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Taste Disks are Induced in the Lingual Epithelium of Salamanders during Metamorphosis

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Abstract

Morphological changes of oral cavity during metamorphosis with special reference to the taste organ were examined in Ezo salamanders (*Hynobius retardatus*) and axolotls (*Ambystoma mexicanum*), and compared with those in bullfrogs (*Rana catesbeiana*). The non-distensible tongue of salamanders changed the structure progressively during metamorphosis: a small area of the rostrum protruded and developed caudally with recession of the flat area of the tongue. The protrusion that developed on the tongue had numerous papillae, as seen in the frog tongue. The apical region of the papillae occasionally had a cell mass similar to the taste disk of frogs (termed a taste disk-like cell mass). On the flat area of the tongue, the barrel-shaped taste buds of larval salamanders were transformed into taste buds with a wider receptor area. The barrel-shaped taste buds decreased progressively during metamorphosis, while taste disk-like cell masses increased. Neuronal labeling with an antibody to neuron-specific enolase and fluorescent carbocyanine dye showed that the taste disk-like cell masses in metamorphosed salamanders were innervated by the glossopharyngeal nerve (nerve IX). Nerve IX responded to taste stimulation as well as mechanical stimulation applied to the rostral tongue. During metamorphosis the salamanders undergo transformation and rearrangement of taste organs on the tongue possibly as an adaptation to the terrestrial environment. **Chem. Senses 22: 535–545, 1997.**

Introduction

Taste buds are the end organ of taste in vertebrates. They are onion-shaped clusters of 50–100 neuroepithelial cells which include chemosensory cells. Sensory cells and some intragemmal cells in the taste bud have direct access to the external milieu through only a limited area of the taste bud, which is called the *receptor area* (Reutter and Witt, 1993). These features occur throughout the classes of vertebrates except for frogs (anurans). Adult frogs have peculiar taste organs, consisting of a flat array of cells with a wider receptor area. These structures are called taste disks (De Han and Graziadei, 1971). The taste disks are embedded in the fungiform papillae, which cause a velvety appearance to the lingual surface. On the other hand, juvenile anurans—tadpoles—do not have a taste disk but instead have ovoid taste buds which are encased in small processes termed premetamorphic papillae (Helff and Mellicker, 1941). The ovoid taste buds in the premetamorphic papillae resemble the taste buds of fish and larval salamanders (urodeles). During metamorphosis they degenerate and seem to be replaced by taste disks (Nomura *et al.*, 1979; Shiba *et al.*,

1980; Zuwala and Jakubowski, 1991). Such comparison of the taste organ among species of amphibians leads us to speculate whether the urodele, if metamorphosed, would develop taste disks to adapt from an aquatic to a terrestrial environment. The structure of the taste bud has been extensively studied by light and electron microscopy, particularly in two species of aquatic salamanders: the mudpuppy (Farbman and Yonkers, 1971; Cummings *et al.*, 1987; Delay and Roper, 1988) and the axolotl (Fährmann, 1967; Toyoshima *et al.*, 1987; Nagai, 1993). Nonetheless, it is not known whether the urodeles are capable of developing taste disks in the oral cavity, because the salamanders used for those structural studies are neotenic and hence do not undergo metamorphosis. In the present study morphological changes in the oral cavity of urodeles during metamorphosis were studied in Ezo salamanders (*Hynobius retardatus*), which had metamorphosed under natural conditions, and axolotls (*Ambystoma mexicanum*), which were artificially metamorphosed by administration of thyroxine.

We found that during metamorphosis, the oral cavity of salamanders was transformed and developed numerous taste buds that were not seen in larval salamanders. These new taste organs resembled the taste disk of frogs. The presence of a nerve supply in the new types of taste organ was examined by using antisera to neuron-specific enolase (NSE), which is a specific marker for neuronal tissues. Furthermore, carbocyanine fluorescent dye, 1,1′-dioctadecyl-3,3,3′-tetramethylindocarbocyanine perchlorate (diI), was used to label the nerve and target cells, if any, for innervation (Finger and Böttger, 1990; Nagai, 1993).

Some of this work has been reported in abstract form (Ido *et al.*, 1995; Takeuchi *et al.*, 1997).

Materials and methods

Animals

Embryos of the Ezo salamander (*Hynobius retardatus*) were collected in the south-east of Hokkaido (Japan) in the early spring and raised in our laboratory (Shizuoka University) with a light–dark cycle of 15:9 h at room temperature. They were fed tubifex worms *ad libitum*. The larvae metamorphosed in 6–7 months after hatching, by which time they had grown to 6.0–7.6 cm in body length and 1.5–1.9 g in body wt. Developmental stages of the larvae were determined according to Iwasawa and Yamashita (1991).

Larvae of the axolotl, *Ambystoma mexicanum*, of the 'wild' and 'eyeless' strains were obtained from the Indiana University Axolotl Colony and maintained in our laboratory. They were fed tubifex worms and dog food pellets *ad libitum* and raised until ~1 year of age. At this stage the axolotls had grown to ~15 cm in body length.

Neotenic axolotls were artificially metamorphosed by treatment with thyroid hormone. To this end the raised larvae were grouped together in groups of four and each group was housed in a polypropylene container (25 × 38 × 14 cm) with rearing solution (50% Holtfreter's saline diluted 10-fold with tap water without chloride) (Asashima *et al.*, 1989), in which thyroxine (T4) was dissolved to give a concentration of 0.1 mg/l. We used this bath application method instead of i.p. injection of T4 (Kühn and Jacobs, 1989) because the injection often induced incomplete metamorphosis and many larvae died during metamorphosis. The solution was replaced every other day during T4 treatment. Metamorphosis was complete in 24–52 days, which was defined as the time when the axolotl's gill slits closed completely. A group of axolotls in the process of metamorphosis were killed at 12–26 days after the start of T4 treatment. During the metamorphosis, the container was kept at 20°C and the level of rearing solution was lowered gradually to facilitate breathing through the nostrils.

Tadpoles of the bullfrog (*Rana catesbeiana*) were purchased from a commercial source and raised at room temperature in our laboratory. The morphological changes of their taste organs were compared with those of salamanders. Their developmental stages were determined according to Taylor and Kollros (1946).

Tissue processing

Animals at different developmental stages were anesthetized in 0.2% MS222 (tricaine methanesulfonate; Sankyo, Japan) for 20–30 min and were perfused transcardially with a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Instead of the paraformaldehyde fixative Zamboni's fixative (4% paraformaldehyde, 0.2% picric acid, 0.65% NaCl in 0.1 M PB, pH 7.4) was used for immunohistological study with NSE.

To study the morphology of the oral cavity, epithelial tissues of the oral floor were dissected out from fixed specimens and dehydrated. To quantify the number of taste organs, serial sections (20 μm) of the tissue embedded in

paraffin were cut in 21 Ezo salamanders and all the sections were examined to count the taste organs. In both Ezo salamanders and axolotls some tissues were embedded in plastic (Technovit 7100, Kulzer & Co. GmbH, Germany) and semi-thin sections (2–3 μm) were cut. Sections were stained with cresyl violet for light microscopic examination. The receptor area of the axolotl taste buds was quantified on selected semi-thin sections that were cut longitudinally through the middle of the area in five taste buds. The area was measured by a tablet-operative video micrometer (Olympus, VM-30). Student's *t*-test was used for statistical analysis.

For the immunocytological study, epithelial tissues of the oral cavity were dissected out from perfused animals and fixed in Zamboni's fixative for 6 h at 4°C. The tissues were then immersed overnight in PB (pH 7.4) containing 15% sucrose at 4°C and embedded in gelatin. Sections 25 μm thick were cut on a sliding microtome (Jung type, Yamato Koki, Japan) with a freezing unit (model MA-102, Komatsu-Solidate Co. Ltd, Japan). Immunostaining was performed on free floating sections using the rabbit polyclonal antiserum against rat NSE (#16625, Polysciences, Inc., USA). Following incubation with the antibody (dilution 1:12 000) for 24–48 h at 4°C, the sections were processed using a streptavidin–biotin–peroxidase kit (Histofine SAB-PO-R, Nichirei, Japan). Labeling was visualized using diaminobenzidine (DAB) as the chromogen and observed by light microscopy with differential interference optics.

The fluorescent carbocyanine dye dil (D-282, Molecular Probes, USA) was used to study not only the presence of nerve fibers in the taste organs but also their synaptic contact with a particular cranial nerve. The dye translocates in axons by lateral diffusion along the cell membrane and labels certain taste bud cells trans-synaptically (Finger and Böttger, 1990; Nagai, 1993). A post-trematic branch of the glossopharyngeal nerve (Nagai and Matsushima, 1990) was cut close to the ganglion. The oral floor and the nerve *in toto* were dissected out from fixed salamanders. The method of application of the dil to the nerve is described elsewhere (Nagai, 1993). After incubating the tissue in a 37°C oven for 2–3 months, the tissue labeled with dil was subjected to examination either in whole mounts or in sectioned tissues. Sections of 50–100 μm thickness were cut on a Vibratome (PL1000, Technical Products International Inc., USA) or a sliding microtome. Specimens were first examined by means of a fluorescence microscope (BX-FLA, Olympus, Japan) equipped with the rhodamine filters. Some selected specimens were further studied with a confocal laser scanning microscope (LSM 410, Zeiss, Germany).

Electrophysiological recordings

Neural activity of the glossopharyngeal nerve was recorded in metamorphosed axolotls when chemical and mechanical stimuli were applied to the rostral part of the oral floor. Methods of neural recordings and stimulation are described in detail elsewhere (Takeuchi *et al.*, 1994). Briefly, in anesthetized animals the peripheral end of the glossopharyngeal nerve was exposed in the caudal end of external mandibular levator and hooked on bipolar platinum wire electrodes. The overall activity of the nerve was differentially amplified and recorded on a thermal array recorder RTA-1200M (Nihon Kohden, Tokyo, Japan). Taste stimuli and distilled water rinses were alternatively presented to the rostral part of the oral floor through a peristaltic pump with a flow rate of 10–12 ml/min. Stimulus duration was 20 s and was separated by 100 s water rinses. The chemical stimuli were distilled water solutions of reagent grade NaCl, KCl, $CaCl_2$, quinine hydrochloride (quinine) and citric acid. For mechanical stimulation, the oral floor was gently touched with a plastic probe (1 mm diameter).

Results

Morphological changes of the oral floor

In larval Ezo salamanders and axolotls, the oral floor is flat and covered with lingual epithelium on which numerous taste buds are seen. Therefore, the epithelium is termed a larva-type tongue, although it does not extend like a mammalian tongue (Figure 1A). Ezo salamanders started metamorphosis (i.e. reduction of the gills and fins) at developmental stage 64 at the earliest or at a couple of stages later, and completed the metamorphosis at stage 68. Because of variation among animals in starting metamorphosis, the morphological change of the oral floor is explained in the following four phases instead of using developmental stages. In the first phase (Figure 1A) a small area of the oral floor between the mandibular arch and the rostrum of larva-type tongue protruded. In the next phase the protrusion pushed up the rostrum of the tongue and merged in the tongue (Figure 1B). In the following two phases, the protrusion expanded caudally with recession of the flat oral floor (Figure 1C,D). Caudal to the emerged

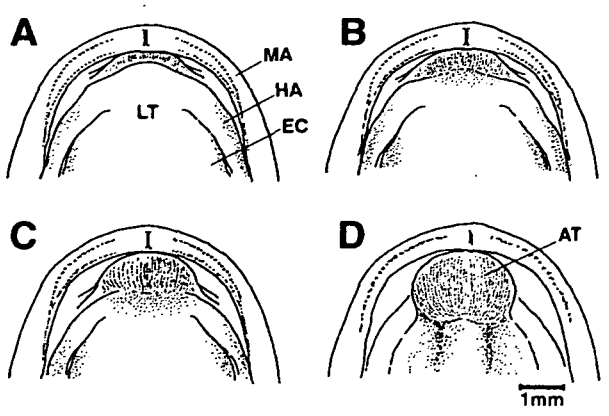

Figure 1 Drawings of the oral floor of Ezo salamanders during metamorphosis. **(A)** Phase 1. The protrusion of the oral floor emerges between the mandibular arch (MA) and the rostrum of a larva-type tongue (LT) supported by the hyoid arch (HA) and the first epibranchial cartilage (EC). **(B)** Phase 2. The protrusion develops. **(C)** Phase 3. The protrusion develops further and invades the larva-type tongue expanding to the caudal. **(D)** Phase 4. The protrusion, termed an adult-type tongue (AT), occupies a large area of the oral floor. Scale bar = 1 mm for A–D.

protrusion, the oral floor sank down, where the larva-type tongue remained. The emerged protrusion was a bulbous shape of ~2.5 mm in diameter. The bulbous protrusion was termed an adult-type tongue, although it did not develop to be distensible like a frog's tongue (see Figure 2). The adult-type tongue had rostrocaudally oriented grooves on the surface, which were covered with thick epithelium (Figure 1D, see also Figure 3B). In artificially metamorphosed axolotls, similar morphological changes of the oral floor occurred, but the bulbous protrusion was approximately five times larger than in Ezo salamanders.

Taste organs in the oral cavity

In premetamorphic Ezo salamanders and axolotls the taste buds were a barrel-shaped cluster of cells embedded in the thin lingual epithelium (Figures 3A and 4A). Metamorphosis transformed these taste buds into two morphologically distinct types. One type occurred in the adult-type tongue, while the other occurred in the larva-type tongue, which is caudal to the adult-type tongue.

The adult-type tongue had a thick epithelium with numerous papillary structures which occasionally encased a barrel-shaped taste bud on their apical end (Figures 3B,C and 4B,C). The receptor area of the adult taste bud was wider than that of the taste buds in the premetamorphic Ezo salamanders and axolotls (e.g. compare Figure 3A,C). In axolotls, the receptor area was significantly wider in postmetamorphic taste buds than in premetamorphic taste

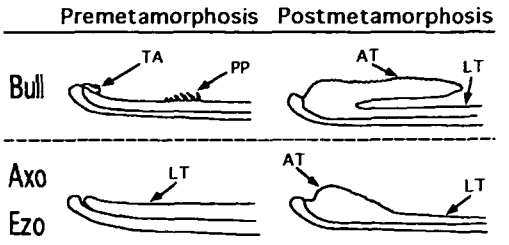

Figure 2 Schematic drawings of the oral floor of amphibians viewed in sagittal sections. The oral floors of bullfrog (Bull) and two species of the salamander (axolotl, Axo; Ezo salamander, Ezo) are depicted in the upper and lower panel respectively. A large mobile tongue develops in postmetamorphic bullfrogs, but not in the salamanders. TA, taste anlage; PP, premetamorphic papillae; AT, adult-type tongue; LT, larva-type tongue.

buds (37.9 μm versus 11.1 μm, $n = 5$, $P < 0.0001$ by two-tailed t-test). This may involve an increase in the cell size and number in the apical region of the taste bud, but the present light microscopic observation was not sufficient for quantitative measurement. The wide receptor area and the papillary structures encasing the taste bud are reminiscent of the taste disk and fungiform papillae of frogs (Figure 4G). Therefore, the taste bud in the adult-type tongue are termed a taste disk-like cell mass in the present study. Similarity of the taste disk-like cell mass to the frog taste disk was more evident in artificially metamorphosed axolotls than in naturally metamorphosed Ezo salamanders: in metamorphosed axolotls the papillary structure was surrounded by elevated ridges and deep troughs and the taste disk-like cell mass was composed of elongated cells (Figure 4C). On the other hand, the epithelium of the larva-type tongue in postmetamorphic Ezo salamanders and axolotls had no papillary structures. Cuboidal or barrel-shaped taste buds were embedded in the flat lingual epithelium, as seen in premetamorphic salamanders (Figures 3D and 4D). However, the receptor area of postmetamorphic taste buds was significantly wider than that of premetamorphic taste buds (in axolotls, 33.7 μm versus 11.2 μm, $n = 5$, $P < 0.0001$).

The number of taste organs

Was the taste disk-like cell mass transformed from the taste bud in the larval salamanders during metamorphosis or was it generated *de novo*? As a cue to answer this question, the number of taste organs in the oral floor was counted on the serial sections of the tissue from Ezo salamanders. The taste buds as well as the taste disk-like cell mass were counted on the oral floor from the rostrum to the level of the caudal end of the eyeballs. For chronological comparison of the taste

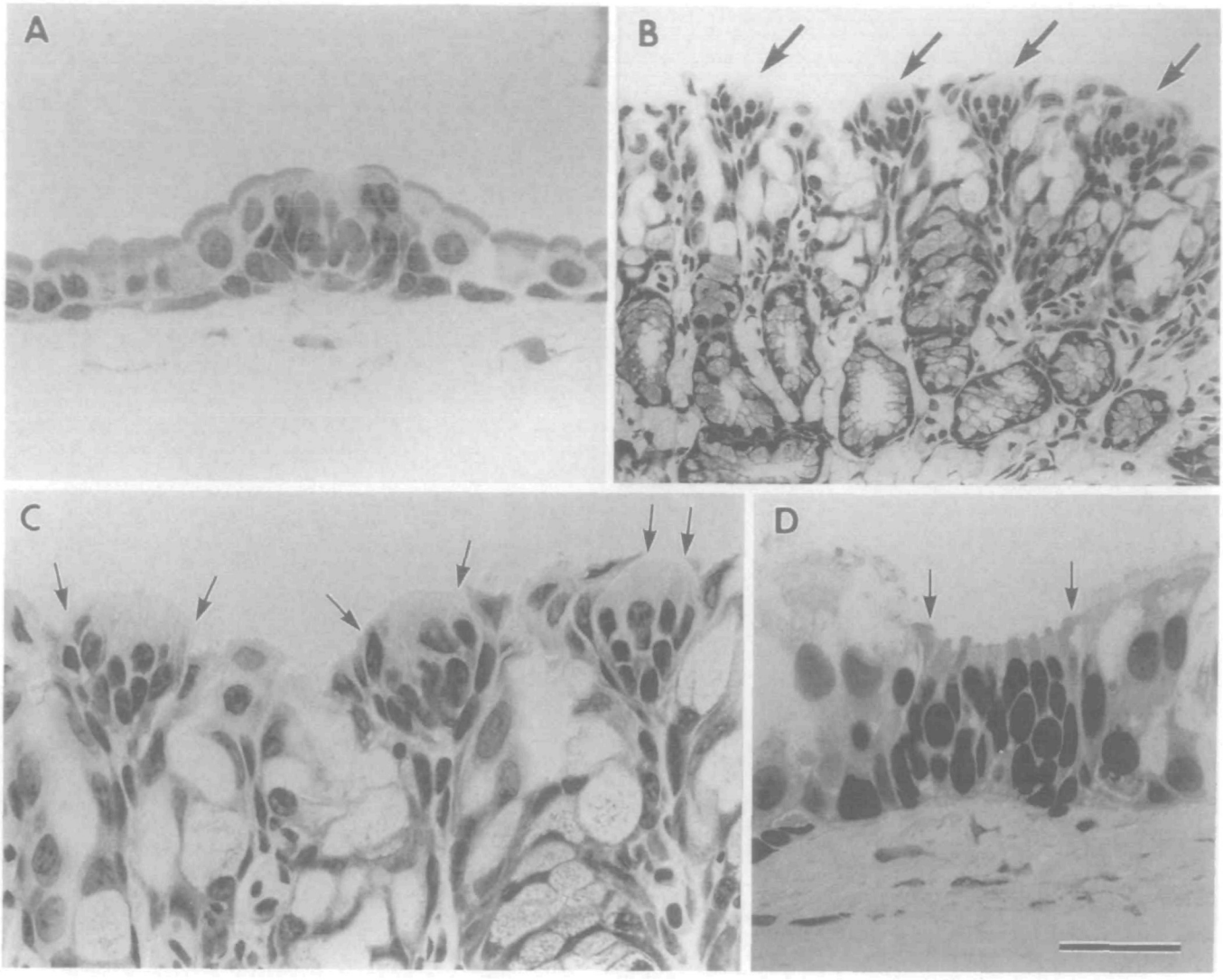

Figure 3 Cross sections of the lingual epithelium in Ezo salamanders in premetamorphic phase **(A)** and postmetamorphic phase **(B–D)**. The lingual epithelium as well as the taste buds undergo morphological changes. (A) Barrel-shaped taste buds are embedded in thin lingual epithelium. (B) Thick lingual epithelium of the adult-type tongue has papillary structures containing a taste disk-like cell mass (arrows). (C) The lingual epithelium shown in B is viewed at a higher magnification. The taste disk-like cell mass has a wide receptor area (thin arrows). (D) Cuboidal taste bud with a wide receptor area (thin arrows) in the larva-type tongue. Scale bar = 50 μm for A, C and D; 100 μm for B.

organ an additional two phases were defined: phase 0 (pre-metamorphic phase) is equivalent to developmental stage 63, which is just before the metamorphosis starts, and phase 5 (post-metamorphic phase) is equivalent to stage 68, which is right after the metamorphosis completed. The taste buds in the larva-type tongue progressively decreased during metamorphosis (Figure 5). This was due to not only reduction of the larva-type tongue in area but also decrease in density of the taste buds (25.3/mm^2, n = 4, in phase 0; 12.2/mm^2, n = 7, in phase 5), which occurred almost in parallel with reduction of the area (see Figure 2). On the other hand the taste disk-like cell mass increased during metamorphosis (Figure 5). In metamorphosed Ezo salamanders the sum of the taste disk-like cell masses and the taste buds exceeded the number of taste buds in pre-metamorphic phase (285 + 38 = 323 > 171; see the mean values in Figure 5). This may show that the taste disk-like cell masses were not simply transformed from the taste buds in the larva-type tongue, but that some of them were generated *de novo*.

Innervation of the taste organs

In metamorphosed salamanders the immunostaining by antisera to NSE revealed that fascicles of nerve fibers extended toward the taste disk-like cell mass, the basal area of which was strongly immunopositive (Figures 6A,B).

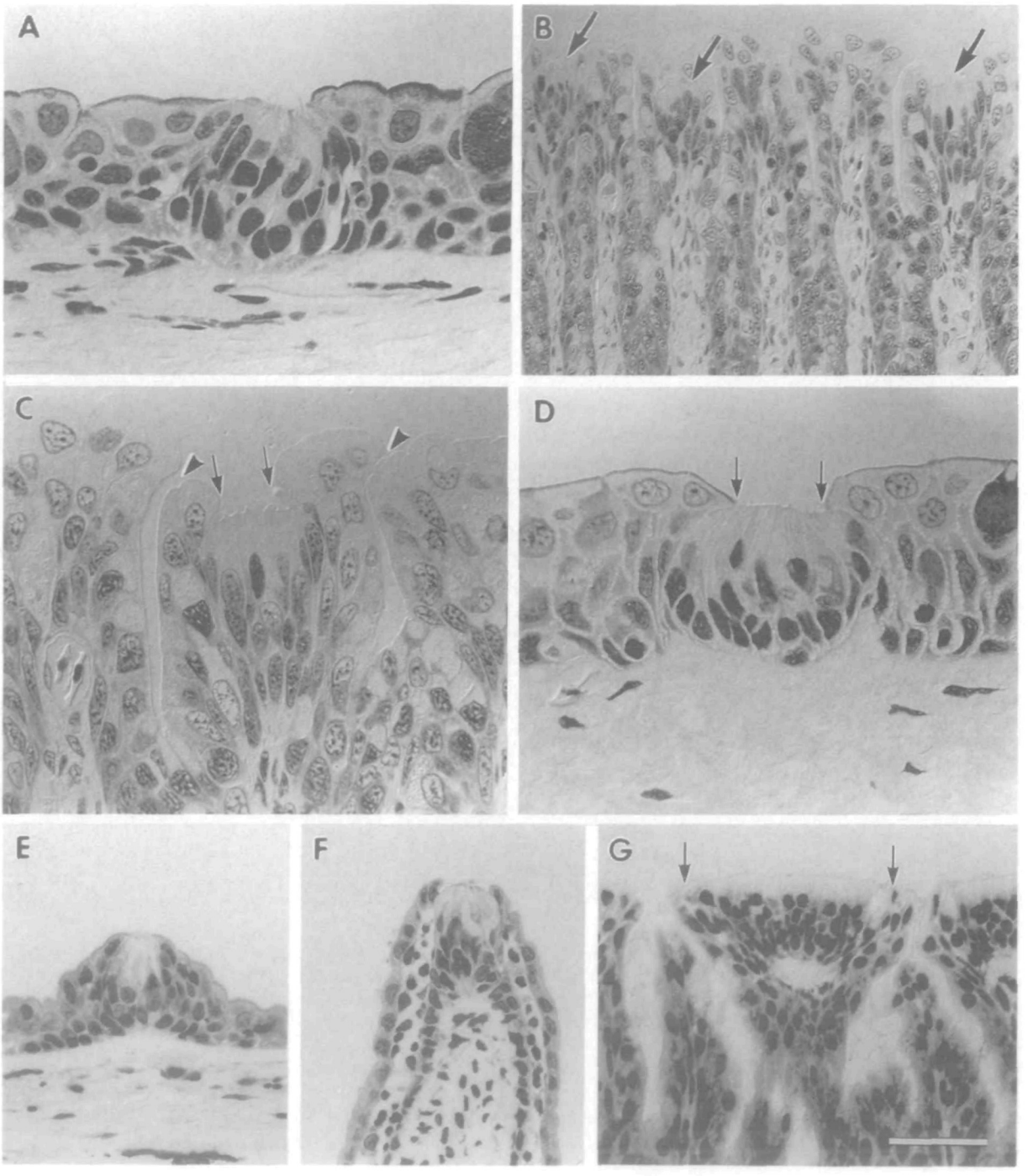

Figure 4 Cross sections of the lingual epithelium of axolotls and bullfrogs. **(A–D)** Axolotls in premetamorphic phase (A) and postmetmorphic phase (B–D). (A) Barrel-shaped taste bud embedded in thin lingual epithelium. (B) Thick lingual epithelium of the adult-type tongue has papillary structures encasing a taste disk-like cell mass (arrows). (C) A single taste disk-like cell mass shown in B is viewed at a higher magnification. The cell mass has a flat and wide receptor area (thin arrows). The papillary structure is surrounded by troughs (arrow heads). These morphological features resemble taste disks of frogs (see **G**). (D) Taste bud in the larva-type tongue is barrel-shaped as seen in the taste bud of premetamorphic axolotls, but with a wider receptor area (thin arrows). **(E–G)** Bullfrogs in tadpoles at stage 17 (E, F) and adult (G). (E) Taste bud in the flat oral floor. (F) Taste bud in a premetamorphic (finger-like) papilla. (G) Taste disk in adult bullfrog has a flat and wide receptor area (thin arrows). Scale bar = 50 μm for A, C–G; 100 μm for B.

However, it did not reveal which cranial nerve innervates the cell mass. Therefore, carbocyanine dye dil was applied to the glossopharyngeal nerve, which innervates a large area of the lingual epithelium of axolotls (Nagai, 1993). Numerous cell masses in the adult-type tongue in metamorphosed Ezo salamanders were strongly labeled with dil (Figure 7A). In

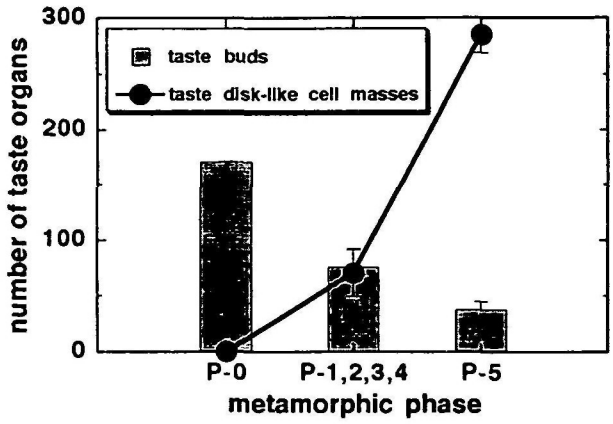

Figure 5 Graphs of the number of taste organs at different stages of development in Ezo salamanders. The taste buds (bar graph) and taste disk-like cell masses (line graph) were counted in a premetamorphic phase (P-0), metamorphic phases (P-1,2,3,4) and a postmetamorphic phase (P-5). With progress in the metamorphosis the taste buds decreased, while the taste disk-like cell masses increased. The values on the bar graph are the mean (+ SEM) calculated from 4–10 animals, while those on the line graph are the mean (+ SEM) calculated from 2–8 animals.

cross-sectioned tissues the nerve plexus was seen at the base of the cell mass (Figure 7B,C). In the cell mass an apical process of labeled cell extended to the receptor area, which is characteristic of the taste receptor cell (Figure 7C).

The glossopharyngeal nerve responses in metamorphosed axolotls

The glossopharyngeal nerve (nerve IX) of metamorphosed axolotls responded to solutions of salts, acid and quinine, when the solutions were applied to the adult-type tongue (Figure 8). The nerve also responded vigorously when the chemosensitive area on the tongue was touched gently with a probe. This electrophysiological experiment suggests that the IX nerve innervates taste receptor cells in the taste disk-like cell mass and other cellular substrates involved in mechano-transduction.

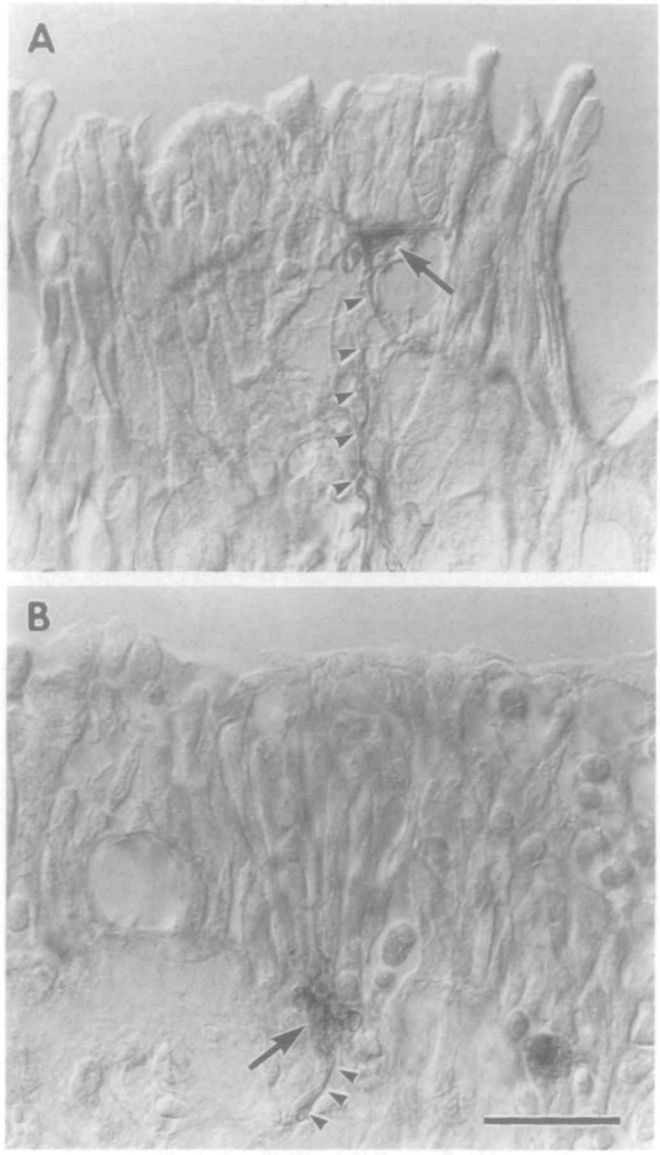

Figure 6 Immunocytochemical demonstration of neuron-specific enolase (NSE) in the taste disk-like cell mass in metamorphosed salamanders. **(A)** Ezo salamander; **(B)** axolotl. The basal region (arrow) of the taste disk-like cell mass is strongly immunopositive. Fascicles of fibers (arrow heads) extend upward to the epithelium to innervate the cell mass. Scale bar = 50 μm for A and B.

Discussion

Taste buds of ovoid shape seem to occur throughout all classes of vertebrates. A single exception would be frogs: they have flat and disk-like taste organs, which are called taste disks. However, the present study showed that disk-like taste organs occur also in the urodeles, when metamorphosed. The development of amphibian taste organs was studied in several species of anurans (Helff and Mellicker, 1941; Nomura *et al.*, 1979; Shiba *et al.*, 1979; Shiba *et al.*, 1980; Zuwala and Jakubowski, 1991), while the urodele taste buds during metamorphosis were systematically studied, to our knowledge, for the first time in the present study.

Morphological changes of taste organs during metamorphosis in amphibians are summarized in the drawing represented in Figure 9. In larval Ezo salamanders and axolotls the taste organ is a barrel-shaped taste bud with a narrow receptor area, which is embedded in the flat lingual epithelium. The taste organ in tadpoles is noted as the taste

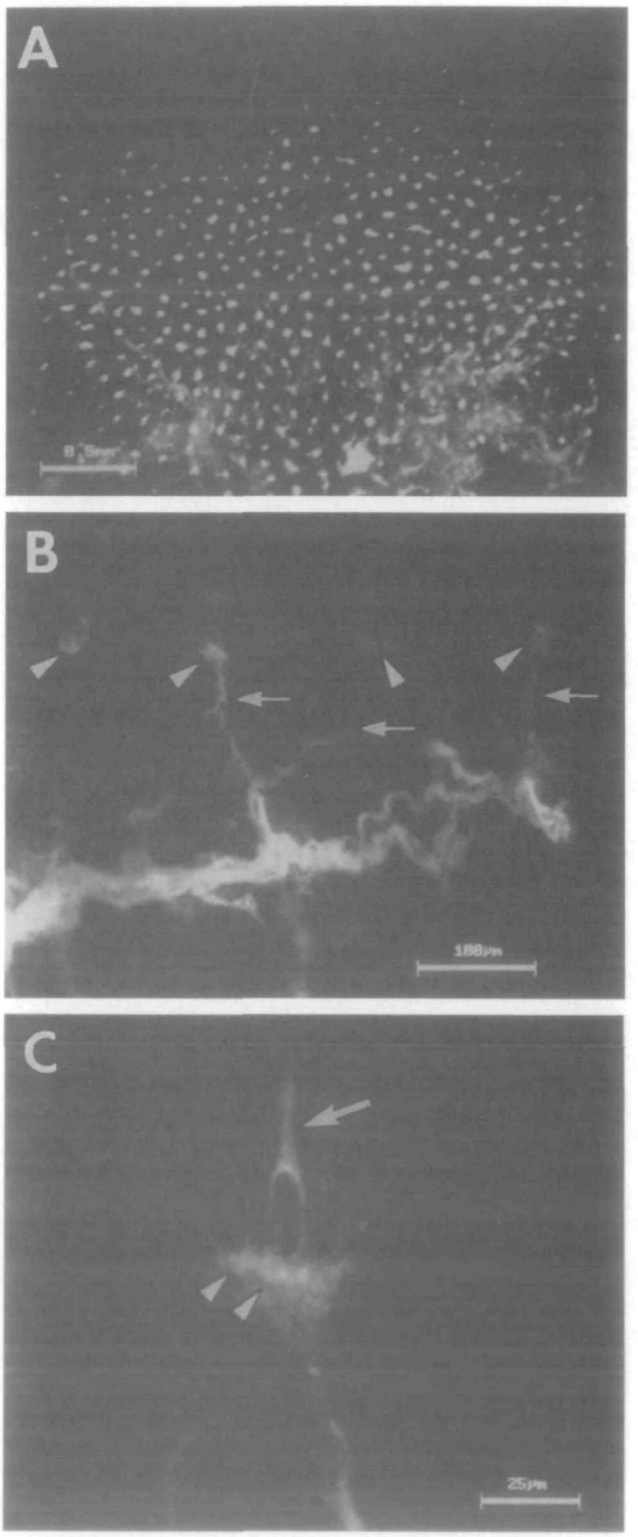

Figure 7 Confocal laser scanning micrographs showing innervation of taste disk-like cell masses in the adult-type tongue of postmetamorphic Ezo salamander. **(A)** Photomicrograph of the dorsal surface of the adult-type tongue, viewed by a low-power objective in a wholemounted preparation. A large portion of the tongue is shown. Compare with Figure 1D to visualize the contour of the protruded tongue. On the photomicrograph, the rostrum of tongue is oriented on the top. The taste disk-like cell masses with bright fluorescence were counted on several optical slices and amounted to ~480 in an area of 8 mm^2. Scale bar = 500 μm. **(B)** Cross section of the tongue. Fascicles of nerve fibers (arrows) extend toward the taste disk-like cell masses embedded in the epithelial cell layer. The basal region of the cell masses (arrow heads) are brightly fluorescent. Scale bar = 100 μm. **(C)** Cross section of a single taste disk-like cell mass. Note that a taste receptor cell with an apical process (arrow) is brightly fluorescent with dil transneuronally diffused through the basal plexus (arrow heads) of nerve fibers. Scale bar = 25 μm.

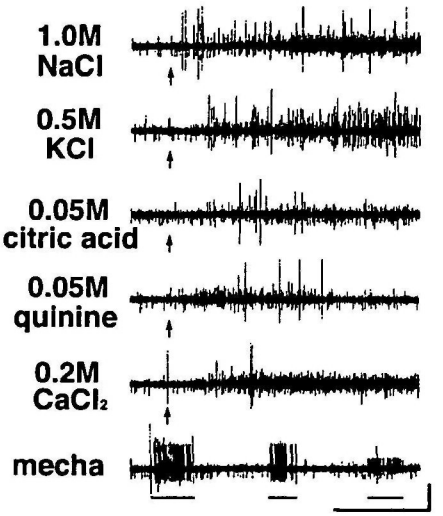

Figure 8 Glossopharyngeal nerve responses of the metamorphosed axolotl to chemical (1.0 M NaCl, 0.5 M KCl, 0.05 M citric acid, 0.05 M quinine and 0.2 M $CaCl_2$) and mechanical stimulation. Arrows show the onset of chemical stimulation. Horizontal bars show the time when mechanical stimulation was applied to three different locations (the rostral end, the top and the caudal end of the protrusion; see Figure 2) on the surface of adult-type tongue. Since neural responses were recorded from whole nerve, action potentials of a different amplitude are shown in these records. Scale bar = 5 s (horizontal) and 100 μV (vertical).

bud encased in a premetamorphic papilla (Helff and Mellicker, 1941) (see Figure 4F). The tadpole also has numerous taste buds in the flat oral floor (Figure 4E); and these taste buds are barrel-shaped. The barrel-like shape of the taste organ is, to some extent, similar to the taste buds in fish (Reutter, 1978, 1993) and therefore seem to be common in aquatic animals. Metamorphosed amphibians develop the papillary structures on the rostral part of the tongue (adult-type tongue), which encase the taste disk-like cell mass with a wide receptor area. Such papillary structures seem to be generated to adapt to terrestrial life, because the South African clawed toads (*Xenopus laevis*), which remain aquatic even after metamorphosed, do not develop the papillary structures but have barrel-shaped taste buds in the

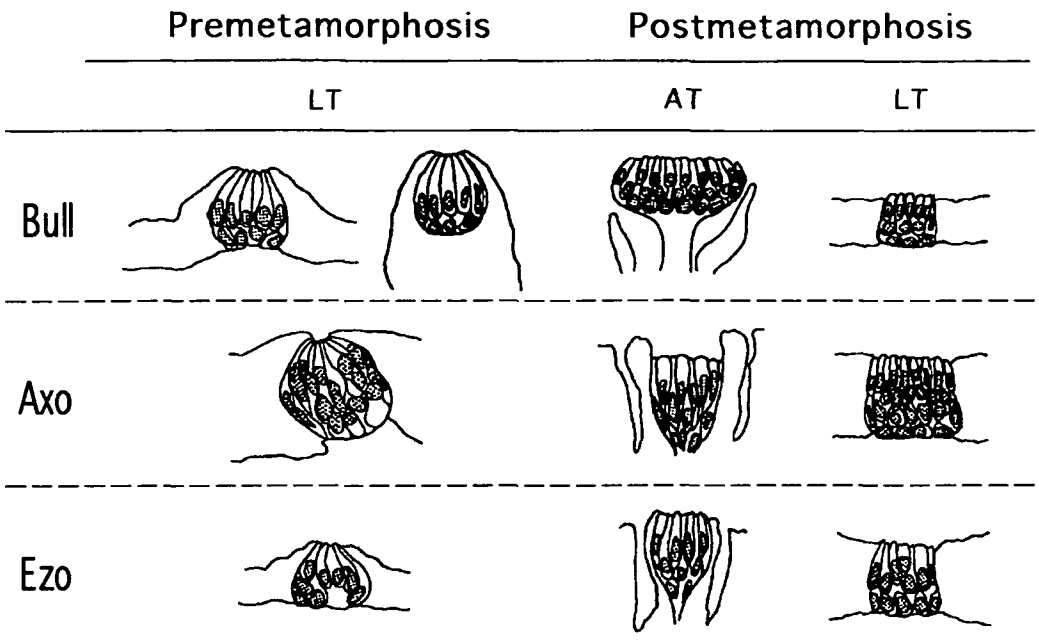

Figure 9 Schematic drawings of morphology of the taste organs in amphibians. The taste organs in the oral floor are shown in pre- and postmetamorphic phases of bullfrogs (Bull), axolotls (Axo) and Ezo salamanders (Ezo). LT, larva-type tongue; AT, adult-type tongue. See text for details.

undulated oral floor (Shiba *et al.*, 1979). However, some salamanders, even though terrestrial, may not develop the papillary structure. Early study on the development of taste organs in salamanders (*Ambystoma maculatum*) by Stone (1940) showed that the taste buds that had been in the adult stage for 2 months were barrel-shaped and had a wider receptor area. No description of the papillary structures is found in the literature, but there is a brief description: 'as the salamander approach metamorphosis, the lingual epithelium begins to thicken and the taste buds become larger and flatter'. Therefore, not the formation of papillary structures but a wider receptor area of the taste organ may be universal among terrestrial amphibians.

In the present study we examined the glossopharyngeal innervation of the taste disk-like cell masses only in post-metamorphic phase. The cell masses in early metamorphic phase such as phase 2 (see Figures 1B and 5) may not be innervated by the IX nerve but the facial nerve (VII), because Samanen and Bernard (1981) suggested that the VII nerve in the mudpuppy (*Necturus maculosus*) innervates the distal tongue, where the taste disk-like cell masses start to develop in Ezo salamanders and axolotls during metamorphosis. Another possibility is that the cell masses developed without innervation at early phase; transplanting tongue tissue in salamanders showed that taste buds develop long before the gustatory nerves develop (Stone, 1940), and raising the presumptive oropharyngeal region of axolotls in culture showed that taste buds are generated in the complete absence of neural elements (Barlow *et al.*, 1996). The sequence of innervation to those cell masses during metamorphosis is certainly worth studying in light of the neuronal control of taste bud development (Oakley, 1993).

The glossopharyngeal innervation of the taste disk-like cell masses was also shown by electrophysiological recordings (Figure 8). The present experiment was not developed to quantitative examination of taste responses, while in larval axolotls, salts are most effective among four basic taste stimuli (Takeuchi *et al.*, 1994). In mammals the taste responses of peripheral nerves, particularly the response to NaCl, are related to whether or not the animals inhabit in a dry, xerophytic environment (Hill and Mistretta, 1990). Therefore, it would be interesting to compare the IX nerve responses of larval and metamorphosed axolotls. Such a comparative study of chemosensitivity is now under way in our laboratory. The IX nerve of metamorphosed axolotls also responded to mechanical stimuli applied to the tongue, as was observed in larval axolotls (Takeuchi *et al.*, 1994). Merkel cells, known to be associated with the mechanosensory function of afferent nerves (Diamond, 1986), were seen in the lingual epithelium outside of the taste buds (non-taste epithelium) in larval axolotls (Nagai

and Koyama, 1994). In the metamorphosed axolotls the cells immunopositive to NSE were found in the non-taste epithelium (data not shown). These are possibly Merkel cells, because Merkel cells found in the frog taste organ are also immunopositive to NSE (Toyoshima and Shimamura, 1988). Therefore, Merkel cells in the non-taste epithelium may be associated with mechanosensitivity of the IX nerve in both larval and metamorphosed axolotls.

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