J. Steroid Biochem. Molec. Biol. Vol. 39, No. 4B, pp. 661–669, 1991
Printed in Great Britain.

0960-0760/91 $3.00 + 0.00

CONSEQUENCES OF REMOVING THE VOMERONASAL ORGAN

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Summary—In the last decade, research in our laboratories has focused on the effects of deafferentation of the mammalian chemosensory vomeronasal organ (VNX). Many different assays have been conducted and the results of some are briefly reviewed in this contribution, including the effects of VNX on neuroanatomical assessments using histochemistry (lectin binding) and immunohistochemistry (LHRH), male mouse and prairie vole ultrasonic vocalizations and hormone surges in response to cues from females, male mouse courtship and sexual behavior, territorial marking and inter-male aggression, the production of a puberty-altering substance found in mice, activation of reproduction in female voles (who generally do not exhibit estrous cycles) and maternal behaviors by female mice, including aggression directed toward intruder males. In some instances, the otherwise detrimental effects of VNX can be overcome by experience prior to deafferentation, especially in assays that are dependent upon expressions of behavior. In other situations, experience may have little impact on amelioration of the effects of VNX. The essential conclusions of this work focus our attention on reproductive physiology and behavior and a role for the vomeronasal organ in the perception of pheromones that modulate these functions.

INTRODUCTION

Guided primarily by their noses, mammals have been enormously successful in penetrating and adapting to a wide variety of environmental conditions over the past 75 million years. This impressive record of diversification and success must be at least partially due to the continued evolutionary refinement of chemosensory communication and its exploitation to influence the behavior and neuroendocrine status of conspecifics via pheromones [*cf* 1]. Commencing with the seminal work of Winans and her colleagues [2, 3], much research on small mammals has repeatedly and convincingly demonstrated the role of the chemosensory vomeronasal organ (VNO) in the perception of pheromones and in the modulation of hormonal and behavioral responses to these substances (for reviews, see Refs [4–7]). The extent to which humans share this chemosensory heritage with small mammals is the subject of other papers in this issue. In this contribution we review some of the research that has been conducted in our laboratories during the last decade.

Work with the vomeronasal system of small mammals has explored the multifaceted interactions between different sensory systems in the nose, hormone titers in the brain and in the blood, and behaviors known to be influenced by chemical signals, e.g. territorial marking, aggression and reproductive physiology and behavior. Our general paradigm is to study the behavioral and/or neuroendocrine effects of pheromones and social interactions comparing animals which possess a VNO and those which do not. Many years ago, a procedure was perfected for removing the VNO via an oral approach [8]. After enlarging the incisive foramen with a dental burr, the entire VNO can be removed (VNX). VNX spares the olfactory epithelium, but it may affect substance-P containing (trigeminal?) fibers [9] and a few luteinizing hormone releasing hormone (LHRH) immunoreactive nerve fibers and perhaps cell bodies presumably of nervus terminalis that reside within the VNO [10, 11]. Whether peripheral endings of nervus terminalis are sensitive to chemosensory stimuli is another matter [*cf.* 12]. In this paper, we will review some of the effects of VNX, beginning with a discussion of the importance and

Proceedings of the International Symposium on Recent Advances in Mammalian Pheromone Research, Paris, France, 6–9 October 1991. Sponsored by the EROX Corporation.

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methodology of histological verification of VNX.

Histology and lectin binding in the vomeronasal system

The olfactory and vomeronasal epithelia are unique among neural tissues in that they regenerate throughout life (for a review, see Ref. [13]). Therefore, VNX must be histologically verified in every VNX subject, since a residual portion of vomeronasal epithelium can apparently maintain or restore the function of the vomeronasal system. A number of neuroanatomical or histochemical consequences of VNX are available for inspection. For example, if successful, VNX results in the total collapse of the glomerular layer of the accessory olfactory bulb (AOB); no glomeruli can be found. In cases of incomplete VNX, many glomeruli can be found, but more importantly, the animal typically appears unaffected by the surgical intervention. As little as 3–5% of the VNO neuroepithelium that may remain within the nasal cavity after surgery appears to be sufficient to maintain glomeruli and normal responsivity, provided that neural contact is maintained between the VNO and the AOB [14]. Thus, standard histological examination of thionine-stained AOB served for many years as the primary mechanism for verifying the adequacy of surgery; however, AOB glomeruli are at times difficult to visualize. A more sensitive assay was preferred and was developed.

Another consequence of VNX is a change in histochemical reactivity. Stimulated by the results of Key and Giorgi [15], experiments were conducted with the olfactory bulbs/AOBs of cat, mouse, guinea pig, musk shrew, rat, prairie vole, pine vole and opossum. The sectioned tissue was reacted with peroxidase conjugated soybean agglutinin (SBA); it bound to the vomeronasal nerves in each instance (see Fig. 1; reviewed in Ref. [16]). Staining was evident from the vomeronasal epithelium to the AOB. No staining was observed in the bulbs of catfish or starling, who lack a vomeronasal nerve, or, surprisingly, in garter snakes, who possess a fully developed AOB. In adults of species where staining was observed in the vomeronasal nerves, it was absent from the main olfactory bulb (these results differ from those of Riggott and Scott [17], who demonstrated binding of SBA to both vomeronasal and olfactory nerves).

We followed polyacrylamide gel separation and Western blots of VNO membranes by incubation with SBA and found one labeled band of $\sim$200 kDa. SBA binds *N*-acetyl galactosamine (GalNac) and to a lesser extent galactose. Peanut lectin binds galactose, but does not bind to vomeronasal nerves, which suggests that the SBA is binding to GalNac. Consistent with this idea, preincubation of AOB sections with GalNac eliminated binding of SBA. We concluded that SBA binds to GalNac [18].

Both the olfactory and vomeronasal neuroepithelia experience neuronal turn-over, yet the sites of axonal termination (their respective bulbs) are contiguous. Therefore, the turn-over process should demand a high resolution projection system. The membrane-bound glycoprotein in the vomeronasal nerve that is recognized by SBA may serve as a cell-surface marker, effectively segregating vomeronasal from olfactory nerves. Regardless of its function, SBA staining in the AOB disappears subsequent to complete VNX [see Fig. 1(B)]. Hence, SBA staining is used to verify VNX.

SOME EFFECTS OF VNX

Hormone secretions and behaviors of males

Hormone responses of males to females. The idea that male mammals achieve and maintain a steady state of endocrine activity following puberty has been strongly challenged by data from a wide variety of small-mammal species. The results unequivocally demonstrate that hormone secretion in males is quite sensitive to

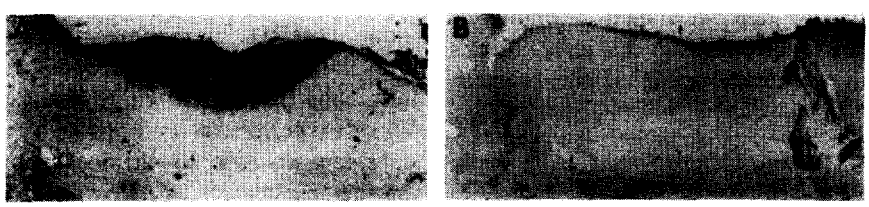

Fig. 1. Guinea pig olfactory bulb, including the AOB, after unilateral VNX. (A) SBA stain in the vomeronasal nerve and glomerular layer of the AOB on the intact side. (B) SBA stain is absent ipsilateral to VNX.

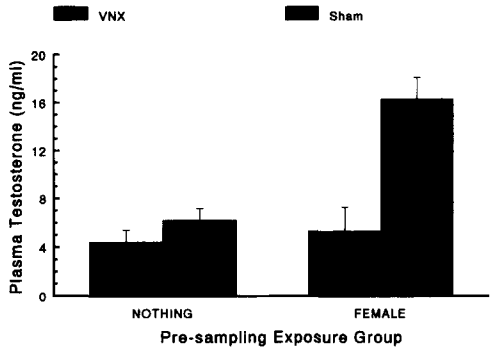

Fig. 2. After recovery from surgery, adult male mice were either left alone in their cages or were exposed to an anesthetized female for 30 min. Immunoreactive plasma testosterone was determined by RIA. Note the presence of a surge in the SHAM-males exposed to females and its absence in VNX-males, who otherwise had normal levels of testosterone [21].

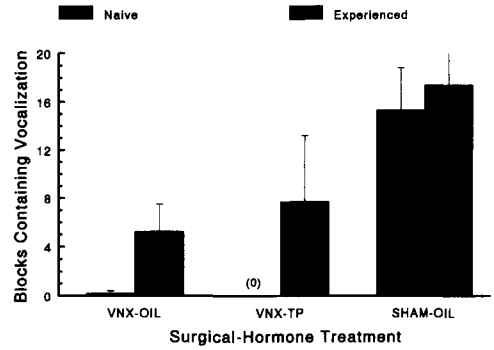

Fig. 4. 70 kHz vocalizations from male mice exposed to an anesthetized female mouse were monitored in 3-min tests that were divided into 5-s blocks. Each block containing at least 1 vocalization was noted. The male mice either were sexually naive or had 3-min exposures to a female on each of 8 consecutive days prior to surgery. Males were injected with testosterone propionate (TP) for at least 10 days prior to testing (150 μg in 0.5 ml of peanut oil daily) or with vehicle.

stimulation provided by the opposite sex. In general, chemical signals from females cause neuroendocrine responses in males leading to increased levels of circulating hormones. For example, many male rodents exhibit a surge in LH followed by an increase in testosterone upon exposure to a novel female or her urine [19]. Subsequent to VNX, male house mice failed to display these changes after exposure to an anesthetized novel female mouse or her urine [20, 21] (see Fig. 2).

Recently, studies were expanded to include male prairie voles, *Microtus ochrogaster*. Two experimental factors, sexual history and surgery, were examined for their influence on steroidal responses to a 30-min exposure to an anesthetized female. Sexually-inexperienced and sexually-proven adult males were subjected to either VNX or SHAM surgery and then tested.

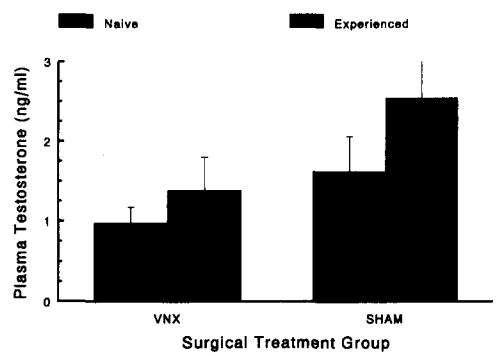

Fig. 3. Male voles resided with an anesthetized female vole for a total of 30 min before blood was obtained for hormonal analysis. Immunoreactive plasma testosterone was determined by RIA. Overall, SHAM-males had higher levels of testosterone than did the VNX-males. The naive VNX-male voles had the lowest levels of testosterone while the experienced SHAM-males had the highest levels. The interaction between surgery and history was not significant.

SHAM-males had significantly higher levels of testosterone in their blood after 30-min of exposure than did VNX-males (Fig. 3). There were no statistically significant main or interaction (with VNX surgery) effects of sexual experience on these responses. At this time, we are uncertain whether androgen levels in SHAM-male voles increased or whether those of the VNX-males decreased.

The effects of VNX are not totally pervasive; when paired with an awake female, male house mice exhibited a surge in LH in spite of the sensory loss [20]. Apparently, cues from a variety of other sensory avenues, when available, can evoke hormonal responses to unanesthetized, interacting females.

Ultrasonic vocalizations. Male house mice emit 70-kHz precopulatory vocalizations in the presence of female mice or their chemical cues [22]. The behavior is affected by VNX [8]; the severity of the deficit depends upon prior experience of the male. Males which had interacted with females prior to VNX surgery emitted more ultrasonic vocalizations than did males which had been isolated from females (Fig. 4). During exposure to the females, the males apparently learned to associate the odors of females with a stimulating sexual encounter. Some of the learning is presumably mediated by an interaction between the olfactory and vomeronasal systems such that subsequent to VNX in experienced males, olfactory cues maintain the behavior [23].

Although the vocalization responses to females depend upon the presence of circulating gonadal steroids [24], treatment of VNX-males

with testosterone had no restorative value. Hormone-treated, inexperienced, VNX-males failed to vocalize in the presence of an anesthetized female. Testosterone treatment also failed to further increase the level of vocalizations elicited from experienced VNX-males relative to oil-injected VNX-males (Fig. 4). Thus, decreased hormonal responses to females after VNX (see above) is an unlikely explanation for the failure of VNX-males to vocalize. Rather, it appears that the negative effects of VNX on vocalizations result from the inability of inexperienced males to detect and/or decode the salient chemical cues from females, and from perceptual deficits for some of the relevant cues in experienced males.

More recently, work was expanded to investigate vocalization responses by male voles in the presence of a female or her chemical cues. Two experimental factors, sexual history and surgery, were examined for their influence on the production of ultrasonic vocalizations. As was observed in mice, male voles emitted more ultrasonic vocalizations if they had had adult experience with a female vole, relative to gender-naive male voles (Fig. 5). The production of vocalizations by male voles was disrupted by VNX (Fig. 5), which also is consistent with the observations noted in tests of VNX-male mice. However, the extent of the VNX-induced deficit appeared to be unaffected by the presence or absence of social/sexual experience (Fig. 5).

Male sexual behavior. Sexual behavior of male house mice is impaired by VNX [25], but the effects are maximized if VNX occurs prior to any adult contact with females [23]. Other factors experienced during interactions with females, including learning, apparently are capable of overriding the otherwise detrimental effects of VNX on male sexual behavior. Similar observations have been reported for hamsters [26].

In one study VNX and SHAM surgical procedures were conducted on 1-day-old house mice which were then tested for nest-odor preferences, the ability to locate their mother's nipples and to suckle. Tests were repeated over several days and body weights were also recorded daily. No significant effects were detected after neonatal VNX for any behavioral or growth measures, relative to groups of mice who received SHAM surgery or no surgery [23, 27]. As adults, each male was paired for 20 min with a female while sexual behavior was recorded. Detrimental effects of neonatal VNX were observed for every measure of male sexual response [27]. The number of mounts with intromission during the 20-min test provides an illustration (see Fig. 6). After the test, the males were housed with the females for 2 weeks. The tests were then repeated with another set of un-mated females. Very similar results were obtained (Fig. 6). As others have done, we too concluded that VNX (in this instance in the neonatal mouse) had significant negative and enduring effects on male sexual behavior. During repeated trials VNX-male guinea pigs extinguished their characteristic head-bobbing response to urine from female guinea pigs [28]. Much like Halpern *et al.* [29] deduced from studies of snakes, we also concluded that stimulation of the vomeronasal organ, conceivably by non-volatile molecules [30], appeared to be inherently reinforcing (for contrary evidence see Ref. [31]). Perhaps reinforcing properties of

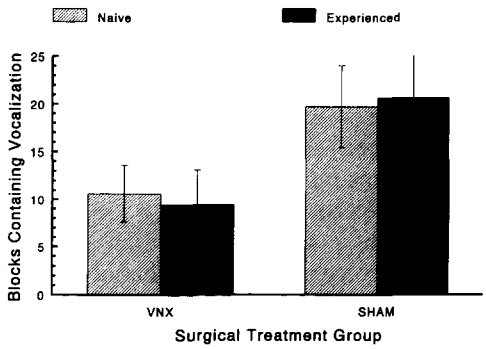

Fig. 5. Ultrasonic vocalizations from male voles exposed to an anesthetized female vole were monitored in 5-min tests that were divided into 5-s blocks. Each block containing at least 1 vocalization was noted. The male voles either were sexually naive or had had prior interactions with a female vole prior to the surgical treatment indicated.

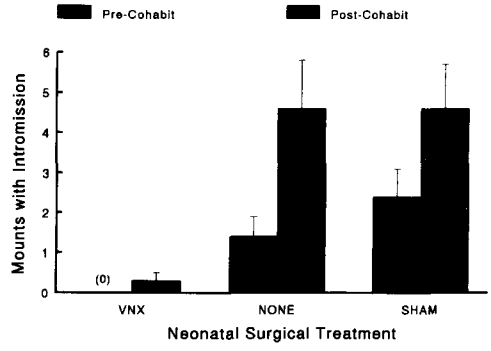

Fig. 6. Number of mounts with intromission in 20-min sex tests are plotted for VNX-, SHAM- and unoperated-male mice. Tests of inexperienced males were conducted during the initial exposure to a female. The same males were tested after 2 weeks of cohabitation with a female. Note the severity of the deficit in the VNX-mice.

stimulation of the vomeronasal system can account for the decreased reproductive effort of male mice. This issue was directly addressed in another experiment. This experiment examined whether sexual behavior in sexually-experienced male house mice would decline and then extinguish in repeated trials after VNX. It apparently does (Fig. 7).

Three groups of sexually-experienced male mice were formed: 2 underwent VNX while the third served as a SHAM control. Members of the VNX-1 group and those of the SHAM group commenced twice-weekly 20-min sex behavior tests 2 weeks after surgery. Early in testing, VNX-males may have been relying upon other sensory input to mediate sexual behavior; there was no difference between the SHAM and VNX-1 groups during the first 3 weeks of trials. Over the course of repeated testing, however, sexual behavior in the VNX-males declined; by the 6th week, sex behavior of males in the VNX-1 group was significantly lower than in the SHAM group. As inferred from the results of tests of members of the VNX-2 group, the diminution did not result from the mere passage of time or other direct sequelae to surgery; levels of sexual behavior in the males of group VNX-2 during 3 weeks of testing did not significantly differ from the SHAM group (Fig. 7). We reiterate the previous conclusion: stimulation of the vomeronasal system during sexual behavior may be inherently reinforcing and may arouse the male or may provide some of the motivation to engage in sexual behavior.

Territorial marking. In tests of sexually-experienced male house mice, Clancy *et al.* [25] noted few effects of VNX on urine-marking by male mice; however, experiments conducted in our laboratories suggested significant deficits after VNX, especially in sexually naive males [32, 33] (see Fig. 8). Although experience with females prior to VNX increased marking relative to the inexperienced males, the behavior continued to remain depressed [32]. As noted above for other behaviors, experience with females appeared to be an essential ingredient in the full expression of territorial marking.

Inter-male aggression. VNX significantly reduced or eliminated aggression by male house mice [23, 25, 33, 34]. The extent of the deficit was determined in part by the amount of inter-male aggression experienced prior to VNX. Males without such experience were more severely affected by the sensory loss [35] (see Fig. 9). The mechanisms underlying these effects remain to be elucidated.

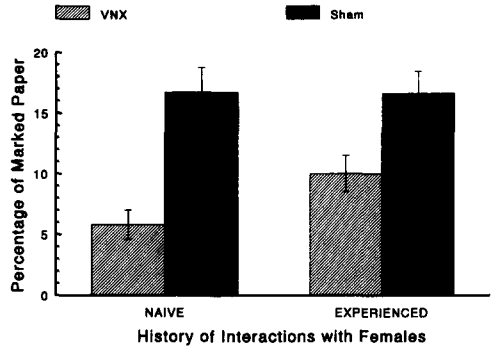

Fig. 8. Male mice were placed for 20 min on paper in an arena divided by a screen with a female on the other side. Total percentage of urine-stained paper was estimated by use of an ultraviolet light (mouse urine is fluorescent). Mice were either experienced with females prior to the indicated surgery or were sexually naive.

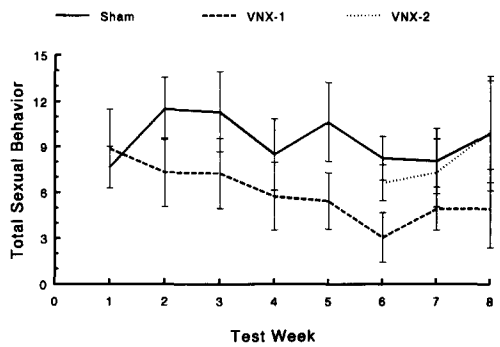

Fig. 7. Sex behavior in 3 groups of male mice: SHAM, VNX-1 and VNX-2. Each male was monitored with an estrous female for 20 min. Attempted mounts, mounts, intromissions and ejaculations were summed for each male to form "total sexual behavior". The average of two weekly tests was calculated. The group means are indicated.

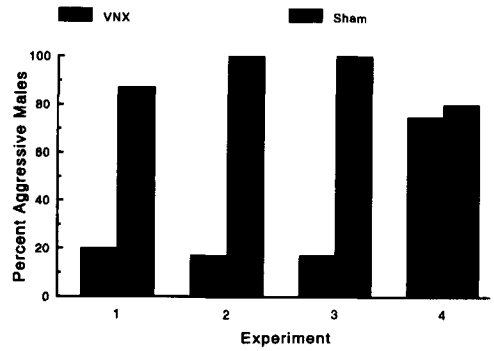

Fig. 9. Percentage of aggressive males in 5-min tests of inter-male aggression in 4 experiments. Expt 1: neonatal surgery. Expt 2: adult males experienced 3 5-min bouts of aggression before surgery. Expt 3: males of Expt 2 had 3 more "training" bouts of aggression, which did not alter the outcome. Expt 4: 8 bouts of aggression "training" were experienced before surgery and testing.

HORMONE SECRETION AND BEHAVIOR OF FEMALES

Female reproductive patterns are orchestrated by complex interactions between sensory information, hormonal factors and behavioral associations. Many different types of hormonal and behavioral responses of females can be observed. We will review the role of the vomeronasal system in some female–female interactions, its control of the activation of reproduction in an induced-ovulator species and its apparent lack of influence with one notable exception, viz. aggression on maternal behaviors.

Puberty-modulating interactions

Crowded female house mice produce a substance in their urine that inhibits sexual maturation in other developing females [36]. The cue that signals a crowded environment among adult females and also triggers production of the puberty-delay substance appears to be chemosensory. VNX of each member of a crowd of females eradicated the puberty-delay substance from the cage of crowded females; the onset of puberty in other developing females was not delayed by exposure to urine collected from group-housed VNX-females [37]. Hence, detection of a crowded condition and subsequent production of a chemical cue that delays the onset of puberty in developing female mice appears to rely upon a functional vomeronasal system. In contrast, adult male house mice continue to release puberty-accelerating compounds in their urine after undergoing VNX [37].

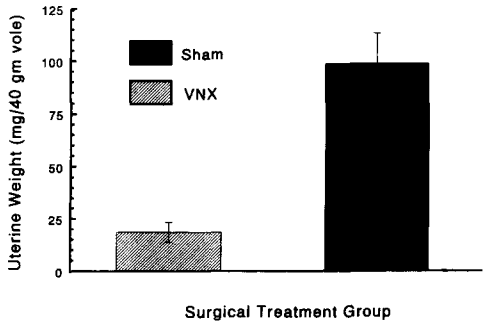

Fig. 10. Subsequent to surgery, female prairie voles were paired with males for 12 h then were isolated in the male's cage for an additional 42 h (for additional details, see Ref. [42]). Uteri were then removed and weighed. VNX either delayed activation of reproduction beyond the length of the exposure or, more likely, prevented activation from occurring (also see Ref. [41]).

Reproductive activation in induced ovulators

Adult female prairie voles normally do not exhibit an estrous cycle; changes in physiology and behavior that normally accompany the reproductive effort are usually activated only in response to the presence of an adult male prairie vole [38]. Uterine hypertrophy, increases in ovarian weight, ovulation and actual mating behaviors appear subsequent to pairing a female with a male. Chemical cues alone from males are sufficient to initiate many of the physiological responses [39]. For example, urine from a male that is placed on the nose of a female can induce significant increases in uterine weight [40]. The female vole's VNO plays a crucial role in the male-induced activation of reproduction [41] (see Fig. 10). Furthermore, evidence was obtained to suggest an interaction among parity, age and activation. Females may have a critical period for activation to sensitize the reproductive neuroendocrine axis: nulliparous females who passed through this period without reproductive activation did not show total activation at a later time in their life-span, whereas primiparous females of the same age exhibited normal activation.

Importantly, experience in the form of a previous reproductive activation did not allay the effects of VNX in primiparous female voles [42] (Fig. 11). These results contrast with those of previous studies suggesting that males of some rodent species, when allowed reproductive experience prior to VNX, can utilize other sensory systems to mediate subsequent

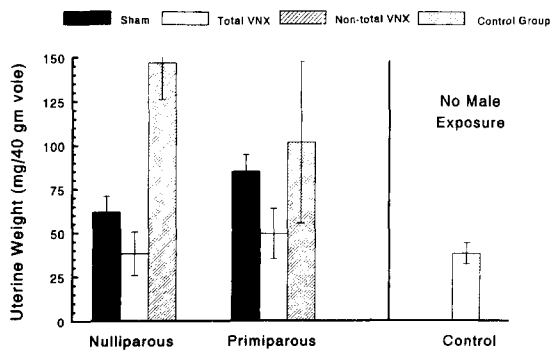

Fig. 11. Mean standardized weights of uteri from female voles exposed to chemical cues from male voles for 54 h (for more details see Ref. [42]). The voles were either nulliparous or primiparous prior to SHAM or VNX surgery. After the experiment, histological assessments, conducted without knowledge of individual organ weights, revealed an incomplete-VNX in 4 nulliparous and 2 primiparous females. The results of these non-total VNX shown separately. The average standardized organ weights from a group of unexposed nulliparous females (CONTROL) is included for comparison.

reproductive responses. The VNX-induced deficits in reproductive activation seen in female voles imply that endocrine responses of female voles to social cues of a chemical nature are mediated via the VNO, regardless of previous sexual experience. As a group, VNX-females had lighter uterine weights and exhibited fewer episodes of lordosis and copulation than did the SHAM-females (previous research indicated that VNX- and SHAM-females elicited equivalent levels of interest from males [41]). In female voles, these reproductive measures are normally supported only by a sufficient titer of circulating estrogen [43]. This suggests that stimulation of the vomeronasal system leads to an increase in the level of circulating estrogen, via unidentified neural mechanisms. Interestingly, quite minimum afferent structure in the VNO apparently can support chemosensory-induced activation of female reproduction; females with incomplete VNX underwent activation (Fig. 11).

LHRH immunoreactivity in the AOB

VNX affects distribution and levels of immunoreactive LHRH in the AOB [44]. Female prairie voles underwent unilateral VNX and their brains were processed for LHRH immunoreactivity. On the intact side, LHRH-positive fibers were visualized in the superficial layers of the AOB looping among the glomeruli. Many months after unilateral VNX, these LHRH-containing looping fibers were absent (which may be a direct result of the collapse of the glomerular layer). The density of LHRH fibers on the dorsal surface of the AOB increased ipsilateral to VNX relative to the intact side.

Some females were exposed to a male opposite a wire divider or to male urine. In females exposed to males, the side ipsilateral to VNX had more LHRH fibers, especially in the deeper layers of the AOB, relative to the intact side.

In these experiments, the LHRH could not have been in the vomeronasal nerve—it was absent after unilateral VNX. Most likely, the source of the LHRH was nervus terminalis [10, 11]. Perhaps, through an interaction with nervus terminalis, stimulation of the vomeronasal system releases LHRH [45]. After loss of vomeronasal inputs, chemosensory-induced release or LHRH may be decreased or absent and hence may appear as an accumulation of LHRH.

Maternal behaviors

VNX prior to mating neither affected nest-building by gestational house mice, nor fecundity, litter size, pup weights, retrieval of pups or discrimination of own from alien pups in primiparous mice [23, 37; *cf* 46], but VNX remarkably eliminated the display of maternal aggression [47]. Generally, lactating mice are highly aggressive toward intruders, especially unfamiliar males. This behavior may be regulated, at least in part, by chemical cues [48]. Aggression was abolished when VNX occurred either prior to mating or during gestation. Experience with aggression prior to VNX may have allayed some of the effects of VNX in some of the females [23, 47] (see Fig. 12), but for the most part, experience with maternal aggression prior to VNX was not adequate to sustain aggression during subsequent encounters with strange males after VNX. The mechanisms underlying the effect of VNX remain to be explored, but appear to result from loss of specific sensory inputs rather than from complex processing within the central nervous system, as has been suggested [49, 50].

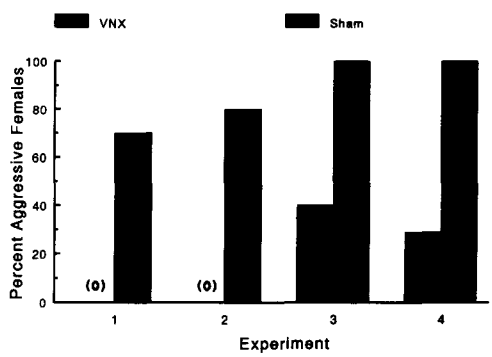

Fig. 12. Percent of aggressive lactating mice in 5-min tests in 4 experiments. Expt 1: females had surgery before experience with an adult male. Expt 2: surgery was conducted after mating (N.B. prior experience with males did not ameliorate the effects of VNX). Expt 3: aggressive SHAM-females of Expt 1 underwent another surgery prior to re-mating. Expt 4: as in Expt 3, primiparous females experienced with maternal aggression underwent surgery prior to a second mating. Similar results were obtain using 30-min tests[47].

CONCLUSIONS

Numerous behavioral and physiological effects of VNX of the vomeronasal system have been observed. Many of these effects appear to result from changes in neuroendocrine responses [*cf.* 51]. Most of the mechanisms underlying these changes are as yet unknown and await the results of future experiments.

Acknowledgements—This work was supported by grants from the National Science Foundation and institutional support. The many long-term collaborators on these projects are deserving of sincere thanks. These include Jay Bean, Gary Beauchamp, Yair Katz, Jay Labov, Joel Maruniak, John Nyby, Judy Wellington and Linda Wysocki.

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