

The Brief Notes section of this issue is printed by photolithography.

DIFFERENTIATION OF TASTE BUDS IN ORGAN CULTURE

ALBERT I. FARBMAN. From the Department of Anatomy, Northwestern University, Chicago, Illinois 60611

INTRODUCTION

It is well established that the structural integrity of mammalian taste buds is dependent on an intact innervation. This is based on the following lines of evidence. Experiments in which gustatory nerves were severed resulted in degeneration of taste buds (5, 8, 9, 14, 17). If gustatory nerves were allowed to regenerate into the areas they previously innervated, taste buds reappeared (8, 10, 18); usually this occurred only under the influence of gustatory nerves, and not in response to other sensory or motor nerves (10, 18).

During development, taste bud cells begin to differentiate only after nerves have grown into the epithelium (4). In transplant experiments fragments of embryonic tongue grafted into the anterior chamber of the eye grew and differentiated in essentially the same way as the normal tongue *in situ*, but taste buds did not appear because there was no innervation (6, 15).

The nature of the interaction between the developing or regenerating nerve and the presumptive taste bud epithelium is not known. The preliminary experiments described in this report were begun in order to develop a model system in which taste bud development could be manipulated and studied in more detail, with the ultimate aim of characterizing, on a molecular basis, the factors responsible for the differentiation of oral epithelial cells into gustatory receptors.

MATERIALS AND METHODS

In these studies two different strains of rats were used, a Long-Evans strain (from stock rats bred and maintained at the Strangeways Laboratory, Cambridge,

England) and a strain of Sprague-Dawley albinos (from Holtzman Company, Madison, Wis.). After pilot studies on 14–20-day fetuses, the 16–17 day fetus was selected for the experiments described in this report. 12 pregnant females in day 16 or 17 of gestation were killed by ether overdose or by crushing the cervical vertebrae. Fetuses were removed under sterile conditions, decapitated, and the mandible was dissected from the head. A small piece of tongue about 2 mm square and 0.5 mm thick, including the circumvallate papilla and its immediate surroundings, was excised and stored briefly at room temperature in the medium to be used for incubation.

The cranial cavity of each fetus was opened and the brain removed. The floor of the cranium was bisected in the sagittal plane. In each half, the foramen through which the IX and X cranial nerves pass was located in the floor of the posterior cranial fossa. With the point of a cataract knife, a cut was made in the cranial base beginning at the foramen and continuing medially and laterally. By separating the two fragments, the IX and X cranial ganglia were exposed, dissected out, and placed in the incubation medium at room temperature.

Preparation of Culture Vessels

Two glass Falcon dishes (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) were enclosed in a 100 mm Petri dish containing three to four sheets of sterile filter paper moistened with 5–10 ml of sterile saline. Each Falcon dish contained a platform, 1 cm square, of stainless steel grid raised about 2 mm above the bottom of the dish. About 1.5 ml of medium was dropped into the culture vessel so that its level reached the surface of the grid platform. Two or three ganglia were placed on a piece of Millipore filter (Millipore Corporation, Bedford, Mass.) and the tongue fragment was de-

posited on top of the ganglia so that the epithelial side was uppermost and the mesenchyme side in contact with the ganglia. The filter was then laid on the platform. Control explants included tongue fragments without ganglia. A total of 75 cultures (60 tongue and ganglia and 15 controls) were done in these experiments.

Preparation of Medium

Several media and additives were tried; the most successful was made up as follows. The basic medium consisted of 50% Waymouth's medium 752/1 (to which NaHCO_3 was added) and 50% newborn calf serum; this was enriched by 15 mg % ascorbic acid, 750 mg % glucose (final concentration, including the glucose in Waymouth's medium), penicillin (50 units/ml), and streptomycin (50 $\mu\text{g}/\text{ml}$). In addition, small amounts of a crude, distilled water extract of male mouse submaxillary gland, containing nerve growth factor (2), were added. The total protein in this extract was estimated, and the extract was added to the medium in concentrations of 2–9 μg total protein per ml of medium. The Petri dishes were placed in a modified anaerobic jar, gassed with a mixture of 60% O_2 , 5% CO_2 , and 35% N_2 , and incubated at temperatures varying between 35° and 37°C.

The explants were examined every 2–3 days, the medium was changed, and the cultures were gassed as above.

After periods varying from 7 to 15 days, all explants were fixed in Zenker's acetic acid or Bouin's fluid and processed for routine paraffin sectioning. Sections were cut at 7–8 μ and stained with hematoxylin and eosin.

RESULTS

At the time of explantation, the circumvallate papilla was clearly visible on the tongue surface with the aid of a dissecting microscope. Histologically, the papilla was covered with two to three layers of epithelial cells (Fig. 1), but no taste buds were present; the absence of taste buds had been confirmed in other studies (15), and in this laboratory with the electron microscope (7). The core of the papilla consisted of cellular mesenchyme, within which bundles of nerve fibers were seen. Proliferation of epithelium from the bottom of the trench into the mesenchyme indicated the early formation of von Ebner's glands, and, deep to the papilla, muscle cells were seen as elongated tubes with central nuclei, but with little or no cross-striation.

Most of the explants grew well for up to 15 days. The epithelium thickened and underwent

parakeratosis or keratinization, and, in some areas, grew down into the mesenchyme to form patent ducts and actively secreting glands (Fig. 2); this occurred whether or not ganglia were added to the tongue explants.

Of particular interest in the epithelium were the small, discrete groups of elongated epithelial cells that stained differently from the surrounding epithelia (Figs. 3 and 4). The long axis of these cells was perpendicular to the surface, in contrast to the squamous epithelial cells, and their cytoplasm was pale in sections stained with hematoxylin and eosin; their general shape often resembled that of developing taste buds, and sometimes what looked like a definite taste pore was distinguishable. These taste bud-like structures were seen in nine of the 60 explants of tongue combined with ganglia, but not in those of tongue alone. The number of such structures in a single explant varied from one to six.

The connective tissue in the explants grew well, but muscle cells did not differentiate in the usual way. Instead, multinucleate giant cells of amorphous shape were present; these contained filamentous eosinophilic cytoplasm and no detectable sarcomeres (Figs. 2, 3, and 4). They were thought to be abnormal muscle cells. These multinucleate cells appeared in explants from 16–17-day embryos, but not in those taken from older embryos; in the latter, only necrotic muscle cells were seen.

After several days in culture, many of the neurons in the combined explants contained eosinophilic cytoplasmic zones, some of which were as large as the neuronal nuclei, and some cells died, but the outgrowth of nerve fibers from the surviving cells was usually extensive.

DISCUSSION

The epithelial structures described above have been identified as taste buds on the basis of their histological similarity to normal fetal taste buds. The fact that they appeared only in those cultures containing the added cranial ganglia indicates that the innervation is required for the differentiation of taste buds, as in vivo. Despite the fact that the neurons were not in very good condition and several died, they retained the capacity to induce taste bud differentiation in the tongue fragments. This supports Zalewski's experiments (19) showing that damaged neurons retained the capacity to induce the differentiation of taste buds in vivo.

The major significance of these preliminary

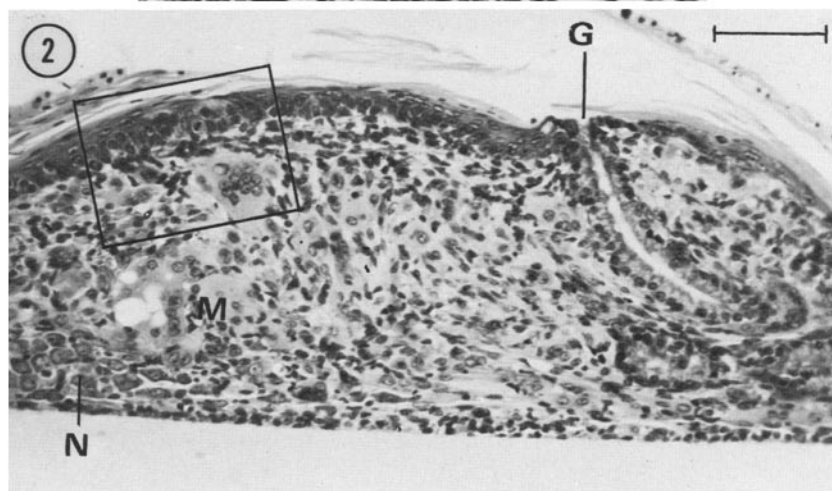
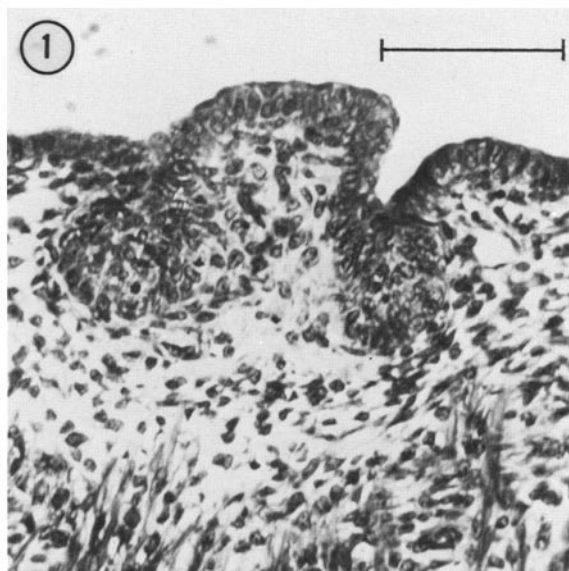


FIGURE 1 Photomicrograph of a section through the circumvallate papilla of a 16 day normal fetal rat tongue. Marker, 100 μ . \times 240.

FIGURE 2 Photomicrograph of a section through a combined explant of tongue and ganglia grown for 8 days in organ culture. A patent duct and glandular tissue are seen on the right side (*G*). Within the boxed area are located three taste buds (enlarged in Fig. 3). Note ganglion cells (*N*) in the lower-left corner of the field. Multinucleate giant cells are also seen (*M*). Marker, 100 μ . \times 150.

studies is that the highly specialized taste bud cells can be induced to differentiate in organ culture under the influence of explanted ganglion cells. Presumably, the influence is mediated by fibers that grow out from the neurons and innervate the epithelium, as has been shown *in vivo* (4). It is likely that only a small number of the total population of neurons from the IX and X cranial

ganglia is usually concerned with taste bud innervation, and these may be the only ones capable of inducing taste bud differentiation. The small number of taste buds that differentiated in these experiments could be explained by a low probability of fibers reaching tongue epithelium under the conditions used. It should be pointed out, however, that because no effort was made to disclose

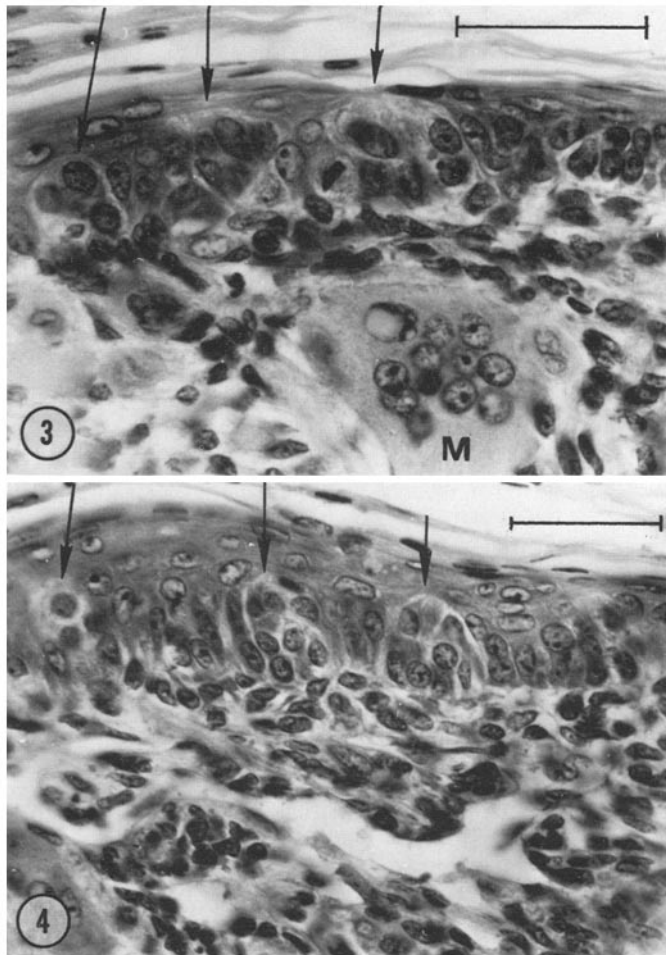


FIGURE 3 The boxed-in area in Fig. 2 at a higher magnification. The three taste buds are conspicuous (arrows); the one on the right has an apical pore. A mitotic figure is adjacent to it on the left. Note multinucleate cell (*M*) in the mesenchyme. Marker, 50 μ . \times 500.

FIGURE 4 Photomicrograph of a combined explant of tongue and ganglia grown for 8 days in organ culture. Several taste buds (arrows) are seen. Marker, 50 μ . \times 200.

the nerve pathway, it is not certain that nerve processes actually reached the epithelium. Experiments are now under way to attempt to increase the numbers of taste buds induced. Other methods will be used to determine whether contact between nerve processes and epithelial cells must occur before taste buds differentiate. It is hoped that the organ culture method can be used as a model system to study taste bud differentiation. Such an *in vitro* model system can be easily manipulated and makes it possible to study problems relating to the nature and specificity of the nerve-cell interaction during taste bud development. More-

over, this system, along with those devised by others to investigate the formation of neuromuscular junctions *in vitro* (1, 3, 11, 12, 13, and 16), permit the study in depth of fundamental problems concerning the roles of induction and cell recognition in development.

This work was begun while I was a Visiting Scientist at the Strangeways Research Laboratory, Cambridge, England. I am deeply indebted to Dame Honor B. Fell, former Director of that laboratory, for her helpful advice and excellent suggestions during the experiment and in preparation of the manuscript. I am

grateful also to Mrs. Christine L. Yang for expert technical assistance.

Dr. Farbman is a recipient of a Research Career Development Award from the United States Public Health Service, No. K 3 DE-17,654.

This study was supported by a National Institute of Health Grant, No. NS-06181 and a grant from the Chicago Dysautonomia Society.

Received for publication 26 August 1971, and in revised form 8 October 1971.

REFERENCES

1. BORNSTEIN, M. B., H. IWANAMI, G. M. LEHRER, and L. BREITBART. 1968. Observations on the appearance of neuromuscular relationships in cultured mouse tissues. *Z. Zellforsch. Mikrosk. Anat.* **92**:197.
2. COHEN, S. 1960. Purification of a nerve-growth promoting protein from the mouse salivary gland and its neuro-cytotoxic antiserum. *Proc. Nat. Acad. Sci. U. S. A.* **46**:302.
3. CRAIN, S. M. 1968. Development of functional neuromuscular connections between separate explants of fetal mammalian tissues after maturation in culture. *Anat. Rec.* **160**:466.
4. FARBMAN, A. I. 1965. Electron microscope study of the developing taste bud in rat fungiform papilla. *Develop. Biol.* **11**:110.
5. FARBMAN, A. I. 1969. Fine structure of degenerating taste buds after denervation. *J. Embryol. Exp. Morphol.* **22**:55.
6. FARBMAN, A. I. 1971. Differentiation of foetal rat tongue homografts in the anterior chamber of the eye. *Arch. Oral Biol.* **16**:51.
7. FARBMAN, A. I. 1972. Development of the taste bud. In *Handbook of Sensory Physiology*. L. M. Beidler, editor. Springer-Verlag, K. G., Berlin. In press.
8. GRIFFINI, L. 1887. Sulla riproduzione degli organi gustatorii. *Rc. Ist. Lomb. Sci. Lett.* **20**:667.
9. GUTH, L. 1957. The effects of glossopharyngeal nerve transection on the circumvallate papilla of the rat. *Anat. Rec.* **128**:715.
10. GUTH, L. 1958. Taste buds on the cat's circumvallate papilla after reinnervation by glossopharyngeal vagus and hypoglossal nerves. *Anat. Rec.* **130**:25.
11. NAKAI, J. 1969. The development of neuromuscular junctions in cultures of chick embryo tissues. *J. Exp. Zool.* **170**:85.
12. PETERSON, E. R., and S. M. CRAIN. 1968. Reinnervation of denervated mammalian skeletal muscle in vitro. *Anat. Rec.* **160**:408.
13. SHIMADA, Y., D. A. FISCHMAN, and A. A. MOSCONA. 1969. Formation of neuromuscular junctions in embryonic cell cultures. *Proc. Nat. Acad. Sci. U. S. A.* **62**:715.
14. TORREY, T. W. 1934. The relation of taste buds to their nerve fibers. *J. Comp. Neurol.* **59**:203.
15. TORREY, T. W. 1940. The influence of nerve fibers upon taste buds during embryonic development. *Proc. Nat. Acad. Sci. U. S. A.* **26**:627.
16. VENERONI, G., and M. R. MURRAY. 1969. Formation *de novo* and development of neuromuscular junctions *in vitro*. *J. Embryol. Exp. Morphol.* **21**:369.
17. VON VINTSCHGAU, M., and J. HONIGSCHMIED. 1877. Nervus Glossopharyngeus und Schmeckbecher. *Arch. Gesamte. Physiol. Menschentiere.* **14**:443.
18. ZALEWSKI, A. A. 1969. Combined effects of testosterone and motor, sensory, or gustatory nerve reinnervation on the regeneration of taste buds. *Exp. Neurol.* **24**:285.
19. ZALEWSKI, A. A. 1970. Continuous trophic influence of chromatolysed gustatory neurons on taste buds. *Anat. Rec.* **167**:165.