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OLFACTORY AND VOMERONASAL DEAFFERENTATION OF MALE HAMSTERS: HISTOLOGICAL AND BEHAVIORAL ANALYSES

SARAH S. WINANS and J. BRADLEY POWERS

Department of Anatomy and Neurosciences Laboratory, University of Michigan, Ann Arbor, Mich. 48109 (U.S.A.)

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SUMMARY

Deafferentation of the vomeronasal system by cutting the vomeronasal nerves severely impaired mating behavior in 44% of male hamsters over a 1–2 month period of postoperative testing, but the remaining males mated normally after the surgery. Damage to the main olfactory bulbs, concomitant to vomeronasal nerve cuts, did not account for this behavioral difference. Subsequent deafferentation of the olfactory system by intranasal infusion of zinc sulfate solution (5 g $ZnSO_4 \cdot 7H_2O$ in 95 ml 0.5% NaCl) had no effect on intromission or ejaculation latencies of sham vomeronasal cut males but eliminated mating behavior 2 days after treatment in males with bilateral vomeronasal nerve cuts. Some of these males recovered the behavior in 1–3 weeks of post zinc sulfate testing. Histological analyses of the olfactory mucosa in 7 males on day 2 after zinc sulfate showed that 89–97% of the mucosa had been destroyed in 6 out of 7 of the males and 78% in the seventh. We conclude that destruction of the vomeronasal system irreparably reduces arousal necessary for mating in some hamsters but in other males sufficient arousal for this behavior to occur is mediated through the olfactory system, presumably in conjunction with other sensory inputs. Subsequent removal of the olfactory input in these animals eliminates the behavior.

INTRODUCTION

Loss of normal social behaviors after removal of the olfactory bulbs has been demonstrated in several mammalian species[6,70]. Subsequent investigations were initiated in the mouse[16], rat[7,35], and hamster[12,13,39,54] to determine whether the behavioral deficits resulted from loss of olfactory sensation alone or loss of central nervous system circuits through the olfactory bulbs. It has also been recognized, but not studied experimentally, that complete bulbectomy inevitably damages more than one component of the peripheral nervous system[2]. In addition to destroying axons from

receptors of the olfactory mucosa, bulbectomy damages the vomeronasal nerves, nervus terminalis and nerves to the septal olfactory organ[4]. Since the functions of these systems are not known, disruption of any one of them might contribute to the observed changes in social behavior.

In order to evaluate the behavioral effects of selective destruction of the primary olfactory (OLF) system, several groups of investigators have damaged the OLF mucosa by infusion of a zinc sulfate (ZS) solution through the nasal cavities, with modifications of a technique described by Alberts and Galef[3]. ZS produces coagulation necrosis of the OLF epithelium in the rat[66], mouse [44], rabbit[48], dog[26], ferret[67] and monkey[60] (for review of much of this work, see Takagi[68]), but quantitative evaluation of the amount of OLF epithelium destroyed by ZS has not been performed in these histological studies, or in behavioral studies which use this technique. This is obviously an important issue in interpreting the results of behavioral experiments which utilize ZS to produce anosmia.

Although bilateral OLF bulbectomy reliably abolishes all mating behavior in the male hamster[50], intranasal ZS treatment has produced conflicting results. In our laboratory, hamsters receiving intranasal infusions of ZS consistently mated normally[54,55], but Devor and Murphy[12] and Lisk et al.[39] reported loss of the behavior in their similarly treated animals. In addition, Doty and Anisko[13] temporarily eliminated sexual behavior in male hamsters by injecting procaine hydrochloride into the nasal cavities. In all of these studies the animals were determined to be anosmic on behavioral testing.

In considering these inconsistent results we hypothesized that differences in the techniques used in the various laboratories might produce differential impairment of the vomeronasal (VN) and OLF epithelia, and that deafferentation of the VN system, or of both OLF and VN systems, might be necessary for the loss of mating behavior to occur. Although there was no direct evidence at that time for such a functional role for the VN system, its anatomical connections from the olfactory-like VN epithelium to the accessory olfactory bulb (AOB), from the AOB to the corticomedial amygdala, and thence to the medial hypothalamus, supported the hypothesis that it functioned in sensory modulation of sexual behavior[57,72].

Because the VN nerves pass along the medial side of the OLF bulbs and are closely applied to the bulbar surface, these nerves are usually damaged in complete bulbectomies. In order to evaluate the results of interrupting VN input, we cut the nerves in the midline, producing only minor damage to the main olfactory bulbs (MOB). With this technique we could deafferent the VN system independent of, or in combination with, deafferentation of the main OLF system with ZS.

Study of male hamsters with VN nerve lesions showed that this system is important for mating behavior in this species. Forty per cent of the males stopped mating after we cut the nerves and, although ZS treatment alone had no effect, when it was applied to the remaining 60% of VN deafferented animals, all of these males with combined VN and OLF deafferentation failed to copulate[55].On the basis of these results we concluded that the OLF and VN systems interact in some way to mediate normal sexual behavior. To determine whether this interaction was a physiological

mechanism or a product of our lesioning techniques, we analyzed the completeness and the selectivity of our deafferentation procedures. The results of this histological analysis are reported in Section I.

To determine whether copulatory impairment was temporary or permanent, we studied the mating behavior of our deafferented animals over a substantial post-treatment period. These data are presented in Section II. In addition we assessed whether mating behavior returned in our VN plus OLF deafferented animals. Recovery of OLF discrimination[3,12,54] and recovery of mating behavior[12,39] have been reported after ZS treatment alone and it has been suggested that this might reflect regeneration of functional OLF epithelium. In fact, interest in possible regeneration of the OLF mucosa in mammals was first aroused by reports that humans treated with intranasal ZS, a treatment used to provide immunity against poliomyelitis, became anosmic and later recovered their sense of smell[15]. Subsequently regeneration of OLF epithelium after ZS treatment has been observed histologically in mammals. When it occurs, it apparently requires two weeks or longer[44,48,60].

GENERAL METHODS

Standard procedures were adopted for all animals with respect to housing, treatment with ZS, cutting the VN nerves and perfusion for histological analysis.

Housing

Male Syrian hamsters weighing approximately 100 g were purchased from Con Olson (Madison, Wisconsin) or Engle's Laboratory Animals (Farmersburg, Indiana). They were housed individually, with lab chow and water freely available, in air-conditioned rooms with a 14-h light, 10-h dark illumination cycle.

Zinc sulfate treatment

We infused ZS through the nasal cavities according to a procedure modified from Alberts and Galef[3]. A hamster anesthetized with ether was placed on its back on an inclined plane with head down. 0.5 ml of a 1-day-old ZS solution (5 g $ZnSO_4 \cdot 7H_2O$ in 95 ml of 0.5% NaCl) was introduced into the posterior part of the nasal cavities through the nasopharyngeal meatus with a hook apparatus which has been described previously[54]. Drainage from the cavities at the nares was promoted by gentle suction from an aspirator moved quickly in front of the nostrils during infusions and for about 10 sec thereafter. Each animal was anesthetized for only 2–3 min for the entire procedure. 0.5% NaCl was administered in an identical fashion as a control procedure in some animals.

Perfusion

The survival period between ZS treatment and perfusion was 2 days for animals which we prepared for study of the OLF and VN epithelia and 8–30 days for the males we studied behaviorally. Animals with VN cuts alone were observed for 4–8 weeks after surgery before sacrifice. At the time of perfusion the hamster was anes-

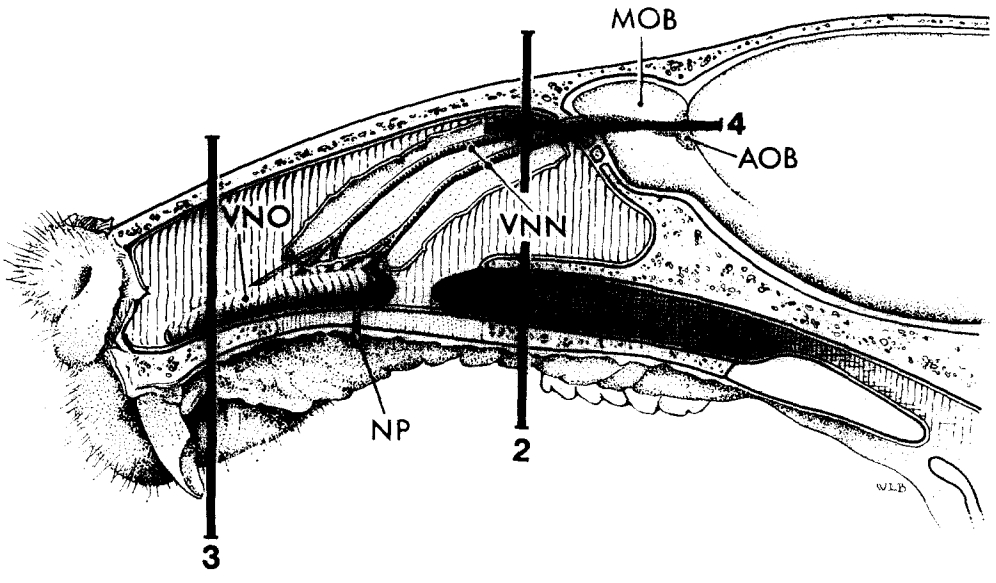

Fig. 1. Parasagittal section through hamster nasal cavity. Exposure shows left side of nasal septum and left lateral surface of brain. Mucosa covering the septum has been dissected away in part to expose the vomeronasal nerves (VNN) leading from the vomeronasal organ (VNO), along the medial surface of the main olfactory bulb (MOB), to terminate in the accessory olfactory bulb (AOB). NP, nasopalatine canal. 2, 3 and 4 are planes of histological sections in Figs. 2, 3 and 4, respectively.

thetized with sodium pentobarbital and perfused through the left ventricle with 0.8% saline followed by 10% formalin in 0.8% saline.

Vomeronasal nerve cuts

The VN nerves in the hamster pass through the cribriform plate at the rostral end of the MOB's, course directly back along the medial surface of each bulb, about midway between the dorsal and ventral surfaces of the bulb, and terminate in the AOB (Fig. 1). We attempted to cut these nerves close to their entrance into the cranial cavity through the cribriform plate. Hamsters anesthetized with 75 mg/kg sodium pentobarbital were secured in a stereotaxic holder. A 5 mm hole was drilled in the frontal bones over both OLF bulbs. The dura was punctured and open McClure Ultra Micro scissors were lowered in the midline. One blade entered the medial surface of each OLF bulb, and when the tips touched the cribriform plate, the scissors were raised slightly and closed, catching the VN nerves between the blades. The midline venous sinus was also cut by this procedure. The resulting bleeding was gradually stopped with gentle pressure on a Gelfoam pledget, and the skin was sutured over the Gelfoam.

Two different sham procedures were adopted for behavioral studies. The animals were prepared identically to those in which the nerves were cut except that in Sham 1 animals, only the venous sinus between the bulbs was cut; the scissors were not lowered into the OLF bulbs. This procedure controlled to some extent the trauma of blood loss. In the Sham 2 procedure, a 22 gauge hypodermic needle was lowered into the medial side of each bulb in an effort to damage the surface layers of the MOB without

severing the VN nerves. With this technique we hoped to mimic the MOB damage concomitant to the VN nerve cuts.

SECTION I: HISTOLOGICAL STUDIES

Our first objective was to ascertain the appearance, the extent and the distribution of any pathological changes in the OLF and VN mucosae after our ZS treatment. We prepared coronal sections through the nasal cavities of normal and treated animals and found that the OLF epithelium, but not the VN epithelium, had been severely affected by the ZS treatment. Second, to determine the completeness of our VN nerve cuts and the extent of MOB damage after the cuts, we studied horizontal sections of the OLF bulbs and VN nerves in situ in most of the animals in which we had cut the nerves during behavioral studies. In 7 of the 36 males in which we cut the nerves, some or all of the OLF bulb tissue was prepared in the coronal rather than the horizontal plane. On these sections the extent of MOB damage (and in some cases the condition of the AOBs) could not be analyzed in the same manner as for the other 29 hamsters.

Histological procedures

In all animals the cranium was decalcified with the brain in situ. Following perfusion, the mandible and all soft tissues, with the exception of the mucosa on the oral surface of the hard palate, were removed. The cranium was decalcified in formic acid (25% for specimens with teeth and entire nasal cavity intact, and 5%, approximately 1 *N*, for specimens in which only the OLF bulbs and surrounding bone were to be analyzed). Sodium citrate (7.5%) was added to the 25% formic acid according to the procedure by Lillie[38]. When sodium oxalate (5%) mixed with a test quantity of the formic acid failed to produce a calcium oxalate precipitate (usually about 4 days in acid), the decalcified cranium was transferred to 5% sodium sulfate for 14 h, then washed in running tap water for 24 h before embedding. Tissues were either embedded in egg yolk mixed with 12% gelatin (2 parts egg yolk; 1 part gelatin; a modification of Method 9 outlined by Ebbeson[14]) for sectioning on a freezing microtome, or dehydrated and embedded in paraffin. Egg-gel embedding was ideal for preparation of horizontal sections of OLF bulb to be stained with cresyl violet, but paraffin embedding for hematoxylin and eosin stained sections was found to be far superior for analysis of the OLF and VN mucosae.

For histological study of these epithelia, 7 hamsters were killed 2 days after ZS treatment. Four were embedded in egg-gel, cut in 40 μm coronal sections on a freezing microtome, and sections at 240 μm intervals were stained with cresyl violet. Tissues from the 3 other animals were paraffin embedded, cut at 10 μm, and sections 200 μm apart were stained with Harris' hematoxylin and eosin.

For analysis of the VN nerve cuts and OLF bulb damage, the decalcified cranium with bulbs in situ were sectioned horizontally. Frozen sections of egg-gel embedded tissue were cut at 40 μm and sections 240 μm apart were stained with cresyl violet. Paraffin embedded material was sectioned at 10 μm sections 200 μm apart were stained with cresyl violet or thionin.

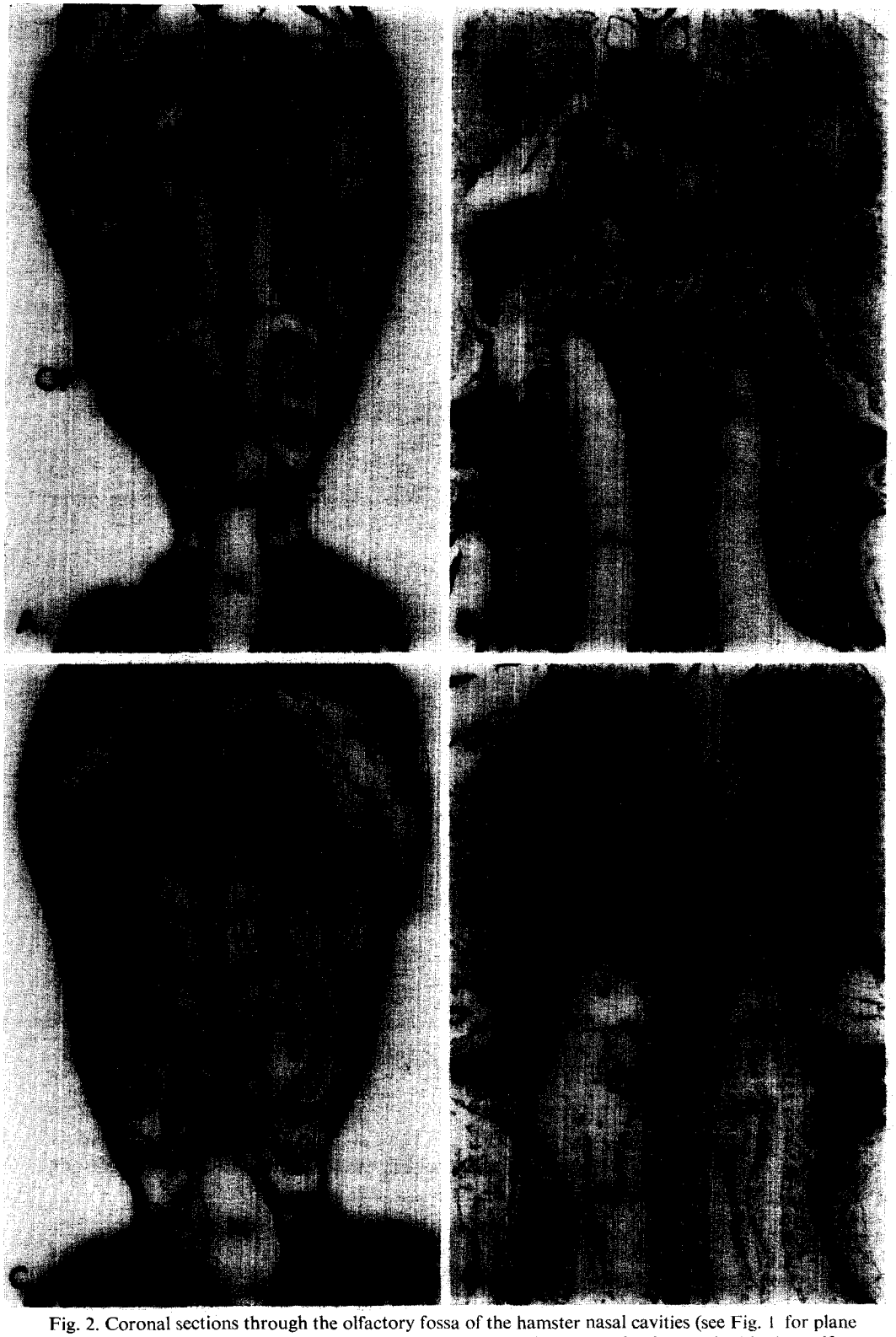

Fig. 2. Coronal sections through the olfactory fossa of the hamster nasal cavities (see Fig. 1 for plane of section); A and B, from an untreated animal; C and D, from an animal treated with zinc sulfate 2 days prior to sacrifice, showing the sloughing epithelium. OB, olfactory bulb; OF, olfactory fossa; N, nasopharyngeal meatus; e, olfactory epithelium; l, lamina propria; n, olfactory nerve fascicles in the lamina propria. Hematoxylin and eosin.

Analysis of histological material and Results

1. Olfactory and vomeronasal mucosae after zinc sulfate. Two days after infusion with ZS the OLF mucosa in the 7 males studied was necrotic and most of the epithelium was in the process of sloughing from the underlying lamina propria. Fig. 2A and B illustrates the appearance of normal OLF epithelium in the posterior part of the nasal cavities (olfactory fossa, level of these sections indicated on Fig. 1) in contrast to the sloughing epithelium from a ZS treated animal (Fig. 2C and D). In many areas where the necrotic epithelium was completely detached (as in Fig. 1C), the underlying lamina propria appeared to be bare of any epithelial covering. However, in some regions a layer of single cells remained. Whether these were basal cells of the sloughed epithelium or cells which had migrated onto the bared lamina propria could not be determined. The lamina propria of these animals contained all of the elements observed in the normal animal but the overall staining was lighter. This appeared to be due to (1) substantially decreased uptake of eosin in the cytoplasm of the cells of Bowman's glands and (2) the vacuous appearance of the nerves. The latter was probably a reflection of the swelling of the degenerating axons in the nerve bundles.

In some parts of the nasal cavities, however, 'normal appearing' epithelium remained attached to the lamina propria. For quantitative analysis of the amount of epithelium that appeared normal, 18 standard sections were traced from a normal control specimen with a microprojector. These standard sections showed the configuration of the turbinates at approximately 1 mm intervals from the external nares to the posterior limit of the nasal cavities. The tracings were 15 × projections of the normal sections, and most corresponded to levels through specific anatomical landmarks in the cavities, as described by Adams and McFarland[1], e.g., the orifice of the VN duct, the rostral end of the septal window, etc. With the aid of a microscope, the distribution of OLF mucosa over the septum and turbinates at each of these 18 levels was plotted onto the standard sections. The total 'length' of the epithelium on each of these drawings was measured with a Keuffel and Esser map measure and the sum total from the 18 standard levels represented 100% of the OLF mucosa.

Areas of degenerating (sloughing or absent) OLF epithelium and normal appearing OLF epithelium in each experimental animal were then plotted onto a set of the standard drawings from microscopic observation. The length of the normal appearing epithelium in these ZS treated animals was measured on the drawings with the map measure, summed for all sections, and expressed as a percentage of the total OLF epithelium in a normal animal. Quantitative analysis revealed that from 3% to 22% of the epithelium was histologically normal at the light microscopic level in the 7 animals studied. The actual percentage for each animal was 3%, 5%, 9%, 10%, 10%, 11% and 22%. Thus, in most animals 10% or less of the OLF mucosa had escaped the destructive effect of zinc sulfate 2 days post treatment. In all of the hamsters this normal OLF mucosa was found in the dorsolateral part of the OLF fossa, primarily on ectoturbinates 1 and 2.

In contrast to the sloughing OLF epithelium, the VN epithelium of the ZS treated animals appeared normal throughout its length (Fig. 3). The ducts entering

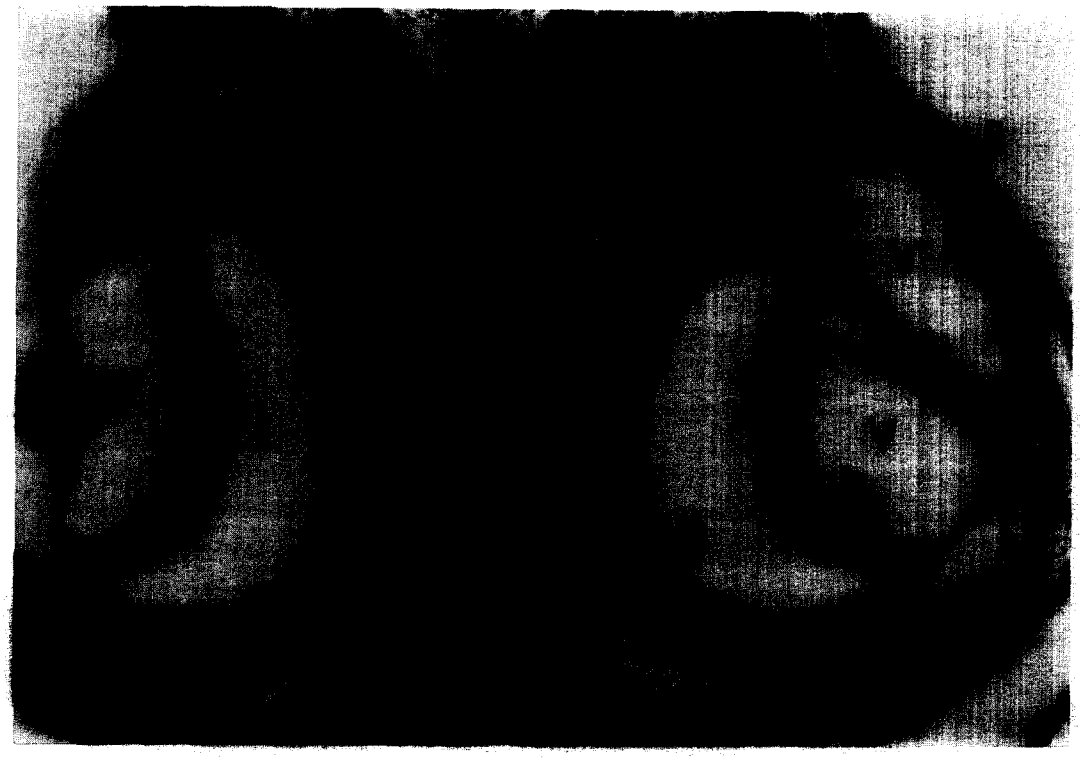

Fig. 3. Coronal section through the vomeronasal organs (VNO) of a hamster treated with ZS 2 days prior to sacrifice. (For plane of section see Fig. 1.) R, receptor cells (bipolar neurons); S, supporting cells; L, lumen of VNO; V, vascular plexus; B, bony capsule of VNO. Hematoxylin and eosin.

the organs were patent; the supporting cells at the surface of the sensory epithelium and the thick neuronal layer were without signs of necrosis in both cresyl violet and H and E stained material.

2. Evaluation of the vomeronasal nerve cuts. Fig. 4A illustrates a horizontal section through an OLF bulb with a normal VN nerve passing along the medial side of the MOB and terminating in the AOB. Horizontal sections through the OLF bulbs of hamsters in which we cut the VN nerves showed the changes illustrated in Fig. 4B. In these animals neither the nerves nor the glomerular layer of the AOB, in which the nerves terminate, were visible on any of the sections. The surfaces of the AOBs showed gliosis replacing the glomeruli. These changes were observed bilaterally in all hamsters reported as 'VN deafferented' in the behavioral portion of this study. Nine additional animals were studied but were eliminated from the behavioral data because glomeruli were found on the surface of the AOB on one or both sides. The VN nerves and AOBs of the sham operated males appeared normal. In the group in which we cut the venous sinus, no damage to VN nerves, AOB or MOB was apparent under the light microscope. In sham animals in which we inserted a hypodermic needle into the MOBs, the VN nerves and AOBs were intact. Slight damage was observed in the glomerular and external plexiform layers of the MOBs but as a con-

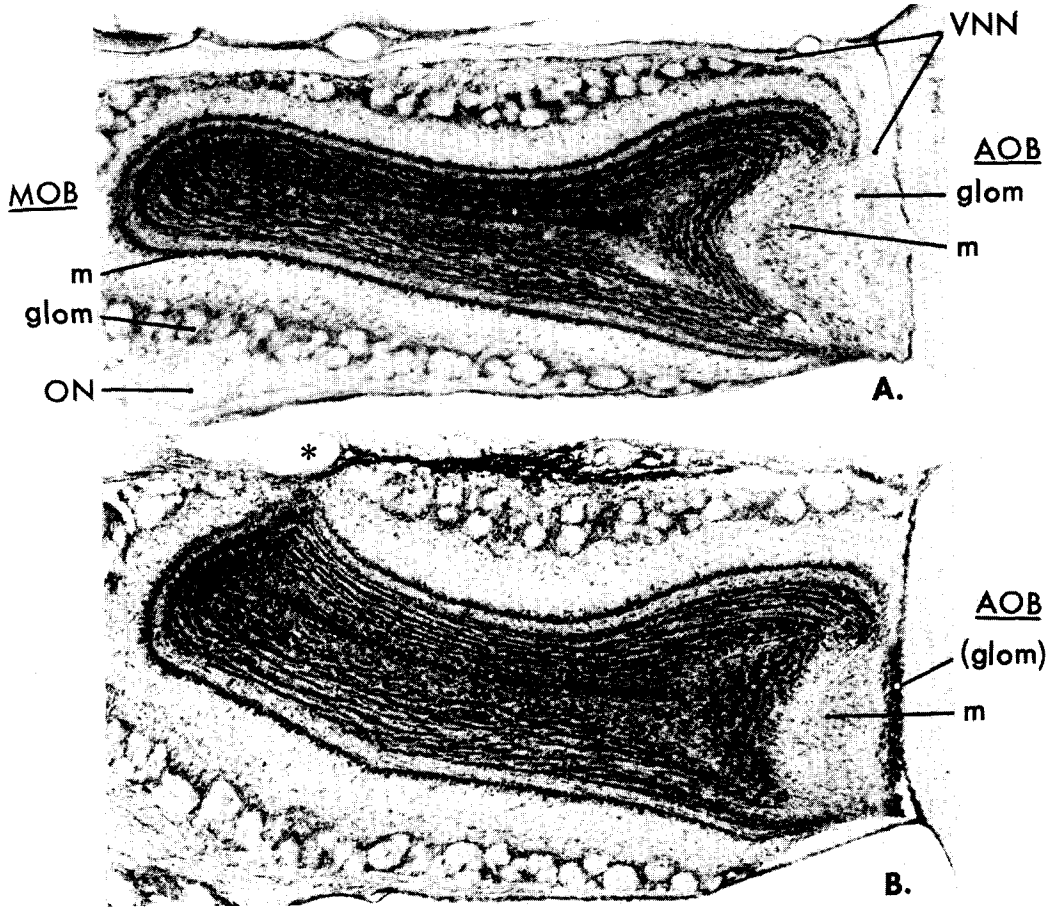

Fig. 4. Horizontal section through olfactory bulb of hamster. (For plane of section see Fig. 1.) A: normal. B: vomeronasal nerve cut 6 weeks prior to sacrifice. Abbreviations: VNN, vomeronasal nerve; AOB, accessory olfactory bulb; ON, olfactory nerve layer; MOB, main olfactory bulb; glom, glomerular layer; (glom), gliosis replacing glomerular layer of AOB; m, mitral cell layer; *, point of damage to MOB created by surgical procedure to cut VNN. Cresyl violet.

trol procedure, this was not entirely satisfactory, in that it produced less damage to the MOBs than that observed in most of our VN nerve cut males.

Our VN nerve cutting procedure caused damage to the MOBs which varied from almost imperceptible to severe in different animals.

The damage was quantitatively assessed by plotting the area of tissue loss and the surrounding necrosis on standardized tracings of horizontal sections at 5 levels through the bulbs. The area of the lesion was plotted on all 5 standard sections from microscopic observation for each animal. Areas of damage were then measured with a planimeter on each section, summed for all 5 sections, and expressed as a percentage of the total area of normal bulb tissue (total area of the 5 standard sections). In 29 specimens, our VN nerve cutting technique produced damage, on an average, to 9.0% of the main bulb tissue. The standard error of the mean was 1.1% and the range

was 1.3% to 26.6%. These measures include both cell loss and the surrounding gliosis in both bulbs.

SECTION II: BEHAVIORAL STUDIES

Testing procedures

Male hamsters selected for the behavioral study had ejaculated within 10 min on at least 2 of 4 mating pretests, which were conducted every 3–4 days during the 2 weeks preceding the experiment. Receptive females for all behavioral tests had been ovariectomized and implanted with a subcutaneous silastic tube (ID = 1.542 mm; OD = 3.175 mm, 10 mm long) containing estradiol benzoate. Subcutaneous injections of 500 μg of progesterone were given 3–5 h prior to the tests and females to be used with experimental males were checked for lordosis responses with stud males.

For each behavioral test the experimental male was adapted to a clean, clear Plexiglas box (24 cm × 30 cm × 21 cm high) for 5 min. All latencies were measured from the introduction of the female at the end of the adaptation period. Mounts, intromissions and ejaculations were recorded on a Rustrak event recorder. A test continued for 10 min or until the male achieved 2 ejaculations. Tests were conducted in a lighted room in the midpart of the animal's dark cycle.

The experimental protocol

The protocol, including a summary of the results, is shown in Fig. 5. Vomeronasal nerve cuts or sham cuts (described in Section I) were performed 1 day after the last pretest. Postoperative mating tests were conducted on days 3, 6 and 10 after surgery. A VN deafferented animal was determined to have a 'severe deficit' if it failed to show any mounts, intromissions or ejaculations on 2 out of these 3 postoperative tests. Testing of these severe-deficit males continued twice a week for a total of 4 weeks. In addition, a subgroup of 7 of these animals was tested again 8 weeks after surgery.

Hamsters which ejaculated on at least 2 of the 3 postoperative tests, on the other

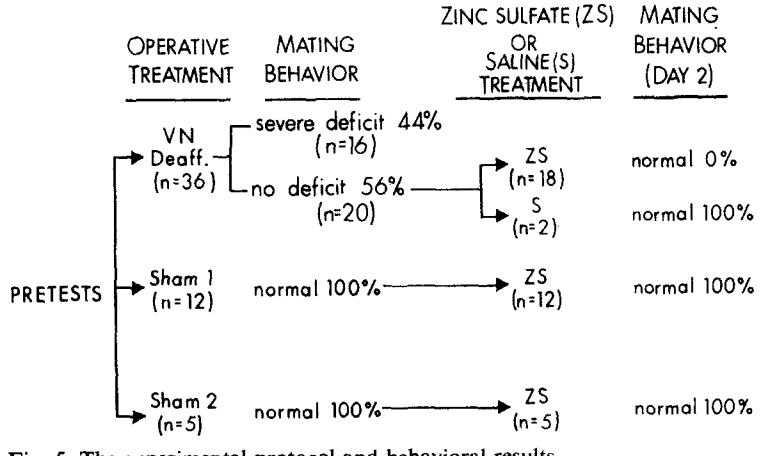

Fig. 5. The experimental protocol and behavioral results.

hand, were classified as 'no deficit', and subsequently received ZS or a saline control treatment. All but 6 received this treatment 4 days after the third mating test; the 6 were treated immediately after that test. The number of mating tests following ZS treatment varied. Fourteen of the 20 no-deficit animals were tested on days 2, 8, 15, and 22 after ZS infusion. The remaining 6 were killed for histological analysis prior to the completion of this testing sequence.

Behavioral results

1. Effect of VN nerve cuts on mating behavior. (a) Sham VN nerve cuts. Thirteen out of 17 operated males ejaculated on all 3 postoperative tests. Four ejaculated on 2 of these 3 tests. Two days after ZS treatment, when olfactory impairment is maximal, all hamsters ejaculated twice. This is consistent with our previous observations of the mating behavior of normal male hamsters after ZS[54]. Following this test the majority of these sham-operated males continued to mate on weekly tests over the next month. One male failed to mate on all tests and 2 others had a single test during which they exhibited no copulatory behavior.

(b) VN nerve cuts. Based on their copulatory performance during the first 3 postoperative tests, 16 of the 36 males (44%) in which the VN nerves had been cut bilaterally were categorized as exhibiting severe behavioral deficits. Nine of these hamsters showed no mounts, intromissions or ejaculations; 7 mated on only 1 of the 3 tests. This severe behavioral deficit was not a transitory phenomenon. Fourteen of the 16 males failed to mate on all subsequent tests (over a 1 month period for 7 animals; 2 months for the other 7). Two hamsters mated sporadically over a month period (1 mounted on 2 tests; the other intromitted on 2 tests). The severe deficit in these 16 hamsters (44%) was a striking contrast to the behavior of the remaining 20 VN deafferented animals (56%). Thirteen of these males ejaculated on each of the first 3 tests after surgery. The other 7 animals ejaculated on 2 of the 3 tests and all but 2 of these males showed mounting, or mounts and intromissions, on the third test.

When mating was observed in the VN deafferented males, the basic temporal pattern appeared normal. This was true for the severe-deficit as well as the no-deficit animals. The latency (X $\pm$ S.E.M.) to the first ejaculation (EL) was 314.06 $\pm$ 35 sec on the last 2 pretests for the 16 severe deficit males; the mean EL's of the few animals which ejaculated after surgery were 317.67 $\pm$ 91 sec (n = 3) on test 1 and 369.0 $\pm$ 190 sec (n = 2) on test 3. We did not record individual instances of grooming, sniffing or other related behaviors for quantitative analysis but we consistently observed a tendency for the males who were not engaging in normal mating to bite the hind-limbs and tail of the female. We had previously observed this same kind of mouthing and biting in both neonatally and adult bulbectomized male hamsters[71].

To evaluate whether VN nerve cuts differentially affected males which were either sluggish or vigorous maters preoperatively, we compared the pretest performance of (a) the severe-deficit males with (b) those males which had ejaculated at least once on 2 of the first 3 postoperative tests (a subgroup of the 'no deficit' group, designated 'minimal deficit') and (c) those animals which had ejaculated twice on all

TABLE I

Differential effects of vomeronasal deafferentation on mating behavior of male hamsters

IL, mean intromission latency (sec) ± S.E.M.; I/E, mean number intromissions to ejaculation ± S.E.M.; EL, mean ejaculation latency (sec) ± S.E.M.

Deficit	*Preoperative*			*Postoperative*								
				1			*2*			*3*		
	IL	*I/E*	*EL*	*IL*	*I/E*	*EL*	*IL*	*I/E*	*EL*	*IL*	*I/E*	*EL*
(a) Severe	153.84 ± 28.22	15.16 ± 1.69	314.06 ± 34.88	214.0 ± 59.59	12.67 ± 2.03	317.67 ± 90.79	—	—	—	134.5 ± 5.5	10.0 ± 5.0	369.0 ± 190.08
(n)	(16)	(16)	(16)	(4)	(3)	(3)	(0)	(0)	(0)	(2)	(2)	(2)
(b) Minimal	166.05 ± 40.31	15.81 ± 2.46	264.4 ± 24.6	120.33 ± 24.9	14.33 ± 2.98	274.89 ± 39.14	97.86 ± 10.58	10.0 ± 1.59	326.0 ± 68.92	206.0 ± 51.59	15.6 ± 4.73	377.6 ± 49.51
(n)	(9)	(8)	(8)	(9)	(9)	(9)	(7)	(6)	(6)	(7)	(5)	(5)
(c) None	88.0 ± 14.35	15.45 ± 1.62	262.82 ± 36.23	101.27 ± 20.32	11.45 ± 1.11	201.82 ± 30.53	132.45 ± 27.84	13.55 ± 1.77	261.64 ± 35.5	114.55 ± 18.09	13.64 ± 1.45	255.55 ± 33.59
(n)	(11)	(11)	(11)	(11)	(11)	(11)	(11)	(11)	(11)	(11)	(11)	(11)

3 tests ('no deficit'). These comparisons, shown in Table I, indicate differences in pretest ILs and ELs which suggest that the males with severe postoperative deficits were those which had been slow to mate prior to surgery, but analyses of variance failed to demonstrate significant differences.

We also examined the possibility that differential damage to the MOBs, secondary to the VN nerve lesions, might account for the behavioral differences between the severe (a) and no-deficit (b and c) animals. We compared % cell loss and % total damage (cell loss plus gliosis) to the MOBs for all animals on which the quantitative data were available (16 severe and 13 no-deficit). These groups did not differ with respect to cell loss but differences in total damage were significant ($P < 0.05$; t-test). This reflects a group difference in the direction of larger lesions in the severe deficit group. This statistical difference rests on the fact that the two animals with the largest lesions to the MOBs are in the severe deficit group. This is not surprising in light of the effects of bilateral bulbectomy in male hamsters. What is surprising is that these two animals are the only ones that fall outside the range of the lesions to the MOBs in the no-deficit group. In other words, eliminating these 2 males leaves 2 groups of animals with strikingly different behavioral characteristics but which span the same range, up to 16%, in terms of MOB damage accompanying deafferentation of the VN organ.

2. Combined VN and OLF deafferentation. Twenty-three males showed no mating behavior deficits after VN deafferentation; 18 of these were treated with ZS and 5 with saline. None of the ZS treated animals mated on Day 2. This total absence of mating after VN and OLF deafferentation stands in contrast to the normal mating of all hamsters which we have observed on Day 2 after ZS alone. Seven of the ZS animals failed to recover any components of mating behavior throughout the 22 days of post-treatment testing. Another 7 males showed recovery: 4 after 3 weeks and 3 after 1 week. The saline control animals continued to mate normally after this treatment. Three of these 5 animals had to be eliminated because their VN nerve cuts were imcomplete.

DISCUSSION

We conclude that the VN system is a major component of the neural systems that control sexual behavior in male hamsters, and that the VN system acting in conjunction with the primary OLF system comprises an essential neural substrate for mating in these animals.

Deafferentation of the OLF system with our ZS technique destroyed approximately 90–95% of the OLF epithelium but caused no histologically identifiable damage to the VN organ. Males treated with ZS showed no impairment in mating behavior on day 2 after treatment. On the other hand, histologically verified VN nerve cuts, which result in concomitant damage to less than 10% of the MOB on the average, produced a severe behavioral deficit in 44% of the animals, and deafferentation of both VN and OLF systems abolished mating in all males.

The persistence of these deficits varied. In the VN deafferented animals with

severe behavioral deficits, 87% showed no recovery of mating behavior. The persistence of the deficit after ZS in the combined VN and OLF deafferented animals was not as striking. One half of these animals showed recovery of some component of mating behavior by the end of the testing period.

Because histological evaluation of ZS treated males revealed destruction of less than 100% of the OLF mucosa, one might postulate that the remaining receptors mediated the normal behavior we observed after ZS and that our combined deafferentation procedure merely produced a complete OLF deafferentation. Although this would be consistent with the interpretation of other behavioral experiments which used ZS[12,39], several factors argue against this explanation.

If mating in our hamsters was maintained by the remaining OLF epithelium, we must assume that in other laboratories ZS infusion can destroy 100% of the epithelium. This is improbable since histological studies of OLF epithelium after ZS treatment[48,60,66], as well as the results reported here, have consistently reported patches of remaining normal OLF epithelium. It is likely that such patches have remained in all male hamsters whose mating behavior has been studied after ZS[12,39,54], but that these animals were anosmic on behavioral testing because the normal-appearing mucosa had been physiologically poisoned. If this was the case, then the differences observed in mating behavior were not due to differences in the amount of OLF epithelium affected but might have been a result of general debilitation of animals which did not mate, or involvement of the VN organ by ZS administration. In view of the results of our behavioral study, it is reasonable to suggest that ZS techniques in other laboratories affected the VN epithelium, but no direct evidence was presented by which to evaluate this possibility.

Another factor which argues against the hypothesis that our combined lesions were simply a complete OLF deafferentation is that the 8 males in this study which stopped mating permanently after VN nerve cuts had damage to as little as 3.5% of the MOBs bilaterally (range, 3.5%–21.3%; mean, 11.2%). Thus, if sexual behavior is dependent upon the primary OLF system, it is difficult to understand why these animals with minimal damage stopped mating, or why they did not recover. If only the particular area of the OLF bulb which we damaged is responsible for mediating the OLF signals necessary for sexual behavior, why did 56% of the males, with MOB lesions of the same size range and in the same part of the bulbs continue to mate?

On the other hand, if the VN organ input is of major importance in sexual behavior, why do only some of the VN deafferented animals stop mating? To answer this we compared the severe and no-deficit hamsters with respect to (1) the quantity of concomitant MOB damage and (2) the correlation of postoperative deficit with preoperative mating behavior. The no-deficit and severe-deficit groups of males were not significantly different with respect to cell loss in the MOBs but with respect to cell loss plus gliosis in the MOBs, the severe deficit group had significantly larger lesions ($P < 0.05$; t-test). Since our combined deafferentation data clearly demonstrate that the 2 systems work together to mediate copulatory behavior, is is not surprising that the animals with larger lesions showed a greater deficit. As pointed out in Section II above, what was surprising was the complete overlap of the two populations with

regard to lesion size, excepting 2 of the severe deficit animals with the largest lesions.

The correlation of postoperative deficit with preoperative mating behavior showed no significant differences between the postoperatively defined groups with respect to their preoperative behavior, although an analysis of variance yielded an F value which was close to significance on the comparison of preoperative intromission latencies. This suggested that the less easily aroused animals were more severely affected by the VN nerve cuts.

As a working hypothesis we propose that both the VN system and the OLF system are contributing to an arousal mechanism which when appropriately activated leads to copulatory behavior, and that the VN system provides the greater part of the input to this arousal system. This is reflected in the fact that arousal thresholds can be reached in all animals in the absence of OLF input. In contrast, eliminating the VN system reduces arousability to a level which cannot be overcome in some males (severe deficit) whereas in others the threshold can be reached but OLF cues are necessary. That is, subsequent elimination of these OLF cues by combined deafferentation results in a complete loss of mating behavior.

In this study we observed recovery of mating behavior in some of our combined deafferented animals. Devor and Murphy[12] and Lisk, et al.[39] reported recovery of copulatory behavior in their ZS treated males. Additionally, the return of OLF function as reflected in other performance measures has been noted in numerous studies[3,12,15,39,54]. In many instances this recovery has been suggested to result from regeneration of the OLF epithelium after ZS induced degeneration. This suggestion is based on studies which provide histological documentation of regeneration after ZS application in the mouse[44], rabbit[48] and monkey[60]. But the assumed applicability of these histological data to behavioral recovery is not necessarily warranted. Differences in the composition of the ZS solutions used may be critical.

First, differences in the vehicle for ZS may alter its action. In the histological studies cited above regeneration was observed after treatment with 1% ZS dissolved in water. Smith[66], however, failed to find regeneration in the rat after application of 1% ZS dissolved in 0.5% sodium chloride, and most behavioral studies have employed ZS in saline solution. Experimental evaluation of this question was attempted by Schultz and Gebhardt[61]. They provided indirect evidence for increased degeneration and/or decreased regeneration after treatment of monkeys with ZS in saline as compared to ZS in water.

Schultz and Gebhardt also suggested that the molecular concentration of the ZS solution was important. This is of interest because many behavioral studies employed concentrations greater than the 1% used in the histological studies. We are currently looking for regeneration in hamsters after administration of our ZS solution (5 g $ZnSO_4 \cdot 7H_2O$ in 95 ml of 0.5% NaCl; this is 2.9% ZS rather than the 5% reported previously, which did not take into account the water of hydration) and to date we have seen little evidence for restoration of normal OLF epithelium in these animals.

Indirect evidence that little or no regeneration occurs after treatment with concentrations of ZS in this range is provided by Margolis et al.[42] and Ferriero and

Margolis[18], who treated mice with 0.17 *M* (2.8%) ZS. They observed a rapid and dramatic loss of carnosine (the olfactory protein) in the OLF bulbs with no subsequent recovery and concluded that either the regenerated OLF epithelium was not capable of carnosine synthesis or that the epithelium had failed to regenerate. If regeneration does not occur after treatment with concentrations of ZS greater than 1%, perhaps the behavioral recovery observed after such treatment depends on restoration of function in the patches of olfactory epithelium not destroyed by ZS. Ideally we should understand at both the histological and functional levels, differences in species susceptibility, dosage (molecular concentration and number of applications), solvents (vehicles) and delivery mechanisms, in order to assess the effect of the ZS technique for producing OLF deafferentation.

If deafferentation of both VN and OLF systems is necessary to eliminate mating behavior in all male hamsters, how do these two systems function together to mediate the behavior in the normal animal? This is difficult to answer primarily because of our total lack of experimental evidence for mechanisms of function of the VN organ. Estes[17] has proposed specific mechanisms for stimulation of the VN organ by substances passing through the nasopalatine canal. This proposal, based on his observation of ungulate urine testing behavior, is equally plausible for numerous species of mammals in which the VN organ opens into or near the nasopalatine canal through the palate[17]. But in the hamster the nasopalatine canal passes through the incisive foramen posterior to the closed, caudal end of the VN organ and is therefore more likely to admit volatile substances to the OLF epithelium than to the VN organ[1] (see also Fig. 1 above). The VN organ in the hamster is probably stimulated via the nares by substances in the fluid medium on the nose or in the mouth, which may reach the nose by flowing up the philtrum in the upper lip[17]. In light of our findings with combined deafferentation, the ability to turn off mating behavior in male hamsters by clamping shut the nostril opposite the side of a unilateral bulbectomy[12] would support the suggestion that the VN organ is stimulated via the nasal cavities.

The hypothesis that the VN organ functions in identifying appropriate sexual partners or in mediating copulatory behavior has appeared previously in a variety of papers dealing with anatomical[41,57,59,72], physiological[47,69], and behavioral[17,43,52] aspects of the VN organ in mammals. Matthes[43] and Planel[52] both attempted to test the hypothesis experimentally in male guinea pigs after VN nerve destruction.

Matthes[43] found that VN nerve section did not decrease the peculiar head nodding (perhaps a form of Flehmen) of the male when a female, or objects with which the female had been in contact, were presented. Although he concluded that the VN organ is not a special smelling organ for sexual odors, there is no reason to assume that depriving an animal of a sensory pathway will eliminate its behavioral attempts to stimulate that sensory system.

Planel[52] observed a decreased tendency to seek out a female in 67% of the animals with verified nerve cuts and concluded that the VN system was probably important, but not crucial, for normal mating behavior in the guinea pig.

The chemical substances which are most important in stimulating the VN and OLF systems prior to and during mating in hamsters are presumed to be components

of the female hamster vaginal secretions[8,27,49], one of which may be dimethyl disulfide[64]. Whether these vaginal substances stimulate the VN organ, the OLF epithelium, or both is not known. Perhaps the OLF system functions as a distance receptor mechanism which instigates males to find and pursue females. The resulting physical proximity between the sexes might be a necessary condition for adequate stimulation of the male's VN system. This would promote mating in the wild but be unnecessary for mating to occur in our laboratory testing situations in which the female is placed directly with the male and provides visual, auditory, tactile and thermal, as well as OLF stimuli.

The VN system, including the peripheral organ and its central connections, has been studied anatomically in amphibians, reptiles and mammals. Although the function of the receptor organ is an enigma, its morphology has been carefully studied in a wide variety of vertebrates with both the light[28,29,30,46,51] and electron microscope[20,31,32,63]. In 1912, McCotter described the termination of the VN nerve axons in the mammalian AOB[45], but only recently has it been established[59,72] and confirmed[5,36,56] that the AOB and MOB have separate central projections. Efferents from the amygdaloid nuclei receiving AOB input (medial and postero-medial cortical) in turn project to diencephalic areas which are critical for mating behavior in several species[9,10,22,24,25] and which contain high densities of testosterone concentrating cells[58]. These findings further implicate the VN system in reproductive behavior mechanisms.

The contribution of the VN system to mating behavior in rodents other than hamsters has not been studied directly. However, the differential effects of surgical[35] and ZS induced[7] anosmia on male rat sexual behavior may be due to interfering with VN function to different degrees. Additionally, mating behavior changes after lesions at one of several points along the central VN pathway suggest that VN system damage can produce behavioral deficits in male rats[19,21].

Likewise, in hamsters, surgical disconnection of both VN and OLF projections by cutting the lateral OLF tract (LOT)[11,40] can mimic the effect of either combined peripheral deafferentation (reported here) or of bilateral bulbectomy[50]. However, LOT cuts which interrupt VN projections, but are sufficiently caudal to spare OLF input to the OLF tubercle and rostral pyriform cortex in hamsters, produce animals that can apparently smell and that exhibit considerable arousal and sexual interest in receptive females, but they do not mount, intromit or ejaculate[11]. This is consistent with our findings after VN nerve cuts (in preparation). We have found that male hamsters which fail to mate after VN nerve cuts will nevertheless respond to female hamster vaginal secretions swabbed on the side of an OLF testing arena and spend a great deal of time during a mating test sniffing and mouthing the female.

The OLF tubercle and pyriform cortex, which receive projections of the MOB, were spared in the animals with LOT lesions[11]; these areas, in turn, project to the dorsomedial thalamus and the lateral hypothalamus[23,34,37,53,62,65]. Thus the central pathways of the VN and OLF systems provide connections through which they may serve both specific signalling and arousal mechanisms in mediating behavior in the male hamster. Our data indicate that these two systems function together in that capacity in a manner that remains to be elucidated.

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