CHEMICAL SIGNALS AND VOMERONASAL SYSTEM FUNCTION IN AXOLOTLS (*AMBYSTOMA MEXICANUM*)

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1. INTRODUCTION

Most tetrapods, the group of vertebrates that includes amphibians, reptiles, and mammals, possess separate olfactory and vomeronasal systems. Why? What are the functions of these two anatomically distinct chemosensory systems? Although the vomeronasal system is often presumed to be specialized for mediating responses to pheromones and the olfactory system assumed to respond to "general odorants" that are not pheromones, this dichotomy does not hold up for any group of vertebrates studied to date. In mammals, some pheromonal effects are mediated by the olfactory system (Dorries et al., 1997; Cohen-Tannoudji et al., 1989), and the vomeronasal organ responds to some general odorants (Sam et al., 2001). In squamate reptiles the vomeronasal system is critically involved in both foraging and in responding to pheromones (Halpern and Kubie, 1984). In salamanders, the vomeronasal organ has recently been suggested to play a role in foraging (Placyk and Graves, 2002), and both the olfactory and vomeronasal organs respond to pheromones (Toyoda et al., 1999; Park and Propper, 2002). Thus, the vomeronasal system cannot be considered to be a labeled line for pheromone processing (Shepherd, 1985), although certainly many pheromonal effects are mediated by the vomeronasal system (see Halpern and Martínez-Marcos, 2003, for a recent review).

An alternative hypothesis suggests that the vomeronasal system mediates responses to large, high-molecular-weight molecules that are not volatile in air, and that the olfactory system responds to molecules that have a sufficiently low molecular weight to become airborne and contact the olfactory epithelium (Halpern and Kubie, 1980; Wysocki et al., 1980). This hypothesis is attractive, as it might explain why the vomeronasal system has been lost repeatedly in aquatic and arboreal tetrapods, including in crocodilians and birds (Parsons, 1959, 1967, 1970), some cetaceans (Lowell and Flanigan, 1980; Oelschläger and Buhl, 1985), manatees (Mackay-Sim et al., 1985),

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several species of bat (Bhatnagar, 1980), and some primates (Smith et al., 2001, 2002). Further, if mammalian pheromones tend to be high-molecular-weight molecules (Singer, 1991; Nevison et al., 2003), this hypothesis might also explain why many pheromone effects in mammals are mediated by the vomeronasal system.

The vomeronasal system is generally present in aquatic amphibians (Eisthen et al., 1994; Eisthen, 2000), an observation that seems to contradict the molecular-weight hypothesis, as volatility is not relevant in water. To date, the pheromones that have been isolated from amphibians are all peptides and proteins (Kikuyama et al., 1995; Rollmann et al., 1999; Wabnitz et al., 1999; Yamamoto et al., 2000), and the only study demonstrating selective stimulation of the vomeronasal organ used stimuli that were 3 kDa and larger (Wirsig-Wiechmann et al., 2002). Perhaps the vomeronasal system of amphibians detects large molecules, and the olfactory system responds to smaller molecules.

Nevertheless, some data concerning the presence of the vomeronasal system in aquatic vertebrates remain difficult to explain. For example, the vomeronasal system has been lost in proteids, a family of aquatic salamanders (Seydel, 1895; Anton, 1911; Farbman and Gesteland, 1974; Eisthen, 2000). Without a firm understanding of the relative functions of the olfactory and vomeronasal systems, the causes of the loss of the system in this group of animals remain mysterious. Another anomalous detail concerns the presence of the vomeronasal system in teleost fishes: although distinct vomeronasal organs and accessory olfactory bulbs are clearly absent in teleosts (Eisthen, 1992, 1997), members of the family of genes encoding mammalian vomeronasal odorant receptors are expressed in the olfactory epithelium of goldfish and pufferfish (Cao et al., 1998; Asano-Miyoshi et al., 2000). Thus, we are far from a solid understanding of the organization and function of the vomeronasal system in vertebrates.

In our laboratory, we are studying chemosensory-guided behavior and the neurobiology of chemosensory systems in axolotls (*Ambystoma mexicanum*), a species of salamander that is aquatic throughout life. Through our studies, we hope to determine the relative functions of the olfactory and vomeronasal systems in axolotls, and in aquatic salamanders in general. We expect that this work will help us understand the function of the vomeronasal system in amphibians and, more generally, in aquatic vertebrates.

We have chosen to study axolotls for several reasons. First, axolotls are hardy, and are easily maintained and bred in the laboratory (Armstrong et al., 1989). Unlike many salamanders, axolotls will court and breed year-round in the laboratory. Axolotls are larger than many salamanders, making the surgeries and dissections for tract-tracing and recording from live cells fairly simple; they also have large cells, simplifying experiments involving single-cell physiology. Finally, axolotls are essentially a subspecies of tiger salamander (Shaffer, 1993), and much of the available data concerning neuroanatomy and olfactory system physiology in tiger salamanders (e.g., Herrick, 1927, 1948; Firestein and Werblin, 1987, 1989; Kauer, 2002) can be applied directly to axolotls.

2. ANATOMY OF NASAL CHEMOSENSORY SYSTEMS IN AXOLOTLS

In axolotls, as in most other salamanders, the vomeronasal organ consists of a pouch that protrudes from the lateral edge of the nasal cavity (Figure 1). The vomeronasal organ

Figure 1. Cross section through the snout of an axolotl, decalcified and stained with cresylecht violet. The midline of the snout is shown on the left, lateral is to the right, and the dorsal surface of the snout is toward the top. This section is taken through the point at which the vomeronasal organ connects with the nasal cavity. The vomeronasal organ is lined with vomeronasal sensory epithelium (vom), and the medial portions of the nasal cavity are lined with olfactory epithelium (olf).

of axolotls is relatively small, extending approximately 10% of the total length of the nasal cavity. In cross-section, the organ is wider than it is tall.

The structure of the olfactory and vomeronasal epithelia differ considerably (Eisthen et al., 1994). In axolotls, as in other vertebrates, the nasal sensory epithelia contain sensory neurons, supporting (sustentacular) cells, and basal cells. The olfactory epithelium of axolotls contains both ciliated and microvillar receptor neurons, and the two types of neurons are found in homogeneous clusters throughout the epithelium. In contrast, vomeronasal receptor neurons terminate in microvilli, although a rare ciliated dendrite can be found in the vomeronasal epithelium. In both the olfactory and vomeronasal epithelia, sustentacular cells terminate in short processes; the vomeronasal epithelium contains a second class of supporting cells with a large surface that is covered with highly active, motile cilia. These cells may function to move mucus and odorants into the relatively small vomeronasal organ and across the sensory epithelium.

As illustrated in Figure 2, the axons of most olfactory receptor neurons project to the main olfactory bulb, at the rostral pole of the telencephalon; the axons of the vomeronasal neurons project to a distinct accessory olfactory bulb on the lateral edge of the telencephalon, immediately caudal to the main olfactory bulb. The central projections of the main and accessory olfactory bulbs have been described in tiger salamanders by Herrick (1927, 1948) and by Kokoros and Northcutt (1977), and we assume that the projections are the same in axolotls; similar projections have also been described in other salamanders (Herrick, 1933; Schmidt and Roth, 1990). Briefly, as illustrated in Figures 2A and B, the afferents from the main olfactory bulb project via medial and lateral tracts to the septum, striatum, and medial amygdala, and to the dorsal, lateral, and medial pallia (the homologue of the mammalian hippocampus). The accessory olfactory bulb projects to the lateral amygdala (Figure 2D); as in mammals and reptiles, different portions of the
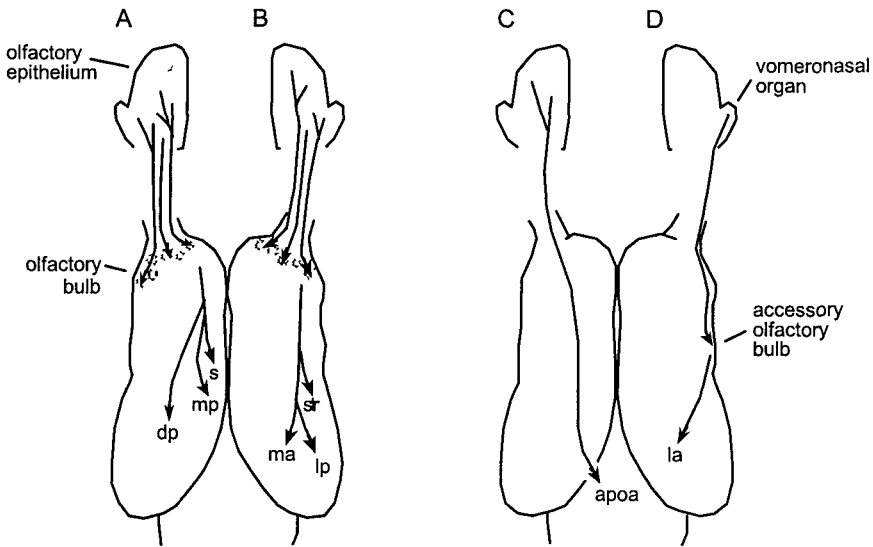
Figure 2. Schematic diagram of the nasal cavities and forebrain of a salamander, illustrating the central projections of the olfactory and vomeronasal systems in dorsal view. Anterior is toward the top of the figure, and only ipsilateral projections are shown. The medial (A) and lateral (B) olfactory tracts arise from the olfactory bulb. (C) The extra-bulbar olfactory pathway bypasses the olfactory bulb and projects directly to the anterior preoptic area. (D) The accessory olfactory bulb, which receives input from the vomeronasal organ, projects to the lateral amygdala (la). Other abbreviations: apoa = anterior preoptic area; dp = dorsal pallium; lp = lateral pallium; mp = medial pallium; ma = medial amygdala; s = septum; str = striatum. Based on descriptions in Herrick, 1927, 1933, 1948; Kokoros and Northcutt, 1977; and Schmidt and Roth, 1990.

amygdala receive input from the main and accessory olfactory bulbs (reviewed in Halpern and Martínez-Marcos, 2003). Finally, axolotls appear to possess an extra-bulbar olfactory pathway (Szabo et al., 1991; Hofmann and Meyer, 1992), in which axons of olfactory receptor neurons bypass the olfactory bulb and project directly to the anterior preoptic area (Fig. 2C; unpublished observations).

3. CHEMOSENSORY-GUIDED BEHAVIOR IN AXOLOTLS

Unfortunately, axolotls are almost extinct in the wild, and little known is about their behavior and ecology in natural settings; nevertheless, it seems reasonable to assume that many aspects of their behavior are similar to those of other neotenic populations of tiger salamanders. We are investigating the roles of chemosensory cues in guiding reproductive and foraging behavior in axolotls in the laboratory, with the goal of using these cues in neurobiological experiments to examine the relative functions of the olfactory and vomeronasal systems.

We have observed axolotls courting in the laboratory, and the basic sequence of courtship behaviors in axolotls appears to be similar to that which has been described for

other ambystomids (e.g., *A. talpoideum*, Shoop, 1960; *A. tigrinum*, Arnold, 1976). The initial stage in courtship consists of the male nudging the female with its snout, first along the flank and then in the cloacal region. This leads to the "waltz" behavior described by Shoop (1960) for *A. talpoideum*, in which each animal holds its snout near the other's cloaca as the pair moves in a circle. After several minutes of waltzing, the male moves away from the female, which follows closely behind, often touching his tail with her snout. During this stage of courtship, the male slowly shakes the posterior parts of the body and tail from side to side with an undulating motion. Arnold (1976) calls this behavior "vent shuffling", and it resembles the "hula dance" performed by male red-spotted newts (*Notophthalmus viridescens*; Verrell, 1982). Unlike newts, however, the female also shakes her tail while following, although not as consistently as does the male. After the female has followed the male for a brief period, the male pauses and lowers his cloacal area while vigorously shaking his abdomen and tail. He deposits a spermatophore on the substrate, and then moves forward approximately one body length. The female follows. Once she is positioned with her cloaca over the spermatophore, the female lowers her cloaca to pick up the spermatophore while rapidly shaking her tail. At this point her behavior is almost indistinguishable from that of a male depositing a spermatophore. After the female has retrieved the sperm cap, the two animals move independently. Eventually, nudging and waltzing may be re-established. This stage of courtship is usually briefer during the second and subsequent encounters, and the female shortly begins following the male again. The entire process of courtship and sperm transfer is generally repeated several times during the course of an encounter.

Females fertilize their eggs internally, then lay most or all of their eggs in a spawn that may contain several hundred eggs. Given that in natural conditions females probably spawn only once in a year, males may be under considerable pressure to discriminate gravid females, which are full of eggs and ready to spawn, from those that have recently spawned or are otherwise not in breeding condition. In contrast, males can mate successfully at 2- to 3-week intervals (Armstrong et al., 1989), and females may not need to discriminate between males that have spawned recently and those that have not.

As a first step toward understanding the role of chemical cues in courtship behavior, we have conducted experiments to determine whether sexually-mature male and female axolotls respond differently to odorants from conspecifics in different reproductive conditions (Park et al., 2004). We presented both male and female axolotls with whole-body odorants from same-sex conspecifics and from opposite-sex conspecifics that had spawned recently or had not spawned recently, as well as a clean-water control, and measured both general activity and the frequency and duration of hula displays by subjects. We found that in males, exposure to whole-body odorants from females led to an increase in general activity, regardless of the reproductive condition of the odorant donor. Intriguingly, males that had no prior experience with adult females were more active than experienced males when exposed to odorants from females; because of our housing regime, males that had not been paired with a female prior to testing had no experience with odorants from sexually-mature females, so we do not know whether the effect we observed requires behavioral interactions with females, or simply exposure to chemical cues from females. We found no differences in the frequency or duration of hula displays by males in any testing condition. Among female subjects, exposure to odorants from conspecifics did not affect general activity, but hula displays were performed only in response to exposure to odorants from males. No difference in the frequency or duration of hula displays was observed in response to odorants from males

that had spawned recently or not. We found that both sexually experienced and inexperienced females performed hula displays when exposed to whole-body odorants from males, suggesting that odorants from male axolotls could function to increase sexual receptivity in female axolotls, as has been demonstrated in other salamanders (Houck and Reagan, 1990; Rollmann et al., 1999). Overall, our results demonstrate that both male and female axolotls can use odorant cues to discriminate the sex of conspecifics, but do not indicate whether either males or females can distinguish the reproductive status of opposite-sex conspecifics based on chemical cues.

The role of chemosensory systems in foraging behavior in aquatic salamanders has been a topic of little research, although chemical cues have been shown to play a role in prey-catching in a terrestrial species (David and Jaeger, 1981; Lindquist and Bachmann, 1982; Placyk and Graves, 2002). Axolotls have well-developed lateral line systems that include both mechanosensory and electrosensory organs (Münz et al., 1984). Given that salamanders eat live prey, the lateral lines presumably play a major role in helping axolotls locate and capture prey. Nevertheless, axolotls raised in the laboratory on a diet of food pellets appear to use chemosensory cues to locate pellets on the substrate. When searching for food pellets, axolotls engage in a stereotypical behavior in which the snout is held at a sharp angle to the substrate, presumably to bring the dorsally-located nostrils
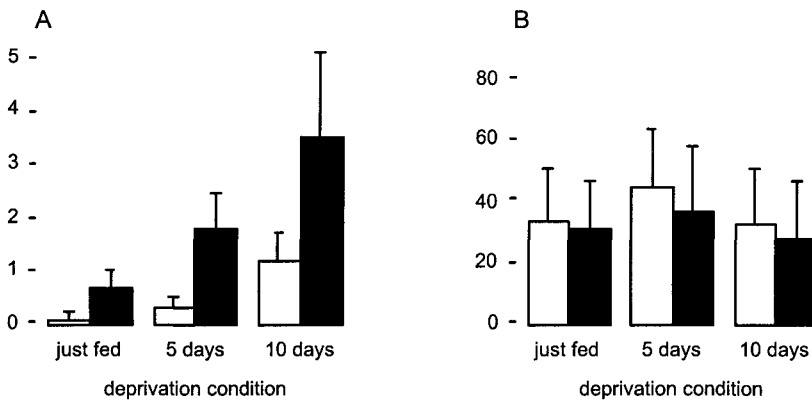

Figure 3. Chemosensory cues play a role in foraging in axolotls. Ten adult axolotls of both sexes were tested immediately after feeding, after 5 days, and after 10 days of food deprivation, in an aquarium containing clean Holtfreter's solution, the standard medium for maintaining axolotls (white bars), or in Holtfreter's solution containing food odors (black bars). Prior to the first test, each subject was fed commercial salmon pellets (Rangen Inc., Buhl, ID) *ad lib*. Thirty to ninety min later, the subject was placed in clean 10-gal aquarium containing Holtfreter's solution and its activity recorded. Each animal was tested again after 5 and 10 days, and then retested later using the same procedure, except that 250 ml food suspension (3 g food pellets mixed into 250 ml dH$_2$O and filtered) was added to the aquarium. (A) The frequency of searching the substrate for food pellets increased in the presence of food odors (two-way repeated measures ANOVA, $F_{1,54} = 5.91$, $P = 0.018$), and with food deprivation ($F_{2,54} = 3.61$, $P = 0.034$). No interaction between these factors was observed ($F_{2,54} = 0.66$, $P = 0.52$). Searching was defined as holding the snout such that the angle between the lower jaw and aquarium floor was more than 30° for at least 5 sec. (B) Overall activity, measured as the number of times a line down the middle of the aquarium was crossed, was not affected by the presence of food odors ($F_{1,54} = 0.11$, $P = 0.75$) or by food deprivation. ($F_{2,54} = 0.20$, $P = 0.82$). Again, no interaction was observed ($F_{2,54} = 0.01$, $P = 0.99$).

closer to the substrate. As demonstrated in Figure 3, axolotls engage in this behavior more when food deprived than when recently fed, and more in the presence of the odorants from food pellets than in a clean aquarium. We plan to use similar odorants to examine the roles of the olfactory and vomeronasal systems in foraging in axolotls.
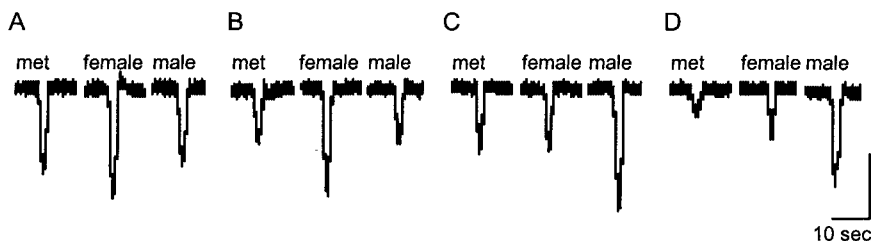
4. PHYSIOLOGY OF THE OLFACTORY AND VOMERONASAL EPITHELIA IN AXOLOTLS

Although tiger salamanders have long been used as a model for studies of odorant processing in the olfactory epithelium and bulb (e.g., Kauer and Moulton, 1974; Mackay-Sim and Kubie, 1981), few researchers have studied the physiology of the vomeronasal organ and accessory olfactory bulb in ambystomids. We have examined voltage-activated currents in olfactory and vomeronasal receptor neurons in axolotls, and find that the currents in olfactory receptor neurons are like those described in larval tiger salamanders (Firestein and Werblin, 1987). In general, vomeronasal receptor neurons have the same complement of currents, although, as in mice (Liman and Corey, 1996), the kinetics of the calcium current differ between olfactory and vomeronasal neurons. Specifically, vomeronasal neurons possess a rapidly-inactivating calcium current (Figure 4), but a sustained calcium current is found in olfactory receptor neurons. Thus, the vomeronasal receptor neurons in axolotls appear to be both morphologically and physiologically similar to those described in other vertebrates.

We have used electro-olfactogram (EOG) recording techniques to examine odorant responses in the olfactory and vomeronasal epithelia of adult axolotls (Park and Eisthen, 2003; Park et al., 2003, 2004). Overall, we have found the odorant responsiveness of the two epithelia to be remarkably similar: for example, both epithelia respond vigorously to amino acids, such as L-methionine (Figure 5). In conjunction with the study of behavioral responses to conspecific odorants described above, we also examined EOG responses to the same stimuli (Park et al., 2004). Because the magnitude of odorant responses varies across the sensory epithelium due to such factors as differential distribution of odorant receptors (Mackay-Sim et al., 1982), we recorded from eight sites on the olfactory epithelium and two on the vomeronasal epithelium. As shown in Figure 5, in both males and females, olfactory and vomeronasal EOG responses were larger in response to whole-body odorants from individuals of the opposite sex than from individuals of the same sex as the subject. In males, odorants from gravid and recently-spawned females elicited differential responses from one location each in the olfactory and vomeronasal epithelia, but no differences in response magnitude were recorded at the other eight locations examined. Among females, we found a curious effect of social experience on EOG responses: at some locations in the olfactory epithelium, EOG responses to odorants from both male and female conspecifics were larger in females that had no experience with adult males than in those that did. Responses in the vomeronasal organ also appeared to differ between experienced and inexperienced females, but our data set was small and this difference did not prove to be statistically significant. Overall, these results indicate that in axolotls both the olfactory and vomeronasal systems may be involved in discriminating the sex and reproductive condition of conspecifics, and that social or sexual experience alters responsiveness to some chemical cues from conspecifics.
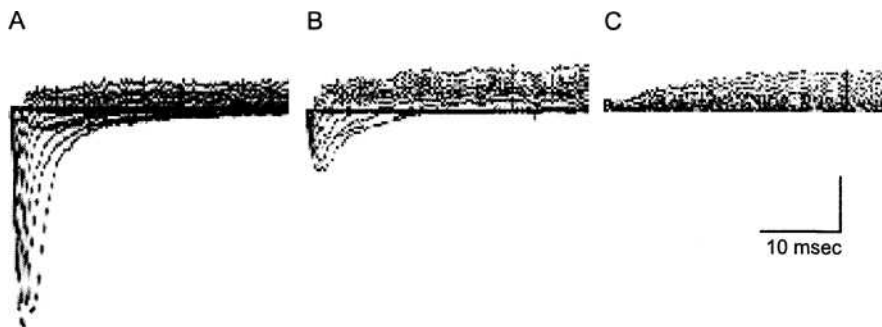
Figure 4. Whole-cell currents recorded from an axolotl vomeronasal receptor neuron in an epithelial slice. Currents were elicited by applying a series of voltage pulses, from -80 mV to +60 mV, in 10 mV increments. V_{hold} = -80 mV. (A) Currents recorded in standard amphibian physiological saline, before any channel blockers were washed onto the slice. (B) Currents recorded 3 min after 10 μM tetrodotoxin (TTX) was washed onto the cell. TTX blocks voltage-gated sodium currents, revealing a second inward current. (C) Currents recorded 9 min after a solution containing both 10 μM TTX and 1 mM $CoCl_2$ was washed onto the slice. Cobalt blocks calcium currents, demonstrating that the rapidly-inactivating inward current shown in panel B was carried by calcium.

Figure 5. Electro-olfactogram (EOG) responses recorded from the olfactory and vomeronasal epithelia in axolotls. Odorants consisted of 100 μl of 1 mM L-methionine (met), of water containing whole-body odorants from sexually-mature adult females (female), or of water containing whole-body odorants from size-matched, sexually-mature adult males (male). (A) Responses recorded from the olfactory epithelium of an adult male; (B) responses recorded from the vomeronasal organ of the same individual. (C) Responses recorded from the olfactory epithelium of an adult female; (D) responses recorded from the vomeronasal organ of the same female. Note that in all cases, responses elicited by odorants from opposite-sex individuals are larger than those elicited by odorants from same-sex individuals. Adapted from Park et al., 2004.

Centrifugal feedback has been shown to shape responses in the retina and inner ear in vertebrates (Akopian, 2000; Ashmore et al., 2000). Similarly, we have found that peptides, presumably released by the terminal nerve, modulate electrophysiological activity and odorant responses in the olfactory epithelium of axolotls (Park and Eisthen, 2003; Park et al., 2003). Given that the cells of the terminal nerve receive input from olfactory areas in the forebrain (Yamamoto and Ito, 2000), it seems possible that the terminal nerve functions to specifically enhance and suppress responding to various olfactory cues; for example, perhaps the terminal nerve increases responding to

conspecific chemical signals during courtship and mating behavior. A complete understanding of peripheral processing of chemical signals will have to include a consideration of the role of the terminal nerve in shaping responses to these signals.

5. CONCLUSIONS

We are studying the anatomy and physiology of the olfactory and vomeronasal systems in axolotls with the goal of determining the behavioral functions of these two chemosensory systems in aquatic amphibians. Our anatomical studies demonstrate that the vomeronasal epithelium of axolotls is much like that of other tetrapods. Other studies indicate that the projections from the olfactory and vomeronasal epithelia into the central nervous system are separate through several synapses, suggesting that these chemosensory systems serve different functions. Our electrophysiological experiments have not revealed striking differences in odorant responsivity between the olfactory and vomeronasal epithelia, but we are just beginning to work in this area and cannot draw yet strong conclusions about the relative quality or strength of odorant responses in these sensory epithelia. We have begun to show that odorant cues play a role in both foraging and in social behavior in axolotls; we hope that by combining neurobiological and behavior studies, we will be able to fully understand the ways in which chemosensory stimuli are processed to mediate behavior in axolotls.

Given that separate olfactory and vomeronasal systems are present in amphibians and in amniotes, the vomeronasal system must have been present in the last common ancestor of these two groups, and this animal is now thought to have been fully aquatic (Panchen, 1991; Lebedev and Coates, 1995). An understanding of the function of the vomeronasal system in aquatic amphibians may help shed light on the factors that led to the evolutionary origin of the vomeronasal system.

6. ACKNOWLEDGMENTS

7. REFERENCES

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