are not exhibited by the female hamsters. Hence, the prepubertal male hamster brain and adult female hamster brain may have similar neural characteristics that do not permit chemosensory facilitation of a behavioral response.

The significant increases in circulating levels of testosterone in animals exposed to FHVS in the present experiment are also in agreement with the existing literature (Macrides, Bartke, Fernandez, and D’Angelo, 1974; Pfeiffer and Johnston, 1992). The present data suggest that adults exposed to FHVS respond with a greater amount of testosterone secretion compared to the FHVS-exposed juveniles. The ability to initiate a transient increase in testosterone secretion may facilitate the greater amount of sexual behavior observed in adult male hamsters than that observed in juvenile males (Meek et al., 1997). In female rats, hormonal treatments that result in episodic increases in estrogen prior to a behavioral test lead to a greater amount of lordosis than treatments that provide constant levels of estrogen (Kow and Pfaff, 1975; Södersten, 1985). Therefore, the transient increase in testosterone 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 FHVS stimulation, prepubertal and adult male hamsters 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 hamster to perform the full repertoire of male reproductive behaviors is not due to a lack of neuronal activity in response to the pheromonal cues present in FHVS.

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induce c-fos in limbic areas regulating male hamster mating behavior. *NeuroReport* 4, 871–874.


