Pre-exposure to Female Chemosignals or Intracerebral GnRH Restores Mating Behavior in Naive Male Hamsters with Vomeronasal Organ Lesions

Jenne M. Westberry and Michael Meredith

Department of Biological Science, Program in Neuroscience, Florida State University, Tallahassee, FL 32306, USA

Correspondence to be sent to: Jenne Westberry, Department of Biological Science, Program in Neuroscience, Florida State University, Tallahassee, FL 32306, USA. e-mail: Westberry@neuro.fsu.edu

Abstract

Chemosensory input is essential for mating in male hamsters and the vomeronasal organ is critical to mating in naive males. In studies to investigate the convergence of vomeronasal chemosensory input and the neurohormone gonadotrophin releasing hormone (GnRH), we have unexpectedly found that pre-exposure to pheromone-containing chemosignals from female hamsters will also eliminate mating deficits normally seen in naive male hamsters with vomeronasal organs removed (VNX). In the present studies, naive-intact and naive-VNX male hamsters were given intracerebroventricular injections of GnRH or saline and exposed to female pheromones found in hamster vaginal fluid (HVF) or to water 40 min prior to a 5 min mating test. VNX males given saline injections and exposed to water had severe mating deficits, but VNX males given saline injections and exposed to HVF mated normally. As shown previously, males given GnRH injections and exposed to water also mated normally. HVF exposure prior to a mating test apparently acted to compensate for the lack of vomeronasal input in these males.

Key words: gonadotrophin releasing hormone, hamster, mating, pheromone, vomeronasal organ

Introduction

Most mammals have two nasal chemosensory systems, the main olfactory system and the vomeronasal system. Chemosensory input from one of these sources is required to sustain mating in males of several species, including golden hamsters. Bilateral olfactory bulbectomy (Murphy and Schneider, 1970), or combined peripheral olfactory and vomeronasal lesions (VNX) eliminate mating in male hamsters, regardless of their level of prior sexual experience (Powers and Winans, 1975; Meredith *et al.*, 1980; Meredith, 1986). Peripheral zinc sulfate lesions of the olfactory epithelium alone do not impair mating (Winans and Powers, 1977), but bilateral lesions of the vomeronasal organ (VNO) do create mating deficits in naive male hamsters given a 5 min mating test (Meredith, 1986; Fernandez-Fewell and Meredith, 1995). These data suggest that main olfactory input alone is not sufficient for mating, and that the VNO is necessary for normal mating behavior in naive male hamsters. Male hamsters that have had sexual experience prior to VNX do not show mating deficits (Meredith, 1986; Pfeiffer and Johnston, 1994), indicating that some change has occurred as a result of experience such that main olfactory input is sufficient to sustain mating. In the present experiments we show that prior exposure to female chemosignals (female hamster vaginal fluid—HVF) eliminates mating deficits in sexually inexperienced (naive) male hamsters with vomeronasal (VN) lesions: animals that normally have severe mating deficits in short-term mating tests. HVF has both sexual attractant (Johnston, 1974) and sexual stimulant qualities (Johnston, 1975) and contains components that can be classified as pheromones, largely acting via the VN system in intact males (Clancy *et al.*, 1984). HVF alone will induce mounting and investigative behaviors by naive-intact male hamsters when placed with inappropriate partners (Johnston, 1975; Macrides *et al.*, 1984; Singer *et al.*, 1984).

In addition to chemosensory input, gonadotrophin releasing hormone (GnRH) is also important for mating in males. In the case of naive-VNX males with impaired mating behavior, GnRH injected intracerebrally can substantially restore mating behavior (Fernandez-Fewell and Meredith, 1995), supplementing olfactory chemosensory input, and substituting for, or bypassing the necessity for, vomeronasal (VN) input. The experiments described here confirm those previous findings that naive-VNX male hamsters show deficits in mating behavior and that intracerebroventricular (i.c.v.) GnRH injections eliminate those deficits.

Both experience and GnRH appear to alter the effectiveness of olfactory chemosignals in supporting mating behavior, possibly by changing the effectiveness of

transmission from olfactory sensory input to brain regions concerned with mating. Olfactory and vomeronasal pathways supporting mating converge at the medial amygdala, which can be activated by either system (Fernandez-Fewell and Meredith, 1994, 1998; Fewell and Meredith, 2002). We propose that increasing activation in this common pathway by prior stimulation with HVF may permit onward transmission of chemosensory input during subsequent interactions with a female, sufficient for normal mating behavior in naive-VNX males.

Materials and methods

Animals used in these experiments were sexually inexperienced (naive) adult (2–3 month) male Golden hamsters (*Mesocricetus auratus*), bred in our laboratory, and maintained on a long photoperiod (a partially reversed 14 L/10 D light cycle). The animals were group-housed in clear plastic cages (44 × 21 × 18 cm) containing bedding with food and water *ad libitum*. All experimental protocols were approved by the Animal Care and Use Committee at Florida State University.

Intact and VNX males were all surgically implanted with guide tubes, injected with GnRH or saline (treatment) and exposed to either HVF or water (exposure). The four groups of intact animals were: males given i.c.v. GnRH and exposed to HVF ($n = 6$), males given i.c.v. GnRH and exposed to water ($n = 6$), males given i.c.v. saline and exposed to HVF ($n = 6$), and males given i.c.v. saline and exposed to water ($n = 6$). The four groups of VNX animals were: males given i.c.v. GnRH and exposed to HVF ($n = 6$), males given i.c.v. GnRH and exposed to water ($n = 5$), males given i.c.v. saline and exposed to HVF ($n = 6$), and males given i.c.v. saline and exposed to water ($n = 5$). For all VNX animals, a midline incision through the palate exposed the bony VN capsules. The natural openings in the palatal bones were extended rostrally with a dental drill, and then a 'U' shaped groove was made with the drill forward and across the midline, to connect the two openings. Forceps were used to break the medial palatine process of the maxillary bones to disconnect the capsules at the caudal end. The capsules were then separated by pressing at the midline suture with a scalpel. The final connection with the palate was broken by drilling anterior to the rostral connection. Each capsule containing one VNO was then removed with small forceps separately. The palatal incision was closed with three to five sutures and sealed with cyanoacrylate adhesive. The noses of the VNX animals were collected and postfixed separately and later decalcified and sectioned for verification of complete VNO lesions. Serial sections through the VNO region were examined and animals with any more than a disconnected fragment of VN sensory tissue were eliminated from the study.

Three to 5 days after VNX surgery, guide tubes (28 ga., Plastics One) were implanted in the left lateral cerebral ventricle. After an additional 3–5 days animals were given intracerebroventricular injections and behavioral testing. Thirty minutes prior to behavior testing, GnRH (50 ng in 2 μl) or saline vehicle (2 μl) was pressure injected through a cannula (33 ga.) inserted into the guide tube of the implanted freely moving animal. The animal was momentarily restrained by hand; the cap on the guide tube was removed and replaced with the injection cannula. The cannula was first filled to the tip after being connected to a long tube, containing the solution to be injected, and attached to a picospritzer. New cannulae were used for each experiment and individually calibrated before use by adjusting the duration of the picospritzer air-pulse at standard pressure until 2 mg of distilled water (2 μl) was ejected onto a filter paper placed on the pan of an analytical balance. The full dose was pressure injected, in three pulses spaced 30 s apart. The cannula was left in place for 90 s after the last pulse to allow the injected material to diffuse away from the tip of the cannula, preventing it from being sucked back up into the guide tube as the cannula was withdrawn. A cap with a wire stylet (dummy cannula) was used to seal the guide tube before and after use.

For exposure to HVF or water, animals were placed in a 3 l glass chamber (14 × 14 × 25 cm) with continuous 2 l/min air-flow, for 10 min to allow for acclimation. After the initial 10 min period, either diluted HVF (1:10 in distilled water) or distilled water was introduced periodically from a 1 ml syringe via a tube connected into a well machined in the aluminum block that protruded into the chamber at one end. One milliliter of stimulus (HVF or water) was delivered to each animal over a 40 min period, in the following temporal pattern designed to maintain the animal's interest in the well (when HVF used) for at least 50% of the first 20 min of the exposure time. HVF or water (0.02 ml) was presented every 3 min for the first 20 min and then every 5 min for the last 20 min. The same pattern was used for all animals. All animals made contact with the stimulus and most consumed some, whether water or HVF.

Behavior was scored manually by an observer using a computer keypad. Recorded behaviors included grooming, sniffing and licking at or close to the well (within 1 cm), sniffing at the back or the front of the chamber, sniffing and licking the walls of the jar, flank marking, escape-scrabbling and sleeping. These behaviors were also placed into three broad categories for analysis: Specific chemosensory investigation, Non-specific investigation and Vegetative behavior. Specific chemosensory investigation included sniffing in the air towards the well where the stimulus was presented (within 1 cm) and contact (sniffing and licking) at the well. Non-specific investigation included all sniffing behavior not directed toward the well such as sniffing and licking at the sides of the chamber. Vegetative behaviors included grooming, sleeping and stereotyped scrabbling at the walls of the chamber. The test chamber and stimulus delivery system were cleaned with detergent between uses. The

chamber and the aluminum and stainless steel end cap were heated and dried in a vacuum oven to remove any odors from the prior test animal or from HVF. Fresh syringes and tubing were used for each animal. Separate identical chambers were used for HVF and water exposure so that the water chamber never contained HVF.

After exposure, the animal was placed in a clean cage for 40 min, then given a mating test. For mating tests, a receptive female was first placed into a clean cage for 1 min, then the male was added and the timer began. The male was left in the cage with the receptive female until it achieved five intromissions, or for a maximum of 5 min. During the 5 min mating test, several aspects of mating behavior were recorded including numbers of correctly and incorrectly directed mounts and number of intromissions. As in our previous studies (Meredith, 1986), we use a composite measure of mating behavior, intromissions per minute, calculated from the time of introduction of the male to the fifth intromission (or 5 min). This is a useful measure of overall sexual performance which reflects both the latency and 'intensity' of mounting and intromissions. After the mating test, animals were euthanized and their brains prepared for immunocytochemical detection of Fos protein expression. The results of the Fos studies are reported in a separate publication.

Statistical analysis

Because we were interested in possible interactions between the effects of HVF exposure and GnRH treatment, we used separate two-way ANOVAs in the intact and VNX groups to analyze the effects of two factors: treatment (GnRH versus saline) and exposure (HVF versus water). An additional two-way ANOVA was used to analyze the interaction of HVF pre-exposure and surgical preparation (intact versus VNX) in the absence of GnRH. A conservative value

($P < 0.01$) for significance was applied because the same data were analyzed twice by these additional tests.

The data on time spent in the chamber prior to the mating test in each category of behavior—'specific investigation', 'non-specific investigation' (not at the stimulus well) and 'vegetative behaviors'—were analyzed together in a two-way ANOVA for each group (intact and VNX). There were no overall significant differences in these behaviors due to GnRH treatment (data not shown). Data for each group and each individual behavior within the three categories were inspected but no additional differences related to treatment, exposure, or surgery were evident, and the data on non-specific and vegetative behaviors were not analyzed further.

Because attention to the stimulus during pre-exposure in the chamber might be a factor in the animal's subsequent mating behavior, we used two-way ANOVAs for each group to analyze the effect of treatment and exposure on investigation time at the stimulus well (specific investigation). A clear effect of HVF on specific investigation time was evident in these within-group tests (see Results).

Results

Mating performance: intact males

There were no significant differences in mating performance among the intact males. Two-way analysis of variance (ANOVA) of all treatment (GnRH versus saline) and exposure (HVF versus water) groups of intact males revealed no significant effects. All animals achieved five intromissions with no significant differences in intromissions per minute (Figure 1A).

Mating performance: VNX males

Two-way ANOVA of all treatment and exposure groups of VNX males revealed an overall effect of exposure to HVF ($P < 0.001$, $F = 18.317$, d.f. = 1,16) and an overall effect of
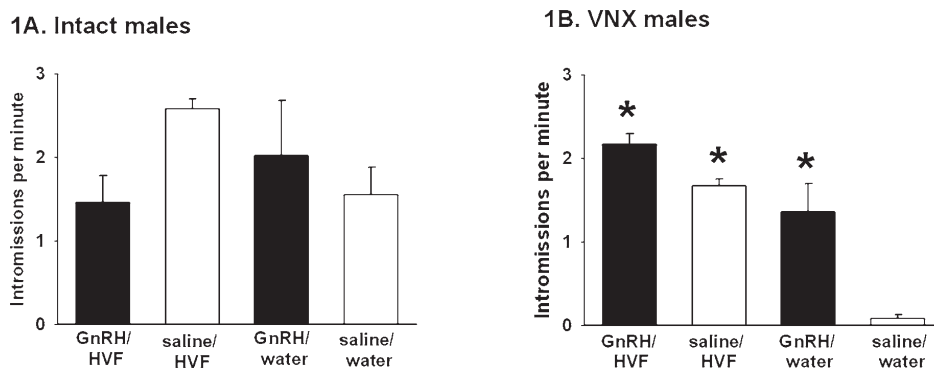

Figure 1 Mating behavior of naive-intact males **(A)** and naive-VNX males **(B)** in the 5 min mating test, occurring 40 min after exposure to HVF or water (mean and standard error). (A) Intact males: there were no significant differences in intromissions/minute for any group. Neither GnRH injection nor HVF pre-exposure had a significant effect on behavior compared with the control saline/water group. See text for statistical details. (B) VNX males: VNX males exposed to water and given a saline injection showed severe deficits in mating behavior. VNX males given saline injections and pre-exposed to HVF mated normally. As previously described, GnRH relieved mating deficits in naive-VNX males. There were no significant differences between the three groups with normal mating behavior. Asterisks indicate significant ($P < 0.01$) difference from saline/water group. See text for statistical details.

GnRH injection ($P = 0.006$, $F = 10.083$, d.f. = 1,16), but the interaction between treatment and exposure was not significant according to our conservative criteria. Tukey *post hoc* tests revealed significant differences in mating performance. All VNX males exposed to HVF had higher intromissions per minute than those exposed to water, regardless of prior injection (GnRH/HVF, $P < 0.001$; saline/HVF, $P < 0.001$).

Among VNX males exposed to HVF, there was no significant difference between the GnRH/HVF and saline/HVF groups: both groups met the criterion of five intromissions in approximately the same time (Figure 1B). Among VNX males exposed to water, those injected with GnRH (GnRH/water) achieved significantly more intromissions per minute, then those injected with saline (saline/water, $P = 0.006$).

Among VNX males injected with saline, those exposed to water (saline/water) failed to mate, as expected for untreated naive-VNX males. Of these saline-injected water-exposed VNX males, two of the five achieved one intromission, while the others had none. Unexpectedly, those males exposed to HVF for 40 min, 40 min before the mating test, but not injected with GnRH, achieved significantly more intromissions per minute (saline/HVF, $P < 0.001$) and mated normally. Their performance was not significantly different from GnRH-injected, HVF-exposed males. In Figure 1B, asterisks indicate groups whose performance was significantly different from saline-injected, water-exposed males according to these *post hoc* tests. There were no differences in performance between the three groups that performed at higher levels than saline-injected, water-exposed controls.

Mating performance: all saline-injected males (intact and VNX)

An additional two-way ANOVA comparing the effect of HVF exposure on intact and VNX males injected with saline showed a significant overall effect of surgery with HVF having a greater effect on VNX males than on intact males ($P < 0.001$, $F = 36,82$, d.f. = 1,16), and a significant effect of HVF exposure ($P < 0.001$, $F = 13.79$, d.f. = 1,16) with a significant interaction ($P < 0.001$, $F = 18.82$, d.f. = 1,16) confirming the difference in the effect of HVF on VNX and intact males. In Tukey *post hoc* tests, VNX males exposed to HVF had significantly higher intromissions per minute than those exposed to water ($P < 0.001$), but there was no significant increase with HVF exposure in intact males. Within HVF-exposed males injected with saline (saline/HVF), there was no difference in mating performance between intact and VNX males.

Behavior in chamber

We also recorded and analyzed the behavior of the males in each group during exposure to HVF or water in the exposure chamber (Figure 2). For specific investigation at the stimulus well, separate two-way ANOVA tests were run to examine the effect of treatment (GnRH versus saline)
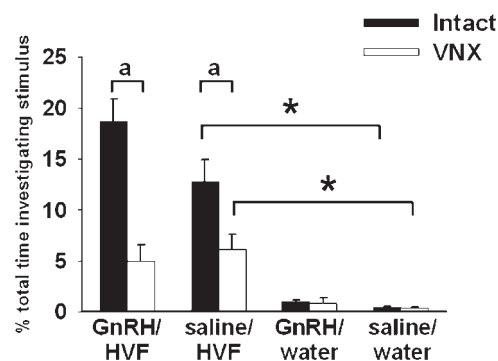

Figure 2 Graph showing time spent investigating the stimulus well as a percentage of time in the exposure chamber (mean and standard error). Both intact and VNX males exposed to HVF spent more time investigating the stimulus well than those exposed to water ($P < 0.001$ for both intact and VNX; indicated by asterisks for saline-injected males; *post hoc* tests following separate ANOVAs for intact and VNX males). There was also an increase with HVF exposure in GnRH-injected males. This and other significant differences between groups are described in the text, but not indicated on the graph to avoid confusion. See text for statistical details. The increase in investigation time in HVF-exposed VNX males injected with saline (saline/HVF) was associated with the increase in mating behavior reported here, but the larger increase in investigation time in intact males injected with saline was not accompanied by any significant increase in mating. Intact males spent significantly more time investigating the HVF than VNX males, regardless of whether they were injected with GnRH or saline ($P < 0.001$ for both GnRH/HVF and saline/HVF; indicated by letter 'a': *post hoc* tests following an ANOVA including all HVF-exposed males).

and exposure (HVF versus water) within each surgical group (intact or VNX). In intact males, there was a significant overall effect of treatment ($P = 0.001$, $F = 14.418$, d.f. = 1,20), a significant overall effect of exposure ($P < 0.001$, $F = 1073.007$, d.f. = 1,20), and a significant interaction between treatment and exposure ($P < 0.001$, $F = 817.468$, d.f. = 1,20). Tukey *post hoc* tests revealed that within both GnRH and saline groups, investigation time was significantly greater in males exposed to HVF (GnRH/HVF and saline/HVF, $P < 0.001$ for both). The HVF effect on saline-injected males is indicated by an asterisk in Figure 2. These tests also revealed that within HVF-exposed intact males, investigation time was significantly greater in those males with GnRH injections than those with saline injections (GnRH/HVF, $P < 0.001$).

In VNX males, there was also a significant main effect of exposure ($P = 0.006$, $F = 10.083$, d.f. = 1,20) and a significant interaction ($P = 0.004$, $F = 10.665$, d.f. = 1,20), but no overall effect of GnRH. Tukey *post hoc* tests revealed that within both GnRH and saline groups, investigation time was significantly greater in males exposed to HVF (GnRH/HVF and saline/HVF, $P < 0.001$ for both), but GnRH did not further increase investigation in HVF-exposed VNX males. GnRH injection in water-exposed males (GnRH/water) did significantly increase 'investigation of water' ($P = 0.008$). As previously reported, there was an increase in mating behavior for GnRH-injected VNX males, without HVF

pre-exposure (Fernandez-Fewell and Meredith, 1995). HVF exposure increased investigation time in both intact and VNX males in the absence of GnRH, and in VNX males this was associated with the increased mating behavior reported here.

A third two-way ANOVA was conducted on all HVF-exposed males (intact and VNX) comparing treatment (GnRH versus saline) and surgery group (intact versus VNX) to look at the effects of VNX. This two-way ANOVA indicated that intact males spent significantly more time overall investigating HVF than did naive-VNX males, regardless of whether the animal was injected with GnRH (GnRH/HVF, $P < 0.001$) or saline (saline/HVF, $P < 0.001$; Tukey *post hoc* tests: indicated by 'a' in Figure 2). There was also a significant overall main effect of treatment ($P = 0.008$, $F = 8.605$, d.f. $= 1,20$), with GnRH producing more investigation, and a significant interaction ($P = 0.001$, $F = 14.537$, d.f. $= 1,20$) because the GnRH effect on investigation was limited to intact animals (GnRH/HVF, $P < 0.001$; revealed by Tukey *post hoc* tests). There was no significant difference in investigation of HVF between VNX animals injected with GnRH (GnRH/HVF) and those injected with saline (saline/HVF) (Figure 2).

Discussion

Unlike previous studies, animals here were exposed to HVF or water shortly before the mating tests. The new finding in these experiments was that naive male hamsters with vomeronasal organs removed (VNX) mated normally (not different from naive-intact males) with a receptive female when they were exposed to a female chemosignal, HVF, 40 min prior to the mating test. Those VNX males exposed to water in the same way failed to mate normally. None achieved more than one intromission in 5 min and their behavior was significantly impaired compared with the intact males. The results also confirmed our previous finding (Fernandez-Fewell and Meredith, 1995) that i.c.v. GnRH relieves mating deficits following bilateral VNX (Meredith, 1986).

The 'priming' effect of HVF was not critically dependent on prolonged investigation of HVF in the chamber. The VNX males spent significantly less time sniffing and contacting the well containing HVF than did the equivalent intact males. They all made contact with the HVF (Figure 2), but an extensive investigation did not appear to be necessary for subsequent mating.

Exposure of male hamsters to HVF results in increased Fos protein expression along the VN pathway, particularly in the medial amygdala (Fiber *et al.*, 1993; Fernandez-Fewell and Meredith, 1994). This was also true for the animals whose behavior is reported here and those data are reported elsewhere (Westberry and Meredith, 2003). Fos is used in these mapping experiments to mark cells with increased neuronal activity, not necessarily to mark changes in synaptic strength. The increase in Fos expression due to HVF exposure is not limited to those males with an intact VNO, presumably because main olfactory input converges on VN pathways (Fernandez-Fewell and Meredith, 1994, 1998). The increase in Fos expression in response to HVF reflects short-term changes (activation) in the VN pathways important for mating. This activation might reduce the additional level of input required to stimulate the system during the subsequent mating test. Thus, the olfactory input into the VN pathways could become sufficient to allow mating to occur in VNX males exposed to HVF prior to the mating test.

In these experiments, GnRH injection in intact males exposed to HVF was followed by a small, non-significant decrease in mating performance compared with saline-injected intact males. A similar effect was seen in previous behavioral tests using a single GnRH injection (Fernandez-Fewell and Meredith, 1994). In an earlier experiment using repeated, alternating GnRH and saline injections (Meredith and Howard, 1992), the reduction in mating of intact males by GnRH was significant. This result may reflect a change following VNX in brain circuits affected by GnRH, in addition to the simple absence of VN input.

Sexual experience prior to VNX eliminates mating deficits, presumably by producing long-term changes in circuits in the brain so that main olfactory input has more influence on areas important for mating: sufficient to compensate for the lack of VNO input (Meredith, 1986, 1998; Fewell and Meredith, 2002). In these studies, prior exposure to the female pheromones found in HVF or GnRH injection also were enough to eliminate mating deficits caused by the lack of a VNO, but probably not by producing long-term changes in mating circuits in the brain. In each case, the mechanism may be an increase in excitability in the central VN circuits, such that the male can respond to other cues from the receptive female. In as far as chemosensory input is essential, one of these cues is likely to be main olfactory input.

Acknowledgements

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