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Embryonic taste buds develop in the absence of innervation

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SUMMARY

It has been hypothesized that taste buds are induced by contact with developing cranial nerve fibers late in embryonic development, since descriptive studies indicate that during embryonic development taste cell differentiation occurs concomitantly with or slightly following the advent of innervation. However, experimental evidence delineating the role of innervation in taste bud development is sparse and equivocal. Using two complementary experimental approaches, we demonstrate that taste cells differentiate fully in the complete absence of innervation. When the presumptive oropharyngeal region was taken from a donor axolotl embryo, prior to its innervation and development of taste buds, and grafted ectopically on to the trunk of a host embryo, the graft developed well-differentiated taste buds. Although grafts were invaded by branches of local spinal nerves, these neurites were rarely found near ectopic taste cells. When the oropharyngeal region was raised in culture, numerous taste buds were generated in the complete absence of neural elements. Taste buds in grafts and in explants were identical to those found in situ both in terms of their morphology and their expression of calretinin and serotonin immunoreactivity. Our findings indicate that innervation is not necessary for complete differentiation of taste receptor cells. We propose that taste buds are either induced in response to signals from other tissues, such as the neural crest, or arise independently through intrinsic patterning of the local epithelium.

Key words: axolotl, taste bud, innervation, explantation

INTRODUCTION

A fundamental problem in the development of the peripheral nervous system is elucidation of the mechanism by which precise contacts are made between sensory ganglion neurons and their peripheral targets, receptor cells. Do arriving nerve fibers determine the positions of receptors or do receptor cells specify the pattern of innervation?

The taste system has long been used as a model for studying trophic interactions between sensory nerves and peripheral receptor organs (Vintschgau and Honigschmied, 1876; Landacre, 1907; Stone, 1940; Torrey, 1940; Wright, 1955, 1964; Fujimoto and Murray, 1970; Hosley et al., 1987b; Whitehead et al., 1987; Kinnman and Aldskogius, 1988). However, the majority of these studies have dealt with regeneration in adult animals, rather than the role of innervation during embryonic development. Taste receptors, which have recently been shown experimentally to arise directly from the local epithelium (Barlow and Northcutt, 1995; Stone et al., 1995), typically appear in the oral cavity and pharynx late in embryogenesis, contemporaneous with or slightly following the appearance of cranial nerve fibers in the oropharyngeal epithelium (Landacre, 1907; Farbman, 1965; Whitehead and Kachele, 1994). This coincidence of timing has led numerous researchers to suggest that nervous contact induces taste bud differentiation (e.g. Landacre, 1907; Torrey, 1940; Farbman, 1965; Munger, 1977; Hosley et al., 1987a,b). Implicit in this interpretation is the assumption that innervation pattern dictates the distribution of taste buds within the oropharyngeal epithelium.

In an early experimental test of the role of innervation in embryonic taste cell differentiation, Stone (1940) grafted the presumptive lower jaw region of an early salamander embryo, prior to contact by sensory nerves, to the trunk of a host embryo at a location remote from potential innervation by cranial nerves. These grafts developed as lower jaws with teeth, tongue and taste buds. However, the degree to which taste buds were innervated was not determined, leaving open the question of whether taste cells developed independently of innervation, or were induced by contact with inappropriate spinal nerves. More recently, Oakley and coworkers (Hosley et al., 1987a,b) denervated rat tongues during a critical period in postnatal development. Although a small percentage of taste buds did form despite the presumed absence of sensory neurites, the majority of taste buds failed to differentiate. These researchers concluded that taste bud differentiation required contact by nerves. This neural induction model was examined further in recent studies showing that tongue papillae, within which mammalian taste buds will develop, appear prior to contact of the papillae by nerve fibers (Farbman and Mbiene, 1991; Whitehead and Kachele, 1994). However, in these studies taste buds were found in papillae only after the arrival of sensory nerves, leading these authors to suggest that although the epithelium produced papillae without neuronal contact, taste bud formation was still nerve dependent.

In order to definitively test whether nervous contact is

necessary for induction of taste bud differentiation, we have employed both in vivo and in vitro approaches. We used embryos of an ambystomatid salamander, the axolotl, since they are extremely resilient to surgical manipulation, and amphibian tissue survives well in culture. Amphibian taste buds are easily recognizable due to their characteristic morphology and the presence of a class of serotonergic cells within each bud (Toyoshima and Shimamura, 1987; Kuramoto, 1988; Delay et al., 1993). Furthermore, we found that an antiserum directed against the calcium binding protein, calretinin, selectively labels a large number of cells within each taste bud, making calretinin immunoreactivity a reliable marker of taste cells. We report that taste buds develop normally when the presumptive oropharyngeal region is transplanted to an ectopic location on the flank of host embryos (as reported by Stone, 1940), and although spinal nerves invade ectopic grafts, ectopic taste cells appear to differentiate in the absence of nervous contact. When the oropharyngeal region is isolated prior to its innervation and raised in culture, taste buds differentiate both morphologically and molecularly in the complete absence of neuronal elements, and do so at densities comparable to those found in situ. We conclude that, contrary to the prevailing model of taste bud development, taste cell differentiation is independent of neuronal contact.

MATERIALS AND METHODS

Pigmented and albino axolotl embryos were obtained from the Indiana University Axolotl Colony, and were maintained in artificial fresh water (AFW: 50 mg/l $CaSO_4$, 5 mg/l $MgSO_4$, 4 mg/l KCl, 1 mg/l NaH_2PO_4) at 28°C. Embryos were staged according to the table of Bordzilovskaya et al. (1989).

Ectopic transplantation of the presumptive oropharyngeal region

Prior to removal from their egg capsules, embryos were placed in 0.2% formalin in AFW to kill fungus. Embryos were then rinsed in AFW, transferred to sterile 100% Holtfreter's solution (HS) with penicillin and streptomycin (400 μg/ml each), and removed from the egg jelly using fine forceps. Dejellied embryos were placed in grooves in plasticine-lined dishes filled with 100% HS. Surgeries were modified from Stone (1940). Briefly, the presumptive oropharyngeal region was removed from pigmented embryos with tungsten microneedles and transplanted ectopically to a region of the trunk of albino hosts from which the ectoderm had been removed (Fig. 1A). The presumptive oropharyngeal region comprises the ventral portion of the mandibular and hyoid arches immediately posterior to the developing forebrain, including the endoderm, ectoderm and intervening mesenchyme of this area. Transplantations were isochronic and were performed using embryos from stages 22 to 33, prior to invasion of the oropharyngeal region by cranial nerve fibers (Northcutt and Brändle, 1995). Experimental embryos were allowed to develop until 2-3 days posthatching (stage 43) and were anesthetized and fixed for immunofluorescence, normal histological, or SEM analysis.

Tissue culture of oropharyngeal explants

Stage 35 to 36 embryos were dejellied as described above, and anesthetized in sterile 100% MMR (modified Ringer's; Gimlich and Gerhart, 1984) with 0.04% ethyl 3-aminobenzoate methanesulfonate (MS-222, Sigma Chemical Co., St. Louis, MO). The oropharyngeal regions of anesthetized embryos were removed, transferred through 5-6 changes of sterile 60% L15 culture medium with 0.5% BSA, 1% Fungibact and 50 μg/ml gentamycin, and placed in individual 1 cm culture wells containing this medium without BSA (L15; Fig. 1B). We initially cultured explants in L15 with fetal calf serum and *Xenopus* embryo extract. However, we found that tissue and taste bud morphology were greatly improved in L15 alone, and subsequently used this medium exclusively. Explants were allowed to develop until intact control embryos had hatched (stage 41) or reached posthatching stage 43, approximately 6 and 9 days respectively at room temperature. At this time, explants were fixed for immunofluorescence, histology, or SEM.

Immunofluorescence

Hatchling larvae with ectopic grafts were anesthetized in MS-222 and both experimental larvae and explants were fixed overnight in 4% paraformaldehyde at 4°C. Tissue was then rinsed in 0.1 M phosphate buffer (PB) and cryoprotected in 20% sucrose in PB for 30 minutes to several hours, depending upon the size of the tissue. Frozen sections were cut at 25 μm for larvae and 20 μm for explants, and collected on warm gelatin-subbed slides. Sectioned material was processed for immunofluorescence using conventional methods. Primary antibodies were used at the following dilutions: rabbit anti-calretinin, 1:2000 (Swiss Antibodies, Bellinzona, Switzerland); rabbit anti-serotonin, 1:2000 (INC-Star, Stillwater, MN); mouse anti-acetylated alpha-tubulin (AT), 1:2000 (Sigma Chemical Co., St. Louis, MO); and mouse 3A10 (neurofilament associated antigen), 1:10 (Developmental Studies Hybridoma Bank, Iowa City, IA). Although both 3A10 and AT recognize sensory neuronal cell bodies as well as their neurites, in a double labeling experiment AT was found to label larger numbers of fine peripheral fibers than 3A10. Subsequently, the experimental tissue was processed with AT to detect the presence of neuronal elements. Immunoreactivity was detected directly with appropriate fluorescein- or rhodamine-conjugated secondary antibodies (1:200, Jackson ImmunoResearch Laboratories, West Grove, PA), or indirectly with a biotinylated secondary antibody (1:200, Vector Laboratories, Burlingame, CA) and fluorescein-conjugated streptavidin (1:100, Chemicon, Temecula, CA). Immunofluorescent images were viewed and collected using either a Spectrasource CCD camera, a Biorad MRC 600 or a Noran Odyssey confocal microscope, and were contrast enhanced and pseudocolored in Adobe Photoshop. In the figures shown here, we consistently use green for calretinin, red for acetylated tubulin and blue for serotonin immunostaining, regardless of the actual secondary antibody used.

Whole-mount immunocytochemistry

Embryos that had received oropharyngeal grafts were allowed to develop until stage 41 or 43, anesthetized as described above and fixed in Dent's fixative (80% methanol, 20% DMSO) overnight at room temperature. The tissue was then immersed in 3% H_2O_2 in Dent's fixative to block endogenous peroxidase activity. Subsequently, tissue was processed immunohistochemically using the acetylated tubulin antibody (as above), and the peroxidase anti-peroxidase method with diaminobenzadene as a chromagen. Reacted tissue was dehydrated, cleared in benzyl alcohol/benzyl benzoate and viewed using conventional bright-field microscopy.

Collection of serial section images and counting of taste buds

Continuous serial sections of three grafts and four explants immunostained with anti-calretinin were viewed using a Dage SIT-650 silicon intensified target camera, and stacks of images were digitized using NIH Image 1.57. Subsequently, images were analyzed to ascertain both the endodermal surface area and number of taste buds within each graft or explant. Endodermal epithelial cells possess numerous large yolk granules, which make these cells distinct from ectodermal epithelium. The contour of the luminal or endodermal surface of each

section was measured, and an estimate of the total surface area was obtained by multiplying the sum of the contours by the section thickness. The presence of taste buds was tallied during perusal of the images on the computer monitor.

Normal histology

Experimental larvae and explants were prepared according to the methods of Barlow and Northcutt (1995). Briefly, the tissue was fixed in 4% glutaraldehyde, rinsed, dehydrated through a graded ethanol series and embedded in methacrylate resin (Historesin, Leica, Heidelberg, Germany). Sections were cut at 8 μm, and counterstained with 0.1% cresyl violet.

Scanning electron microscopy

Larvae and explants were processed following the method of Northcutt and Brändle (1995). Animals and tissue were fixed in 4% glutaraldehyde, rinsed thoroughly in deionized water, dehydrated through a graded ethanol series, critical point dried in liquid CO_2, mounted on stubs, and gold-palladium sputter-coated.

RESULTS

Identification of endogenous taste buds

In axolotls, taste buds are identifiable as rosettes of fusiform cells located exclusively within the oropharyngeal epithelium (Northcutt et al., 1996). The characteristic onion shape of these sense organs, as well as the tuft of apical microvilli extruding through the taste pore, make them easily recognizable in situ (Fig. 2A). We have also found that antibodies directed against the calcium binding protein, calretinin, selectively bind a subset of primarily fusiform cells within each taste bud (Fig. 2B). Furthermore, a class of basal cells within the taste buds of salamanders contain serotonin (Delay et al., 1993) (Fig. 2C). Thus positive immunostaining within the oropharyngeal epithelium for either calretinin or serotonin is a reliable indicator of the presence of taste buds in axolotls. In addition, numerous elongate solitary cells are calretinin immunopositive, and may be mechanoreceptors (Nagai, 1993; Nagai and Koyama, 1994). This latter solitary cell type is readily distinguishable from calretinin-positive aggregates of taste cells (Figs 4B, 6A).

Axolotl taste buds are innervated by branches of the facial (VII), glossopharyngeal (IX) and vagal (X) cranial nerves (Northcutt et al., 1996). Synaptic contact of peripheral taste cells by these nerve fibers can be visualized using antisera against acetylated alpha-tubulin (AT). Typically, numerous nerve fibers ramify within the basal zone of each taste bud, and fine AT-positive fibers travel among taste cells toward the apex or pore region of each bud (Fig. 2B,C).

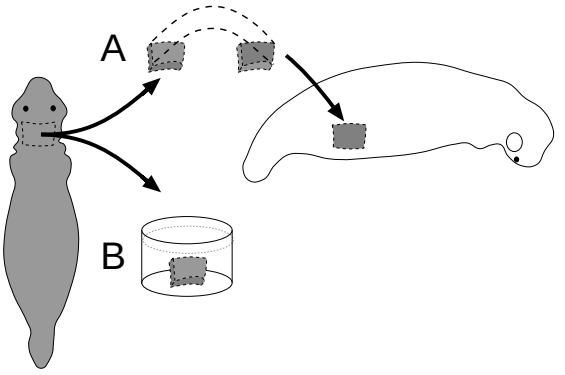

Fig. 1. Schematic diagram of experimental manipulations. (A) Transplantation of the oropharyngeal region. The ventromedial oropharynx was removed from a pigmented donor embryo between stages 22 and 35 and grafted, with the endoderm (stipple) facing outward, to a wound made in the flank of an equivalently staged albino embryo. Experimental animals were raised until early larval stages (stage 41-43). (B) Isolation of the oropharyngeal region in vitro. The ventromedial oropharynx was removed from stage 35 to 36 embryos and placed in sterile medium in 1 cm tissue culture wells until intact controls had reached stage 41 to 43.

Taste buds develop in grafts in the absence of appropriate innervation

To test whether taste bud development requires appropriate innervation, we performed heterotopic grafting experiments. The presumptive oropharyngeal endoderm, as well as underlying mesenchyme and ectoderm, were removed as a block of tissue from pigmented embryos, and grafted to wounds made in the flanks of albino host embryos of similar stages (after Stone, 1940; Figs 1, 3A,B). Taste cells are first evident within the oropharyngeal epithelium by stage 40, one day prior to hatching (Barlow and Northcutt, 1995). The cranial nerve fibers that will innervate taste buds typically reach the epithelium between stages 38 and 39 (Northcutt and Brändle, 1995), prior to the appearance of taste receptors. To disrupt the normal contact of taste cells by ingrowing fibers, transplantation was done between stages 22 and 33, several days before both the onset of innervation of the oropharyngeal region by cranial nerves and the differentiation of taste buds. Experimental embryos were allowed to develop beyond hatching (stage 41), at which time control animals are known to possess taste buds.

Typically, grafts underwent substantial morphogenesis such that the endodermal portion was overgrown by the ectoderm (Fig. 3A,B). Since taste buds in axolotls arise from oropharyngeal endoderm (Barlow and Northcutt, 1995), we predicted that taste buds would be evident within this internalized epithelium. Upon examination of transverse serial sections through ectopic grafts, abundant well-differentiated taste buds were encountered throughout the endodermal epithelium (Fig. 4) of 94% of experimental animals (n = 33, Table 1). Ectopic taste buds were identical to those found in situ, in that they were onion-shaped clusters of cells (Fig. 4C) comparable in size to those found in situ (compare with Fig. 2A), a subset of which were immunoreactive for calretinin (Fig. 4B,D) and

Table 1. Percentage of oropharyngeal grafts and explants with taste buds

	% Grafts/explants with taste buds	
Stage of development	in vivo	in vitro
Stage 41 in vivo, or 6-7 days in vitro	NA	100% (14)
Stage 43 in vivo, or 9-10 days in vitro	91% (34)	100% (19)

Note: Taste bud tallies are based on counts of aggregates of calretinin-immunopositive fusiform cells. Sample sizes are in parentheses.

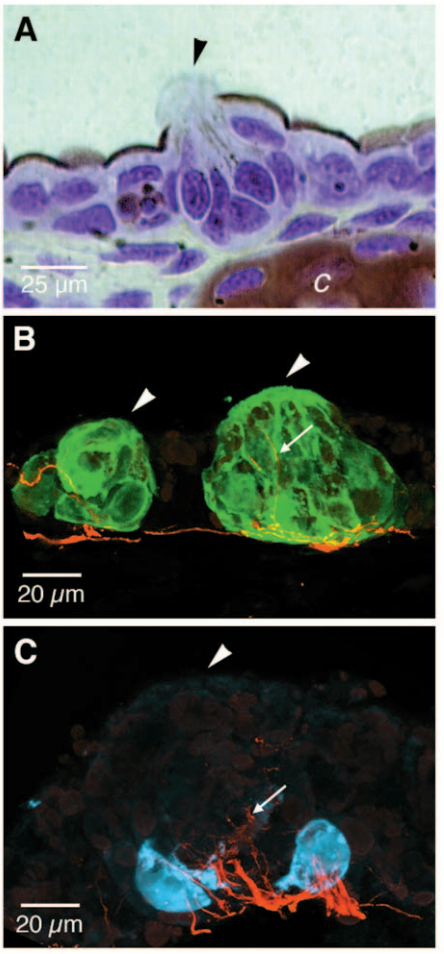

Fig. 2. Transverse sections of endogenous taste buds from stage 43 larvae. (A) A typical taste bud is shown located within the epithelium of the floor of the oropharyngeal cavity, immediately above a cartilaginous branchial arch (*c*), with apical microvilli extruding through the taste pore (arrowhead). (B) A projection of confocal optical sections through a frozen section processed with both anti-calretinin (green) and anti-AT (red) antisera. Numerous calretinin-immunoreactive taste cells are evident in two adjacent taste buds (arrowheads: taste pores). Many AT-positive fibers are shown invading each of the taste buds (red and yellow, small arrow), with the most dense AT immunostaining found at the base of each bud. (C) A projection of confocal sections through a frozen section processed for serotonin (blue) and AT (red) immunoreactivity. Serotonergic cells surround the base of the taste bud, although only two are evident in this plane of section. A chalice of AT-positive fibers forms around and within the base of the bud, with some fine fibers (small arrow) extending toward the apex (arrowhead).

serotonin (Fig. 4E). Numerous calretinin-positive solitary cells were also evident throughout the grafts (Fig. 4B,D).

The degree to which ectopic grafts were innervated varied among preparations. Some grafts were extensively innervated, whereas others received virtually no innervation. In some larvae processed in whole mount for AT immunocytochemistry, spinal nerves, which normally innervate the skin and muscle of the trunk, were seen invading the oropharyngeal graft (Fig. 5). When examined in cross section, the majority of immunoreactive fibers ramified primarily in the ectodermal epithelium of the graft (Fig. 4B), with only rare neurites detected in the endodermal epithelium lining the lumen of each graft. Occasionally, immunopositive neurites were seen near ectopic taste buds, however, spinal nerve fibers were not seen forming close contacts with taste buds, i.e., resembling those formed in situ by cranial nerves (Fig. 2B,C). Furthermore, when the lipophilic dye, DiI, was used to trace the projection patterns of spinal nerves within grafts, DiI labeled fibers were found throughout each graft, but did not label ectopic taste buds transsynaptically (data not shown), as has been demonstrated to occur in axolotls (Nagai, 1993; Northcutt et al., 1996). These findings suggest that although spinal nerve fibers invade ectopic oropharyngeal grafts and may be found near taste buds, ectopic taste buds are not actually innervated by spinal nerves. However, we could not eliminate the possibility that spinal neurites might transiently contact taste cell precursors, inducing taste bud differentiation without forming permanent contacts. To rule out this hypothesis, we raised oropharyngeal explants in the complete absence of innervation, using an in vitro protocol.

Taste buds develop in vitro in the total absence of innervation

To generate oropharyngeal explants devoid of neuronal elements, the donor region described in the grafting experiments was removed from stage 35 or 36 embryos, and placed in culture for 6-10 days until intact control embryos had reached stage 41 to 43. The explants healed rapidly, with the ectoderm partially overgrowing the endodermal region within 3 hours of surgery, such that the explants formed smooth rectangular blocks of tissue with one face clearly covered with endoderm. As the culture period progressed, the shape of the explants became increasingly triangular (Fig. 3C) as a result of elongation of the cartilaginous elements of the lower jaw, and a distinct endodermally lined lumen formed in each of the explants (Fig. 6A). Immunofluorescent examination of serial sections revealed the presence of numerous, well-differentiated taste buds in 100% of explants (n = 33; Table 1), of a size similar to those found in the oropharynx of intact larvae (compare with Fig. 2). Taste buds were present as early as day-6 to -7 postexplantation (14/14 explants). As in ectopic grafts, taste buds in explants displayed both calretinin and serotonin immunoreactivity (Fig. 6). In addition, in some older explants, the endodermal epithelium lining the central lumen was exposed, revealing the well-differentiated apical microvillar processes of the taste cells extruding through the taste pore (Fig. 3D). Calretinin immunopositive solitary cells were also evident throughout the endodermal epithelium of the explants (Fig. 6A). Occasionally, a small number of AT-immunopositive apical processes, distinct from immunoreactive nerve fibers, were encountered in cultured taste buds (Fig. 6A,B). Although we did not observe this staining characteristic in endogenous taste buds, the degree to which the apical AT staining was specific to cultured taste buds was not determined.

All explants developed taste buds, and the vast majority (85%; n = 33) were completely free of neuronal elements, as evidenced by the lack of AT or 3A10 immunostaining. Only five explants from one experiment possessed small aggregates of AT-positive cell bodies and neurites, and in these explants

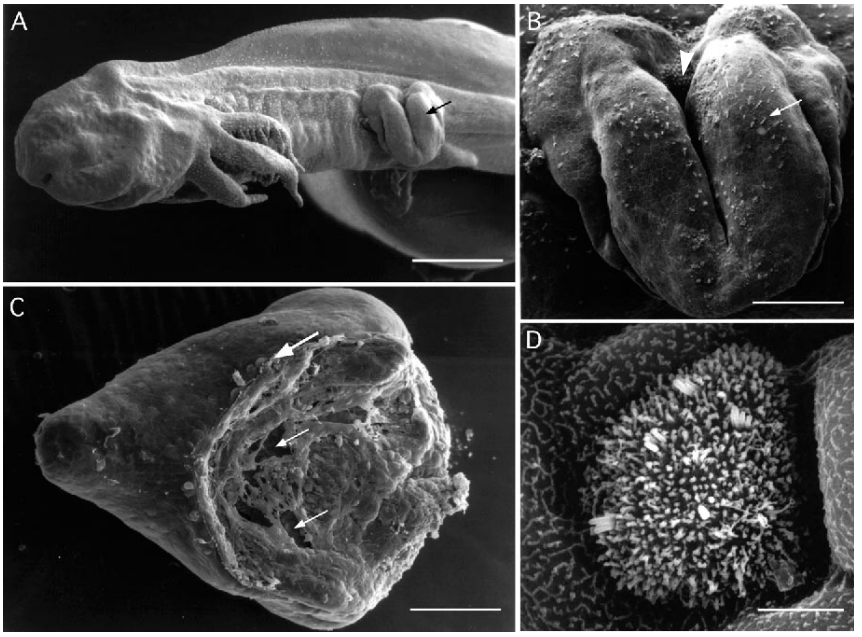

Fig. 3. Scanning electron micrographs of a larva with an oropharyngeal graft (A,B), an oropharyngeal explant (C) and a taste bud from an explant (D). (A) A low power image of a stage 43 larva which had received a graft (arrow) at stage 28. (B) Higher power image of the graft in A. The graft is covered with an ectodermal epithelium, including ciliated cells (e.g. small arrow), from the donor tissue. The endodermal epithelium, where the taste buds reside, can be seen at the opening to the lumen of the graft (arrowhead). (C) An oropharyngeal explant raised in culture for 9 days. Anterior is left. Fibrous cells obscure the border between the ectoderm and endoderm (large arrow) as well as most of the endodermal epithelium. The lumenal endoderm is visible through small holes in the fibrous cell layer (small arrows). (D) The apical processes of a taste bud found in the endodermal epithelium of an explant cultured for 15 days. At this late stage, the endodermal epithelium is typically free of fibrous cells. The larger microvilli most likely represent the apical specializations of the receptor cells within each bud. Scale bars: A, 1 mm; B, 250 μm; C, 200 μm; D, 5 μm.

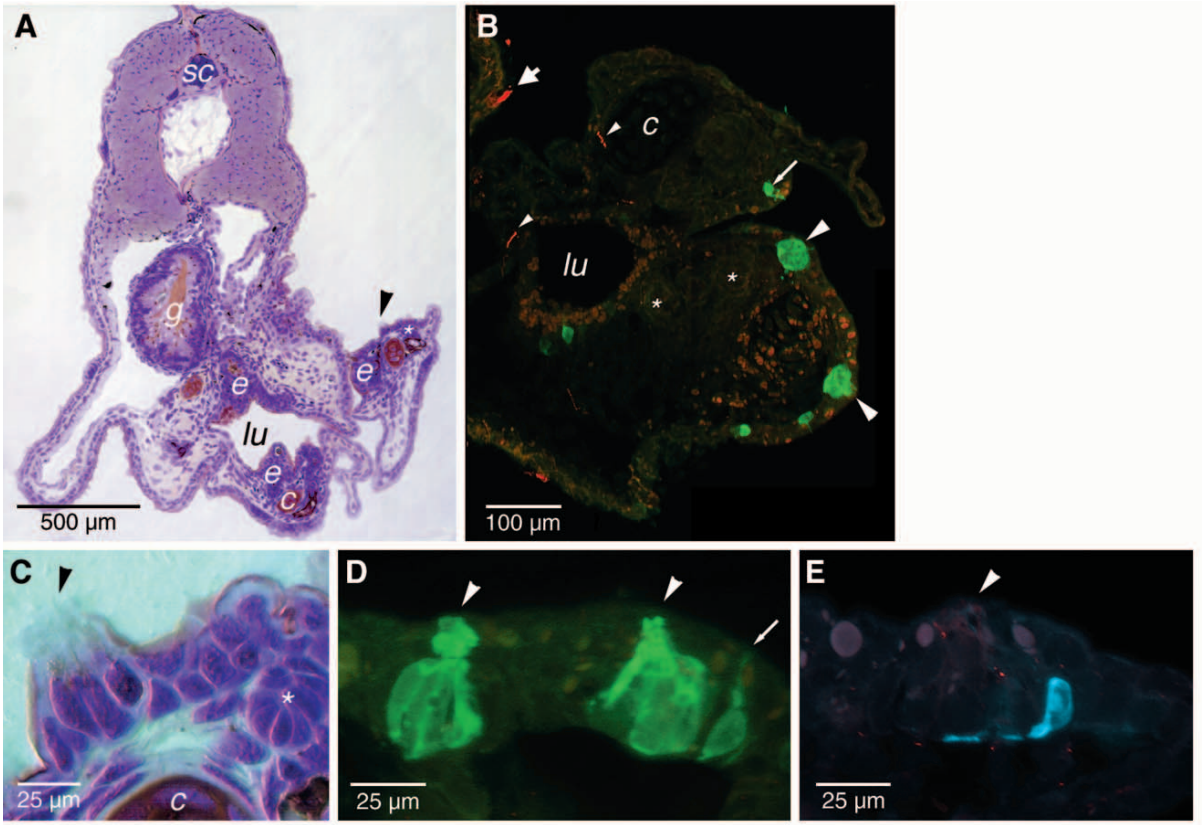

Fig. 4. Photomicrographs of transverse sections of oropharyngeal grafts (A,B) and taste buds within them (C-E). (A) An 8 μm plastic section through the trunk of a stage 43 larva that had received an oropharyngeal graft at stage 28. The graft (lower right) has formed a lumen (*lu*), along which are numerous patches of endoderm (*e*). Also arising from the graft tissue are numerous cartilaginous elements (*c*) and tooth primordia (*), as well as one taste bud (arrowhead). *g*, gut; *sc*, spinal cord. (B) A photomontage of confocal projections showing the distribution of calretinin-immunoreactive taste buds (large arrowheads) and solitary cells (one of several, small arrow), and the location of AT-positive elements, such as the ciliated ectodermal cell in the host epithelium (large arrow) and the sparse distribution of neurites (small arrowheads) in the graft. (C) A high magnification DIC photomicrograph of the well-differentiated taste bud shown in A, located immediately above a cartilaginous element (*c*). The arrowhead indicates the apical microvilli extruding from the taste pore. (D) Two calretinin-immunoreactive taste buds (arrowheads: apical taste pore) and one solitary cell (small arrow) are shown in the endodermal epithelium of an ectopic oropharyngeal graft in this photomicrograph collected using a cooled CCD camera. (E) Serotonergic cells are also found in ectopic taste buds within oropharyngeal grafts. (arrowhead: apical taste pore).

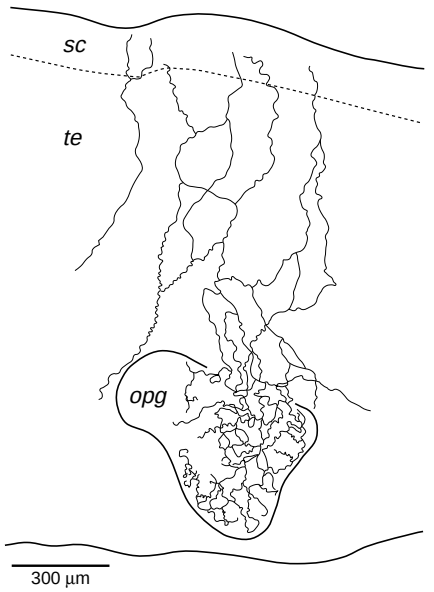

Fig. 5. A camera lucida drawing of the trunk of a stage 43 larva with an oropharyngeal graft (opg), processed in whole-mount for AT immunostaining. Numerous nerve fibers of spinal origin (sc: spinal cord) descend into the graft and ramify extensively in its more anterior (right) portion. Adjacent spinal nerves reticulate within the trunk epithelium (te). Anterior is right.

neuronal fibers were not seen contacting serotonin- or calretinin-immunopositive taste cells. These neurons probably represent a portion of the anteroventral lateral line ganglion (gAV), arising from the AV placode (Northcutt et al., 1994), which, by stage 35, has begun to elongate ventrally as a sensory ridge along the mandibular arch (Northcutt et al., 1994). In this particular experiment, portions of the AV placode were accidentally removed with the oropharyngeal explant.

Scattered ectodermal cells with AT-immunopositive cilia were also found in most explants (Fig. 6B), and served as a positive control for AT staining. These ciliated cells are normally present in large numbers in the ectoderm throughout most of the embryonic period, and are gradually lost during early larval life.

Taste bud distribution in ectopic grafts and cultured explants is comparable to the distribution of taste buds formed in situ

The grafted or explanted region in this study consisted of the ventromedial portions of the mandibular and hyoid arches which give rise to the anterior region of the floor of the oropharyngeal cavity. By stage 41 or hatching, axolotl larvae possess approximately 47 taste buds/mm^2 of endodermally derived epithelium lining this area (R.G. Northcutt, L. Barlow and K. Catania, unpublished observations; Table 2). The distribution of taste receptor organs is irregular: they are located on the top surfaces of each arch but the epithelium lining the lateral walls of each arch is devoid of taste receptors. Furthermore, the number of receptors is highest anteriorly in the region derived from the mandibular arch, and declines posteriorly.

Comparable receptor cell densities were encountered in oropharyngeal grafts and explants. An average density of ectopic taste buds was obtained by reconstructing serial cryosections of three grafts and four explants immunostained for calretinin. The average density of taste buds in the endodermal epithelium of grafts was somewhat less than that in the intact oropharyngeal floor, whereas the average density of taste buds in oropharyngeal explants more closely approximates the density in intact animals (Table 2). Taste buds were found only in the endodermal regions of grafts or explants and were not encountered in ectoderm, consistent with the location of these receptors in situ. The tendency of taste buds to be clustered within the central endodermal regions of grafts and explants suggests that wound healing at the edges of each explant or graft may interfere with the generation of taste buds.

DISCUSSION

Taste bud differentiation is independent of contact by neurites

Contrary to long prevailing models of taste receptor development (Vintschgau and Honigschmied, 1876; Landacre, 1907; Torrey, 1940; Hosley et al., 1987b), we have demonstrated experimentally that taste buds can develop in the absence of nerve contact. Taste buds differentiate fully in ectopic grafts in which no contact by spinal nerve fibers was observed, and in explants that are completely devoid of neuronal elements. In both cases, these taste buds resemble those found in control animals: they are cohesive aggregates of fusiform cells with apical microvilli that extrude through a prominent taste pore located within the endodermal epithelium. Like control taste buds, both ectopic and cultured taste buds possess calretinin- and serotonin-immunopositive cells. Furthermore, taste buds were generated in grafts and explants at numbers and densities comparable to those found in situ, and with a developmental time course similar to that of intact control animals.

Although nerve fibers were found occasionally near ectopic taste buds, it is unlikely these associations represent specialized contacts. First, DiI labeling of spinal nerves failed to label ectopic taste buds (unpublished observations), as would be

Table 2. Comparison of the number and density of endogenous taste buds with those of oropharyngeal grafts and explants

Oropharyngeal region	Taste bud number (mean ± s.e.m.)	Taste bud density (per mm^2) (mean ± s.e.m.)
Mandibular arch (I)*	28	56
Hyoid arch (II)*	17	38
Combined arches (I, II)*	58	47
Grafts (I, II)† (n = 3)	18.3±0.7	29.0±7.4
Explants (I, II)† (n = 4)	21.3±3.1	36.7±5.9

*Control taste bud numbers are from an SEM photomontage of the oropharyngeal epithelium of one larva.

†Taste bud counts from experimental tissue are based on the presence of aggregations of calretinin-immunopositive cells within endodermal epithelia.

expected if synaptic contacts had formed (Finger and Böttger, 1990; Nagai, 1993; Northcutt et al., 1996). Secondly, AT immunostaining of ectopic taste buds did not reveal the extensive chalice of nerve fibers typically found in association with endogenous taste organs (Fig. 2B,C). Nonetheless, we could not eliminate the possibility that transient contact by spinal neurites was sufficient to induce taste bud differentiation in ectopic grafts. This was of particular concern, since inappropriate nerves have been shown in association with adult taste buds after removal of appropriate gustatory innervation (Kinnman and Aldskogius, 1988). However, our finding that cultured oropharyngeal explants possessed well-developed taste buds in the complete absence of neural elements indicates that differentiation of these receptors is indeed independent of nervous contact.

Our conclusions contradict those made from experimental studies in newborn rats, in which denervation of the tongue during a critical period of postnatal development resulted in the apparent failure of induction of the majority of taste buds (Hosley et al., 1987a,b). However, in these studies only the presence of taste pores, which represent the terminal phase of taste organ differentiation (Guth, 1957; Farbman, 1965), was used to assess the presence of taste buds. This methodology would fail to detect taste buds that had undergone substantial differentiation, but had simply not formed taste pores, as has been shown to be the case with denervated taste buds in adult mammals (Whitehead et al., 1987). Furthermore, over long postnatal time periods in rats a small number of fully differentiated taste buds did form in denervated tissue (Hosley et al., 1987a,b), suggesting innervation was not necessary for taste cell differentiation, but may be crucial for long term trophic support of receptor organs.

The mechanism of embryonic genesis of peripheral receptor cells in general remains elusive. Based primarily upon descriptive studies, it has been proposed that induction by nerves is required for a number of epithelial receptor cell types, including Merkel cells (Breathnach, 1980), electroreceptors (Vischer et al., 1989), and neuroepithelial bodies (Lauweryns and Van Lommel, 1983). However, some experimental results indicate that several mechanoreceptor cell types will develop in the absence of innervation (Stone, 1931; Tweedle, 1978). Vertebrate teeth (Lumsden and Buchanan, 1986) and whisker follicles of rodents (Andrés and Van Der Loos, 1982), though not strictly considered excitable receptor cells, can also develop in vitro without nerve contact. Furthermore, in our in vivo and in vitro studies, calretinin-immunopositive solitary cells differentiate in the complete absence of innervation. Thus, innervation-independent receptor cell differentiation may be a general property of peripheral sensory systems.

Does peripheral pattern guide the development of sensory neurons?

If the peripheral neurites of cranial nerves do not induce the formation of taste cells, what developmental mechanism is responsible for the precise pattern of connectivity observed between nerves and taste buds? Peripheral receptor primordia may direct the formation of appropriate contacts by guiding nerve fibers directly toward taste cells and/or by trophically supporting only those fibers that contact receptor primordia.

Evidence from many systems indicates that target tissues play an important role in directing innervation patterns of developing neurites. They produce chemical cues that act at some distance to attract or repel developing neurites (for reviews: Tessier-Lavigne, 1992; Kennedy and Tessier-Lavigne, 1995), and express a diversity of trophic factors that regulate neuronal survival (for reviews: Korsching, 1993; Davies, 1994) and peripheral arborization patterns (Arvidsson et al., 1995; Fundin et al., 1995; Wilkinson et al., 1995; and for review: Kennedy and Tessier-Lavigne, 1995).

Several recent findings concerning the development of innervation of taste buds hint that they are specifying the pattern of contact by ingrowing gustatory neurites. First, the development of taste buds or their primordia is independent of innervation. As we have shown here, taste buds in axolotls can develop without nerves. This is also the case for the taste bud-bearing fungiform papillae of mammals; these papillae form prior to contact by neurites in vivo (Farbman and Mbiene, 1991; Whitehead and Kachele, 1994) or in the absence of innervation in vitro (Farbman and Mbiene, 1991). Second, the majority of embryonic cranial nerve fibers appear to grow directly to the fungiform papillae in both rats (Farbman and Mbiene, 1991) and hamsters (Whitehead and Kachele, 1994), suggesting that taste bud primordia may attract these nerves from a distance via production of a diffusible chemical cue. Third, fungiform papillae express brain-derived neurotrophic factor (BDNF) mRNA at a time when cranial nerve fibers reach the papilla epithelium (Nosrat and Olson, 1995). The restricted availability of this trophic factor may play a role in maintaining and stabilizing early neuritic contacts of gustatory sensory neurites with taste bud primordia.

Potential mechanisms involved in early patterning of the taste epithelium

Our findings, and those in mammals (Farbman and Mbiene, 1991; Whitehead and Kachele, 1994) suggest that taste bud primordia are either induced by earlier contact with other embryonic tissue(s), or as a result of intrinsic patterning of the oropharyngeal epithelium. Which tissues are positioned appropriately to induce formation of taste bud primordia? There are two candidates tissues that can be considered: cephalic neural crest and paraxial mesoderm.

Cephalic neural crest has been shown to participate in a number of epithelial-mesenchymal inductive interactions, such as those involved in formation of cartilaginous elements of the facial skeleton and teeth (Hall, 1981; Le Douarin, 1982; Lumsden, 1987). In axolotls, neural crest migration into the oropharyngeal region (stage 23-35; Stone, 1922; Landacre, 1931; Northcutt and Brändle, 1995) precedes taste bud innervation and differentiation (stage 40), and this temporal sequence could explain the ability of our stage 35 grafts and explants to generate taste buds in the absence of innervation. Furthermore, the early distribution of taste buds within the epithelium correlates with the position of the underlying cartilaginous elements of the oropharynx (R.G. Northcutt, L. Barlow, K. Catania, unpublished observations), which are derived from neural crest. The proximity of neural crest deriv-

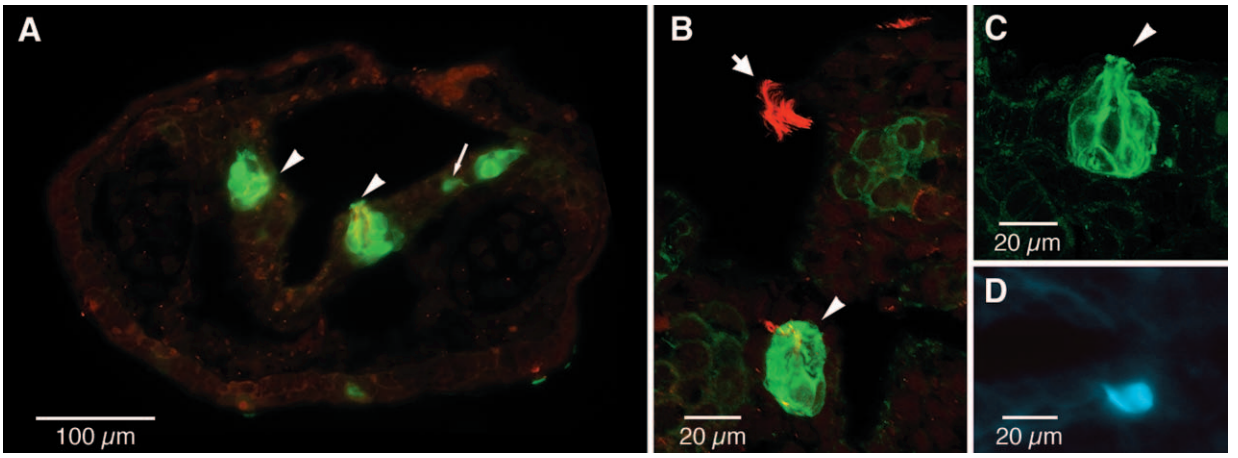

Fig. 6. Transverse frozen sections of oropharyngeal explants raised in culture for 7 or 9 days and processed for calretinin and AT (A-C) or serotonin (D) immunoreactivity. (A) A low power CCD image of a 7 day explant with two calretinin-immunoreactive (green) taste buds with well-defined taste pores (arrowheads). A calretinin-positive solitary cell is also present (small arrow), adjacent to a glancing section through a third taste bud. No AT immunoreactive neurites were detected in this explant, although faint AT immunostaining is evident within the apices of the middle taste bud (yellow). See text for explanation. (B) A calretinin-immunoreactive taste bud (green; arrowhead) and AT-positive cilia of an ectodermal cell (arrow) are evident in this confocal projection from an explant cultured for 9 days. AT immunostaining is present in the taste bud (red, yellow), but no neurites were found in this explant. (C) This confocal projection shows a fully differentiated taste bud from an explant cultured for 9 days. The calretinin-immunoreactive flask shaped cells extrude their apical processes through the taste pore (arrowhead). (D) Taste buds also generate serotonin-immunoreactive cells in culture, as is depicted in this cooled CCD image of a frozen section through an explant cultured for 10 days. The apical region of the taste bud is not evident in this plane of section, although the serotonergic basal cells lie below and to the right of the lumen of the explant.

atives to taste buds prior to taste cell differentiation enhances the argument for neural crest as the inducing tissue. We are currently examining the ability of early oropharyngeal endoderm to generate taste buds in the absence of contact with cephalic neural crest.

Paraxial mesoderm of the head is also appropriately positioned for involvement in the induction of taste cells. It is initially located bilaterally adjacent to the chordamesoderm, and is thought to move ventrally, preceding neural crest migration, between the pharyngeal pouches to form the branchiomeres (Noden, 1988, 1991). Thus paraxial mesoderm is potentially the first embryonic tissue to contact the endoderm of the oropharynx and may induce arrays of taste cell primordia.

Alternatively, the development of taste receptors may not be induced by contact with another tissue but rather may be intrinsic to the endodermal epithelium from which they arise (Barlow and Northcutt, 1995). The delineation of anterior endoderm could represent the first step in commitment of oropharyngeal epithelial cells toward a taste cell fate. Some evidence indicates the anterior-posterior axis of endoderm, like ectoderm and mesoderm, is specified at gastrulation, and that homeobox genes may be involved. For example, *Hoxc5* is expressed in the endodermal lining of the esophagus and posterior pharynx in mouse embryos, whereas the *b5* and *a5* paralogs are primarily restricted to the developing stomach and tracheal endoderm respectively (Gaunt et al., 1990). Such staggered expression patterns could represent a molecular code for axial patterning of embryonic endoderm (Gaunt et al., 1990), implying early specification of head endoderm as distinct from that of the trunk. Once the oropharyngeal zone has been defined, taste cell primordia may be specified via a local signaling mechanism, such as lateral inhibition (Greenwald and Rubin, 1992; Ghysen et al., 1993), in which taste cell precursors inhibit neighboring epithelial cells from acquiring a receptor cell or neuronal fate (for reviews: Greenwald and Rubin, 1992; Artavanis-Tsakonas et al., 1995). However, testing of these hypotheses awaits identification of early molecular markers of taste versus epithelial cell fate and the determination of the timing of taste cell commitment.

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