

Research report

The influence of chemosensory input and gonadotropin releasing hormone on mating behavior circuits in male hamsters

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Abstract

Chemosensory input is important for mating behavior in male hamsters. Chemosignals found in female hamster vaginal fluid activate regions of the brain that receive input from the vomeronasal/accessory olfactory system and are important for mating behavior. Mating or exposure to these chemosignals produces increased Fos protein expression in the amygdala, bed nucleus of the stria terminalis, and medial preoptic area (MPOA). These brain regions contain cell bodies and/or fibers of gonadotropin releasing hormone (GnRH) neurons, suggesting potential relationships between chemosensory systems and GnRH. GnRH is released naturally when male rodents (mice and hamsters) encounter female chemosignals, and intracerebrally injected GnRH restores mating behavior in sexually naive male hamsters after removal of the vomeronasal organs. We report here that the combination of pheromone exposure and intracerebrally-injected GnRH increases Fos expression in the MPOA above the increase seen in pheromone-exposed males, or in males given only the exogenous GnRH. In males with vomeronasal organs removed (VNX), there was also an increment in Fos expression in the MPOA when these pheromone exposed males were injected with GnRH, provided they had previous sexual experience. Males with vomeronasal organs removed and without sexual experience showed increased Fos expression in the medial amygdala when pheromone exposure and GnRH injection were combined, but not in the medial preoptic area.

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1. Introduction

Chemosensory signals can be detected in rodents via the main olfactory system or by the separate vomeronasal/accessory-olfactory system. The sensory neurons of the vomeronasal organ (VNO) have receptors that detect chemical signals from other individuals, some of which can be defined as pheromones (see Ref. [22]). We define pheromone communication as chemical communication of information between individuals of the same species, likely to be to their mutual advantage. Chemosignals that in-

crease the probability of mating and reproduction would fall within this definition.

The VNO is important for mating behavior in male golden hamsters. Sensory information from the VNO passes via the accessory olfactory bulb to the amygdala and other areas of the brain that are important for reproductive behavior. Chemosensory signals received during mating or exposure to pheromone sources, such as hamster vaginal fluid (HVF) activate brain regions along the vomeronasal (VN) pathway and the medial preoptic area (MPOA), resulting in increased Fos-protein expression in activated neurons [17,7,18,10]. A stimulus driven increase in Fos in undamaged mature brain can generally be attributed to neuronal activation, most often due to depolarization [24].

The brain regions activated by chemosensory input also contain cell bodies and/or fibers of gonadotropin releasing

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hormone (GnRH) neurons [12,31], suggesting a possible relationship between chemosensory input and GnRH; outside the role of GnRH as a hypothalamic hypophysiotropic neuropeptide. GnRH is known to influence mating behavior in male rodents [5,6]. In hamsters, sexually inexperienced males with vomeronasal lesions have impaired mating behavior [21], but exogenous GnRH injected into the lateral ventricle can substantially restore mating behavior [8].

GnRH from endogenous sources is implicated in the rapid hormonal response to VN chemosensory input in hamsters and mice: It may be released naturally when male rodents encounter female chemosignals. In male mice, serum luteinizing hormone (LH) levels increase within 5 min in response to exposure to female urine [4]. In male hamsters, exposure to female hamster vaginal fluid produces an increase in testosterone [28]. Both of these responses are dependent on an intact VNO [28,33]. Because GnRH induces LH secretion and subsequent increases in testosterone, these results suggest that chemosensory input, mediated by the VNO, increases intracerebral release of endogenous GnRH, which may then facilitate reproductive behavior. We have previously shown an increase in Fos expression in the MPOA of mating males given i.c.v. injections of GnRH. This increase was greater than the increase in Fos seen in mating males given intracerebroventricular (i.c.v.) saline, or in non-mating males given i.c.v. GnRH alone [1]. We know that the MPOA receives chemosensory input from the VN pathway [9] and is known to be important for mating in males [29].

The goal of the following experiments was to investigate neuronal activation in brain regions known to be activated in mating behavior—when they are influenced by chemosensory signals alone, or by GnRH alone, or when these inputs are combined. We used Fos expression as a marker for neuronal activity in male hamsters exposed to hamster vaginal fluid (HVF) in combination with i.c.v. GnRH or i.c.v. saline. Exposure to HVF in the absence of the female allowed us to investigate the convergence of chemosensory and GnRH influences on neuronal activation (Fos expression) without the complication of Fos expression related to mating itself.

HVF contains pheromones known to act via the VN system [2], and has both sexual attractant [13] and sexual stimulant (aphrodisiac) qualities [14]. It is a sufficient stimulus to induce mounting and other copulatory behaviors in naive male hamsters placed with inappropriate partners [14], such as other male hamsters that have been anesthetized and scented with HVF [2]. Male hamsters exposed to HVF have increased Fos expression along the VN pathway and MPOA [7,11,18].

The pheromonal components of HVF are largely transduced via the VNO [2], although whole HVF also elicits behavioral responses via the main olfactory system [27]. The vomeronasal information passes via the accessory

olfactory bulb (AOB) centrally to the anterior and posterior medial amygdala (MeA, MeP) and posterior medial cortical nucleus (PMCN) of the amygdala (collectively called the VN amygdala) and to the bed nucleus of the stria terminalis (BNST) [15]. These areas then send projections to the medial preoptic area (MPOA). Many of these brain regions, particularly the MPOA, are important for mating behavior in male hamsters [29]. Lesions of the pathway from the amygdala to the MPOA disrupt mating, as do lesions of the MPOA [19,20,29]. The central vomeronasal (VN) pathway is illustrated on the left side of Fig. 1.

The main olfactory system is largely involved in the processing of general odorants, but has potential in-

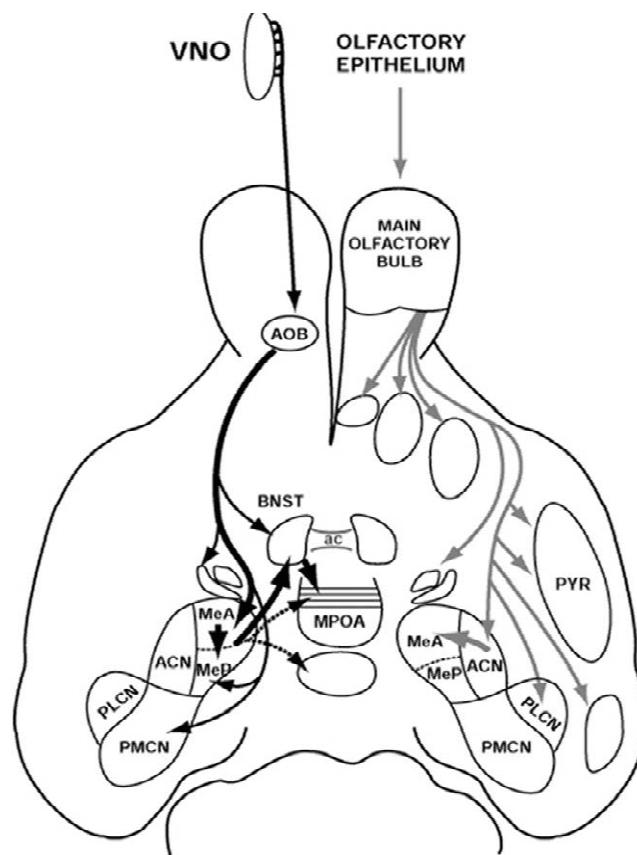


Fig. 1. Projections of chemosensory systems in hamsters. The vomeronasal/accessory olfactory system is shown on the left in this ventral view. The main olfactory projections are shown on the right. The accessory olfactory bulb (AOB) projects heavily to the anterior medial amygdala (MeA) and more lightly to posterior medial amygdala (MeP) and posterior medial cortical nucleus (PMCN) of the amygdala, as well as weakly to the bed nucleus of the stria terminalis (BNST). MeA and MeP project directly and via the BNST to the medial preoptic area (MPOA). Fos expression was measured in each of these areas to determine the effects of GnRH in combination with chemosensory input on neuronal activity. The main olfactory system influences the vomeronasal amygdala regions via projections from the main olfactory bulb (MOB) to anterior cortical nucleus (ACN) and posterior lateral cortical nucleus (PLCN) which have cross projections to medial amygdala (Modified from diagrams used by S. Winans-Newman). (AC=Anterior commissure, PYR=piriform cortex).

volvement in pheromonal chemosensory signaling. Via the main olfactory bulb, olfactory input goes to olfactory (piriform) cortex and to regions of the amygdala including the anterior cortical nucleus (ACN) and postero-lateral cortical nucleus (PLCN), together called the olfactory amygdala [16]. The olfactory amygdala, especially the ACN is an indirect route by which main olfactory signals can influence areas in the VN pathway, because it has projections to the vomeronasal amygdala, especially MeA [3,9]. Via these projections, main olfactory input can influence activity in the MPOA and ultimately, influence behavior. Significantly, sexually experienced male hamsters continue to mate normally after their vomeronasal organs are removed [21]. Because chemosensory input is essential for mating in hamsters [25,30,26], these animals must use main olfactory input for mating. The central olfactory pathway and its connections to the central vomeronasal pathway are illustrated on the right side of Fig. 1. The approximate locations of most of the anatomical areas are illustrated in Fig. 6A.

Here, we investigate Fos expression in response to HVF stimulation and its modulation by GnRH, in the MPOA and medial amygdala of intact males and males with VNOs removed (VNX), both before (naive) and after experience.

2. Materials and methods

2.1. Experimental animals

Animals used in these experiments were adult (2–3 months) male golden hamsters (*Mesocricetus auratus*), bred in our laboratory, and maintained on a long photoperiod (a partially reversed-14L/10D light cycle). The animals were group-housed in clear plastic cages (44 cm×21 cm×18 cm) containing bedding with food and water ad libitum. Experiments 1–3 used, respectively, intact naive males, naive males with vomeronasal lesions (VNX), and sexually experienced VNX-males. Each of these groups of subjects was further divided in to four sub-groups to investigate the effects of exposure to HVF or water, and the effects of injection of GnRH or saline on Fos expression. Initially, there were six animals in each sub-group.

2.2. Experience protocol

For the experienced VNX group, inexperienced male hamsters between 2 and 3 months of age were placed in a clean cage and provided with a naturally cycling behaviorally receptive female and observed mating for 1 h. If males did not achieve three ejaculations they were presented with another receptive female for up to 1 additional hour. Animals were returned to their home cage and considered sexually experienced after this one exposure [21]. All of the males achieved three ejaculations with this

procedure in the first hour. Vomeronasal organs were removed 4–9 days following experience.

2.3. VNO Removal

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 and across the midline with a dental drill to form a ‘U’ shaped groove connecting the two natural 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 into the midline suture with a scalpel. The final, anterior, connection with the palate was broken by drilling rostro-dorsally on the midline anterior to the U-groove. Each capsule containing one VNO was then removed with small forceps, separately. The palatal incision was closed with 3–5 sutures and sealed with cyanoacrylate adhesive. Experiments were conducted at least 6 days following VNX surgery.

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 two animals with incomplete lesions were eliminated from the study, leaving five animals in two groups: saline-injected naive males exposed to water and GnRH-injected experienced males exposed to water.

2.4. Intracerebroventricular injections

3–5 days after VNX surgery, guide tubes (28 gauge, Plastics One) were implanted in the left lateral cerebral ventricle. After an additional 3–5 days animals were given i.c.v. injections, exposed to HVF or water, and their behavior was tested. Thirty minutes prior to chemosensory exposure and behavior testing, GnRH (50 ng in 2 μ l) or saline vehicle (2 μ l) was pressure injected through a 33 gauge cannula inserted into the guide tube of the implanted freely-moving animals.

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. The cannulae were individually calibrated before each use by adjusting the duration of the picospritzer air pulse at standard pressure until 2 mg of distilled water (2 μ l) was ejected onto a filter paper placed on the pan of an analytical balance. For GnRH and saline injections, 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. Fresh cannulae were used for each experiment.

2.5. Exposure and behavior testing

Animals were placed in a 3-l glass chamber (25×14 cm) with continuous 2 l/min air flow for 10 min to allow for acclimation. Then, either diluted HVF (1:10 in distilled water) or distilled water was introduced periodically from a 1 ml syringe via a tube connected to a well machined into the aluminum block attached to the end cap and protruding into the chamber at one end.

A 1-ml volume of stimulus (HVF or water) was delivered to each animal over a 40-min period, in a temporal pattern designed to maintain the animal's interest in the well for at least 50% of the first 20 min of the exposure time. Behavior was scored manually by an observer using a computer key-pad. Recorded behaviors included grooming, sniffing and licking at or close to the well, sniffing at the back or the front of the chamber, sniffing and licking the walls of the chamber, flank marking, escape scurrying and sleeping. These behaviors were also placed into three broad categories for analysis: specific chemosensory investigation, non-specific investigation, and vegetative behavior. 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 scurrying at the walls of the chamber. The test chamber and stimulus testing 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 testing, the animal was placed in a clean cage for 40 min to allow for detectable Fos protein production. Animals were then given a standard 5-min mating test to determine their behavioral readiness to mate. This test was given 5 min before perfusion so that mating behavior would not significantly contribute to Fos expression. Detailed results of the mating behavior studies are presented separately.

2.6. Tissue processing for Fos ICC

Animals were deeply anesthetized with sodium pentobarbital and perfused through the heart with 0.1 M phosphate-buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde. Brains were removed and post-fixed 2–16 h, washed for an hour in 0.1 M PBS, then sectioned

serially on a Vibratome at 50- μ m thickness. Free-floating coronal sections were processed based on protocols described and used by Fernandez-Fewell and Meredith [7,9]. Sections were washed for 30 min in 0.1 M PBS (three washes), then incubated in polyclonal rabbit anti c-fos primary antiserum (1:50,000, Santa Cruz Biotechnology, Inc.), for 16–24 h at room temperature. Sections were then washed in 0.1 M PBS (three washes), incubated in biotinylated goat anti-rabbit secondary antiserum (1:400) for 1 h. Sections were then incubated in avidin–biotin–horseradish peroxidase (HRP) complex (Vector) and diaminobenzidine (DAB) with added nickel chloride for visualization of a black reaction product. Pre-absorption and primary antibody deletion controls with this method showed no nuclear staining (data not shown). As far as possible, tissue from each sub-group of animals was processed together within each experiment. The three experiments were run in sequence and although the processing described here was used for all experiments, tissues from different experiments were not processed together.

Fos-positive nuclei were counted using computer image analysis software (Image-Pro Plus), initially in a single tissue section for each brain-area of interest, carefully selected to be at the same anatomical position in each animal. The areas of interest included central projections of the VN pathway such as the anterior medial amygdala (MeA), posterior medial amygdala (MeP), rostral and caudal posterior medial BNST (pmBNSTr and pmBNSTc, respectively) [7,11], and mid-caudal MPOA. The anatomical positions of the sections chosen for counting were originally selected as representative areas with increased Fos expression in mating animals [7]. In each area Fos-positive nuclei were counted within the border of the neuroanatomical nucleus except for the medial preoptic area, where Fos-positive nuclei were counted within an area bordered by a medio-lateral line dorsally and a dorso-ventral line laterally, as illustrated in Fig. 6B. Additional sections and additional sub regions within the MPOA region were later analyzed separately because GnRH-enhanced Fos expression did not appear to be uniformly distributed within the MPOA. These areas are described with the results.

2.7. Statistical analysis

Initially, Fos data from each brain area were analyzed by two-way analysis of variance (ANOVA) comparing two factors: treatment (GnRH vs. saline) and exposure (HVF vs. water). A significant main effect of exposure would indicate a significant difference in Fos expression with HVF exposure regardless of the effect of GnRH. A significant main effect for treatment would indicate a significant difference in Fos expression with GnRH regardless of the effect of HVF. A significant interaction would

indicate that the effect of GnRH on Fos expression was different in HVF exposed and water exposed males.

In addition to these primary ANOVA analyses within each area, two further ANOVA analyses were conducted. The first used data from HVF exposed males only. This repeated measures (RM) ANOVA compared the effect of treatment (GnRH vs. saline) in all of the different brain areas. The second of these RM ANOVA analyses for the initial data also compared the effect of treatment in all areas, but for water-exposed males only. This ANOVA was performed to examine our hypothesis that GnRH would have no effect on Fos expression in water-exposed males.

The final ANOVA for the initial data analyzed differences between the three surgical groups (intact, experienced-VNX, naive-VNX) in all brain areas for the HVF-exposed, saline-injected males. This ANOVA allowed us to look at differences in the basic HVF response between groups. These cross-experiment comparisons in Fos expression should be interpreted carefully because tissue from different experiments were not processed together.

In the expanded analysis of Fos expression within medial preoptic nucleus (MPN) and magnocellular MPN (MPNmag), ANOVAs compared treatment (GnRH vs. saline) and location (sections +2 to -3) within HVF-exposed males in each surgical group (separately) to identify which sections showed a synergistic effect of GnRH on Fos expression in response to HVF exposure. Another set of RM ANOVAs compared exposure (HVF vs. water) and section (+2 to -3) within saline-injected males for each group to determine the effect of HVF in each group and section (basic HVF response). In an additional two-way RM ANOVA data from saline-injected, HVF-exposed males were used to analyze differences in HVF response across all surgical groups (intact, naive-VNX, and experienced-VNX) for all sections (+2 to -3). This ANOVA looked at the basic HVF response across groups within the subdivisions of MPOA.

For analysis of behavior in the exposure chamber, a two-stage analysis was performed. Initially the data on time spent in each behavior category: '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, naive-VNX, and experienced-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 were inspected but no additional differences related to treatment, exposure, or surgery were evident, so the data on 'non-specific investigation' and 'vegetative behaviors' were not analyzed further. Additional ANOVAs looked only at time spent in 'specific investigation' at the stimulus well. Two-way ANOVAs comparing treatment (GnRH vs. saline) and exposure (HVF vs. water) were conducted for each surgical group. An additional ANOVA including data from HVF-exposed males in all groups was

also conducted to look at differences in investigation time due to surgery and experience.

3. Results

Our primary focus was on the MPOA: the area expected from our experiments in mating animals to show changes in neuronal activity important for chemosensory/hormonal influences on mating. We describe these results first for each experiment, followed by descriptions of the other VN projection areas. The data for MPOA are for a single section at the mid-caudal MPOA level as reported in previous publications [7,9,10]. A more extensive analysis of regions and sub-regions of the MPOA for all experiments follows the description of individual experiments.

3.1. Experiment 1: naive intact males

3.1.1. Fos expression in MPOA

Fos expression in the mid-caudal MPOA was recorded in naive-intact male hamsters from each of four groups: GnRH-injected and exposed to HVF; GnRH-injected and exposed to water; saline-injected and exposed to HVF; saline-injected and exposed to water. The mid-caudal MPOA is strongly activated during mating and is where we previously saw a significant increase in Fos expression in mating males given i.c.v. GnRH compared to those given i.c.v. saline. Fig. 2 shows representative photomicrographs of Fos expression in a coronal section from an animal in each condition. Two-way ANOVA comparing 'treatment' (GnRH vs. saline) and 'exposure' (HVF vs. water) within the MPOA indicated significant increases in Fos expression with HVF exposure (Fig. 3A).

Within the MPOA data, there was a significant overall (main) effect of HVF exposure compared to water exposure ($P < 0.001$, $F = 98.944$, $df = 1, 16$) that was significant in post-hoc tests for both GnRH and saline injected groups (indicated by 'a' in Fig. 3A). There was no significant overall effect of GnRH. However, a significant interaction ($P = 0.001$, $F = 15.78$, $df = 1, 16$) showed that GnRH injection combined with HVF exposure produced a significantly greater increment in Fos than GnRH injection combined with water exposure ($P < 0.001$; Newman-Keuls post-hoc test). This is indicated by the asterisk in Fig. 3A.

These data indicate that exogenous i.c.v. GnRH combined with HVF exposure activated additional neurons in the MPOA that were not activated by either GnRH (water-exposed) or HVF (saline-injected) alone.

3.1.2. Fos expression in areas on the central VN pathway

Fos expression in additional brain regions along the VN pathway was also recorded. Intact males exposed to HVF had significantly greater Fos expression in several brain areas compared to males exposed to water, as indicated by

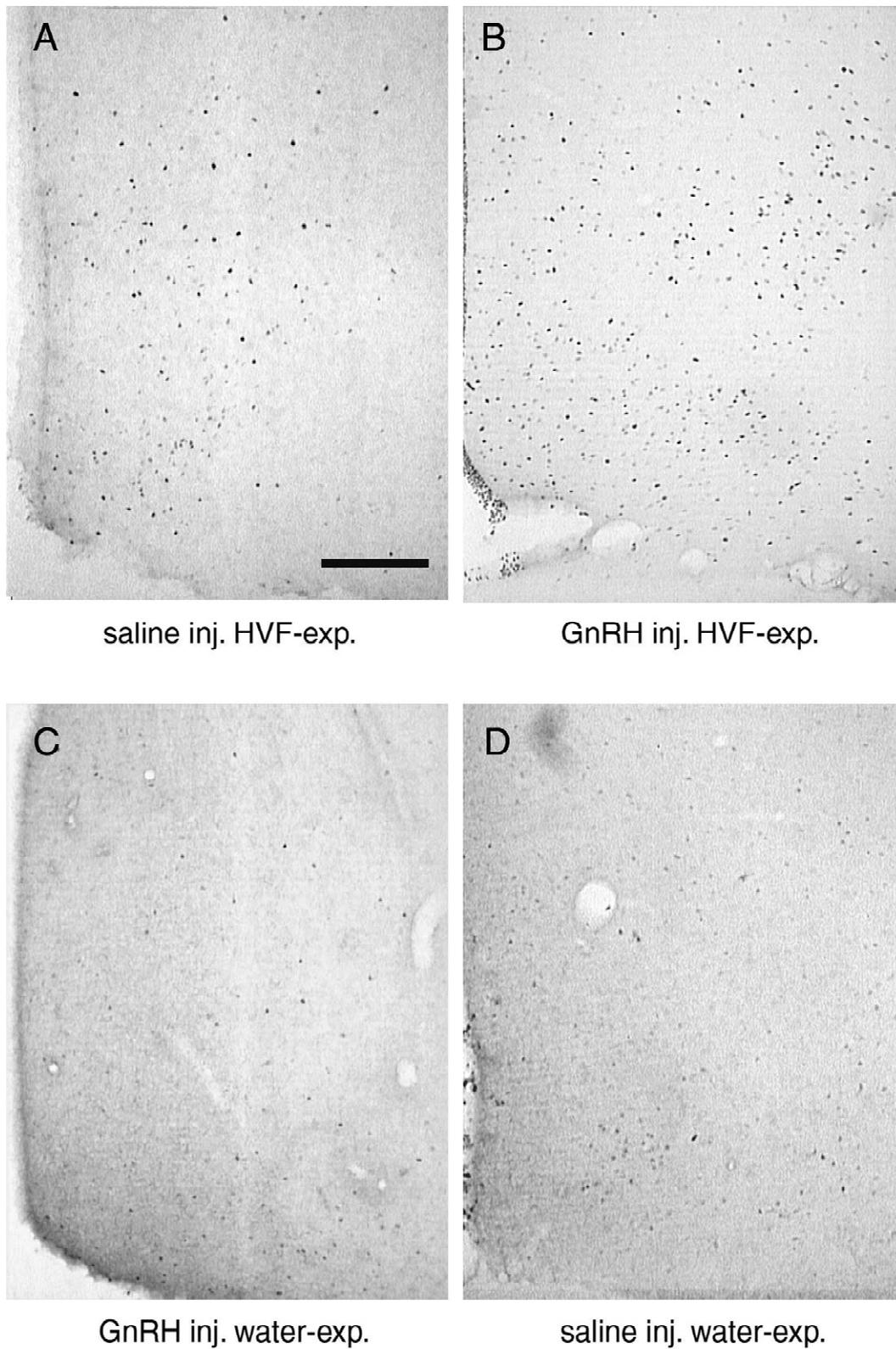


Fig. 2. Representative photomicrographs showing Fos expression in the MPOA in one INTACT male in each treatment and exposure condition. Exposure to HVF significantly increased Fos expression in the MPOA (A). This increase in Fos was even greater when exposure to HVF was combined with i.c.v. GnRH (B). GnRH injection combined with water exposure produced little increased Fos expression (C) compared to the control condition; saline injection and water exposure (D). Midline is to the left in each case. Scale bar=50 μ m.

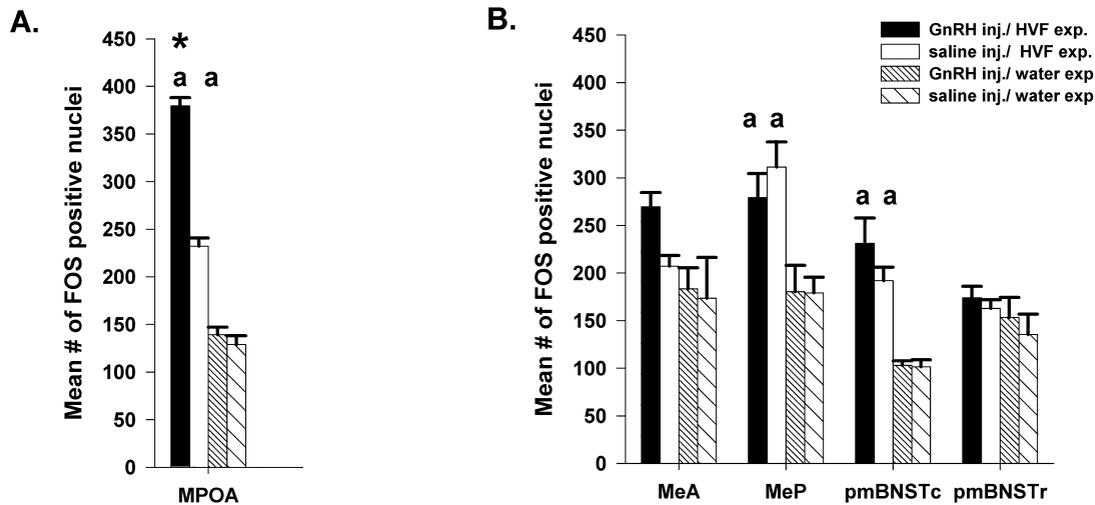


Fig. 3. Fos expression in MPOA and vomeronasal pathway in intact males. Data shown in Figs. 3–5 are mean and standard error (S.E.) for each group and brain area. Probabilities are for post-hoc tests associated with the ANOVAs described in the text. (A) Fos expression in mid-caudal MPOA in the four combinations of treatment and exposure. Males exposed to HVF had significantly higher levels of Fos expression than those exposed to water ($P < 0.001$; indicated by letter 'a'). HVF-exposed males also injected with GnRH had a significant increment in Fos expression compared to those injected with saline ($P < 0.001$; indicated by asterisk). See text for statistical details. (B) Fos expression in four areas along the vomeronasal pathway with each of the four combinations of treatment and exposure. HVF exposure resulted in significantly greater Fos expression in MeP and pmBNSTc ($P < 0.001$; indicated by letter 'a'). There was no incremental effect of GnRH in these areas in intact males.

two-way ANOVA comparing 'treatment' (GnRH vs. saline) and 'exposure' (HVF vs. water) in each brain area. A significant overall increase in Fos expression, attributable to HVF exposure appeared in the caudal posterior medial amygdala (MeP: $P = 0.037$, $F = 5.205$, $df = 1, 16$), MPOA (described above), and caudal postero-medial bed nucleus of stria terminalis (pmBNSTc: $P < 0.001$, $F = 16.430$, $df = 1, 16$). Individual sub-groups of males showing a significant increase with HVF exposure in these areas are indicated by the letter 'a' in Fig. 3B ($P < 0.001$ in Newman–Keuls post-hoc tests). No area other than MPOA showed an extra increment in Fos expression with GnRH. In these tests the increase in Fos expression in the rostral postero-medial bed nucleus of the stria terminalis (pmBNSTr) and anterior medial amygdala (MeA) did not reach significance although significant increases in Fos expression with HVF exposure are generally seen in MeA. All of these areas showed increased Fos expression in mating males in previous experiments.

A separate two-way RM ANOVA compared 'treatment' (GnRH vs. saline) across all brain areas shown in Fig. 3A and B, for HVF-exposed males. There was a significant overall effect of area ($P < 0.001$, $F = 8.512$, $df = 6, 60$) and no overall effect of GnRH. Newman–Keuls post-hoc tests confirmed an increment in Fos expression in GnRH-injected animals exposed to HVF, compared to saline-injected animals exposed to HVF, within the MPOA only ($P = 0.002$) (Fig. 3A; already noted by the asterisk). There were no effects of GnRH on other brain-areas in this test. A similar two-way RM ANOVA comparing treatment and area for water-exposed animals revealed no significant

difference in any brain area due to GnRH, and there was no significant overall effect of GnRH.

3.2. Experiment 2: naive-VNX males

In the previous experiment, intact males had both VN and main olfactory chemosensory input. Removal of the VNX eliminates vomeronasal input, but chemosensory input via the olfactory epithelium and main olfactory bulb is still present. In the next experiments, we wanted to determine if GnRH would enhance Fos activation due to relevant chemosensory input regardless of its source through the main or accessory olfactory (VNO) chemosensory systems. Naive-VNX males show mating deficits [21], but GnRH relieves those deficits [8]. Therefore, we also wanted to determine if this behavioral result was associated with increased Fos expression.

3.2.1. Fos expression in MPOA

Fos expression was recorded in brains of naive-VNX male hamsters that had been exposed to female chemosignals (HVF) in the same manner as described for intact animals. Two-way ANOVA comparing 'treatment' (GnRH vs. saline) and 'exposure' (HVF vs. water) in showed that HVF exposure significantly increase Fos expression ($P > 0.001$, $F = 87.426$, $df = 1, 14$) in MPOA in both saline- and GnRH-injected males ($P < 0.001$ in post-hoc tests; indicated by 'a'), but there was no overall effect of GnRH and no interaction between treatment and exposure. Thus, there was no additional effect of GnRH in this brain area in naive-VNX males (Fig. 4A). Nor did we see a synergistic

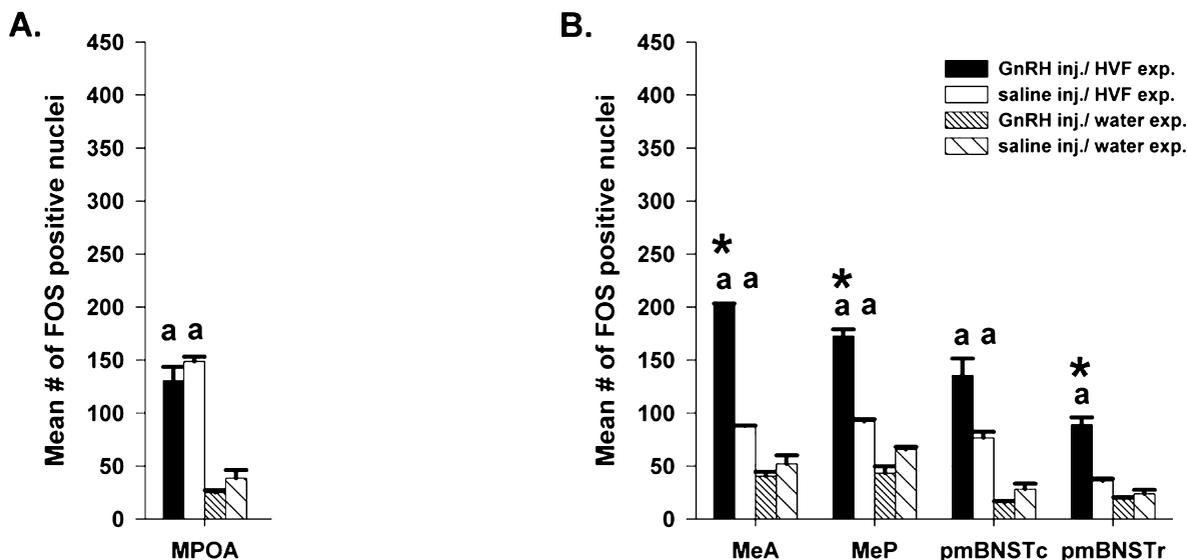


Fig. 4. Fos expression in MPOA and vomeronasal pathway of naive-VNX males (mean, S.E.). (A) Fos expression in mid-caudal MPOA with the four combinations of treatment and exposure. Males exposed to HVF had significantly higher levels of Fos expression than those exposed to water ($P < 0.001$; indicated by letter 'a'). There was no enhancement of HVF response in MPOA by GnRH for these males. (B) Fos expression in four areas along the vomeronasal pathway with each of four combinations of treatment and exposure. HVF exposure resulted in significantly greater Fos expression in MeA, MeP, and pmBNSTr ($P < 0.001$ for each area indicated by letter 'a'). GnRH injection further enhanced Fos expression in MeA ($P < 0.001$), MeP ($P < 0.001$), and pmBNSTr ($P < 0.001$) (asterisks) compared to saline-injected, HVF-exposed males. In pmBNSTr, Fos expression was only significant in response to HVF ('a') when combined with GnRH injection.

effect of GnRH combined with HVF in the MPOA in a two-way RM ANOVA of the effect of treatment across all areas in HVF-exposed males (see below). If neurons sensitive to the combination were activated in MPOA, they were not activated strongly enough to induce an increase in detectable Fos expression.

3.2.2. Fos expression in areas on the central VN pathway

As for intact animals, HVF exposure increased Fos expression in areas of the VN pathway as well as in the MPOA. Two-way ANOVA comparing 'treatment' (GnRH vs. saline) and 'exposure' (HVF vs. water) for each area showed overall significant increases in Fos expression due to HVF in the following areas: MeA ($P < 0.001$, $F = 161.57$, $df = 1, 12$), MeP ($P < 0.001$, $F = 89.924$, $df = 1, 12$), MPOA (described above), pmBNSTr ($P < 0.001$, $F = 33.825$, $df = 1, 12$), and pmBNSTc ($P < 0.001$, $F = 43.028$, $df = 1, 14$). The sub-groups with significant increases due to HVF exposure are indicated by letter 'a' in Fig. 4B ($P < 0.001$ in Newman–Keuls post-hoc tests). There was also a significant overall effect of GnRH in MeA ($P < 0.001$, $F = 161.57$, $df = 1, 12$), MeP ($P < 0.001$, $F = 89.924$, $df = 1, 12$), pmBNSTr ($P < 0.001$, $F = 33.852$, $df = 1, 12$), and pmBNSTc ($P < 0.001$, $F = 43.028$, $df = 1, 12$). These ANOVAs also revealed a significant interaction between treatment and exposure in the MeA ($P < 0.001$, $F = 65.762$, $df = 1, 12$), MeP ($P < 0.001$, $F = 39.327$, $df = 1, 12$) and pmBNSTr ($P < 0.001$, $F = 17.048$, $df = 1, 12$), such that GnRH produced an increment in Fos expression in HVF-

exposed males in each of these areas, above the level in saline-injected HVF-exposed males ($P < 0.001$ in post-hoc tests; indicated by asterisks in Fig. 4B).

An additional two-way RM ANOVA analysis compared 'treatment' (GnRH vs. saline) across all brain areas, including MPOA, for HVF-exposed males. There was a significant effect of treatment (GnRH) in the across-area test ($P < 0.001$, $F = 57.86$, $df = 1, 24$) and a significant interaction ($P < 0.001$, $F = 6.93$, $df = 4, 24$) in that some areas showed a significant increase in Fos with GnRH and the other areas did not (Fig. 4A and B). Newman–Keuls post-hoc tests from this across-area ANOVA confirmed conclusions from the individual area ANOVAs that GnRH injections further increased Fos expression in the MeA ($P < 0.001$), the MeP ($P < 0.001$), and pmBNSTr ($P = 0.012$), but not in the MPOA.

GnRH injection had no effect on any area in water-exposed animals. The second two-way RM ANOVA, comparing 'treatment' and 'area' showed no significant differences in Fos expression between GnRH-injected and saline-injected males for any individual area in males not exposed to HVF.

3.3. Experiment 3: experienced-VNX males

If GnRH potentiation of Fos expression in the MPOA was not seen in the naive-VNX males exposed to HVF because levels of Fos expression were sub-threshold, we reasoned that substitution of experienced-VNX males might reveal an effect of GnRH. Experienced-VNX males

mate normally and in previous published experiments had higher overall Fos expression in response to HVF [10,21]. This could increase the probability that a further potentiation by GnRH would be observed as an increase in Fos expression. After sexual experience, main olfactory input becomes a sufficient chemosensory input for mating in male hamsters. Therefore, main olfactory input may be sufficient to produce measurable GnRH-potentiation of HVF-induced Fos in the VN pathways, and/or MPOA of experienced-VNX males.

3.3.1. Fos expression in MPOA

Experienced-VNX males were grouped as for naive-VNX males, but were given sexual experience prior to the removal of the VNO. Two-way ANOVA comparing ‘treatment’ (GnRH vs. saline) and ‘exposure’ (HVF vs. water) in MPOA revealed a significant overall increase in Fos expression with HVF exposure in the MPOA ($P < 0.001$, $F = 105.80$, $df = 1, 16$). Post-hoc tests showed a significant ($P < 0.001$) increase with HVF for both saline and GnRH-injected males (indicated by ‘a’ in Fig. 5A). There was no significant overall effect of GnRH and no interaction. GnRH did not potentiate the expression in our ‘mid-caudal’ MPOA section by this analysis (Fig. 5A) or by a two-way RM ANOVA comparing treatment across areas in HVF-exposed males (see below). In fact Fos expression was lower in MPOA with GnRH injection compared to saline injection in experienced-VNX males exposed to HVF (indicated by an asterisk in Fig. 5A). This effect was only marginally significant ($P = 0.042$). However, further analysis did reveal significant increases in Fos expression

with GnRH in adjacent regions of the MPOA (see expanded analysis).

3.3.2. Fos expression in areas on the central VN pathway

Two-way ANOVAs comparing ‘treatment’ (GnRH vs. saline) and ‘exposure’ (HVF vs. water) for each area showed that HVF exposure increased Fos expression in all areas for both GnRH and saline injected males ($P < 0.001$ in post-hoc tests; indicated by ‘a’ in Fig. 5B). These regions included the MeA ($P < 0.001$, $F = 30.523$, $df = 1, 12$), MeP ($P < 0.001$, $F = 20.327$, $df = 1, 12$), MPOA, pmBNSTr ($P < 0.001$, $F = 241.876$, $df = 1, 12$) and pmBNSTc ($P < 0.001$, $F = 342.758$, $df = 1, 16$), (Fig. 5B). There was no potentiation of Fos expression by GnRH. In fact there was a significant overall depression of Fos expression in GnRH-injected compared to saline-injected males in MeP ($P = 0.001$, $F = 20.38$, $df = 1, 10$), pmBNSTr ($P < 0.001$, $F = 241.88$, $df = 1, 10$), and pmBNSTc ($P < 0.001$, $F = 799.84$, $df = 1, 10$). These within area tests (described above) also revealed significant interactions in the pmBNSTr ($P < 0.001$, $F = 321.68$, $df = 1, 10$) and pmBNSTc ($P < 0.001$, $F = 241.98$, $df = 1, 10$), such that there was a significantly greater depression of Fos expression with GnRH when combined with HVF exposure than GnRH combined with water exposure (shown by asterisks on graph in Fig. 5B; post-hoc tests P -values given in legend).

The additional two-way RM ANOVA comparing GnRH and saline effects across all areas in HVF-exposed males confirmed an overall depressive effect of GnRH ($P = 0.002$, $F = 1.54$, $df = 1, 32$) and a significant interaction between

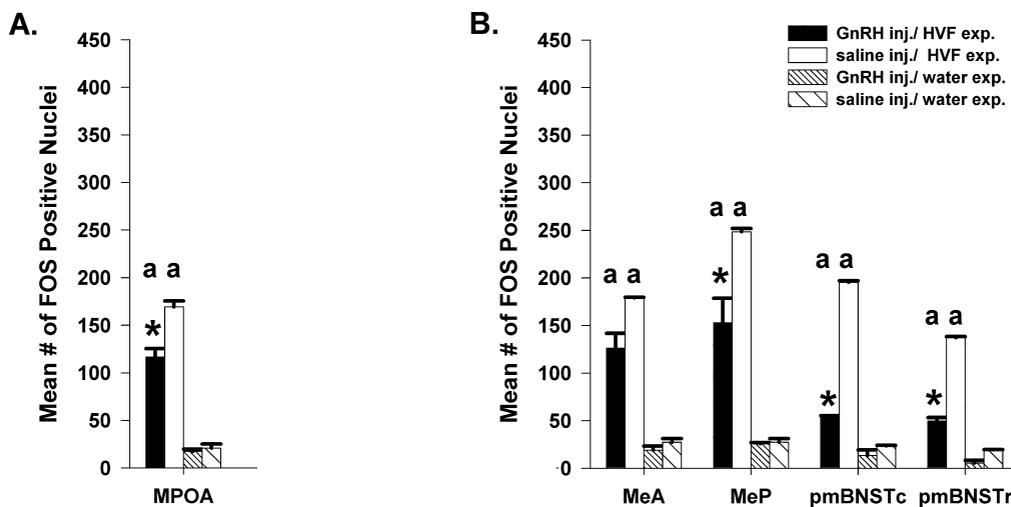


Fig. 5. Fos expression in MPOA and vomeronasal pathway of experienced-VNX males (mean and S.E.). (A) Fos expression in MPOA with the four combinations of treatment and exposure. Males exposed to HVF had significantly higher Fos expression in MPOA than those exposed to water ($P = 0.001$; indicated by letter ‘a’). GnRH slightly depressed Fos response in HVF exposed males in this section ($P = 0.042$; asterisk). GnRH increased Fos expression elsewhere in MPOA (see Figs. 7 and 8). (B) Fos expression in four areas along the vomeronasal pathway with the four combinations of treatment and exposure. HVF exposure resulted in significantly greater Fos expression in all areas (indicated by ‘a’). As in MPOA, GnRH injection depressed Fos expression in MeP ($P = 0.042$), pmBNSTc ($P < 0.001$), and pmBNSTr ($P < 0.001$) (asterisks) compared to saline-injected, HVF-exposed males.

treatment and area ($P=0.021$, $F=3.35$, $df=4$, 32). Newman–Keuls post-hoc tests confirmed significant decreases in Fos expression in HVF-exposed males when given GnRH, in the MeP ($P=0.001$) and pmBNSTc ($P=0.001$). In this across-area test, the decrease in Fos expression in MPOA and pmBNSTr did not reach significance. In water-exposed males, a two-way RM ANOVA comparing ‘treatment’ and ‘area’ indicated no significant effect of treatment (GnRH vs. saline) in animals not exposed to HVF (Fig. 5A and B).

3.4. Comparison across experiments

The final statistical test on the initial data was a two-way RM ANOVA including HVF-exposed, saline-injected animals for all three experiments. The analysis examined the effect of surgical treatment (intact, experienced-VNX, naive-VNX) and brain area (MPOA, MeA, MeP, pmBNSTr, pmBNSTc) on Fos expression. There was a significant overall effect of surgical treatment ($P<0.001$, $F=32.76$, $df=2$, 48) and area ($P<0.001$, $F=8.099$, $df=4$, 48) with no significant interaction. Post-hoc tests showed that Fos expression following HVF exposure in intact and experienced-VNX males across all areas was not different, but for each of these groups, was greater than that for naive-VNX males. Among individual areas, intact males had greater Fos expression than naive-VNX males in MeA ($P=0.02$), MeP ($P<0.001$), pmBNSTr ($P=0.001$), and MPOA ($P=0.04$). The overall across-area difference between experienced-VNX and naive-VNX males was reflected in individual areas only in MeA ($P=0.024$) and pmBNSTr ($P=0.014$). There were no significant differences between surgical groups in Fos expression in pmBNSTc.

3.5. Expanded analysis of Fos expression in MPOA: all experiments

The MPOA is a large brain region containing several subdivisions. In previous experiments, sexually experienced-VNX males had higher levels of Fos expression in the mid-caudal MPOA due to HVF exposure than did naive-VNX males [22,10]. Even with this potentially higher level of activation, in the present experiments. We saw no enhancement of HVF-induced Fos, by i.c.v. GnRH, in the mid-caudal MPOA (the standard region examined in previous experiments). However, when we conducted a more extensive investigation of Fos expression in subregions of MPOA spanning several sections, we found a significant enhancement by GnRH, of HVF-induced Fos within MPOA as we had predicted for experienced-VNX males. This expanded analysis was then applied uniformly to data from all three of the experiments reported here.

We sub-divided the caudal MPOA region into the two established anatomical nuclei, the MPN and the MPNmag [22]. Previous reports have identified the MPN [7] and MPNmag [18] as regions showing increased Fos in intact

males exposed to HVF. To investigate the effects of i.c.v. GnRH combined with HVF exposure, we looked at these two divisions in five additional (50 μ m) coronal sections (designated -3 to $+2$) throughout the mid and caudal MPOA, beginning at the posterior end of the midline anterior commissure (-3) and ending 100 microns ($+2$) behind our ‘standard’ mid-caudal MPOA section. The standard section was designated 0. Thus, the sections more rostral were ‘ -3 ’ (150 microns rostral), ‘ -2 ’, and ‘ -1 ’ (100 and 50 microns rostral). The two sections more caudal were ‘ $+1$ ’ (50 microns caudal) and ‘ $+2$ ’ (100 microns caudal) caudal to our standard section. Fig. 6 shows where these sections are located in relation to our standard mid-caudal MPOA section (0).

Significant Fos activation was seen in the MPN and to a lesser extent in the MPNmag in intact males (experiment 1), naive-VNX males (experiment 2) and experienced-VNX males (experiment 3) due to HVF exposure. The further enhancement of Fos expression with GnRH treatment was seen only in intact and experienced-VNX males. The distribution of ‘extra’ Fos attributable to GnRH appeared different between the two groups such that significant increases occurred in different sections. Even with the extended analysis, naive-VNX males (experiment 2) showed no significant increment in HVF-induced Fos expression due to i.c.v. GnRH, in any subdivision of MPOA, despite the fact that it is these animals that show an effect of i.c.v. GnRH on mating behavior.

In intact HVF-exposed males there was a significant overall effect of GnRH ($P=0.007$, $F=15.688$, $df=1$, 30), in a two-way RM ANOVA comparing ‘treatment’ (GnRH vs. saline) across sections (-3 to $+2$). Fos expression was significantly greater ($P=0.005$, post-hoc tests) in the MPN region of most sections in GnRH-injected intact males, with the exception of the most rostral and most caudal sections (Fig. 7A; asterisks). A second two-way RM ANOVA comparing ‘treatment’ and ‘section’ for MPNmag in intact males indicated a significant overall effect of GnRH ($P=0.002$, $F=28.671$, $df=1$, 30), with significant increments in Fos expression due to GnRH in three sections: ‘ -1 ’ ($P<0.001$), ‘ -2 ’ ($P=0.002$), and ‘ $+1$ ’ ($P<0.001$) (Fig. 8A; asterisks).

Among experienced-VNX males exposed to HVF, there was also a significant overall effect of GnRH ($P=0.025$, $F=12.178$, $df=1$, 20) in MPN with a significant increment in Fos expression due to GnRH in the ‘ -2 ’ section ($P=0.01$) and ‘ $+2$ ’ section ($P=0.031$): That is, not in the same sections as in intact males (Fig. 7B; asterisks). In the MPNmag of experienced-VNX males, there was no significant main effect of GnRH, but a significant interaction ($P=0.002$, $F=5.532$, $df=1$, 20) between ‘treatment’ and ‘section’ indicating a non-uniform response to GnRH across sections. The pattern across sections was again different from that in the equivalent area of intact animals. Only the ‘ -2 ’ section showed a significant increment in Fos ($P=0.008$) attributable to the GnRH (Fig. 8B; asterisks).

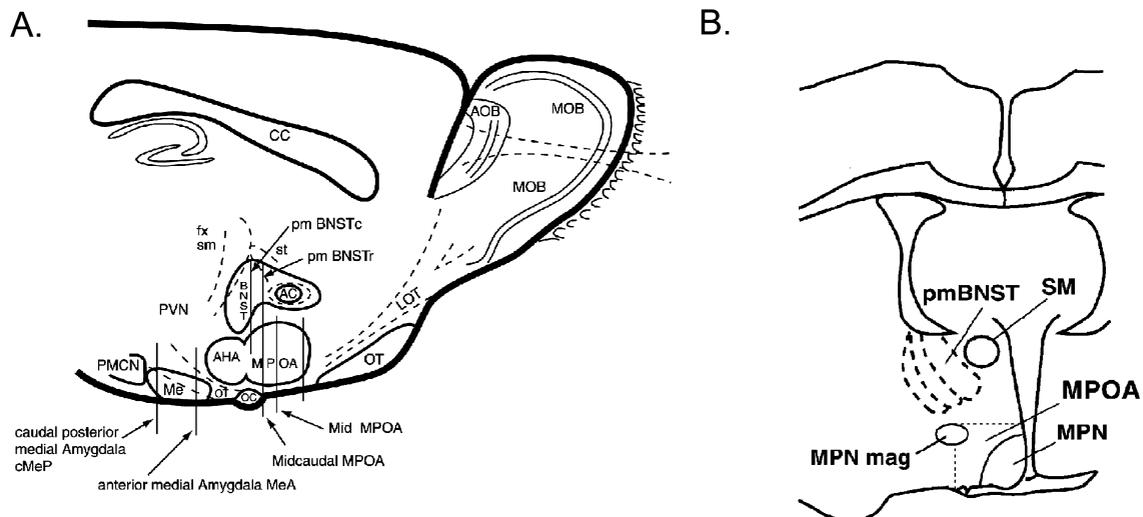


Fig. 6. (A) Sagittal projection illustrating the positions of the additional sections counted for the extended analysis of Fos expression in MPOA. The original mid-caudal MPOA section analyzed in Figs. 3–5 is section ‘0’; drawn approximately to scale. (B) Coronal map of the mid-caudal MPOA section drawn approximately to scale. The square box indicates the original mid-caudal MPOA region counted. The medial preoptic nucleus (MPN) and magnocellular MPN (MPNmag) subdivisions analyzed for the extended analysis of MPOA (Figs. 7 and 8) are also indicated. (LOT=Lateral olfactory tract, ot=optic tract, fx=fornix, Sm/SM=stria medullaris, st=stria terminalis, oc=optic chiasm, PVN=paraventricular nucleus, cc=corpus callosum).

isk). The naive-VNX group showed no overall main effect of GnRH in HVF-exposed males for either MPOA sub-region, and no interaction. Therefore, there could be no significant increase with GnRH in any sub-region of any of the medial preoptic area sections that we counted in naive-VNX males (Figs. 7C and 8C).

To confirm that these regions of MPOA responded to HVF exposure with an increase in Fos expression, separate two-way RM ANOVAs were run on each surgical group (intact; naive-VNX; experienced-VNX) comparing exposure (HVF vs. water) across all sections (+2 to –3) in saline-injected males. There was a significant overall effect of HVF exposure on Fos expression in MPN for each group: intact ($P=0.001$), experienced-VNX ($P=0.001$) and naive-VNX ($P<0.001$). In MPNmag, there was a significant overall effect of HVF exposure on Fos expression for intact ($P=0.010$) and experienced-VNX males ($P<0.001$), but not for naive-VNX males.

A final two-way ANOVA compared the baseline Fos response to HVF exposure with saline injection across all groups (intact; naive-VNX; experienced-VNX) for all MPOA sections (+2 to –3). There were no significant differences in (HVF-induced) Fos expression between the different groups for any section. There were differences between groups in the ability of GnRH to elicit significant increases in Fos expression in various MPOA sections, and in other areas as described above.

3.6. Behavior in the chamber—(intact, naive-VNX, and experienced-VNX males)

We recorded and analyzed the behavior of males in each group during exposure to HVF or water in the exposure

chamber, focusing on the attention paid to the stimulus. For specific investigation at the stimulus well, separate two-way ANOVA tests were run for each experiment (intact, naive-VNX, and experienced-VNX) to examine the effect of treatment (GnRH vs. saline) and exposure (HVF vs. water).

In intact males, there was a significant overall effect of treatment ($P=.001$, $F=14.418$, $df=1$, 20), a significant overall effect of exposure ($P<0.001$, $F=1073.007$, $df=1$, 20), and a significant interaction between treatment and exposure ($P<0.001$, $F=817.468$, $df=1$, 20). Tukey post-hoc tests revealed that within both GnRH and saline groups, investigation time was significantly greater in intact males exposed to HVF (GnRH/HVF and saline/HVF, $P<0.001$ for both). 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, $P<0.001$). Thus, HVF attracted more attention to the stimulus well and GnRH further increased attention in HVF-exposed intact males. Both of these effects are correlated with increased Fos expression in MPOA. However, the increased attention with GnRH was not reflected in increased Fos expression in the central VN pathways: medial amygdala (MeA/MeP) and BNST.

In experienced-VNX males, there was an overall effect of exposure ($P<0.001$, $F=32.456$, $df=1$, 20), but no overall effect of treatment and no interaction. HVF-exposed males spent more time investigating the stimulus well than water-exposed males regardless of GnRH ($P=0.002$) or saline injection ($P<0.001$) and there was a correlated increase in Fos expression (see Fig. 5A and B). Post-hoc test revealed no significant differences in in-

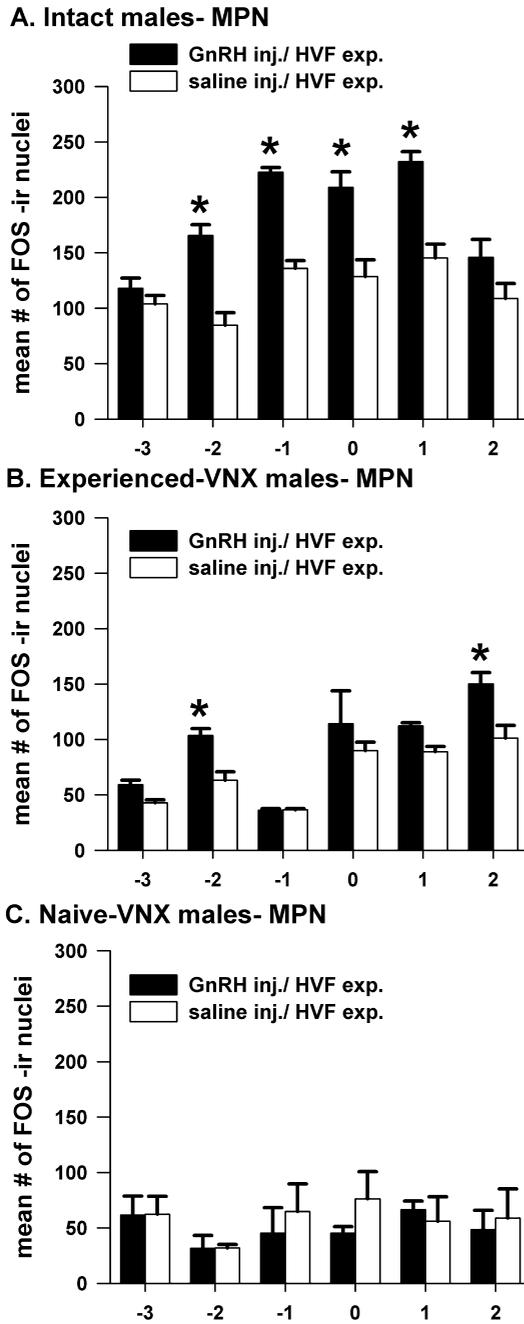


Fig. 7. Fos expression in the medial preoptic nucleus (MPN) in five sections through the MPOA for all three groups of males. Fos expression (mean and S.E.) for HVF exposed males only is shown; Fos expression in water exposed males was not affected by GnRH and these data are omitted for clarity. HVF exposure did produce a significant overall increase in Fos expression in MPN areas for each group (see text). GnRH produced a further increase in Fos expression (asterisks) in four of the sections counted for intact males (A) and in two of the sections counted for experienced-VNX males (B). The pattern of enhancement across sections was not the same for intact and experienced-VNX males. There was no enhancement of Fos expression by GnRH in any section in naive-VNX males (C).

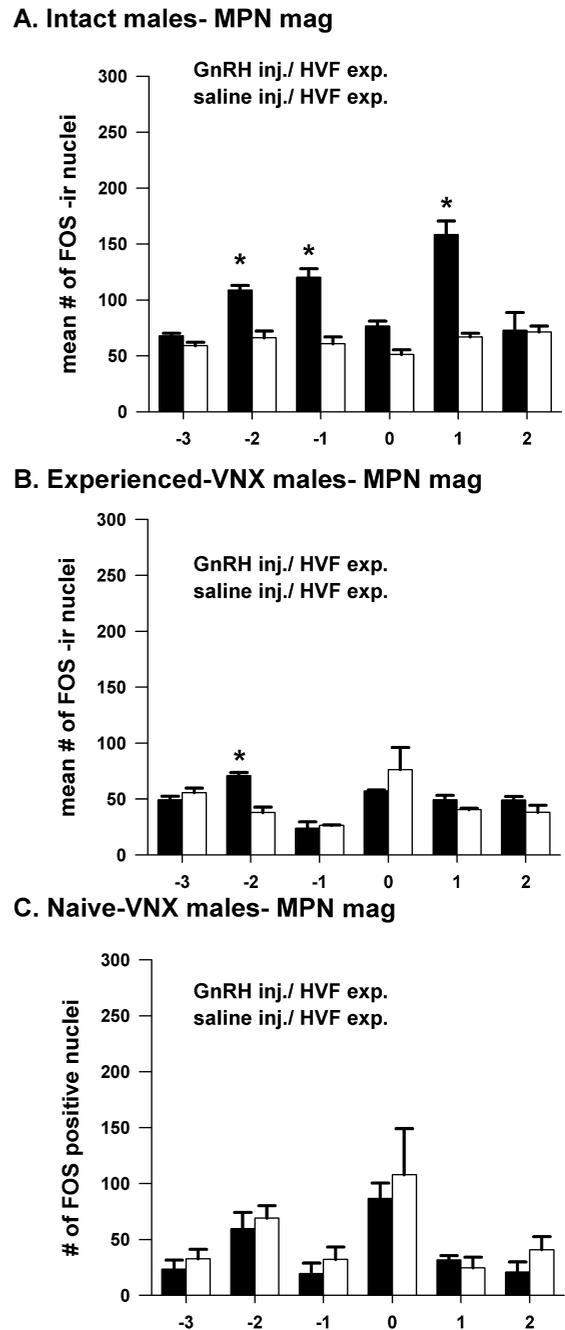


Fig. 8. Fos expression in the magnocellular MPN (MPNmag) region of the MPOA in five sections through the MPOA for all three groups of males. Fos expression (mean and S.E.) for HVF exposed males only is shown; Fos expression in water exposed males was not affected by GnRH and these data are omitted for clarity. HVF exposure did produce a significant overall increase of Fos expression in MPNmag of intact and experienced-VNX males, but not in naive-VNX males. GnRH produced a further increase in Fos expression (asterisks) in three of the sections counted for intact males (A) and in one of the sections counted for experienced-VNX males (B). The pattern of enhancement across sections was not the same for intact and experienced-VNX males. There was no enhancement of Fos expression by GnRH in any section in naive-VNX males (C).

vestigation time between GnRH and saline injected subgroups, but there was a significant decrease in Fos expression in three of the four original brain areas counted in GnRH-injected males.

In naive-VNX males, there was also a significant main effect of exposure ($P=0.006$, $F=10.083$, $df=1$, 20) and a significant interaction ($P=0.004$, $F=10.665$, $df=1$, 20), but no overall effect of GnRH. Tukey post-hoc tests revealed that within both GnRH and saline groups, investigation time was significantly greater for males exposed to HVF ($P<0.001$ for both). The increase in attention with HVF was correlated with increased Fos expression in MPOA and central chemosensory pathways. GnRH did not further increase investigation in HVF-exposed males, so the significant increase in Fos expression in MeA, MeP and pmBNSTr of GnRH-injected HVF-exposed males was not correlated with increased attention. GnRH injection in water-exposed males (GnRH/water) did significantly increase 'investigation of water' ($P=0.008$), compared to the water-exposed, saline-injected males. There was no increase in Fos expression correlated with this increase in attention, in any area counted here.

A final two-way ANOVA compared treatment (GnRH vs. saline) and surgical group (intact, naive-VNX, and experienced-VNX) to look at the effects of surgery and experience on all HVF-exposed males. This two-way ANOVA across groups indicated an overall effect of surgical group ($P<0.001$, $F=9.0678$, $df=1$, 20), but no overall effect of treatment. There was also a significant interaction ($P=0.002$, $F=29.098$, $df=1$, 20). Tukey post-hoc test revealed that intact ($P<0.001$) and experienced-VNX males ($P<0.001$) spent significantly more time investigating HVF than did naive-VNX males. This difference in attention to HVF between experienced and naive-VNX males had no obvious correlate here in increased Fos expression in MPOA, with or without GnRH.

This test also confirmed that among intact HVF-exposed males, investigation time was significantly greater in those males with GnRH injections than those with saline injections ($P<0.001$). Within both the naive and experienced-VNX groups there was no significant difference in investigation time between HVF-exposed animals injected with GnRH and those injected with saline.

3.7. Behavior with receptive females

When given access to receptive females in the standard 5-min mating test, the intact and experienced-VNX males mated normally, with or without GnRH. Naive-VNX males did not mate unless injected with GnRH or pre-exposed to HVF (or both). These results are reported more fully elsewhere [32]. Because the mating tests occurred 5 min before the animals were perfused, they did not contribute to observable Fos expression.

4. Discussion

In this study, investigation of female chemosignals (HVF) was sufficient to activate the central vomeronasal pathway and the MPOA in all three groups of males, intact, experienced-VNX and naive-VNX. In intact and experienced-VNX males, i.c.v. GnRH potentiated the activation in MPOA, although not in exactly the same subdivisions. Even with an extended analysis, naive-VNX males showed no significant increment in Fos expression in MPOA due to i.c.v. GnRH. However, in the medial amygdala and BNST of naive-VNX males, GnRH did produce a significant increment in Fos expression over the levels produced by HVF exposure with saline injection. These same areas showed a significant decrease in Fos expression with GnRH in experienced-VNX males. Except for the intact animals, these changes in Fos expression with GnRH were not accompanied by changes in attention to the HVF stimulus.

These data suggest that intracerebral GnRH can affect neural activity in brain regions associated with reproductively relevant chemosensory input. Unlike the earlier results in mating males, showing a synergistic action of GnRH with exposure to receptive females, we can attribute these new results to a combination of chemosensory input and intracerebral GnRH. We suggest that the differences between groups in the effect of i.c.v. GnRH on Fos expression may reflect changes in brain circuits resulting from removal of vomeronasal input and sexual experience.

4.1. Access of chemosensory input to the MPOA

In the experiments presented here, there was a higher level of Fos expression in all brain areas in experienced-VNX than in naive-VNX males, when exposed to HVF without GnRH injection. However, in these experiments, the difference in Fos expression between the saline-injected HVF-exposed males of the two VNX groups did not reach significance in the MPOA. These results differ from our previous reports where there was a significantly lower level of Fos expression in the MPOA of naive- than experienced-males exposed to HVF [22,10] and no significant increase in Fos expression was detected in the MPOA of naive-VNX males exposed to HVF [10,7]. The present experiments used a different Fos antibody which, judging by background levels of expression appears to be more sensitive than the antibody used in the earlier studies. This higher sensitivity in combination with nickel amplification during Fos processing, and possibly a closer attention to the HVF stimulus when presented in the chamber, could have raised expression above the immunocytochemical detection threshold in the MPOA of naive-VNX males, reducing differences between experienced-VNX and naive-VNX males. In exploratory experiments using shorter exposure to HVF and processing without nickel intensifica-

tion, we have confirmed a significant increase in chemosensory input to MPOA in experienced males. In the results presented here, there was a clear difference between naive and experienced-VNX males in Fos response to HVF in the amygdala and BNST, reflecting the characteristic differences in mating behavior between these groups. These differences are congruent with differences in other species in chemosensory behavior of naive and experienced animals [34]. Both the differences in HVF response (of saline-injected males) and in GnRH response (of HVF-exposed males) suggest differences in the internal state of the circuits involved between these two groups of animals, as discussed further below.

4.2. HVF and GnRH influence on the medial preoptic area

Differences between intact and experienced-VNX males in the pattern of enhancement of Fos expression by exogenous GnRH could be attributed, in part, to: (a) differences in sensory input, the VNX males having no vomeronasal input available; (b) different levels of endogenous GnRH released in response to HVF, which occurs only in intact males; or (c) an increase in attention to the HVF stimulus with GnRH injection, seen only in intact males.

The differences in Fos expression between naive-VNX and experienced-VNX males are more difficult to explain. Our present data indicate no significant difference between these two groups in the level of HVF-induced Fos expression in any section of the MPOA. Only experienced-VNX males, not naive-VNX males, showed the enhanced MPOA Fos expression with i.c.v. GnRH (despite the fact that it is the latter group that show a behavioral effect of i.c.v. GnRH—see below). Because both receive only olfactory chemosensory input, the two groups would not differ in that respect, nor in endogenous GnRH release. Experienced-VNX males paid more attention to HVF in the chamber but there were no GnRH-related differences in attention. With no obvious differences in attention or sensory connections to account for the ability of HVF to activate more MPOA neurons after GnRH injection in experienced-VNX males, we propose that it reflects changes in underlying brain circuits as a result of experience.

The extra neurons activated by GnRH in intact and experienced-VNX males do appear to be dependent on HVF stimulation in that they are not seen in water-exposed GnRH-injected males. However, a reduction in HVF activation does not explain their absence from MPOA in naive-VNX males because there was no general reduction in HVF response in the MPOA of naive-VNX males. Apparently the cells sensitive to GnRH are in a different state or absent in naive-VNX males in that they cannot be seen to respond to the same exogenous GnRH signal. Alternatively they might be exceptionally sensitive to small differences in the level of HVF activation between

these two groups that are not evident in our statistical tests. The former seems more likely than the latter, suggesting that the cells may not be part of the general population of HVF-sensitive neurons in MPOA. We have not yet been able to identify any independent anatomical markers for these neurons. They do not occupy a unique position in the dorso-ventral or medio-lateral dimension of MPOA sections although they do appear in significant numbers in some sections and not others. Of course, their distinction from other MPOA cells may be in their connections from other GnRH-sensitive cells, not in any intrinsic differences.

4.3. Differences between groups in distribution of MPOA Fos expression

The broader rostro-caudal distribution of the GnRH-enhanced Fos expression within the MPOA of intact males compared to experienced-VNX males, may have interesting implications considering the differences between these groups. Chemosensory input in VNX males is limited to the main olfactory system but intact males have both systems functioning. Thus, the spatial pattern of Fos expression may reflect differences in the spatial distribution of olfactory and VN input. Chemosensory input through either system can drive mating behavior and activate the same central VN pathway [21,23,7,9], but they may not have an identical pattern of activation in the MPOA. The GnRH-enhanced Fos expression in MPOA of these two groups had no direct correlate with mating behavior, which was normal in both GnRH- and saline-injected subgroups. However, the susceptibility of MPOA Fos expression to enhancement by GnRH in these two groups does correlate with their ability to sustain mating behavior without exogenous GnRH. On the other hand, GnRH did enhance mating behavior in naive-VNX males compared to saline-injected, water-exposed males but had no observable effects on Fos expression in MPOA. The direct effect of GnRH on behavior in this group was associated with increased Fos expression in medial amygdala and BNST, as discussed below.

4.4. HVF and GnRH influence on brain regions of the VN pathway

As expected from previous experiments [11,18,7,10], HVF exposure increased Fos expression in areas along the VN pathway in all groups whether GnRH or saline injected. In the naive-VNX males there was a further increase in Fos expression with i.c.v. GnRH in pmBNST and in anterior and posterior medial amygdala (MeA and MeP). Possibly these effects of GnRH were not seen in the naive-intact animals because with a higher overall level of Fos expression, further increases were not detectable. The increases in Fos expression with HVF exposure (in all groups) were correlated with increased attention to the stimulus well and presumably reflect increased sensory

input both from the introduction of an overt chemosensory stimulus and increased sampling. The effect of GnRH on Fos expression appears to reflect neither of these factors. In naive-VNX males exposed to HVF, GnRH increased Fos expression without affecting attention (compared to saline-injected males). In water-exposed naive-VNX males, GnRH increased attention but without affecting Fos expression (compared to saline-injected males). The facilitation of mating behavior in this group by GnRH may reflect a synergism with activation in these circuits by female (HVF) that would occur too late to be visible here because of the timing of the mating tests (see below).

Experienced-VNX males also had greater Fos expression in response to HVF than naive-VNX males (both saline injected) in the initial parts of the central vomeronasal pathway (MeA, MeP, and pmBNST). Because olfactory input is able to maintain mating behavior in experienced-VNX males, we suggest that the increased Fos response reflects a greater access of olfactory input to these vomeronasal projection areas after experience. Although not significant here, other data suggests there is also an increased chemosensory input to MPOA. Unlike its effect in naive-VNX males, GnRH significantly decreased Fos expression in the VN projection areas in experienced-VNX males exposed to HVF. The implications of reductions in Fos expression below levels in saline-injected males, are not clear but do not appear to be related to effects of GnRH on attention. Reduced Fos expression was not associated with a significant reduction in attention to the HVF stimulus. We suggest that the changes in brain circuits responsible for increased olfactory activation of vomeronasal amygdala may also alter the sensitivity of these circuits to GnRH.

4.5. Relationship of Fos expression to 'readiness to mate'

Mating in naive-VNX males is substantially restored by i.c.v. GnRH, at short latency [8], and this appears to be reflected in an increase in GnRH-induced Fos expression in the medial amygdala when the combined effects of GnRH and HVF are observed (GnRH/HVF sub-group). When GnRH was given without HVF pre exposure (GnRH/water sub-group), there was no increase in Fos expression due to GnRH, but these males did mate normally in a test with a receptive female 40 min later. At that time, HVF stimulation would be available from the female so we may suggest there would be a GnRH related increase in amygdala activation. The timing of our experiment with the mating test five min before perfusion, however, precludes any observable effect on Fos expression here.

Based on previous results, we expected the GnRH-injected, water-exposed sub-group of naive-VNX males to mate [8] but the saline-injected, HVF-exposed sub-group

was a control group that we had not expected to mate. However, they did mate normally in 5-min mating tests, and the behavioral results are described more fully elsewhere [32]. These animals showed a significant increase in Fos expression in MeA, MeP, pmBNSTc, and MPOA compared to water exposed, saline-injected males (Fig. 4A, B). Thus, these increases could be attributed to HVF stimulation (and perhaps increased attention). Whether there was any sustained increase in electrical activity due to the pre-exposure cannot be determined, but the increased Fos expression does suggest a changed state in these brain regions at the time when mating was tested, compared to the water-exposed, saline-injected males that did not mate.

Experience before VNX enables chemosensory (olfactory) input to drive mating behavior in the absence of GnRH, and this is reflected in a higher level of Fos expression in medial amygdala of (saline-injected) experienced-VNX males here. In both naive and experienced-VNX males, the changes in behavioral outcome imply a change in brain circuit function, and in both cases changes appear to affect the amygdala, as reflected in altered Fos expression. The apparent reversal in the ability of GnRH to enhance amygdala Fos expression in experienced-VNX males may indicate an interaction or conflict between two mechanisms, the action of GnRH and the effect of experience.

Thus, we propose that the enhancement of amygdala Fos expression by GnRH in naive-VNX males may be a short term effect of the peptide on HVF responses in the 'inexperienced' amygdala; perhaps an enhancement of transmission from the olfactory to vomeronasal amygdala. The depression of HVF responses by GnRH in experienced-VNX males may be due to the longer-term effect of experience, perhaps an enhanced chemosensory transmission through the medial amygdala to MPOA, resulting in an altered susceptibility of amygdala circuits to GnRH.

The general similarities between intact and experienced-VNX males in enhanced MPOA Fos responses with GnRH injection may reflect similarities in the access of chemosensory input to MPOA and the local (direct or indirect) action of GnRH. The differences in pattern of GnRH enhancement of Fos expression, and in amygdala and BNST Fos expression, suggest that the circuits delivering chemosensory activity to MPOA differ. On the other hand, GnRH enhancement of Fos expression in the medial amygdala of naive-VNX males reflects the direct GnRH effect on behavior, enhancing mating in this group.

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