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Organ Cultures of Embryonic Rat Tongue Support Tongue and Gustatory Papilla Morphogenesis In Vitro Without Intact Sensory Ganglia

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ABSTRACT

Taste buds on the mammalian tongue are confined to the epithelium of three types of gustatory papillae: the fungiform, circumvallate, and foliate. The gustatory papillae are composed of an epithelium that covers a broad connective tissue core, with extensive innervation to taste bud and nongustatory epithelial locations. Although the temporal sequence of gustatory papilla development is known for several species, factors that regulate initiation, growth, and maintenance of the papillae are not understood. We tested the hypothesis that sensory innervation is required for the initial formation and early morphogenesis of fungiform papillae in a patterned array. An organ culture of the embryonic rat tongue was developed to provide an in vitro system for studying mechanisms involved in fungiform papilla morphogenesis in patterns on the anterior tongue. Tongues were dissected from embryos at 13 days of gestation (E13), a time when the tongue has not yet fully formed and gustatory papillae have not yet appeared, and at 14 days of gestation (E14), when the tongue is well formed and papillae make their initial morphological appearance. Dissected tongues were maintained at the gas/liquid interface in standard organ culture dishes, fed with DMEM/F12 plus 2% B-27 supplement and 1% fetal bovine serum. After 1, 2, 3, or 6 days in culture, tongues were processed for scanning electron or light microscopy, or immunocytochemistry. Tongues cultured from E13 or E14 underwent extensive morphogenesis and growth in vitro. Furthermore, fungiform papillae developed on these tongues on a culture day equivalent to E15 in vivo; that is, after 2 days for cultures begun at E13 and 1 day for those begun at E14. Because E15 is the characteristic time for gustatory papilla formation in the intact embryo, results demonstrate that the cultured tongues retain important temporal information related to papilla development. In addition, fungiform papillae formed in the tongue cultures in the stereotypic pattern of rows. The papillae were large structures with epithelial and mesenchymal cell integrity, and an intact epithelial basement membrane was indicated with laminin immunoreactivity. The cultures demonstrate that gustatory papilla morphogenesis can progress in the absence of an intact sensory innervation. To exclude a potential developmental role for autonomic ganglion cells that are located in the posterior rat tongue, cultures consisting of only the anterior half of E14 tongues were established. Fungiform papilla development progressed in half tongues in a manner directly comparable to whole tongue cultures. Therefore, robust, reproducible development of fungiform papillae in

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patterns is supported in rat tongue cultures from E13 or E14, without inclusion of intact sensory or major, posterior tongue autonomic ganglia. This is direct evidence that papillae will form and develop further in vitro without sensory ganglion support. The data also provide the first detailed account of in vitro development of the entire embryonic tongue. J. Comp. Neurol. 377:324–340, 1997.

Indexing terms: autonomic ganglia; circumvallate papilla; fungiform papilla; taste; taste bud

The lingual taste organs include three gustatory papilla types: fungiform, circumvallate, and foliate papillae, and their resident taste buds. During development of gustatory papillae, circumferential epithelial downgrowths form that encompass underlying mesenchyme; a discrete structure results, composed of epithelium over a core of connective tissue with centrally located nerve bundles. Thus, morphogenesis of the taste papillae requires coordinated interactions among lingual epithelium and mesenchyme, and the tongue innervation.

Although the temporal sequence of papilla development is established for several species (Bradley and Mistretta, 1989), there is little known about mechanisms involved in papilla differentiation, patterning, growth, and morphogenesis (Mistretta, 1991; Mistretta and Hill, 1995). We have tested the hypothesis that sensory innervation is required for initial formation and early morphogenesis of fungiform papillae in a patterned array on the anterior tongue. A limiting factor for prior studies in analyzing regulatory factors that govern gustatory organ differentiation and morphogenesis has been the lack of a suitable in vitro system for investigating development of tongue and associated papillae in a controlled setting. Therefore, we have developed a culture system for tongue and taste papillae, to study participation of epithelial, mesenchymal, and neural tissues in the initiation, morphogenesis, growth, and maintenance of gustatory organs.

Several unique features render the gustatory papillae an especially useful and accessible model system for studying roles of epithelium, mesenchyme, and nerve in organogenesis (Mistretta, 1991). First, the papillae are arranged in patterns and stereotypic locations: The fungiform papillae are in diagonal rows on the anterior tongue, and the circumvallate and foliate reside in the central and lateral posterior tongue, respectively. Second, each papilla type has a distinctive innervation: Fungiform papillae are innervated by the lingual branch of the trigeminal nerve, and taste buds in the fungiform papillae are innervated by the chorda tympani branch of the facial nerve; circumvallate papillae and taste buds are innervated by the lingual branch of the glossopharyngeal nerve; and foliate papillae and taste buds by both chorda tympani (anterior papilla folds) and glossopharyngeal (posterior folds) nerves. In this paper, the chorda tympani branch of the facial nerve, the lingual branch of the trigeminal nerve and the lingual branch of the glossopharyngeal nerve are referred to, collectively, as the sensory lingual innervation. Third, each papilla type has a different number of taste buds, from one to several in fungiform, contrasted with a few to several hundred in circumvallate, depending on the species. Fourth, each papilla is composed of an epithelium that covers a broad connective tissue core, with an extensive innervation to the specialized taste buds within the epithelium. Thus, the tongue provides three papilla types each with unique features, but all representing a special environment that supports taste buds; taste buds do not reside outside of the gustatory papillae on the tongue. Factors that regulate the patterned formation and distinctive histology, the characteristic number of taste buds and the specific innervation of the gustatory papillae are not known.

There are few published reports of in vitro systems that support lingual development. Tongue formation and development reportedly progress in cultures of mandibular processes from embryonic mouse initiated at 9–11 days of gestation (Slavkin et al., 1989; Kronmiller et al., 1991; Chai et al., 1994). However, in these cultures the focus has been on morphogenesis of cartilage and teeth in the mandible, and therefore, details about tongue formation are not provided; in addition, these systems might include ganglionic elements and are not intended to strictly exclude innervation. In a study of in vivo and in vitro gustatory papilla development, cultures of embryonic rat, dorsal tongue slices supported fungiform papilla formation to a limited extent (Farbman and Mbiene, 1991). Similarly, small tongue fragments containing the immature circumvallate papilla of embryonic rat, cultured with and without cranial ganglia, supported papilla maintenance to some degree (Farbman, 1972). It is important to note that none of these studies has provided a detailed evaluation of cultured tongues or reported a system that supports patterned development of papillae.

We have developed a culture system for the entire embryonic tongue to create an environment that permits development similar to that which occurs in vivo, for testing tissue, cell and molecular factors important in papilla morphogenesis, and pattern formation. To learn whether sensory nerves are essential for initial papilla formation, we excluded intact sensory innervation from the system. Therefore, we dissected the early tongue from rat embryos at 13 days of gestation, a time when the tongue has not yet fully formed, nerves are just entering the base of the lingual swellings and gustatory papillae have not yet appeared on the tongue. We also dissected the tongue at 14 days of gestation, when the tongue has acquired its characteristic spatulate form and papillae make their initial histological and morphological appearance. The latter stage permits a test of potential morphogenesis in vitro of papillae that have just begun to differentiate, but are not yet innervated. This paper presents a working organ culture system that not only supports morphogenesis and growth of the embryonic rat tongue, but also, formation, morphogenesis and patterned development of gustatory papillae. Furthermore, results demonstrate that gustatory papillae develop in the absence of intact sensory innervation or major, intralingual autonomic ganglia. Preliminary reports have appeared in abstracts (Mbiene et al., 1994; Mistretta et al., 1995).

MATERIALS AND METHODS

Rat embryos and tissue dissection

Timed, pregnant Sprague Dawley rats were obtained from Charles River breeders. Embryonic day 0 designates the day on which the dam was determined to be sperm positive. Dams at E13 (13 days of gestation) or E14 were used and all experiments were begun between 9 and 11 A.M. of the day when cultures were initiated. For each culture series, the two most proximal (to the vagina) embryos, left and right, were taken as controls for the starting developmental stage (Day 0); the next two embryos were cultured for examination after 1 day in culture (Day 1), the next for examination after 2 days in culture (Day 2), and so on, for Days 3 and 6.

Dams at E13 or E14 are deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight), which anesthetizes the embryos also. Subsequent procedures are conducted using aseptic procedures. An anesthetized embryo is removed and placed in a sterile Petri dish containing Earle's balanced salt solution (EBSS) buffered with 20 mM HEPES (pH 7.4), and gentamicin sulfate (20 (µg/ml). The embryo is decapitated, the head moved to fresh EBSS solution, and the entire tongue is microdissected from the mandible and placed in a small culture well.

In some cultures, to eliminate the possibility of including clusters of autonomic ganglion cells that reside in posterior tongue under the circumvallate papilla (Graziadei and Graziadei, 1978) and at the region where the chorda-lingual nerve trunk enters the tongue (Kuder, 1984), tongues are transected at the distal edge of the intermolar eminence. The size of the anterior half tongues could vary somewhat, depending on the accuracy of cutting precisely at the distal edge of the eminence. The anterior half tongue is then cultured in these experiments. After all embryos are removed and dissected, the anesthetized dam is exsanguinated.

Tongue cultures

Dissected tongues are oriented with dorsal surface upward on small squares of sterile Millipore type HA filter (submerged in EBSS), 0.45 µm pore size. Tongues and filter papers are then removed from EBSS and placed on stainless steel grids in standard organ culture dishes (Falcon 3037). Cultures are fed with a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (DMEM/F12 GIBCO BRL, Gaithersburg, MD), containing 1% fetal bovine serum, 20 µg/ml gentamicin sulfate, and 2% B27 culture supplement (GIBCO). Tongues are maintained at the interface between the gas (5% CO_2 in air) and liquid phases of the culture, and the level of medium is adjusted so that the tissue is wetted, but not covered, by the medium (MacCallum, 1994). Cultures are kept in a humidified incubator at 37°C and the medium is changed daily. After 1, 2, 3, or 6 days in culture, tongues are removed and processed for scanning electron or light microscopy, or immunocytochemistry.

We analyzed tongues from four series of whole tongue cultures begun at E13 and four begun at E14 (each series represents use of one litter); however, numerous prior experiments were conducted to establish optimal culture conditions. Three series of half tongue cultures begun at E14 were also analyzed.

Scanning electron microscopy and thin section light microscopy

For high resolution study of the dorsal tongue surface, cultured tongues were prepared for scanning electron microscopy. Cultures were rinsed briefly in phosphate buffered saline, and fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.025 M cacodylate buffer (pH 7.3) for 6 hours at 4°C. Tissues were then rinsed in buffer and subsequently post-fixed in a sequence of 1% OsO_4, 1% tannic acid, 1% OsO_4 (at 1 hour each). Dehydration was through an ascending series of alcohols, and displacement of alcohol is accomplished with three changes of hexamethyldisilazane (HMDS). Residual HMDS was evaporated in a fume hood overnight, before mounting the tissue on specimen stubs for light sputter coating with gold/palladium.

Cultures to be thin sectioned for histology were rinsed briefly in 37°C, balanced salt solution, subsequently immersed in a 50:50 mixture of 2.5% glutaraldehyde, and 2% OsO_4 in buffered cacodylate (0.1M Na cacodylate, pH 7.4, 4% sucrose, 2 mM $CaCl_2$) for 2 hours at 4°C, and stained en bloc with 2% uranyl acetate (MacCallum et al., 1982). Tissues were then dehydrated in ethanol and propylene oxide, and embedded in epoxy resin. One µm sections were mounted on glass slides and stained with 1% toluidine blue O, pH 9.0.

Immunocytochemistry

To learn whether the epithelial basement membrane remained intact in cultured tongues, immunoreactivity to rabbit anti-human polyclonal laminin antiserum (GIBCO-BRL) was evaluated. The antibody has demonstrated cross-reactivity to rat laminin, and no cross-reactivity with human fibronectin or other human plasma proteins in Western blot analysis (GIBCO technical information). In our laboratory the antibody has been used in tongue and gustatory papillae in embryonic and postnatal rat, and in fetal and postnatal sheep (Mistretta and Haus, 1996) with comparable results.

To examine possible neural elements in tongue cultures, immunoreactions were examined by using a mouse monoclonal antibody to growth associated protein-43 (GAP-43; Boehringer Mannheim, Indianapolis, IN); a polyclonal rabbit antiserum to the 200 kD neurofilament protein (NF-200, Sigma, St. Louis, MO); and, a polyclonal rabbit antiserum directed against choline acetyltransferase (ChAT; Chemicon, Temecula, CA), the enzyme responsible for the final synthetic step in formation of acetylcholine.

Tongue cultures were rinsed briefly in phosphate buffered saline and placed in molds contained embedding medium (O.C.T., Miles Scientific, Elkhart, IN), frozen in hexane cooled by an acetone and solid carbon dioxide bath, and stored at -80°C until sectioned. Sections were cut at 5–10 µm on a cryostat in sagittal plane, thaw-mounted onto gelatin coated microslides, and stored at -80°C until reacted.

Mounted sections were fixed on slides and blocked for endogenous peroxidase staining simultaneously, by exposure to 0.05% hydrogen peroxide in 100% ethanol for 30 minutes at room temperature. Tissues were rinsed and then incubated in a blocking solution that contains 10% normal goat serum (normal horse serum for GAP-43) and 0.3% Triton X-100 in 0.1 M phosphate buffered saline (PBS, pH 7.4) at 4°C overnight. Tissues were removed

from the blocking solution and incubated in: laminin primary antibody diluted 1:3000 in carrier solution of PBS and 1% normal goat serum, for 3 hours at 37°C; GAP-43 antibody diluted 1:4,000, for 72 hours at 4°C; NF-200 antibody diluted 1:8,000, overnight at 4°C; and, ChAT antibody diluted 1:12,000, overnight at 4°C. After thorough rinses in carrier solution, tissues were incubated in biotinylated goat anti-rabbit IgG, diluted 1:300, for 1 hour at 4°C. Tissues were thoroughly rinsed and incubated in horseradish peroxidase (HRP) labeled streptavidin (Kirkegaard and Perry, Gaithersburg, MD) in a 1:500 dilution with carrier for 7 to 15 minutes at room temperature. The HRP label was visualized with diaminobenzidine (DAB kit, Vector, Burlingame, CA) with nickel intensification to produce a black reaction product. Tissues were rinsed in several changes of Tris buffer (pH 7.4), dehydrated through ethanols, cleared in xylene, and coverslipped with Permount (Fisher Scientific, Chicago, IL). Controls were routinely performed by substituting blocking solution for the primary antiserum. Heads from E13–E15 embryos were routinely immunoreacted along with tongue cultures as positive controls.

Data analysis

Scanning electron micrographs of tongues after 1, 2, 3, and 6 days in culture are compared to control tongues taken at the time of culture initiation, and are examined to determine: (1) progress in tongue morphogenesis as evidenced by remodeling of embryonic tissue swellings that form the tongue, and the formation of the intermolar eminence; (2) growth of the tongue, measured from the tip to the junction between oral and pharyngeal tongue components; (3) formation, growth and morphogenesis of fungiform papillae on the anterior tongue; (4) patterned distribution of fungiform papillae; and (5) surface epithelial characteristics of the dorsal tongue.

Thin sections of cultures are examined by light microscopy to determine: (1) the nature and integrity of epithelial and mesenchymal tissues in the cultures; (2) development of fungiform papillae; and, (3) general remodeling of dorsal tongue tissues. Laminin immunoreactions are examined to evaluate: (4) the integrity of the basement membrane of dorsal lingual epithelium and fungiform papilla epithelium. (5) GAP-43, NF-200 and ChAT immunoreactions are studied to learn whether neural elements remain in cultured tongues.

RESULTS

Summary of intrauterine, embryonic tongue development: E13 to E20

Before describing results from embryonic tongue cultures, a brief summary of lingual development is presented. At E13 (13 days of gestation) the two lateral, lingual swellings of the anterior embryonic tongue are still prominent and separated at the distal end by a deep sulcus (Fig. 1). Behind these swellings the tuberculum impar is apparent in the midline. The dorsal lingual epithelial surface is relatively homogeneous in appearance with no signs of papilla formation.

By E14 the tongue has almost doubled in size and there is a pronounced intermolar eminence (Fig. 1). Although the anterior tongue halves have joined to the tip, a deep sulcus demarcates the midline of the tongue. E14 also denotes the day of appearance of small surface mounds that represent incipient fungiform papillae. The E15 tongue exhibits further growth and has acquired a spatulate shape, and the fungiform papillae are now highly distinctive, and positioned in diagonal rows on either side of the lingual midline (Fig. 1). On the posterior tongue the single circumvallate papilla is seen in the midline as a large ovoid swelling. From E16 through E19 (Fig. 1), the tongue again almost doubles in length. The fungiform and circumvallate papillae alter in shape and protrude more on the tongue surface.

During the period between E19 and E20, the dorsal lingual surface acquires a roughened, flaking appearance that obscures the gustatory papillae (Fig. 2A). This appearance is caused by surface epithelial cells that desquamate (Fig. 2B), revealing the underlying nongustatory, filiform papillae. After the period of desquamation, the fungiform papillae once again are readily distinguished, surrounded by filiform papillae (Fig. 2C,D).

Neural elements are apparent at the most lateral edge of the base of the embryonic tongue primordia at E13 (Fig. 3). From a detailed evaluation of the sensory innervation of the embryonic rat tongue it is apparent that there is no mid-tongue innervation at this stage (Mbiene and Mistretta, in preparation). At E14 there is innervation throughout the body of the tongue, in lateral regions but not in the midline; the innervation does not approach the lingual epithelium (Fig. 3). In fact it is not until E16 that extensive epithelial innervation is observed in the embryonic tongue (data not shown).

Whole tongue organ cultures begun at E13

Scanning electron micrographs of control tongues (Day 0), sampled on the day of initiating cultures at E13, are characterized by lingual tissue swellings that have not yet fully fused to form the embryonic tongue (Fig. 4, Day 0). After 1 day in culture, the tongue swellings have fused and the oral portion of the tongue has increased in length from about 700 to 900 μm (Fig. 4, Day 1). The surface epithelium remains smooth and homogeneous with no indication of fungiform papilla development.

By 2 days in culture the oral tongue has assumed an elongate shape and has increased to about 1000 μm in length (Fig. 4, Day 2). The surface of the tongue has undergone extensive morphogenesis from the smooth, homogeneous lingual epithelium at Day 1 to acquisition of surface specializations including numerous fungiform papillae seen in rows on either side of the midline. The dorsal surface of the papillae is about 45 μm in diameter. Tongue cultures initiated at E13 and maintained for 2 days are temporally equivalent to E15 in vivo, the day on which fungiform papillae appear in the intact animal.

After three days in culture (Fig. 4, Day 3) the tongue has not increased in length compared to Day 2. Fungiform papillae are still apparent on the anterior tongue, but appear more closely spaced due to the lack of lingual growth. The papillae remain at about 45 μm diameter, comparable to Day 2. The tongue after 6 days in culture is about 1200 μm in length (Fig. 4, Day 6) and has an undulating surface epithelium that is reminiscent of E20 in vivo. Fungiform papillae are present but more difficult to discern in scanning micrographs than at earlier stages. This is due to the extensive, overall remodeling of the lingual epithelium and comparatively small size of the remaining fungiform papillae.

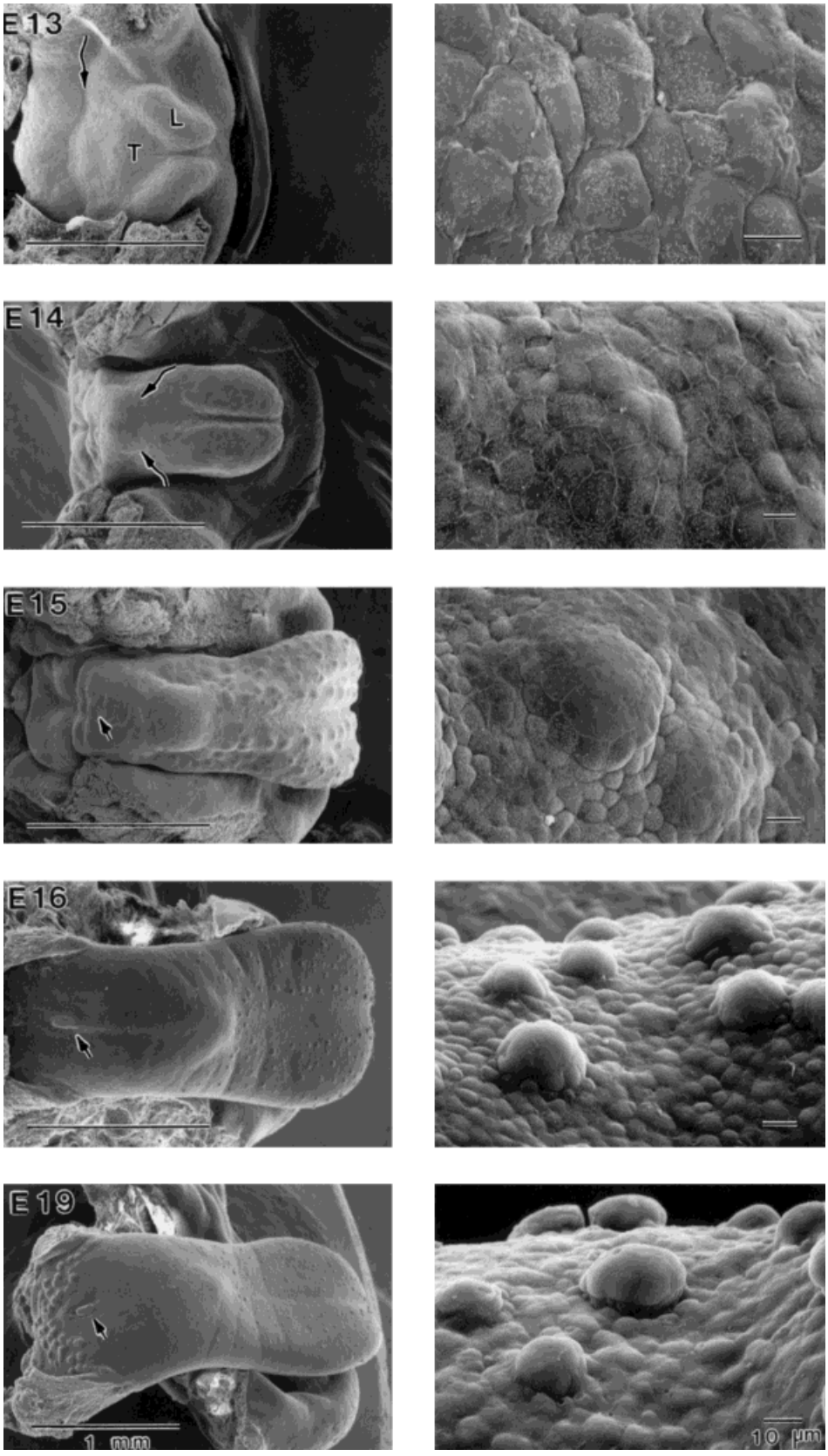

Figure 1

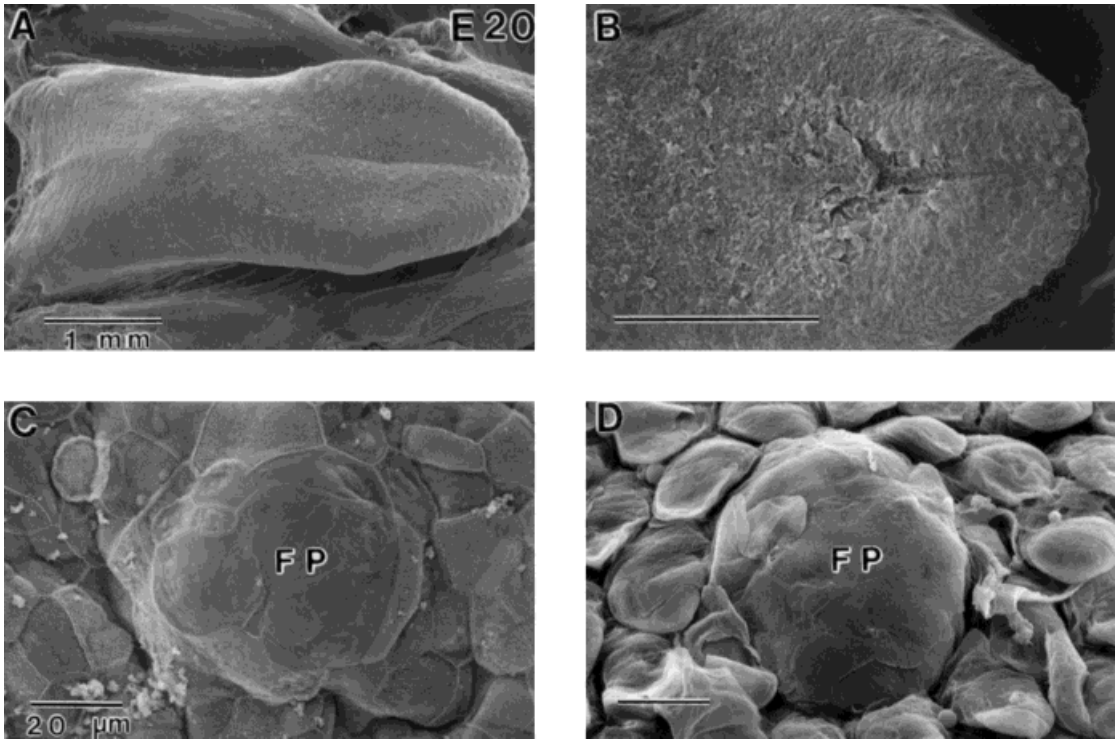

Fig. 2. Scanning electron micrographs of intrauterine rat tongue topography at E20. **A:** Low power micrograph illustrates flaking cells on the dorsal surface of the anterior tongue. **B:** Higher power micrograph of another E20 tongue demonstrates desquamating cells. **C:** Fungiform papilla (FP) and surrounding epithelial squames before surface cells have desquamated. **D:** Fungiform papilla (FP) and surrounding filiform papillae that are revealed after surface cells desquamate. Scale bars = 1.0 mm in A,B, 20 µm in C,D.

Light microscopic examination of thin sections of E13 control tongues illustrates the relatively smooth, dorsal epithelium and large mesenchymal cells (Fig. 5A). After 2 days in organ culture early fungiform papillae are observed, composed of an epithelium covering a broad core of mesenchymal cells (Fig. 5B). Laminin immunoreactivity confirms the intact nature of continuous basement membrane beneath both the nonspecialized lingual epithelium and the papilla epithelium of E13 tongues after two days in culture (Fig. 5C,D). Therefore, in vitro conditions are adequate to sustain basement membrane structure which is crucial for supporting continued morphogenesis of epithelial specializations.

In summary, whole tongue cultures initiated at E13 exhibit tongue growth and morphogenesis, and the development of fungiform papillae in a patterned distribution on a culture day that is equivalent to E15 in vivo (the intrauterine day of appearance of these papillae in the intact animal). The fungiform papillae in organ culture are characterized by a distinctive epithelium and large mesenchymal core, and an intact epithelial basement membrane. These cultures demonstrate that fungiform papillae will consistently develop in tongue organ cultures in the absence of intact sensory innervation.

Fig. 1. Scanning electron micrographs summarizing the intrauterine development of rat tongue and fungiform papillae from 13 days of gestation (E13) through 19 days of gestation (E19). Left column: Entire embryonic tongues in situ on the mandible. Right column: High power micrograph of dorsal lingual epithelium to illustrate fungiform papillae. At E13 the two lateral lingual swellings of the anterior embryonic tongue (L) and the central, posterior tuberculum impar (T) are apparent. The caudal border of the anterior, or oral, tongue is marked with an arrow. The dorsal epithelium is relatively homogeneous with no evidence of fungiform papillae. By E14 the tongue has grown considerably and acquired a spatulate shape. The intermolar eminence is now apparent on the back of the tongue (delimited by arrows). Small eminences on the anterior tongue are the first morphological indication of fungiform papillae. The high power micrograph illustrates one of these eminences. The tongue at E15 is much longer, and fungiform papillae are now apparent in rows on the anterior tongue. Two fungiform papillae are included in the high power micrograph. An arrow points to the single circumvallate papilla on the back of the tongue at E15, 16, and 19. By E16 the fungiform papillae are more raised and distinctive on the lingual surface. At E19 the fungiform papillae have assumed a mushroom like shape that protrudes from the surface of the tongue. Scale bars = 1.0 mm in left column, 10 µm in right column.

Whole tongue cultures begun at E14

Control or Day 0 tongues for E14 cultures are well formed, with fused anterior tongue halves and a distinctive intermolar eminence (Fig. 6, Day 0). On some of the Day 0 tongues, small eminences that are the incipient fungiform papillae are observed, as illustrated in Figure 6.

After 1 day in culture the tongue has increased in length, from about 1200 µm at Day 0 to 1400 µm, and fungiform papillae are well formed and distributed in a patterned array on either side of the midline (Fig. 6, Day 1). This is temporally equivalent to E15 in vivo, when well developed fungiform papillae are characteristically observed in intact embryos. Papilla diameter is about 55 µm.

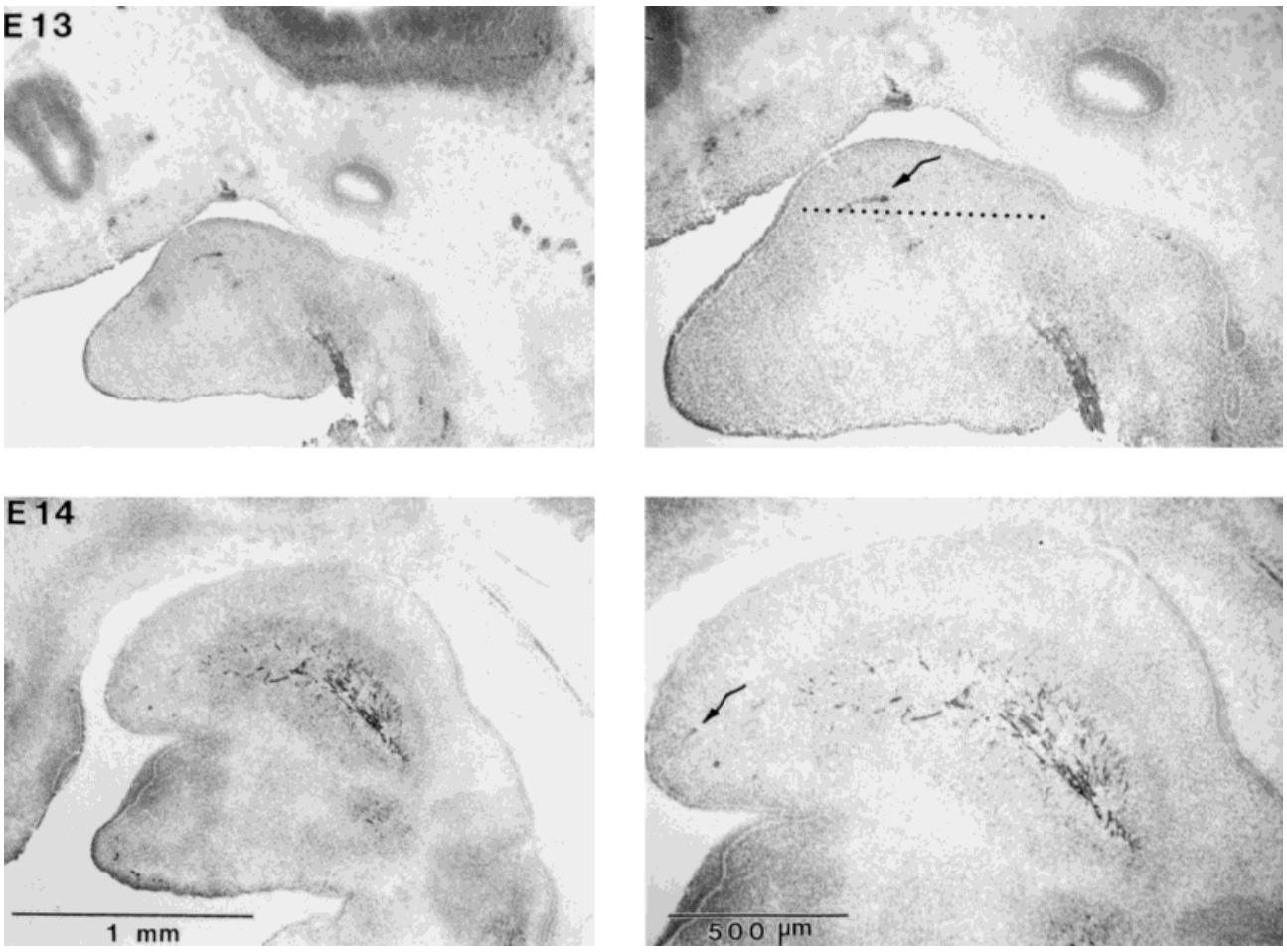

Fig. 3. Light micrographs of embryonic rat tongue in sagittal sections immunoreacted with antibody to neurofilament 200 to examine early lingual innervation. Left column: Low power micrographs; right column, higher power. Top: Section through the lateral portion of the embryonic tongue primordia at E13. In the higher power micrograph, an arrow indicates small nerve bundle at the base of the early tongue. The dotted line demarcates approximate border of tongue tissue from mandible. Bottom: Section through the lateral embryonic tongue at E14. Lingual innervation is apparent throughout the central body of the tongue, but nerves do not yet approach the lingual innervation. In the higher power micrograph an arrow indicates anterior-most extent of nerves. Scale bars at bottom also apply to micrographs above.

There is no appreciable tongue growth after 2 days in culture (Fig. 6, Day 2), but the fungiform papillae remain intact in surface character and distribution. Papilla diameter is apparently smaller compared to Day 1, at about 40 µm, and the interpapillary epithelium appears less smooth.

After 3 days in culture, the fungiform papillae appear more closely spaced and less prominent, apparently due to cessation of tongue growth and further differentiation of the interpapillary epithelium (Fig. 6, Day 3). Tongues after 6 days in organ culture (Fig. 6, Day 6) have an epithelial surface characterized by desquamating cells, similar to the E20 tongue in vivo (see Fig. 2).

Sections of E14 organ cultures at 1 µm demonstrate large, well formed fungiform papillae after 1 and 2 days in culture, and the integrity of the papillae is clearly maintained after 3 days (Fig. 7A). These papillae are similar in light microscopic features to those in E13 cultures at Day 2 (compare with Fig. 5). After 6 days in culture, the E14 tongue epithelium has undergone extensive remodeling and a cornified surface layer is apparent (Fig. 7B). As in E13 cultures, laminin immunoreactivity demonstrates an intact epithelial basement membrane (not illustrated).

In summary, whole tongue cultures initiated at E14 support continued tongue morphogenesis and the development of fungiform papillae in patterned arrays after 1 day

Fig. 4. Scanning electron micrographs of a complete series of embryonic rat tongues cultured at E13. Left column: Whole cultures are illustrated at low power; middle column: the anterior tongue is shown at higher power; right column: high power micrographs of fungiform papilla. **D0:** E13 tongue taken as a control when cultures are established. (Note that this control tongue has not been dissected from the mandible.) Lateral lingual swellings (L) are obvious. The caudal limit of the anterior, or oral, tongue is noted with an arrow in this and subsequent photomicrographs. **D1:** After 1 day in culture the tongue has increased in size and the anterior lingual swellings have fused. Fungiform papillae are not observed on the dorsal surface. **D2:** The tongue has increased in length and numerous fungiform papillae have developed in rows on the anterior tongue. One papilla is illustrated in the high power micrograph. **D3:** Fungiform papillae appear more densely distributed on the anterior tongue because tongue growth has plateaued. General papilla topography is similar to that at D2. **D6:** After 6 days in culture, the lingual surface has an undulating appearance and fungiform papillae are more difficult to discern. Scale bars at bottom apply to micrographs in their column.

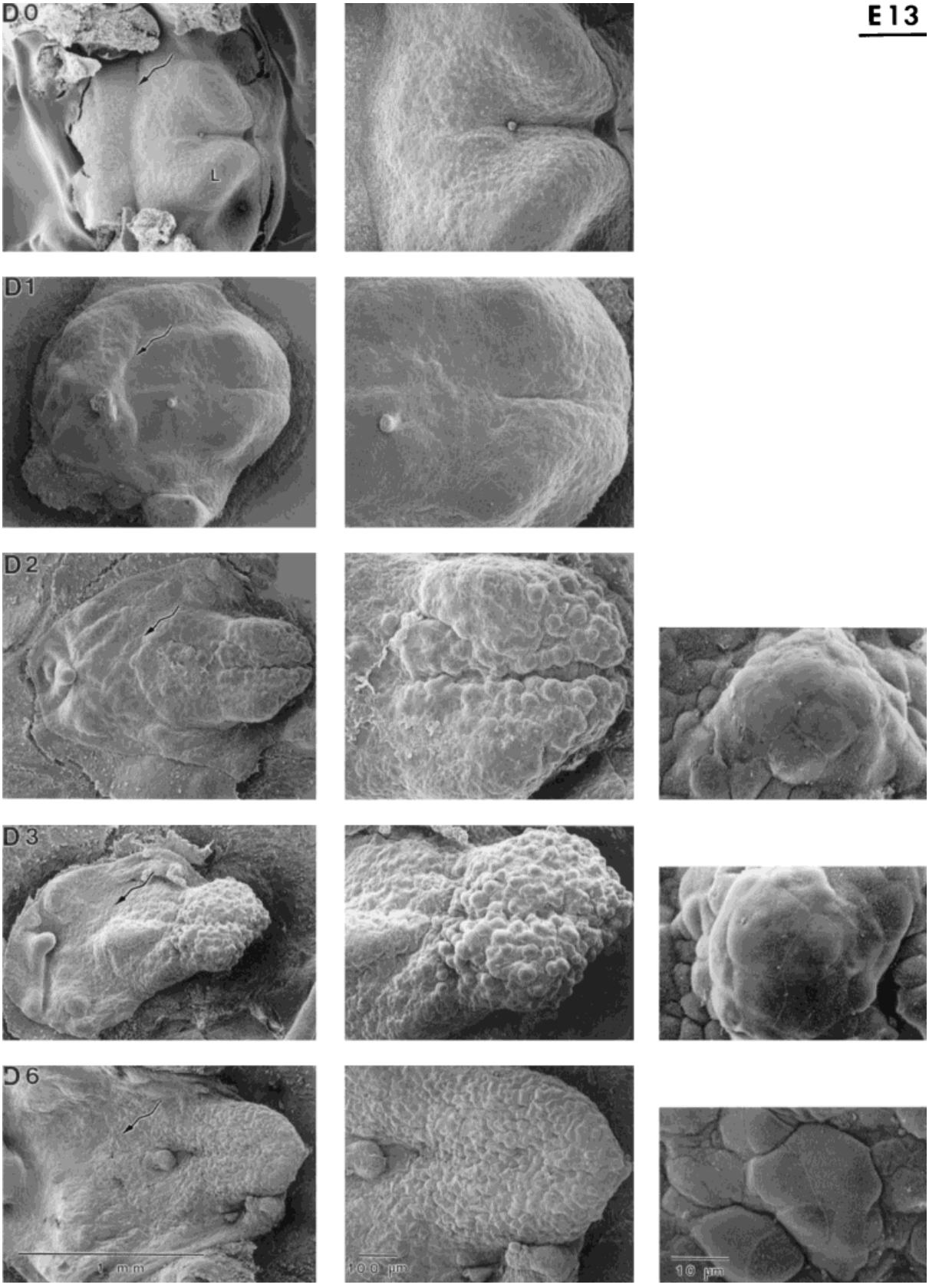

Figure 4

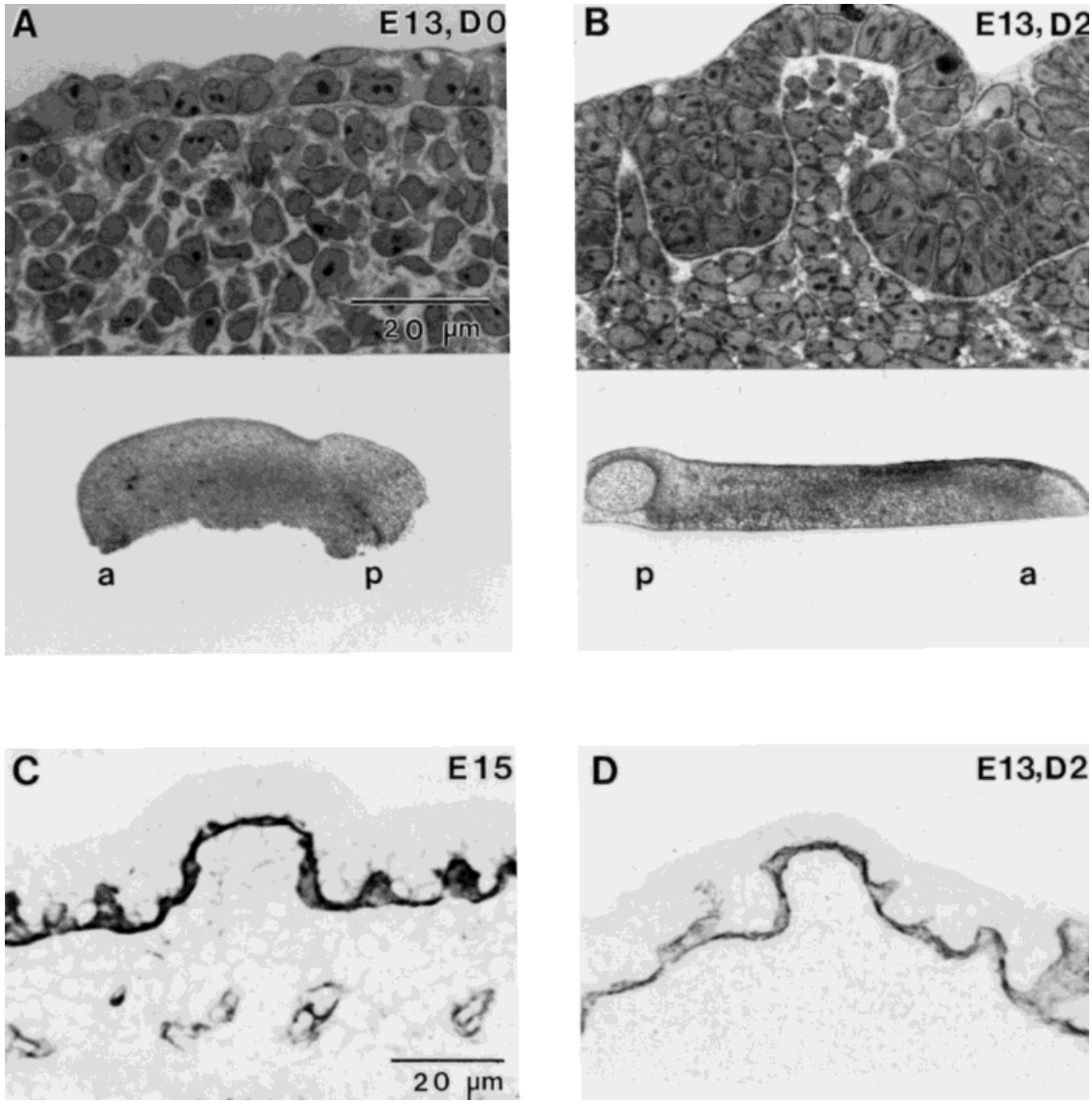

Fig. 5. Light micrographs and immunocytochemistry to illustrate E13 papilla development in culture. **A:** Thin section of the dorsal lingual surface from a control, Day 0 culture, with accompanying low power illustration of the entire cultured tongue (a, anterior and p, posterior tongue). **B:** E13 tongue surface after 2 days in culture, with accompanying low power illustration of the entire cultured tongue. A fungiform papilla is illustrated to demonstrate the well developed papilla epithelium over a broad mesenchymal core. **C,D:** Immunoreactions for laminin in an E15 control tongue, and in an E13 cultured tongue after 2 days (E13, D2), demonstrate an intact epithelial basement membrane in lingual cultures and comparable papilla development in the cultured tongue compared to an "age-matched" control. Scale bar in A applies to B; C applies to D.

in culture, equivalent to E15 in vivo when these papillae typically appear. Papillae are maintained in culture and are large structures characterized by intact epithelia and mesenchymal cells within a broad connective tissue core. As with E13 cultures, the E14 cultures demonstrate gustatory papilla morphogenesis and maintenance in the absence of intact sensory innervation.

Half tongue cultures begun at E14

The presence of intralingual, autonomic neurons in a discrete ganglion has been described in the posterior rat tongue under the circumvallate papilla (Graziadei and Graziadei, 1978). Also, small clusters of ganglion cells are associated with branches of the lingual/chorda tympani nerve complex where it enters the tongue and with branches of the glossopharyngeal nerve (Kuder, 1984). Immunoreactions for NF-200, GAP-43, and ChAT demonstrated a ganglion mass in the posterior tongue region of some of our whole tongue cultures, and in some whole tongue cultures neurites extended within the mesenchyme of the posterior tongue (Fig. 8A). However, the immunoreactivity was generally confined to posterior tongue and never approached the dorsal epithelium and fungiform papillae (Fig. 8A). In fact, neural immunoreactivity was usually not apparent in anterior or posterior tongue in our series (Fig. 8B). Limited sensory innervation that is pre-

sent at E13 and E14 (Fig. 3) degenerates after the tongue is dissected and thereby separated from intact sensory ganglia.

To examine fungiform papilla development in organ cultures that excluded potential neural elements from intralingual ganglia that could remain in posterior tongue, we cultured the transected, anterior half tongue alone. Three experimental series utilizing half tongues, maintained for 3 days in culture, yielded similar results. Data are presented from one of these series with parallel data from companion, "control" cultures of entire tongues.

The control, or Day 0, whole tongue presents a typical E14 tongue topography, with a well developed morphology and small eminences that are the earliest signs of fungiform papillae (Fig. 9, Entire, Day 0). After 1 day in culture, well formed fungiform papillae are distinguished in diagonal rows on either side of the midline furrow (Fig. 9, Entire, Day 1). After 2 and 3 days in culture the fungiform papillae retain a patterned configuration, but appear more closely spaced on Day 3 due to constrained tongue growth (Fig. 9, Entire, Days 2 and 3).

Data from half tongue cultures can be directly compared to the companion, whole tongue cultures in Figure 9. Half tongue cultures from E14 consist of the tongue anterior to the distal edge of the intermolar eminence (Fig. 9, Half, Day 0). As in whole tongues, small raised clusters that are the early fungiform papillae are observed on control, or Day 0, half tongues. After 1 day in culture distinctive fungiform papillae have formed, in the stereotypic patterned array (Fig. 9, Half, Day 1).

To compare numbers of fungiform papillae, we counted papillae at culture Day 1 in the same location on half and whole tongues. Examining the row of papillae just to the left of midline, over a 500 μm distance from the tongue tip, nine papillae were observed in whole, and eight papillae in half tongue cultures (Fig. 9, Day 1, Entire and Half).

After 2 and 3 days in culture, fungiform papillae were maintained quantitatively and qualitatively on half tongues, in a manner directly comparable to whole tongues (Fig. 9, Half, Days 2, 3).

Immunoreactions for NF-200, ChAT, and GAP-43 indicated that half tongue cultures did not have neural elements in association with fungiform papillae (Fig. 10A). Occasional immunoreactive elements were observed (Fig. 10B); however, they were not near the epithelium and were extremely sparse relative to intact tongue (Fig. 10C,D). We consider these neural elements to be of autonomic origin based on their location and appearance.

In summary, cultures of the anterior half of the embryonic tongue, initiated at E14, support fungiform papilla development and continued morphogenesis. Furthermore, the characteristic pattern of fungiform papilla development on anterior tongue is retained. The half tongue cultures provide direct evidence that gustatory papilla develop, continue morphogenesis, and occupy patterned locations in the absence of intact, posterior tongue autonomic ganglia.

DISCUSSION

This study has not only provided a test of the hypothesis that nerves are required for gustatory papilla formation and early morphogenesis, but also has established details of a culture system for the peripheral gustatory organs. Morphogenesis and growth of the dissected, embryonic rat tongue can be supported in a standard organ culture beginning from E13, when the early tongue is first apparent, or from E14, when the tongue is well formed but still morphologically and histologically immature. The culture system excludes intact sensory ganglia and yet supports development of tongue topography, and extensive epithelial and mesenchymal remodeling. To our knowledge these are the first results from cultures of the entire tongue to be described in detail.

Not only is general tongue topography supported in these cultures, but also, gustatory papillae form in a patterned array and grow. Fungiform papillae appear on a culture day equivalent to E15 in vivo; that is, after 2 days for cultures begun at E13 and after 1 day for cultures begun at E14. Because E15 denotes the characteristic timing for gustatory papilla formation in the intact embryo (Mistretta, 1972), our results demonstrate that the molecular and cellular information necessary for timing papilla appearance is retained in vitro. Furthermore, the development of fungiform papillae in organ culture in a stereotypic pattern, in rows on either side of the lingual midline, again indicates that this system mimics in vivo development.

The patterned formation of large numbers of fungiform papillae proceeds in the organ culture in the absence of an intact sensory innervation. In addition, the papillae develop in patterns when the anterior half tongue alone is cultured, which prevents possible inclusion of autonomic ganglia from the posterior tongue. This is direct evidence that taste papillae will form in a patterned distribution and continue to grow, in the absence of intact sensory ganglia or the autonomic ganglia in posterior tongue.

Role of sensory innervation in gustatory papilla formation and morphogenesis

It has been concluded previously that nerves are not required for formation of gustatory papillae, based on early electron microscopic observations documenting appearance of the initial fungiform papilla eminences before nerves approach the lingual epithelium (Farbman, 1965). Other current light microscopic studies, using DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine perchlorate) crystals to label lingual innervation, also suggest that papilla eminences form in the absence of contiguous innervation (Farbman and Mbiene, 1991). In contrast, a recent description of fungiform papilla development in hamster tongue concludes that Lucifer Yellow-labeled fibers of the lingual nerve are already seen in association with fungiform papilla eminences at embryonic day 12 (Whitehead and Kachele, 1994). However, some hamster fungiform papillae at this stage apparently have no nearby nerve fibers and presumably begin to form in the absence of innervation.

Published descriptions of papilla development in vivo, therefore, have not resolved whether nerves are essential for the initial formation of fungiform papillae (Mistretta, 1991; Mistretta and Hill, 1995). Preliminary in vitro data have been reported from slices of dorsal rat tongue epithelium placed in culture at an equivalent of E13 (Farbman and Mbiene, 1991). Although it was concluded that fungiform papillae develop after 2 days in vitro without an intact innervation, only a small number of papillae developed in a random distribution in the cultures, and occasional swollen, sloughing surface cells were reported. This suggests that optimal culture conditions were not yet met.

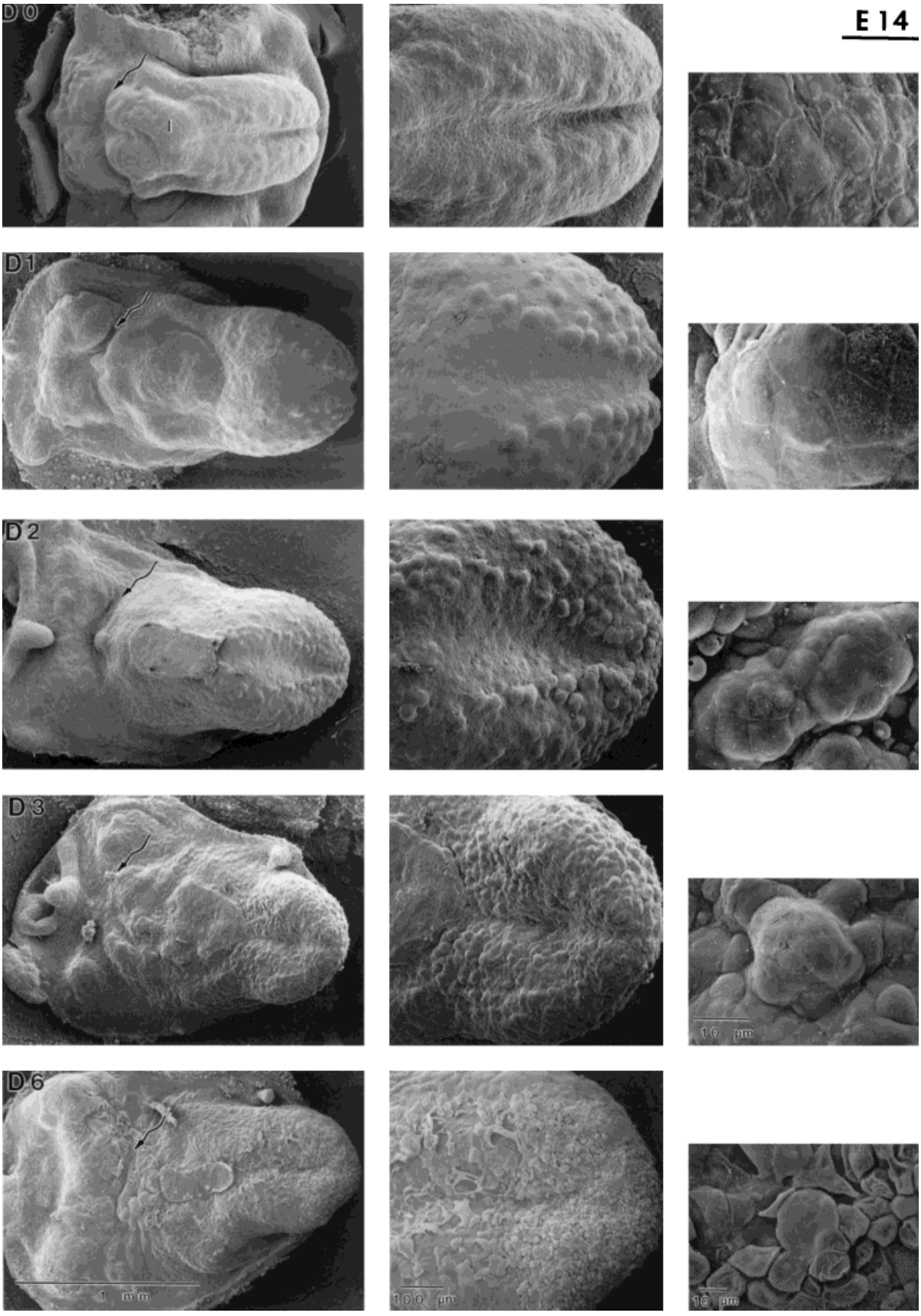

Figure 6

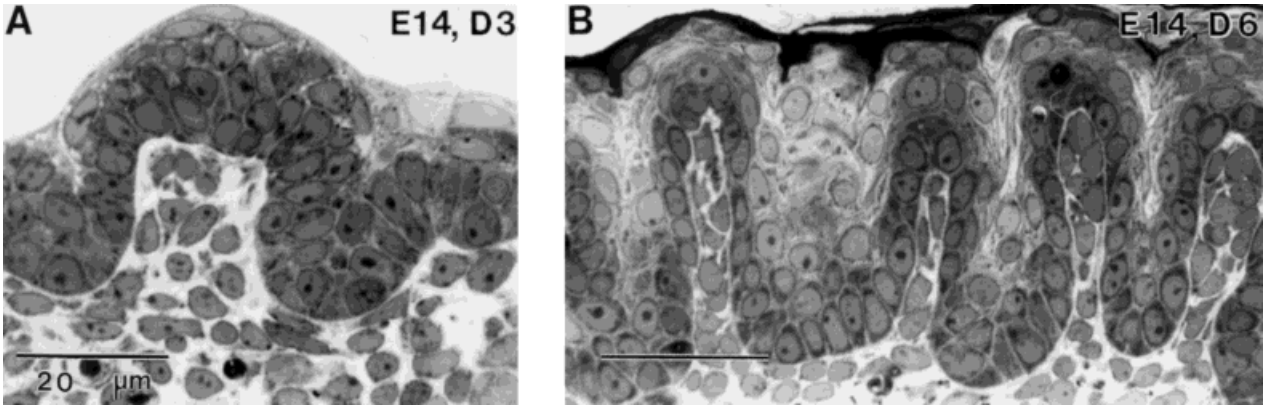

Fig. 7. Light micrographs of fungiform papilla and dorsal tongue epithelium from E14 tongues: **A:** after 3 days in culture (E14, D3); and, **B,** after 6 days in culture (E14, D6). The papilla in A is composed of a multilayered epithelium over a broad mesenchymal core. After 6 days in culture, the tongue epithelium in B demonstrates extensive remodeling and cornified surface layers. Scale bars = 20 µm.

Further, no data were provided on the integrity of the epithelial basement membrane, or on the potential inclusion of neural elements in this study.

Our data demonstrate that an intact sensory innervation is not essential for large numbers of fungiform papilla eminences to form in a stereotypic pattern on the rat tongue. Examination of intact E13 rat tongues, after immunoreactions to label growing terminals, indicates that nerve fibers are within the lateral base of the body of the developing tongue, although not near the dorsal epithelium (Mbiene and Mistretta, 1993). Therefore, we cannot exclude the possibility that a chemical factor is present already at E13 in the rat, and that it diffuses from nerves in basal tongue regions to signal papilla development in the dorsal lingual epithelium. However, it is difficult to imagine how such a diffusible factor would direct the precise spatial formation of more than 100 papillae arranged in rows on only the anterior tongue.

Based on immunocytochemical results in our laboratory (Mbiene and Mistretta, 1993), at E14 the lateral regions of the body of the tongue are innervated, but fibers do not yet approach the lingual epithelium (Fig. 3). Thus, in concert with data from tongue cultures begun at E13, the E14 cultures demonstrate papilla morphogenesis without intact sensory innervation.

In other epithelial appendages, sensory nerves apparently are not essential for initial organ formation. For example, whisker pads from E9 and E10 mouse embryos, cultured in the absence of innervation, generate a patterned array of vibrissa follicles (Andres and Van der Loos, 1983). On the other hand, there are data to support a role for nerves in directing establishment of spatial patterns of vibrissae (reviewed in Holbrook and Minami, 1991).

If sensory nerves do not direct papilla initiation, what factors might play a critical regulatory role? Signals deriving from mesenchymal cells are key in other organ systems. The appearance of hair germs (precursors of hair follicles) in human fetal skin is predated by small, mesenchymal cell clusters at regular intervals under the epidermal basement membrane (Holbrook and Minami, 1991). In developing teeth, a role for the Hox-7 gene, expressed in condensing mesenchyme of the dental papilla, has been proposed (MacKenzie et al., 1991). Furthermore, expression of the Hox-8 gene in the epithelial dental placode suggests participation of this gene in establishing sites of tooth initiation (MacKenzie et al., 1992). Recently, expression of the homeodomain gene Distal-less-3 has been demonstrated in mouse fungiform papillae at E14.5 (Morasso et al., 1995). These genes and those of the PAX family (Kessel and Gruss, 1990; Strachan and Read, 1994) are likely candidates as genetic factors that direct the mesenchymal and epithelial interactions crucial to papilla initiation.

A potential role for extracellular matrix factors in directing gustatory papilla and taste bud morphogenesis has been discussed at length (Mistretta and Haus, 1996), and adhesion molecules have been studied in relation to taste bud (but not papilla) turnover and regeneration (Nelson and Finger, 1993; Smith et al., 1994). Exploration of such factors has not been applied in a culture system.

Although our tongue cultures demonstrate that sensory nerves are not essential for papilla formation, there has

Fig. 6. Scanning electron micrographs of a complete series of embryonic rat tongues cultured at E14. Left column: Whole cultures illustrated at low power; middle column: anterior tongue shown at higher power; right column: high power micrographs of fungiform papilla. **DO:** E14 tongue taken as a control when cultures are established. (Note that this control tongue has not been dissected from the mandible.) The intermolar eminence (I) and caudal border of the anterior tongue (arrow) are noted. Numerous small eminences that represent incipient fungiform papillae are apparent on the anterior tongue. **D1:** After 1 day in culture the tongue has increased in length. Fungiform papillae are now well formed and prominent in rows on either side of the midline. One of the papillae is illustrated at higher power, at right. **D2:** The fungiform papillae are even more distinctive on the lingual surface. Two contiguous papillae are illustrated at right. **D3:** After 3 days in culture the fungiform papillae appear more closely spaced, presumably due to cessation in lingual growth. **D6:** The tongue epithelium is characterized by desquamating cells and fungiform papillae are less readily discerned. Scale bars at bottom apply to the micrographs in their column.

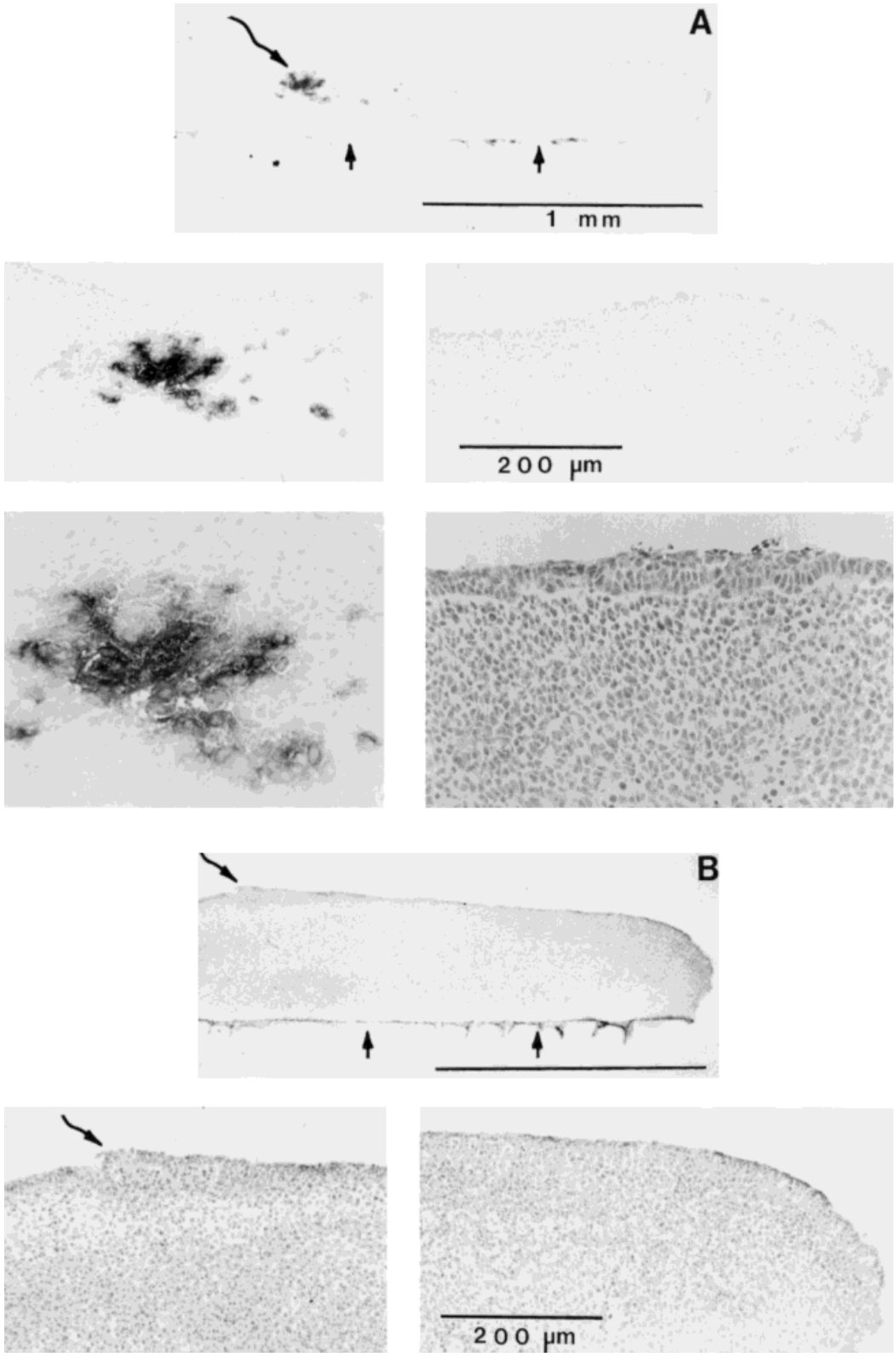

Fig. 8. Light micrographs of immunoreactivity for GAP-43 to illustrate an immunopositive cluster in posterior tongue of some cultures. In all micrographs, the tip of the tongue is oriented toward the right. **A:** E14 tongue after 3 days in culture illustrates a cluster of GAP-43 immunoreactivity in posterior tongue (long arrow in top micrograph). The anterior half of the tongue, however, is devoid of immunoreactivity. Note that some immunoreactive artifacts appear on the filter paper that holds the culture (see two arrowheads). Higher power micrographs underneath illustrate the nature of the GAP-43 immunoproduct in posterior tongue (left) and immunonegative anterior tongue epithelium (right). **B:** E14 tongue after 3 days in culture illustrates absence of GAP-43 immunoreactivity, even in posterior tongue region under the circumvallate papilla (arrow at top of photomicrograph). Arrowheads indicate immunoreactive artifacts along edge of filter paper. Higher power micrographs underneath illustrate posterior tongue epithelium in the region of the circumvallate papilla (left) and anterior tongue (right). (Note: all immunoreacted tissues are lightly counterstained with hematoxylin.)

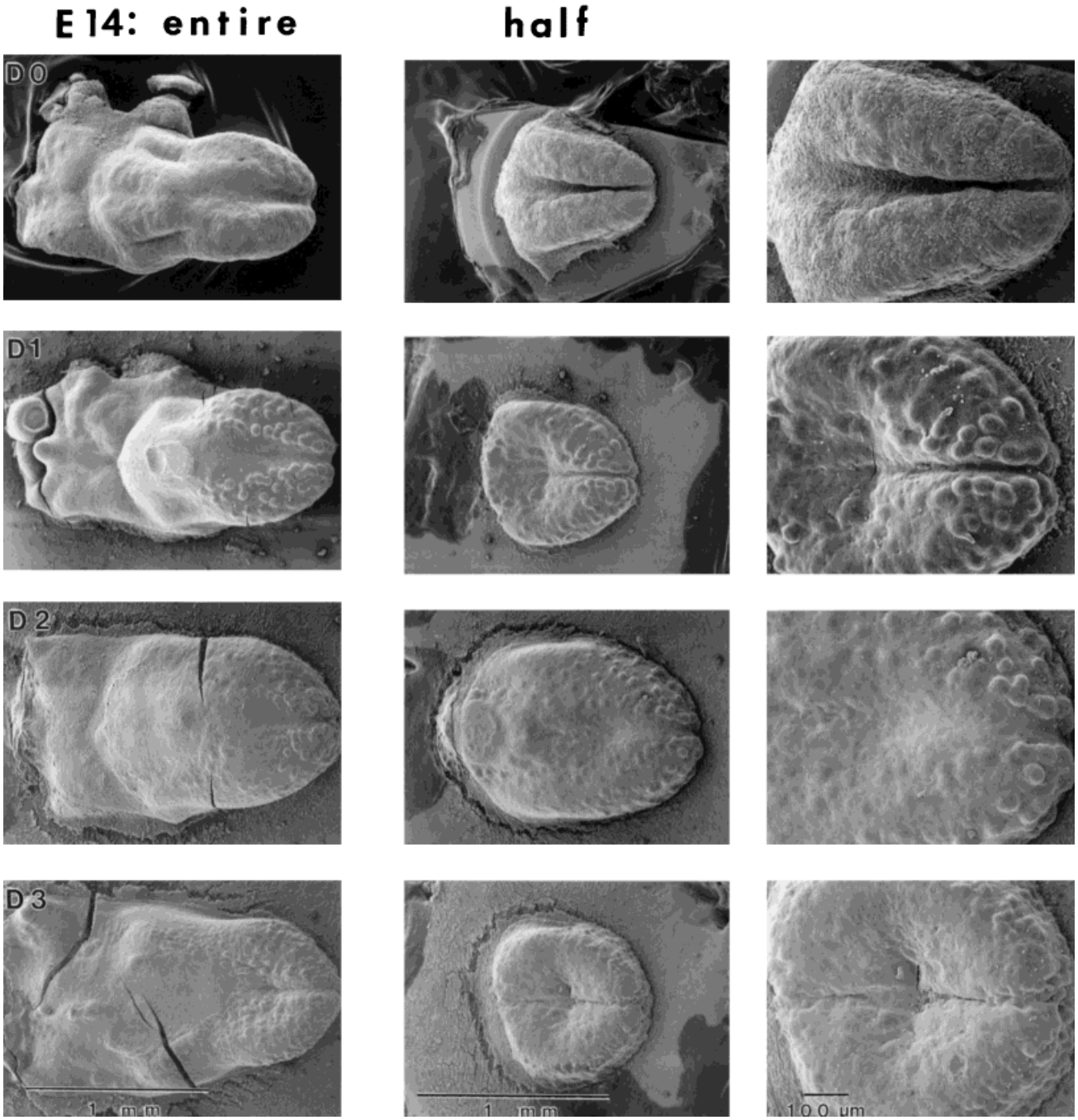

Fig. 9. Scanning electron micrographs of two series of embryonic rat tongues cultured in parallel, beginning at E14. In one series the entire tongue was cultured (E14, entire, left column). In the other series, the anterior half tongue was cultured (E14, half, middle and right columns, low and high power micrographs, respectively). Entire Tongue: DO: The control tongue taken when cultures were established is well formed, with several small eminences on the anterior tongue. D1: After 1 day in culture the fungiform papillae are prominent on the anterior tongue. D2: Prominent papillae continue to characterize the anterior tongue. D3: The fungiform papillae appear more closely spaced. (Note that the transverse clefts at D2 and D3 are artifacts that occurred during mounting of tongues on SEM stubs.) Half Tongue: D0: The control half tongue, taken when cultures were established. D1: After 1 day in culture, fungiform papillae have developed in rows on either side of the midline. Numbers of fungiform papillae on tip of half tongue cultures compare well with those on the entire tongue cultures. D2: Well formed papillae remain numerous on the half tongue cultures. D3: Papillae appear more closely spaced. Scale bars at bottom apply to the micrographs in their column.

been agreement throughout in vivo studies that fungiform papillae are replete with nerves at a time just beyond the eminence stage, when epithelial downgrowths have delineated a distinct connective tissue core for each papilla (Farbman, 1965; Farbman and Mbiene, 1991; Mbiene and Mistretta, 1993; Whitehead and Kachele, 1994). There-

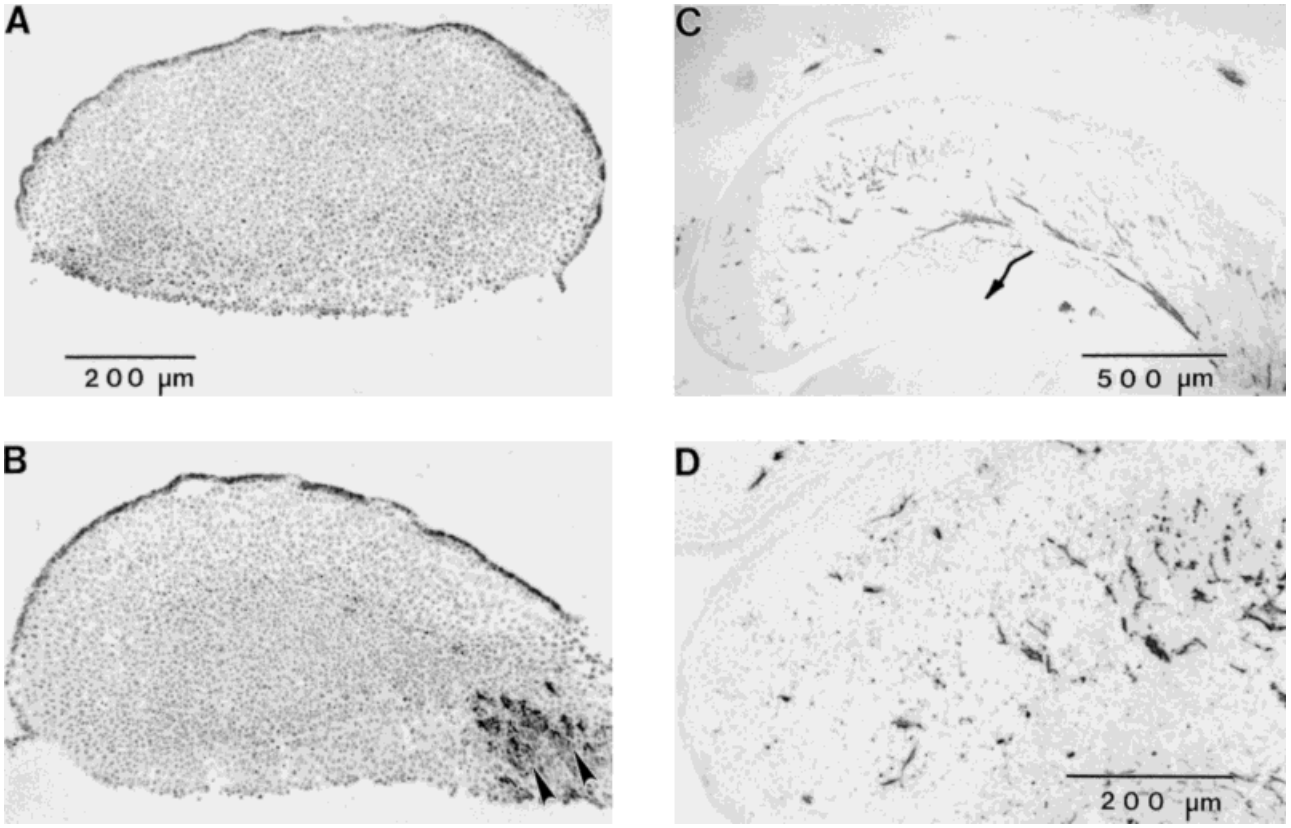

Fig. 10. **A,B:** Light micrographs of immunoreactivity for ChAT in E14 half tongues after 1 day in culture. The tip of the tongue is to the left. Half tongue in A demonstrates absence of immunoreactive neural elements. Cultured half tongue in B illustrates presence of immunopositive elements in posterior-most portion of some cultures (arrowheads at bottom left of figure). (Scale bar in A applies to B, also.) **C,D:** Low and high power light micrographs of immunoreactivity for NF-200, in an intact, embryonic "control" tongue at E15. In contrast to the absence of a neural network in the cultured tongues, extensive lingual innervation is clear in the intact embryo, as demonstrated in this age-matched equivalent to the E14 tongue after 1 day in culture. An arrow demarcates the approximate position at which tongues were cut for half tongue cultures. (Note: all immunoreacted tissues are lightly counterstained with hematoxylin. There is some artifactual immunoreactivity in the surface epithelium of cultures in A and B, where the epithelium has lifted from the slide surface.)

fore, it has been suggested that the fungiform papillae chemically attract their associated innervation (Farbman and Mbiene, 1991), and that nerves have a trophic influence on sustained papilla development (Whitehead and Kachele, 1994).

Cultures of embryonic rat tongues demonstrate that papilla morphogenesis will progress in vitro without sensory innervation, in cultures established at E13 with first papilla formation on culture day 2 (Fig. 4) or in those established at E14 with papilla appearance on culture day 1 (Fig. 6). Papillae mature from early eminences through a stage of distinctive mushroom-shaped morphogenesis. However, after 6 days in culture the fungiform papillae are no longer distinct on the lingual surface, which exhibits extensive epithelial/mesenchymal remodeling characterized by deep epithelial invaginations and tall connective tissue papillae (Fig. 7B). These observations support an essential role for intact innervation in sustaining papilla integrity.

Beyond the issue of sustained papilla integrity in culture is the question of taste bud formation in vitro, which is under active investigation in our laboratory. Taste buds do not develop until E21 in rat tongue (Mistretta, 1972), several days after gustatory papillae first appear. Whereas taste buds will develop in salamander in the absence of innervation (Stone, 1940; Barlow et al., 1996) there are no publications of a direct test of the hypothesis that taste bud induction is neurally dependent in mammals. Brain derived neurotrophic factor (BDNF) is present in rat embryo taste papillae in advance of taste bud formation and has been proposed as a critical tropic molecule in directing innervation to future gustatory regions of the papilla (Nosrat and Olson, 1995). Initial reports of taste bud development in BDNF-null mutant homozygote embryos indicate a quantitative relation between numbers of taste buds and size of gustatory ganglia, and support a role for gustatory innervation in rodent taste bud development if not induction (Mistretta et al., 1996; Oakley et al., 1996).

Autonomic ganglion cells in the posterior rat tongue

Discussions about a potential role for lingual innervation in directing gustatory papilla and taste bud development are usually limited to consideration of the sensory innervation to the tongue: the chorda tympani branch of the facial nerve, the lingual branch of the trigeminal nerve, and the lingual branch of the glossopharyngeal nerve. However, the presence of clusters of autonomic

ganglion cells in the posterior mammalian tongue has been described (Gairns and Garven, 1952; Fitzgerald and Alexander, 1969; Graziadei and Graziadei, 1978; Ferrell and Tsuetaki, 1983; Kuder, 1984). In adult rat, the circumvallate ganglion is composed of about twenty cells near the base of the circumvallate papilla, all with an appearance typical of autonomic neurons (Graziadei and Graziadei, 1978). Other neurons are found within the core of the circumvallate papillae, and in close relationship to serous and mucous glands of the tongue (Gairns and Garven, 1952), but not in the vicinity of fungiform papillae. These circumvallate ganglion cells exhibit no topographical relations with taste buds (Graziadei and Graziadei, 1978). The possibility that these neurons are sensory is ruled out by their pluripolar nature and the presence of numerous synaptic endings on soma and dendrites.

In a study of intralingual parasympathetic ganglia in rat, three locations for ganglion cells were identified (Kuder, 1984). These are around the main duct of the mandibular and sublingual salivary glands; within the mandibular and sublingual salivary glands; and, in the trunk, and occasionally in the apex, of the tongue. Most cells and clusters of cells are within the first two locations; within the tongue, cells are very dispersed, and are in relation to blood vessels and branches of the lingual/chorda tympani nerve. In addition, other investigators have described the occasional appearance of cholinesterase positive ganglion cells in the apex of the rat tongue (Kuder, 1984) and in the anterior tongue region, at the periphery of fungiform papillae (Purwar, 1974). In the latter report it is not possible to determine the frequency of this observation or the precise location of the cells. In general, across various species, it is suggested that the intralingual ganglion cells function as a parasympathetic supply to posterior tongue glands and the lingual vasculature (Fitzgerald and Alexander, 1969; Graziadei and Graziadei, 1978; Kuder, 1984; Hino, et al., 1993).

To eliminate a possible regulatory role of various posterior tongue ganglion cells in fungiform papilla formation, for example through release of some diffusible factor, we cultured E14 half tongues that included only the anterior lingual aspect, distal to the intermolar eminence. Because fungiform papillae developed in comparable numbers and patterns to those in whole tongue cultures, we can exclude a role for the posterior tongue, autonomic ganglion cells in fungiform papilla formation and morphogenesis. However, we cannot exclude absolutely the presence of occasional anterior tongue autonomic cells in the half tongue cultures. It is unlikely, though, that these cells would exert any influence on the stereotypic formation of numerous fungiform papillae in culture.

General viability of cultures

We have cultured the entire embryonic rat tongue for 6 day periods beginning at E13 or E14 (Figs. 4, 6). Throughout this period epithelial and mesenchymal cell viability is maintained. Fungiform papillae are less distinct on the tongue at culture day 6 than on previous days; however, day 6 would be equivalent to E19 or 20 in vivo, a time when surface cells in the dorsal lingual epithelium desquamate to uncover the nongustatory filiform papillae on anterior tongue (Mistretta, 1972; Fig. 2). Thus, the dorsal surface of cultured tongues after 6 days resembles the lingual surface at a comparable age in vivo.

However, our light microscopic observations indicate that large numbers of fungiform papillae are not sustained at the end of the culture periods, and indeed, by 3 days in culture, overall tongue growth has plateaued. Therefore, a number of hypotheses should be considered in relation to long term papilla maintenance. Sensory innervation might be essential for maintenance and later stages of morphogenesis of fungiform papillae in rat tongue. It also is possible that mesenchymal factors participate in initiation and continued morphogenesis of papillae, and that such factors are either no longer present after 6 days in culture or are no longer effective in supporting papilla development. Other factors also may be crucial to sustain tongue growth in culture, for example extracellular matrix molecules such as hyaluronan that maintain tissue macrostructure (Sherman et al., 1994).

Our current study has emphasized morphogeneis of fungiform papillae on the cultured embryonic tongue, because these multiple papillae develop in a pattern. The single, posterior, circumvallate papilla also forms in the embryonic tongue cultures and is apparent in Figures 6 (Day 2) and 8 (Day 3). The epiglottis, an extralingual structure, can also develop in our system. We note, however, that there is not a robust, consistent development of the circumvallate papilla, comparable to fungiform papillae, and we are investigating factors relating to morphogenesis of the posterior tongue papillae in other experiments.

SUMMARY AND CONCLUSION

The data presented here on cultures of the entire embryonic tongue are to our knowledge the first detailed study of lingual and gustatory papilla development in vitro. Robust, reproducible development of fungiform papillae in stereotypic rows is supported whether cultures are established at E13, when the lingual swellings are still apparent and there are no surface or cellular indications of papillae, or at E14 when the tongue is well formed and incipient papillae are apparent on some tongues. Because the dissected whole tongue is cultured, or the anterior half tongue only, the cultures clearly exclude an intact sensory innervation and an intact autonomic, circumvallate ganglion or other posterior tongue, autonomic ganglion cells. This is direct evidence that papillae will form and undergo further morphogenesis in vitro without neural ganglion support.

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