

Interactive report

Pheromonal activation of vomeronasal neurons in plethodontid salamanders

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Abstract

Pheromones from the mental glands of male plethodontid salamanders increase sexual receptivity in conspecific females. The pheromone enters the vomeronasal organ during courtship to produce this effect. Vomeronasal neurons from female *Plethodon shermani* were examined following exposure to male pheromone or saline placed on the nares. Agmatine was used in conjunction with the pheromone to enable immunocytochemical visualization of chemosensory neurons that were activated by the pheromone. Olfactory neurons exposed to pheromone or saline, and vomeronasal neurons exposed to saline did not demonstrate significant labeling. A population of vomeronasal neurons was intensely labeled following exposure to the pheromone. This study suggests that a specific population of vomeronasal neurons in a female plethodontid salamander is responsible for transmitting pheromonal information to the brain to produce modifications in behavior.

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Topic: Olfactory senses

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1. Introduction

Pheromones are important chemosensory signals that modulate reproductive behaviors in many animals. These conspecific-emitted chemicals can act as attractants, behavioral stimulants or neuroendocrine modulators. The vomeronasal system is an accessory chemosensory system that plays an extensive role in the processing of pheromonal information. In many male and female rodents, mating cannot occur or is impaired without a functional vomeronasal organ [18,21,27,28,30,37,38]. Responsiveness of vomeronasal neurons to pheromones has been explored at the single cell level using calcium imaging [17] and patch clamp recording techniques [12,13,26,36]. These tech-

niques give a brief picture of the response of a narrowly selected group of neurons (as in the slice preparation).

In the present study, we have used neuronal uptake of agmatine as an indicator of the responsiveness of vomeronasal neurons in female plethodontid salamanders (*Plethodon shermani*) to male pheromones. Agmatine (1-amino-4-guanidobutane or AGB) is a guanidinium analog that passes through nonspecific cationic channels during neural activation [19,20]. This molecule can be detected by an anti-AGB antibody, allowing visualization of individual vomeronasal neurons that have been pheromonally stimulated. Thus, a permanent histological record can be obtained of stimulated neurons so that quantitative analysis or studies of neural morphology can be performed in the entire neural organ. Agmatine has previously been used to demonstrate odor-stimulated labeling of olfactory receptor neurons in both vertebrates and invertebrates [24,25].

A male plethodontid salamander utilizes proteinaceous

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pheromones [29] during courtship to increase the female's receptivity [10,11]. The male has an enlarged mental gland (located under the chin) that is hypertrophied during the mating season. Pheromones from the mental gland are delivered to the female when the male brings his gland in direct contact with the female's nares. The water-soluble courtship pheromones enter the nasal capsule via the nasolabial groove, and then are diverted laterally to the vomeronasal organ [3]. The goal of the present study was to visualize and map the effect of male plethodon pheromone on female vomeronasal neurons.

2. Materials and methods

2.1. Animals

Ten female salamanders (*P. shermani*) were used as olfactory subjects in this experiment. In addition, approximately 100 male *P. shermani* were collected to obtain pheromone extract from their mental glands. Animals were collected from Wayah Bald (Macon County, NC, USA) during August 2000. Animals were maintained individually, each in a plastic box (31×17×9 cm) lined with moist paper towels and containing crumpled moist towels as refugia. The salamanders were exposed to a 14:10 light/dark illumination schedule and were fed wax worm larvae or fruit flies.

2.2. Isolation of male pheromone

Male salamanders were anesthetized in 4% ether in water and mental glands were excised. Mental gland extract was obtained by placing the glands in 0.8 mM acetylcholine chloride (AcCh) for approximately 60 min. Gland solutions were centrifuged at 12,000 *g* for 10 min at 4 °C. The supernatant was removed, placed in a new vial, and centrifuged for 10 min at 4 °C. The supernatant was again removed, placed in a new vial, and frozen at –80 °C. To ensure maximum removal of AcCh, ultrafiltration with a 3 kDa cut-off was used. This reduced AcCh levels to the picomolar range that is well below the level required for physiological responsiveness. We pooled extracts from all males, and standardized the protein concentration to 2.0 μg/μl. This initial concentration was selected because it elicited female behavioral response in earlier studies (cf. Ref. [29]). The pheromone solution was subsequently diluted, as described below.

2.3. Pheromone application to females

Female salamanders were exposed to either saline or male pheromone. Females from each group (pheromone, *n*=5; saline, *n*=5) were placed in separate plastic containers with dividers so that no more than two animals occupied a chamber. Both the male pheromone extract and

the saline control (0.9% sodium chloride) were mixed 1:1 with 6 mM AGB dissolved in phosphate-buffered saline (PBS) to yield a final agmatine concentration of 3 mM. A 2-μl volume of solution was applied to the female's nares using a P-10 Gilson Pipetman approximately every 2 min over a 45 min period (20 stimulus applications per female). Following pheromone or saline applications, 5 μl of PBS was applied three times over the course of 5 min to female nares to wash away excess AGB.

2.4. Tissue preparation and immunocytochemistry

Immediately following exposure to stimuli, female salamanders were killed by rapid decapitation and heads were immersed overnight in fixative (4% paraformaldehyde–2.5% glutaraldehyde in PBS, pH 7.4). Heads were decalcified in DeCal (Decal Corporation, Congers, NY, USA) for 3 days and cryoprotected in 30% sucrose dissolved in PBS for 2 days. Heads were mounted in M-1 matrix (Shandon, Pittsburgh, PA, USA), frozen and stored at –80 °C until sectioning.

Pairs of heads (one from the pheromone group and one from the saline group) were sectioned together (20 μm) in the coronal plane on a cryostat microtome. Five sets of sections were collected on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA, USA) so that each section in a set was separated by 100 μm. Plastic slide mailers were used for tissue incubations.

For immunocytochemical labeling of AGB, tissues were rinsed in six 5-min changes of PBS, preincubated in 0.2% Triton X-100–1% normal goat serum–0.004% sodium azide in PBS, pH 7.4 for 30 min and then incubated in rabbit anti-AGB antisera (1:4000; Chemicon International,

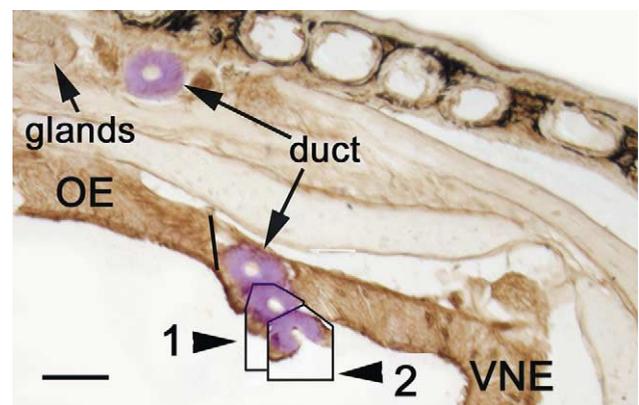


Fig. 1. Coronal micrograph of the dorsocaudal aspect of the vomeronasal and olfactory chambers illustrating the opening of the nasolacrimal duct (pseudocolored violet duct) into the vomeronasal organ. Inserted sections onto the main micrograph of nasolacrimal duct represent the two successive caudal sections (1, 2) of duct with the opening of the duct being the most caudal (2). A dorsal section of duct is also seen at this level since the duct projects rostrally and then hooks slightly caudally to reach the epithelium. Vomeronasal epithelium (VNE) is to the right of the duct and olfactory epithelium (OE) is to the left. Bar=100 μm.

Temecula, CA, USA; lot No. 18112624) for 3 days. One set of sections was labeled with diaminobenzidine (DAB) and another set was labeled with fluorescently labeled secondary antisera. For DAB labeling, tissue was incubated in biotinylated goat anti-rabbit IgG, followed by a horseradish peroxidase–avidin complex (Vector Laboratories, Burlingame, CA, USA) each for 30 min and with six 5-min rinses in PBS between each incubation. Following the final rinses in PBS, the tissue was then immersed in 0.05 M Tris, pH 7.4 for 5 min, then into 0.02% DAB–0.001% hydrogen peroxide in 0.05 M Tris buffer, pH 7.4 for 30 min. The labeling reaction was monitored by observing tissue under a Nikon light microscope. After labeling was completed, tissue was rinsed again in Tris buffer, dehydrated through a series of ethanol dilutions and cleared in xylene. Slides were coverslipped with Permount (Fisher Scientific).

For fluorescent labeling, tissue was rinsed in PBS following incubation in the primary antisera, and incubated in goat anti-rabbit IgG conjugated to Alexa 488 (1:500; Molecular Probes, Eugene, OR, USA) for 30 min. Tissue was rinsed in PBS, counterstained with Hoechst stain and coverslipped with Cytoseal (Electron Microscopy Sciences, Fort Washington, PA, USA).

2.5. Histological analysis

All sections of plethodon nasal cavity were examined with light or fluorescent Nikon and Olympus microscopes. Representative digital images were taken of DAB- and Alexa 488-labeled vomeronasal organ sections from both experimental groups with a SPOT camera (Diagnostic Instruments, Sterling Heights, MI, USA) and Olympus microscope. For DAB-labeled tissues, labeled neurons

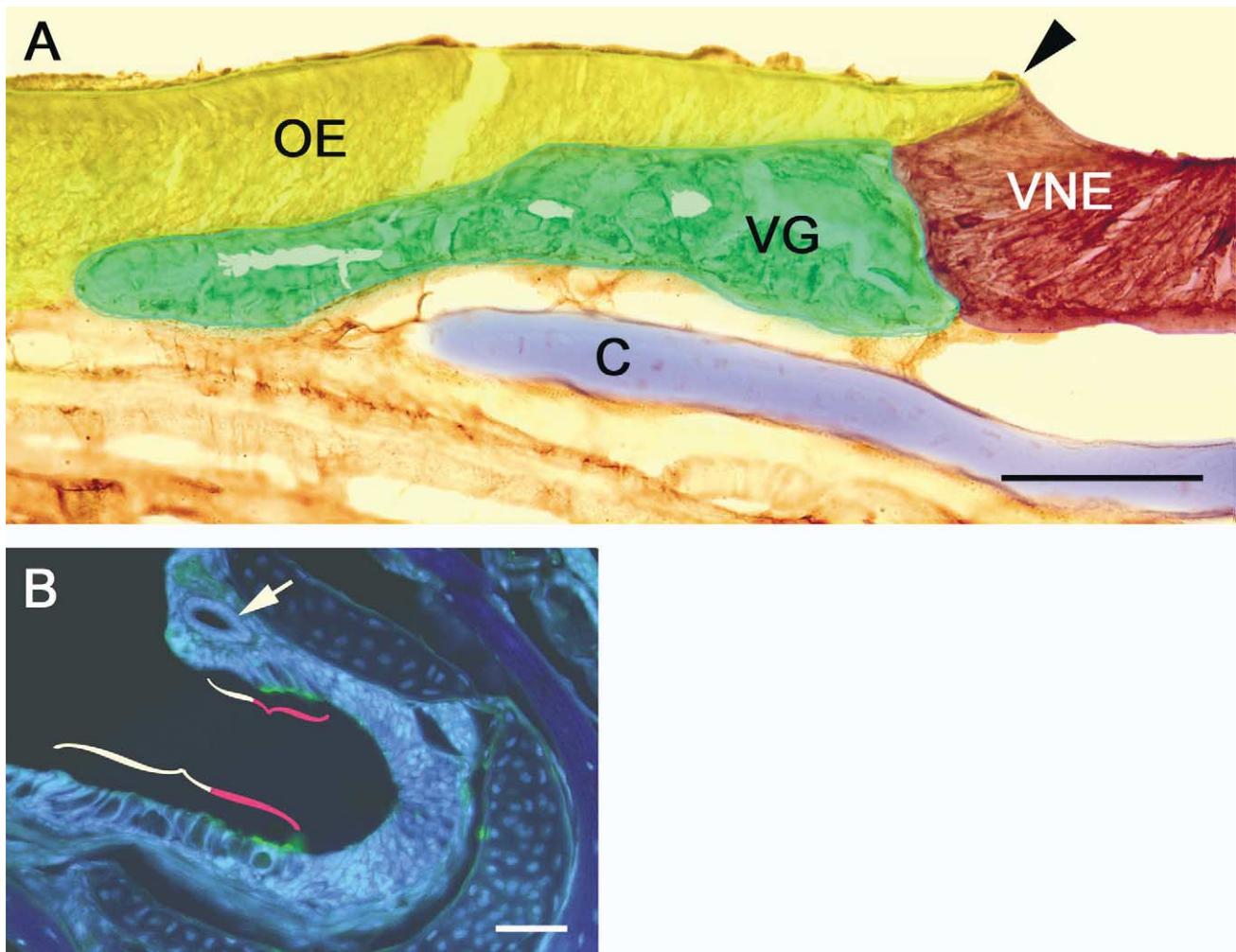


Fig. 2. Micrograph of vascular and glandular tissue surrounding the vomeronasal organ at rostral (A) and caudal (B) levels. In the micrograph in (A) pseudocolorized overlays have been used to designate the various structures. In (A), the olfactory epithelium (OE, yellow) abuts the vomeronasal epithelium (VNE, red) in a thinning taper (arrowhead). Vascular and glandular connective tissue (VG, green) underlies this convergent area. The mucosa is surrounded by a cartilaginous capsule (C, blue). At more caudal levels (B), the vomeronasal sensory epithelium is surrounded by secretory epithelium (brackets) that projects beyond the chamber of the vomeronasal organ (white portion of bracket). Note that AGB label is confined to the surface of only the vomeronasal epithelium (red portion of brackets) at this level. The arrow indicates the nasolacrimal duct. Bars=100 μ m.

were counted separately in each section from both right and left vomeronasal organs with a 20 \times microscope objective. The number of labeled neurons was recorded from all sections containing the vomeronasal sensory epithelium. For neural count comparisons between animals, the cell count data were aligned by using the opening of the nasolacrimal duct into the dorsal wall of the

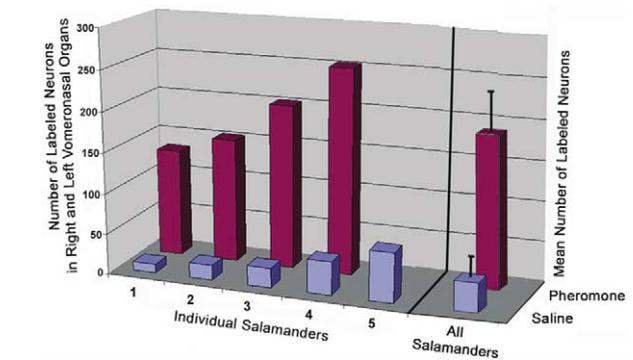
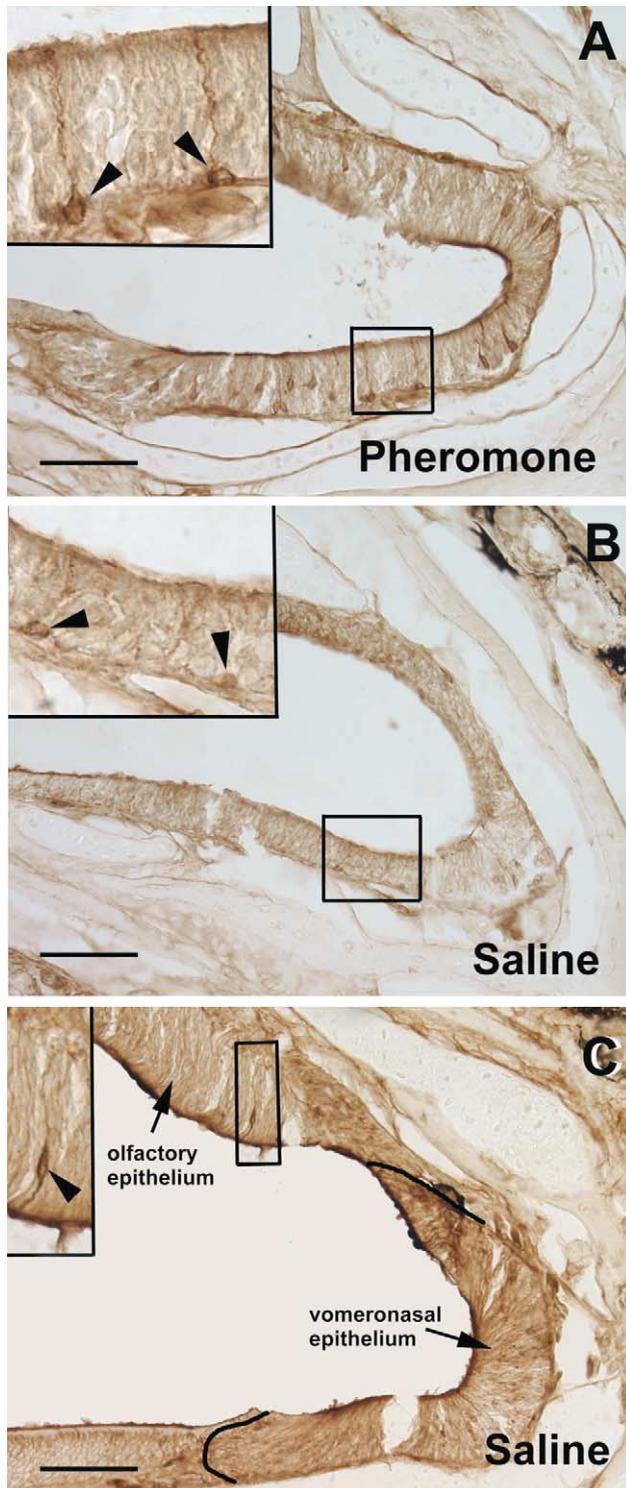


Fig. 4. The total number of labeled neurons (added together from right and left sides) in vomeronasal organs of individual salamanders (left portion of graph) from the pheromone and saline groups, arranged in ascending order. On the right portion of the graph is the mean (\pm S.D.) number of labeled vomeronasal neurons from each group. The tissue from one salamander was too darkly labeled to count cells, thus giving an n of 4 for the pheromone group. The difference in the total number of VNO neurons between pheromone and saline groups was significant ($P=0.0005$).

vomeronasal organ as a standard point of alignment (Fig. 1). Not all vomeronasal organs were exactly the same size, so the number of sections on each slide differed somewhat.

2.6. Statistical analysis

In order to control for variations in the size of the vomeronasal organ, an analysis was first conducted to determine whether vomeronasal organs from the two groups had statistically similar number of sections of vomeronasal sensory epithelium. Sections containing vomeronasal sensory epithelium were counted from each vomeronasal organ to give an n of 10 for each group. A Student's t -test with unpaired variables was used to compare the number of sections from the two groups. This showed that there were no differences in the number of sections in vomeronasal organs from the two groups of animals.

Two statistical analyses of labeled cell counts were performed. The first analysis compared the total number of labeled neurons in right plus left vomeronasal organs between pheromone vs. saline groups. This gave four data points for the pheromone group (one set of tissue was too

Fig. 3. Micrographs of AGB labeling following exposure to the pheromone (A) or saline (B, C). Insets show magnifications of the boxed area in each figure. (A) Following pheromone application, vomeronasal neuron cell bodies (arrowheads) and dendrites are heavily labeled by DAB in the vomeronasal epithelium. (B) Following exposure to saline, very few neurons are labeled and these are labeled very lightly (arrowheads). (C) Some AGB flowed over the olfactory epithelium, as evidenced by dark label on the surface of the epithelium. Black lines designate the separation between the olfactory and vomeronasal epithelia. Very few, if any, olfactory neurons are labeled. Inset shows one olfactory neuron (arrowhead) that is labeled following exposure to saline. Bars=100 μ m.

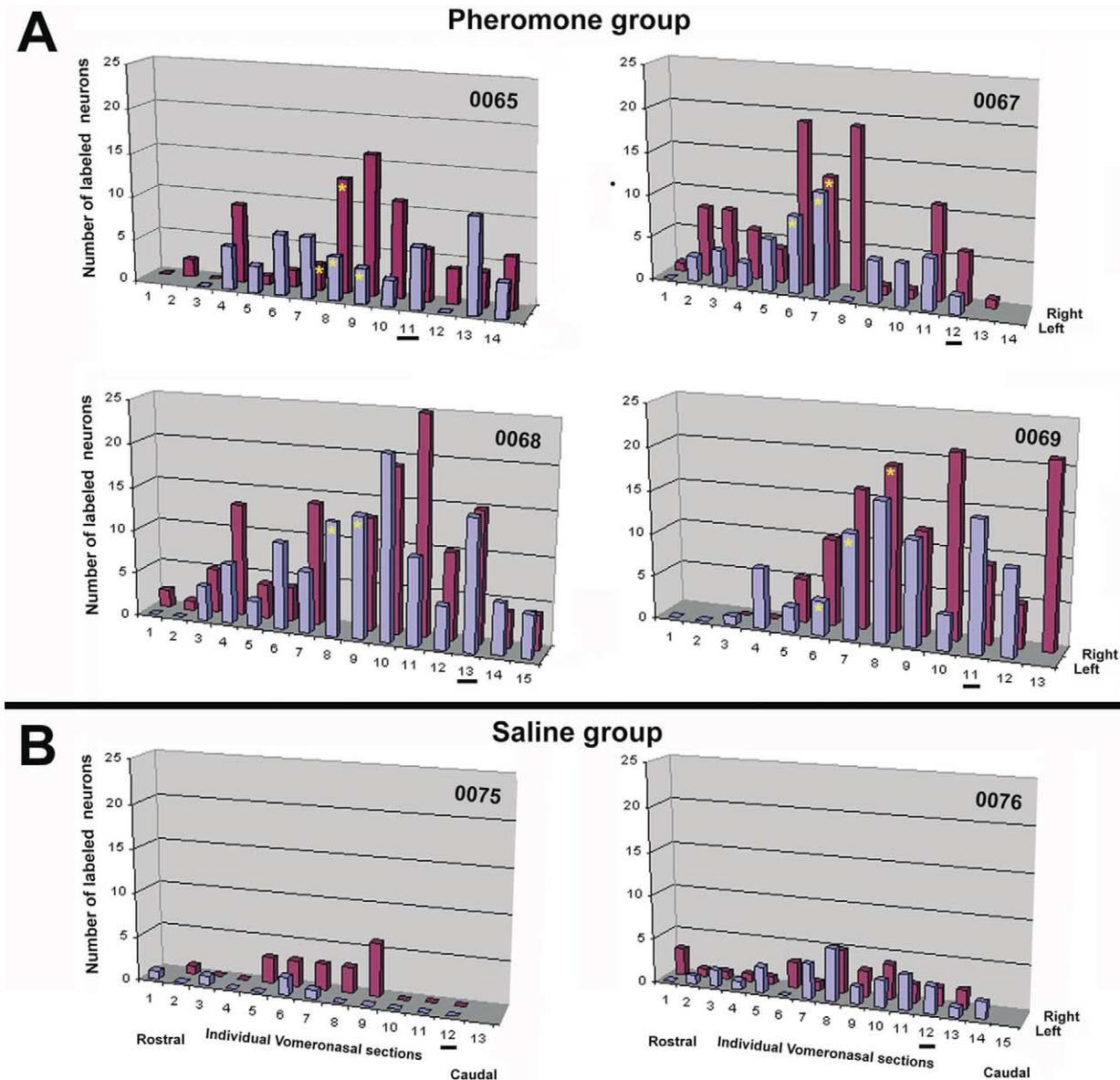


Fig. 5. The number of labeled vomeronasal neurons in the rostral and caudal levels of the vomeronasal organ. Each bar represents counts from one tissue section. The yellow asterisks on the bars indicate the dividing point between designated rostral and caudal sections. Each graph represents data from an individual salamander. Data from all salamanders in the pheromone group and two representative salamanders from the saline group are presented. Animal numbers are shown in the upper right hand corner of each graph. The underlined number on the x-axis represents the section in which the nasolacrimal duct opens into the vomeronasal organ. Sections containing the opening of the nasolacrimal duct are aligned for left and right sides. In the pheromone group (A), there was a general tendency for the presence of more labeled neurons from mid to caudal levels of the vomeronasal organ. In the saline group (B), there was no difference in the number of labeled neurons between rostral and caudal levels.

intensely labeled for analysis) and five data points for the saline group. An unpaired Student's *t*-test was used to compare the total neuron count data between groups.

The second analysis tested for a distribution trend for the neural counts. This was conducted to determine whether there was a difference in the number of labeled neurons between rostral and caudal levels of the vomeronasal sensory epithelium in either of the groups. We conducted this test for purposes of later being able to find cells for electrophysiological recordings. Data consisted of

total neural cell counts for rostral and for caudal regions of vomeronasal organ for each animal. A paired Student's *t*-test was used to compare the neural cell count data between rostral and caudal levels of the vomeronasal organs in each group separately. To calculate the total number of neurons for rostral and caudal levels, the number of tissue sections containing vomeronasal sensory epithelium for each vomeronasal organ was divided in half and neurons were added separately for the rostral half of the sections and for the caudal half of the sections for each

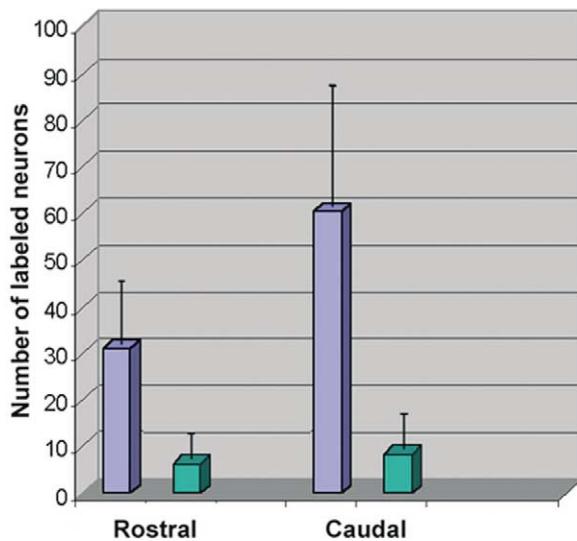


Fig. 6. The mean (\pm S.D.) number of labeled vomeronasal neurons in the rostral and caudal halves of the vomeronasal organs of pheromone and saline groups. The mean number of neurons from the pheromone group is derived from the individual animals shown in Fig. 5, while the mean number of neurons in the saline group is derived from the two animals shown in Fig. 5 plus the three additional animals in the saline group not shown in Fig. 5.

animal (pheromone group: rostral VNO, $n=4$; caudal VNO, $n=4$; saline group: rostral VNO, $n=5$; caudal VNO, $n=5$). All statistical analyses were carried out using online programs at: <http://faculty.vassar.edu/lowry>.

3. Results

Agmatine labeling was found on the surface of the vomeronasal epithelium and sometimes on the surface of the proximal olfactory epithelium. The vomeronasal organ consists of a groove or channel in the lateral wall of the nasal chamber. Substances are directed into the organ via the nasolabial groove. The restriction of substances entering the vomeronasal organ is controlled by vascular and glandular tissues (Fig. 2A and B). In this experiment, we placed a fairly large amount of fluid on the nares of each female and some of the fluid flowed into the olfactory region.

Application of pheromone to female plethodontid salamanders resulted in the robust labeling of a population of vomeronasal neurons (Fig. 3A). Application of saline produced little, if any, faint labeling of vomeronasal neurons (Fig. 3B). Neither pheromone nor saline application produced any significant labeling of olfactory neurons, except for an occasional neuron (Fig. 3C) that may have been labeled because of some type of endogenous activity. The lack of labeling of olfactory epithelium occurred despite the apparent flow of AGB over the most lateral olfactory epithelium, as evidenced by immunocytochemical labeling on the surface of the olfactory epithelium.

There was a significant difference in the total number of labeled neurons (combining right and left vomeronasal organs) between the pheromone (mean=185.3 neurons, S.D.=53.7) and saline (mean=25.8 neurons, S.D.=20.3) groups (Fig. 4; $t=6.2$, $df=7$, $P=0.0002$). In addition, there were significantly more labeled neurons in the caudal (mean=120.9 neurons, S.D.=46.2) vs. the rostral (mean=61.9 neurons, S.D.=15.0) levels of the vomeronasal organ in the pheromone group ($t=-2.48$, $df=3$, $P=0.05$; Figs. 5 and 6). This can be explained by the greater total volume of vomeronasal epithelium in the caudal half of the vomeronasal organ (the caudal half of the organ has approximately twice the volume of the rostral half). The heaviest concentration of labeled neurons appeared to be in the region where the epithelium bends inward, forming a pocket in the caudal vomeronasal organ (Fig. 7). In the saline group however, there was no significant difference in the number of labeled neurons between the rostral (mean=12.6 neurons, S.D.=7.5) and caudal (mean=17 neurons, S.D.=13.5) levels ($t=-1.02$, $df=4$, $P=0.18$).

There did not appear to be a consistent laminar distribution within the vomeronasal organ of labeled neurons. Labeled neurons were found in the basal, middle and superficial layers (Fig. 8). For neurons that responded to pheromone stimulation, label was found in the dendrite, cell body and proximal-most part of the axon. Dendrites appeared to have a corkscrew shape (Fig. 9), suggesting that the epithelial layer was compressed, either from natural physiological causes or from fixation. Not all neurons were labeled with the same intensity (Fig. 10). The labeling intensity may signify the level of activity of the neuron to the stimulus.

4. Discussion

A subset of vomeronasal neurons was activated by stimulation from a species-specific pheromone, using agmatine uptake [19,20] as an indicator of neural activity. Pheromone extract from the mental glands of male plethodontid salamanders (*P. shermani*) activated a dispersed population of vomeronasal neurons throughout the entire vomeronasal epithelium. We estimate that approximately 3% of vomeronasal neurons responded to the pheromone extract. The activated neurons were found in all vomeronasal epithelial lamina (i.e., deep to superficial layers) and were in greatest numbers in caudal regions of the vomeronasal organs of female *P. shermani*. The greater number of labeled vomeronasal neurons in the caudal vomeronasal organ could be explained by the greater volume of vomeronasal epithelium in caudal compared with rostral vomeronasal organ of *P. shermani*. Saline stimulation of vomeronasal neurons failed to produce similar results. The regional distribution data will be used to identify regions of the vomeronasal organ most suitable for conducting patch clamp studies on these neurons. The

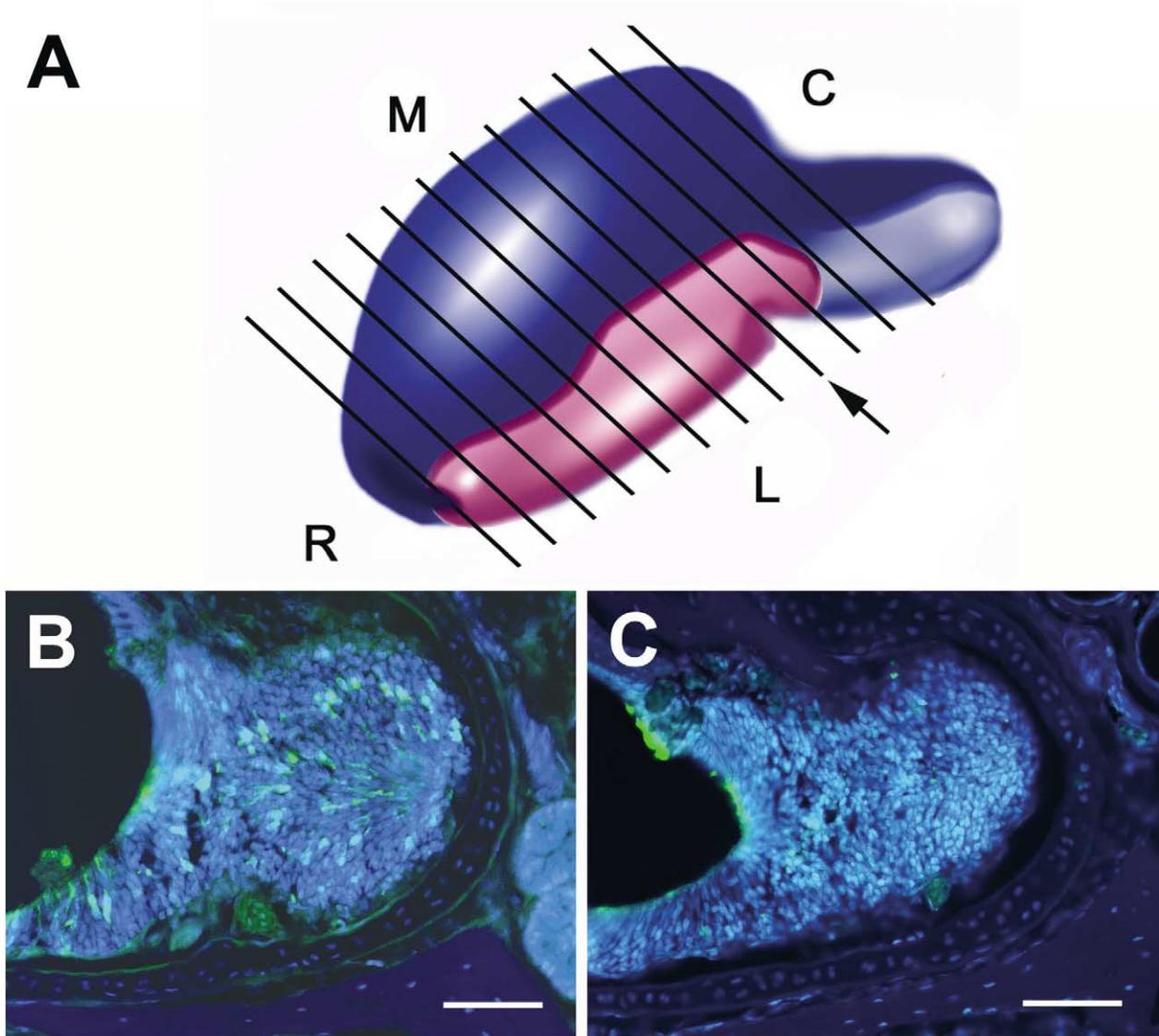


Fig. 7. Orientation of sections through the nasal sac (A) with corresponding fluorescent micrographs of the region in which the vomeronasal epithelium curves inward. The vomeronasal organ (pink) is on the lateral side of the nasal sac (blue). Micrographs are from representative salamanders in the pheromone (B) and saline (C) groups. In this region the vomeronasal epithelium curves medially so that coronal sectioning cuts the epithelium tangential to the surface of a hemispherical pocket at the caudal end of the vomeronasal organ (C). Labeled neurons (green) are dispersed in the tangential section of epithelium from the pheromone-exposed salamander (B), but not the saline-exposed salamander (C). Abbreviations in (A): M, medial; C, caudal, R, rostral; L, lateral. Bars=100 μ m.

use of vomeronasal organ sections from larger regions of epithelium with a greater number of responsive cells will increase the ease of recording.

The distribution of labeled neurons throughout rostral to caudal levels of the vomeronasal organ, as well as in all laminae of the organ, may reflect a possible heterogeneity of compounds within the pheromone extract from male *P. shermani* mental glands. Studies in other species have demonstrated heterogeneity of cell types in both the vomeronasal organ and accessory bulb [9]. This heterogeneity may reflex segregation of response characteristics to various substances. Previous studies on male *P. shermani* mental gland pheromone have demonstrated that, of

the multiple protein components that comprise the pheromone extract, two main proteins account for approx. 85% of all components [6]. These two main proteins are: (a) a 22-kilodalton protein, termed 'plethodontid receptivity factor' (PRF) [29], and (b) a 7-kilodalton protein (P7) [6]. The presence and relative proportion of these two proteins has been highly consistent over multiple years of obtaining gland extracts from *P. shermani* salamanders (Richard C. Feldhoff, unpublished observations). The function of the P7 protein is not yet known. However, experimental tests using a purified solution of PRF showed that female *P. shermani* receiving PRF were more receptive, as compared to females that received only a saline control solution [29].

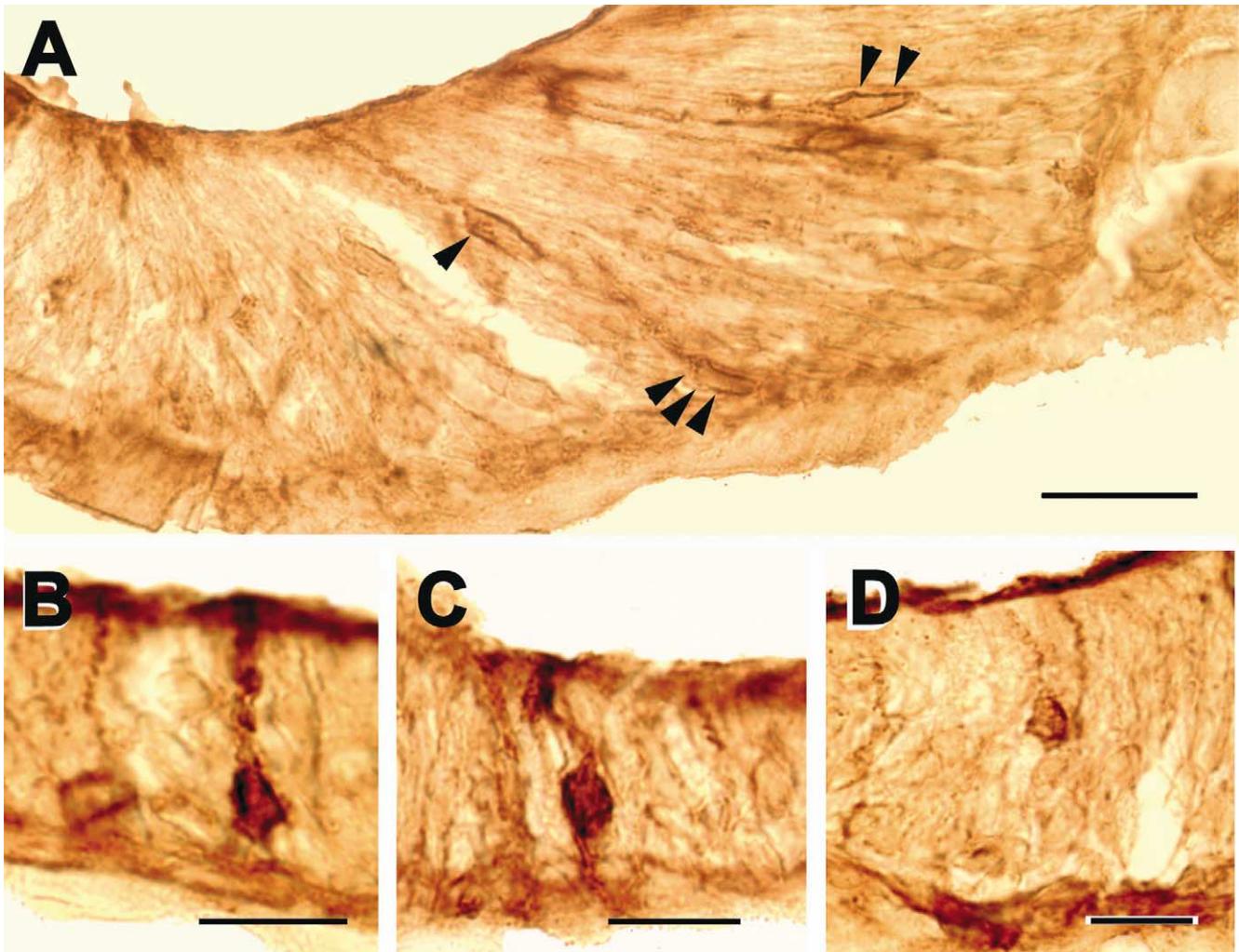


Fig. 8. Micrographs of vomeronasal epithelium showing the laminar distribution of DAB-labeled neurons from pheromone-stimulated salamanders. (A) In a single section of ventral vomeronasal epithelium, labeled neurons can be seen in the superficial (single arrowhead), middle (two arrowheads) and basal layers (triple arrowheads). Additional sections also show labeled neurons in basal (B), middle (C) and superficial layers (D). Bars=25 μ m.

This change in receptivity of female salamanders parallels similar vertebrate responses in which an individual's reproductive behavior or physiology was altered in response to chemical information received from a conspecific [34,37].

The most common neural pathway that mediates this kind of physiological response is via the accessory olfactory system, with initial reception by the vomeronasal organ (e.g., Refs. [1–4,39]). A neural pathway from the accessory olfactory system to the hypothalamus has been demonstrated experimentally for fish, reptiles and mammals (e.g., Refs. [7,15,16,34,39]). In plethodontid salamanders, an initial step in this pathway has been confirmed with the documentation of projections from the VNO to the amygdala [31]. The significance of the accessory olfactory pathway is the well-known connection between pheromonal stimulation and the expression of reproductive behaviors, including increased receptivity [28,32].

Access of the nonvolatile pheromone to the vomeronasal

organ in this plethodontid species is mediated by the capillary action of the nasolabial groove. This groove directs compounds directly into the vomeronasal organ (a feature different from mechanisms in other species [5,8,22,23]). However, with the large amount of fluid applied to the snout in our experiment, some fluid flowed over the lateral areas of olfactory epithelium adjacent to the vomeronasal organ. In contrast with the vomeronasal response to the male courtship pheromones, main olfactory neurons were not visibly labeled by AGB immunocytochemistry. Thus, the low level or lack of olfactory labeling indicates that the components of this salamander courtship pheromone specifically stimulate the vomeronasal organ to a high degree and not the olfactory epithelium.

In many mammalian species, the main olfactory system is capable of detecting certain volatile pheromones [37]. In certain cases, the olfactory system may be used initially to detect particular pheromones in order to activate the mechanisms designed to draw chemicals specifically into

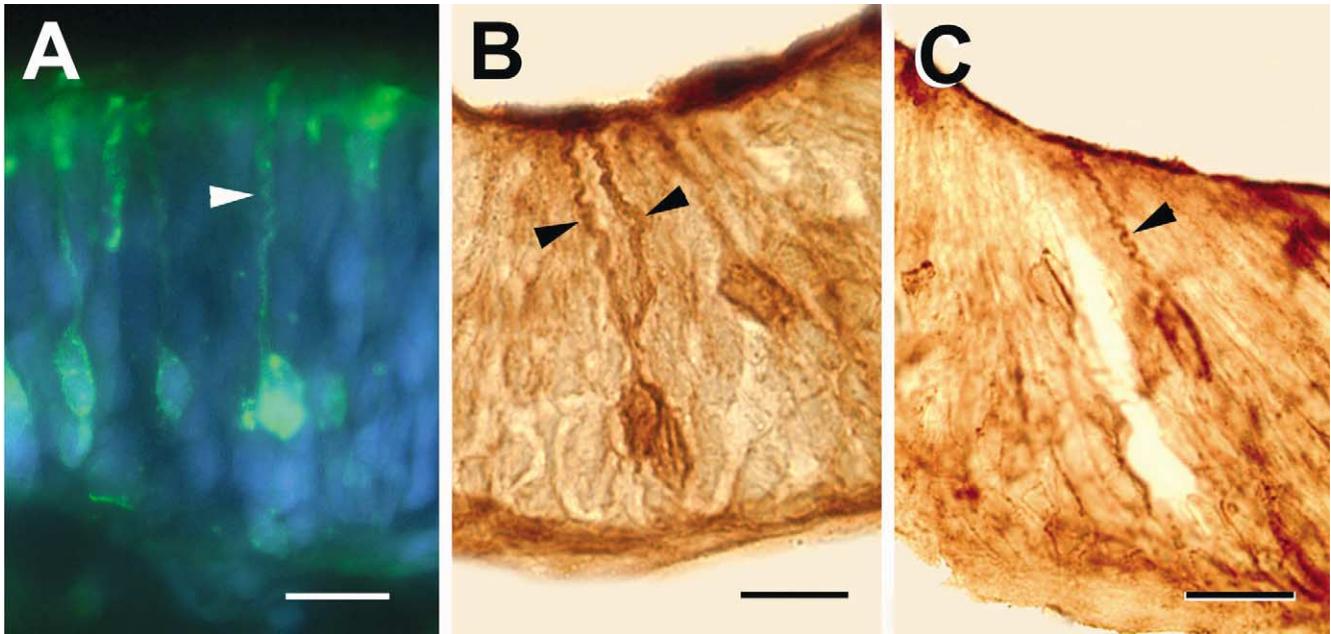


Fig. 9. Fluorescently-labeled (A) and DAB-labeled (B,C) micrographs of vomeronasal sensory epithelium demonstrating the morphological appearance of labeled dendrites from salamanders in the pheromone-stimulated group. In both fluorescently labeled tissue (A) and DAB-labeled tissue (B, C), dendrites displayed a corkscrew morphology (arrowheads). Bars=25 μ m.

the vomeronasal organ. In the case of courtship pheromone from our male salamanders, however, the pheromone is shunted directly to the vomeronasal organ, thus averting the need for initial detection. In mammals, the main

olfactory system may mediate certain complex pheromone-directed behaviors [33,35] that are lacking in amphibian species. In humans, the olfactory system can even mediate pheromone-induced psychological and emotional states without conscious detection of the pheromone [14].

The exact effect of courtship pheromones on the physiology of female *P. shermani* is not known. It is difficult to determine whether the pheromone acts as a sedative or a stimulant without further exploration of the central mechanisms of its action. Perhaps the different protein components of the pheromone solution mediate both sedative as well as stimulatory functions in the female. A determination of the vomeronasal neurons (and their central projections) that are activated by each of the individual protein components of the pheromone solution may further our understanding of the behavioral effects of pheromones on reproductive behavior in plethodontid salamanders.

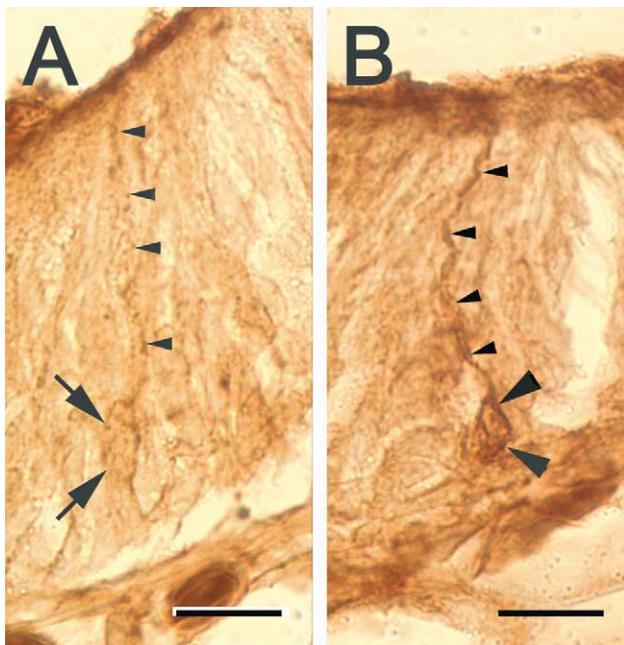


Fig. 10. Micrograph of vomeronasal epithelium from saline-stimulated salamanders demonstrating varying levels of intensity of DAB labeling. In (A), the neural cell body (arrows) and dendrite (arrowheads) are faintly labeled. In (B), the cell body and dendrite are intensely labeled. Bars=25 μ m.

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