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ON THE SPECIFICATION OF TASTE NEURONS IN THE RAT TONGUE

BRUCE OAKLEY

Department of Zoology, The University of Michigan, Ann Arbor, Mich. 48104 (U.S.A.)

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SUMMARY

It is known in mammals that regenerating taste fibers will reform, innervate and maintain new taste buds in characteristic tongue locations—the taste papillae. The ability of non-taste sensory and motor nerve fibers (auriculo-temporal, mylohyoid and hypoglossal nerves) to substitute for a taste (IXth) nerve was evaluated by whole nerve recording and anatomical examination of experimentally innervated rat tongues. The non-taste nerve endings grew into the tongue and were physiologically responsive to cooling and to localized mechanical stimulation of the foliate and circumvallate taste papillae on the tongue. However, unlike the control regenerated IXth nerve these non-taste nerves were unable to (1) reform new taste buds, or (2) innervate, and (3) maintain existing taste buds. It was suggested that taste fibers have physico-chemical properties which permit interaction with groups of specialized cells in the dorsal epithelium of the tongue (taste receptor cell precursors) by a process involving recognition between matched taste fiber and nascent receptor cell.

INTRODUCTION

The developmental factors which govern the formation of a primary afferent fiber's connections are poorly understood. For the analysis of this problem the mammalian taste system has several advantageous features. Among these is the peripheral location of the first synapse—it lies in the tongue between the receptor cells (differentiated from epithelial cells) and the primary afferent fiber. In addition the adult taste system appears to maintain considerable plasticity. Not only do fetal taste buds apparently form under the trophic (nutritive) influence of innervating nerve fibers[5], but also a similar formative influence of taste nerve fibers is readily demonstrable in adult mammals. When adult taste fibers are cut, the taste buds rapidly degenerate, yet the buds readily reform from precursor epithelial cells as the taste nerve fibers grow back and reinnervate the taste papillae[8,11,21]. Moreover, the continual turnover

of adult taste receptor cells (10- to 12-day life span)[2,4] suggests that even with normal undisturbed innervation in the adult, new taste receptor cells are continually developing and differentiating under the formative influences of the innervating nerve fibers.

An initial step in analyzing the rules of fiber–receptor cell connectivity in the taste system is to determine whether particular fibers are specified or 'labeled' as taste neurons prior to innervation of the taste papillae. For example, taste fibers may have unique physico-chemical properties that permit their interaction with nascent receptor cells or they might be distinguished from other nerve fibers only by their ability to grow into taste papillae, *e.g.*, foliate and circumvallate. Or taste fibers might become taste fibers simply because in the normal sequence and timing of developmental events they are the first to arrive at the papillae where taste receptor cells are located. Such speculations on the specification of sensory neurons must be subjected to experimental analysis. Anatomical studies in mammals have reported an absence of trophic effects of skin sensory (auriculo-temporal) or tongue motor (hypoglossal) fibers upon taste buds[6,16]. The functional status of these fibers, including the possibility of functional innervation of taste buds, was not studied. Histological demonstrations of the presence of fibers within the taste papillae cannot, of course, rule on the physiological status of non-taste fibers in this foreign epithelium. Furthermore, the fibers observed in a papilla of an experimentally innervated tongue could be new growth of intrinsic tongue fibers disturbed by the invasion of a test foreign nerve. It is helpful to have electrophysiological recording to clarify these problems. Certainly, the experimental nerves must display a general capacity to regenerate physiologically reactive endings in the tongue before we can attribute to them specific defects in taste function.

In the present experiments, electrophysiological recording was used to evaluate the functional properties of non-taste fibers which grew into the tongue. In addition, the search for non-taste fibers which might have latent but normally unexpressed taste capacities was broadened to include motoneurons foreign to the tongue, *i.e.*, motoneurons of the mylohyoid nerve, which innervate the mylohyoideus, digastricus and transversus mandibularis muscles. The general physiological status of the non-taste nerves was evaluated by recording action potential discharges to cooling and to mechanical stimulation of the tongue. In this manner it was possible to determine that the non-taste fibers could functionally innervate papillae normally associated with taste buds. The specific taste status was determined by examining the ability of non-taste fibers: (1) to form new taste buds, (2) to maintain existing taste buds, or (3) to connect functionally with existing taste buds.

METHODS

Operative procedures

Female Sprague–Dawley albino rats were anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.) for initial nerve suturing and subsequent operations. In 3 experimental groups of 8 rats each, the right IXth nerve was cut near its

entry into the posterior lacerated foramen and the central (proximal) portion avulsed. The distal portion of the right IXth nerve was then joined by a miniature suture[9] to the central portion of one of the following 3 nerves: hypoglossal (XII), (tongue motor); mylohyoid (M-H), (mixed); auriculo-temporal (A-T), (sensory). Owing to bilateral innervation, 88% of the circumvallate papilla taste buds were maintained solely by the left IXth nerve[7,15]. Consequently, if the non-taste nerves from the right side successfully regenerated into the tongue, they would encounter numerous taste buds in the circumvallate papilla. This permitted assessment of the ability of the non-taste nerves to make functional connections with existing circumvallate buds or to reform right foliate taste buds. In 8 control rats the right IXth nerve was cut and sutured together again (control regeneration). Three weeks prior to electrophysiological and histological analysis (which occurred 14–18 months post-suturing), 4 of the 8 rats in each group underwent a second operation in which the left IXth nerve was cut. By this means it was possible to determine whether the non-taste nerves, native and foreign to the tongue, could maintain existing circumvallate taste buds by themselves even if they were unable to reform them *de novo* in the right foliate papillae. At the time of the left IXth nerve transection the initial operative field was reexamined and cleared of possible regrowth from avulsed right IXth nerve fibers in the experimental rats. Only one case of unwanted regrowth was encountered.

Electrophysiological recording

Fourteen to 18 months after nerve suturing the experimental and control rats were reoperated under sodium pentobarbital anesthesia to expose the regenerated nerves for electrophysiological recording. Electrophysiological records were also taken from a minimum of 4 normal rats for each of the following normal nerves: IX, M-H, A-T, XII and lingual (without the chorda tympani). Four of the 24 experimental rats died before recording (2 A-T, 1 M-H, 1 XII). Flaxedil and artificial respiration were routinely employed in conjunction with pentobarbital anesthesia to prevent reflex movements during stimulation. In each case the regenerated nerve was cut central to the suture, the sheath stripped back and the bare whole nerve laid on a pair of 120-μm diameter nichrome or stainless steel wire recording electrodes. In most cases additional records were taken distal to the suture as well. Impulses recorded with these wire electrodes were fed push-pull into a Grass P-511 preamplifier whose single sided output led to a monitoring audio system, an oscilloscope, and a Grass electronic summator with a 0.5-sec time constant. The summator output was recorded by a Grass polygraph inkwriter.

The mechanical stimulus for the skin or tongue was a glass rod with a fine beaded tip. The thermal stimulus was distilled water of varying temperatures applied through a gravity flow system. A small glass tube directed the flow of water onto the skin or tongue. Responses of the normal M-H and A-T nerves to mechanical and thermal stimulation were taken under 3 conditions: (1) normal skin, (2) shaved skin, and (3) bare skin produced by a 5-min application of a depilatory. This application of 'Neet' (5% calcium thioglycolate; see ref. 13), was sufficient to remove the hairs down to the top of the hair canal. A thermocouple connected to one polygraph

channel continuously monitored the temperature of the water to the nearest 0.25 °C. The initial tissue temperature was constant to ± 10% for any given rat and ranged from 29 to 33.5 °C among rats.

Chemical stimuli were applied through the same gravity flow system and warmed to 30 °C to prevent responses to cooling. Each nerve was tested with the following sequence of chemicals: 0.3 *M* NH_4Cl, 0.3 *M* NaCl, 0.3 *M* KCl, 1.0 *M* sucrose, 2.0 *M* glycerol, 0.3 *M* NH_4Cl, 0.01 *M* Na saccharin, distilled water control, 0.01 *M* QHCl (quinine hydrochloride, M.W. 396.91), citric acid (pH 2.5), 0.3 *M* NH_4Cl. Stronger saccharin, quinine, and chloride solutions were occasionally used. Hairy skin was either shaved or depilated before chemical testing.

Histological analysis

At the end of the electrophysiological recording session the rat was perfused first with Ringer's solution, and then with 10% formalin, and the tongue placed in Heidenhain's Susa solution for 48 h. After paraffin embedding, complete 10 μm serial, horizontal sections of the posterior tongue were mounted on slides and stained with Heidenhain's iron hematoxylin. Each section was then examined for taste buds.

RESULTS

Adequacy of regeneration: mechanical and thermal stimuli

Responses to mechanical and thermal stimulation provided proof of the functional viability of the foreign nerves in the tongue; 5 of 6 A-T (auriculo-temporal) nerves, and 7 of 7 M-H (mylohyoid) nerves responded.

Cooling of the shaved or depilated skin produced summated responses in the normal A-T (Fig. 1A) and M-H nerves. Similarly, the normal M-H (Fig. 1F) and A-T nerves responded vigorously to mechanical stimulation of the hairy skin. In mapping the normal cutaneous receptive fields of the A-T and M-H nerves (Fig. 1), it was found that mechanical sensitivity did not extend into the mucous membrane of the mouth or lip. In nerve regeneration the tongue was tested by touch at 3 specific sites: at the posterior folds of the foliate papillae, at a non-taste region of the tongue located midway between circumvallate and foliate papillae, and at the circumvallate papilla (Fig. 1C–E).

Responsiveness of the IXth nerve to mechanical stimulation of the tongue did not noticeably decline as a result of IXth nerve regeneration, *per se* (Fig. 2A, B). Furthermore, the taste papillae continued to be the most effective sites of mechanical stimulation (Fig. 2B–D). The cross-regenerated M-H and A-T nerves were not only highly responsive to mechanical stimulation of the tongue but also displayed the same rank order of effectiveness as the IXth nerve (foliate > circumvallate > other posterior tongue region, Figs. 1C–E, 2F–H). Stimulation of non-taste areas including the area immediately adjacent to the taste papillae was less effective than touching the taste papillae. No responses were observed to mechanical stimulation of the anterior portion of the tongue. The excellent responses to mechanical stimulation of the taste papillae may have resulted from structural features of the papillae (*e.g.*,

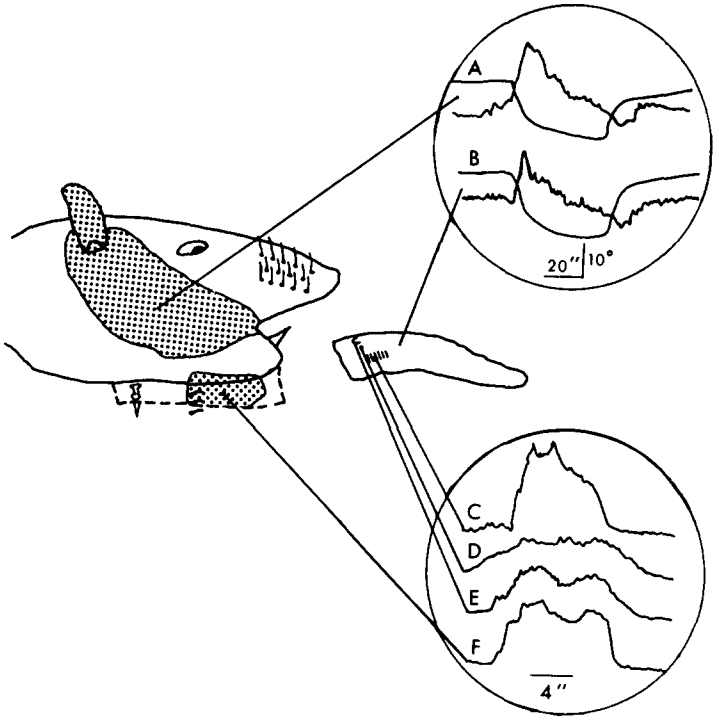

Fig. 1. Schematic drawing of rat head and tongue. Shown on the side of the tongue are 6 parallel slits of the right foliate papillae and on the dorsum the single midline circumvallate papilla. Dotted regions are electrophysiologically mapped cutaneous receptive fields for mechanical stimulation. Also indicated are the sites of stimulation and typical summated responses of normal and cross-regenerated right auriculo-temporal nerves to cooling (A, B) and right mylohyoid nerve to mechanical stimulation (C–F). In B the left IXth nerve was cut. Thermocouple registrations in A and B have initial temperatures of 31° and 30.5 °C, respectively. Time in seconds.

thin keratin), which augmented the mechanical stress on nerve endings, or may have resulted from a greater density or sensitivity of nerve terminals. It was clear that the presence of taste buds was not critical for the occurrence of touch responses, since touch responses were still obvious after the remaining circumvallate taste buds were eliminated by transection of the left IXth nerve, 3 weeks prior to recording (Fig. 2I and J). (There were individual differences in the size of the circumvallate touch response.) In a parallel manner, responses to cooling were also present, even after the left IXth nerve had been cut (Figs. 1B, 2K and L).

The normal and regenerated XIIth nerve preparations only responded to intense mechanical or thermal stimulation. This finding is consistent with the current view of minimal sensory representation in the XIIth nerve[20]. The absence of a touch response in the regenerated XIIth nerve suggests that by itself regrowth along the old IXth nerve pathways to the tongue papillae will not produce mechanical sensitivity in non-taste nerve endings.

No discharges were observed to warming the tongue or skin in any of the non-taste or control nerves. With regard to cooling, the normal IXth nerve was more

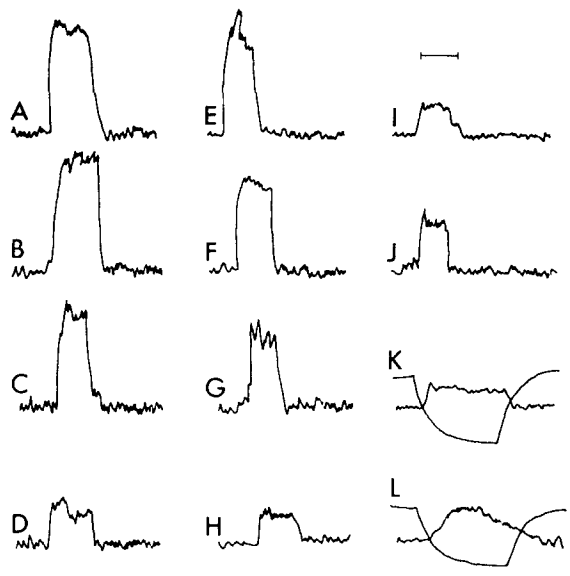

Fig. 2. Tracings of summated nerve responses to mechanical (A–J) and cold (K, L) stimulation. A, normal IXth, foliate. B–D, regenerated IXth with mechanical stimulation of: B, foliate; C, circumvallate; D, other posterior tongue area. E, normal auriculo-temporal nerve, skin of temporal area. F–H, regenerated auriculo-temporal nerve with mechanical stimulation of: F, foliate; G, circumvallate; H, other posterior tongue area. I–L, regenerated mylohyoid nerve: I, circumvallate; J, circumvallate with left IXth cut; K, tongue cooling; L, tongue cooling with IXth cut. The length of the line in I is 10 sec for all records and 12 °C vertically for thermocouple records in K, L. Initial temperature in K, L 30 °C and 30.5 °C, respectively. All recordings central to nerve suture.

responsive than the normal M-H nerve. This was true both for the rate of change of the response (slope) and for the threshold (X-intercept). This comparison across nerves was made with a normalizing procedure that gives a reliable measure of responses to cooling (Fig. 3; see ref. 9). The superior cold response of the IXth nerve was maintained after regeneration. Localized stimulation with fine cold glass rods suggested that some regenerated fibers within the circumvallate and foliate papillae were contributing to the cold responses. On the basis of the responses to mechanical and thermal stimulation of skin and tongue it appears that cross-regenerated non-taste fibers, of normal physiological function, terminated in the taste papillae.

Regeneration: taste

Regeneration of the IXth nerve did not appear to change its relative responsiveness to different chemical solutions (Fig. 4). This is in agreement with previous analyses of the effects of control regeneration upon the chorda tympani taste response[9]. Unless irritated, the shaved or depilated skin of the chin (normal M-H, 4 rats) or temporal region (normal A-T, 4 rats) was quite insensitive to chemical stimulation. When the skin was irritated by scratching, responses to the salts and acid could be obtained. This insensitivity to taste solutions was likewise maintained when the non-taste nerves were deviated from hairy skin and forced to innervate the tongue. No responses to chemical stimulation were recorded from the 20 surviving experimental rats with the exception of one M-H nerve preparation which had the left IXth nerve

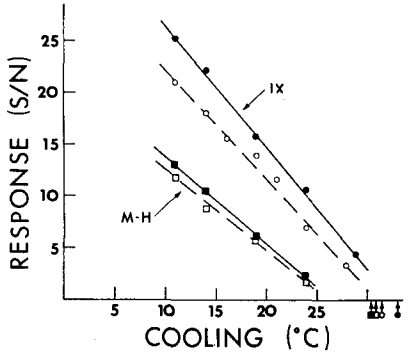

Fig. 3. Neural activity as a function of cooling the tongue. The Y axis is magnitude of peak summated response to transient cooling (S) divided by the average magnitude of the peak-to-peak fluctuations in the summator output during the pre-stimulus period (N). That is, N represents summated neural noise with the tongue at the adaptation temperature. (N was constant for any given preparation and varied with amplifier gain.) The average initial or adaptation temperature is shown by arrows on the X-axis. Filled circles, normal IXth nerve, N = 5; open circles, regenerated IXth nerve, N = 4; open squares, cross-regenerated M–H nerve, N = 4; filled squares, normal M–H nerve, skin cooling, N = 3.

intact. This animal showed a clear response — recorded proximal to the suture — to NH_4Cl and weak responses to other salts and acid. There was no response to sucrose, saccharin or quinine, which are effective stimulants for the IXth nerve. This profile is what one might expect from common chemical sensitivity, *i.e.*, direct stimulation of fiber endings. The lingual nerve, for example, (proximal to the branching out of the chorda tympani) responds to strong chemical stimulation of the tongue and mouth

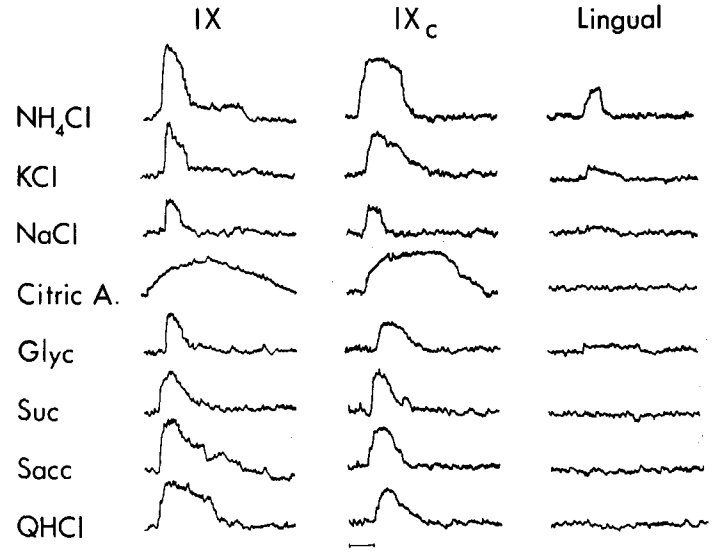

Fig. 4. Tracings of summated nerve responses to chemical stimulation of the tongue. IX, norma IXth nerve responses; IX_c, regenerated IXth nerve responses; lingual, normal lingual nerve central to the chorda tympani responding to 2.0 *M* glycerol and 1.0 *M* salts, including a weak NaCl response. See Methods for other concentrations. Time line, 10 sec.

TABLE I

NUMBER OF TASTE BUDS IN FOLIATE AND CIRCUMVALLATE PAPILLAE

For each nerve-type (rows) counts of taste buds were made on rats with the left IXth nerve intact *(Formation)* and on rats with the left IXth nerve cut 3 weeks prior to histology (*Maintenance*: N = 4 for A–T; N = 3 for M–H and XII). In the control IXth nerve group (IX_c), 4 rats had the left IXth intact and 4 cut. Values are mean number of taste buds at site indicated. Those animals with the left IXth nerve intact had, as expected, numerous taste buds in the circumvallate (M = 611) and left foliate papillae (M = 214).

Right nerve	*Formation: (left IXth intact) right foliate*	*Maintenance: (left IXth cut)*		
		Right foliate	*Left foliate*	*Circumvallate*
A–T	21 (N = 4)	27	19	0
M–H	27 (N = 5)	9	12	0
XII	7 (N = 5)	13	20	0
IX_c	231 (N = 4)	219	17	551

(Fig. 4). There is evidence that the lingual nerve does not innervate fungiform taste buds; it will neither maintain[10] nor reform them (Oakley, unpublished observations). In summary, the electrophysiological results demonstrate that although the non-taste nerves responded to touching the taste papillae, and to cooling, these fibers failed to functionally innervate taste receptor cells. In addition to the foreign cutaneous sensory fibers, the M-H nerve motor fibers to the mylohyoideus, digastricus and transversus mandibularis muscles also failed to make functional connections with taste buds.

The histological results indicated that the regenerated IXth nerves reliably reformed taste buds in the foliate papillae (Fig. 5A), but the non-taste nerves lacked the capacity to reform taste buds in either the foliate or circumvallate papillae (Table I, Fig. 5C and D) even though the non-taste fibers and found their way to the correct sites. (The few taste buds remaining in the right foliate papillae were no more numerous than those few normally maintained by the chorda tympani nerve; Table I[10,15].) In the experimental animals with the left IXth nerve intact, the circumvallate papilla contained numerous taste buds (Fig. 5B) and cross-regenerated nerve fibers responsive to cooling and touch, but not to taste. When the left IXth nerve was cut, no taste buds remained in the circumvallate papilla 3 weeks later. Even though non-taste nerves were present, they were unable to maintain the circumvallate taste buds (Table I, Fig. 5D). In summary, counts of taste buds from serial sections of the posterior region of the tongue demonstrated that the non-taste nerves could neither form new taste buds nor maintain existing taste buds.

DISCUSSION

Fibers in the regenerated non-taste nerves were physiologically responsive to cooling and to mechanical stimulation of the circumvallate papilla and thus were literally in a position to form functional connections or trophic connections with existing circumvallate taste buds. Consequently, the failure of the non-taste nerve

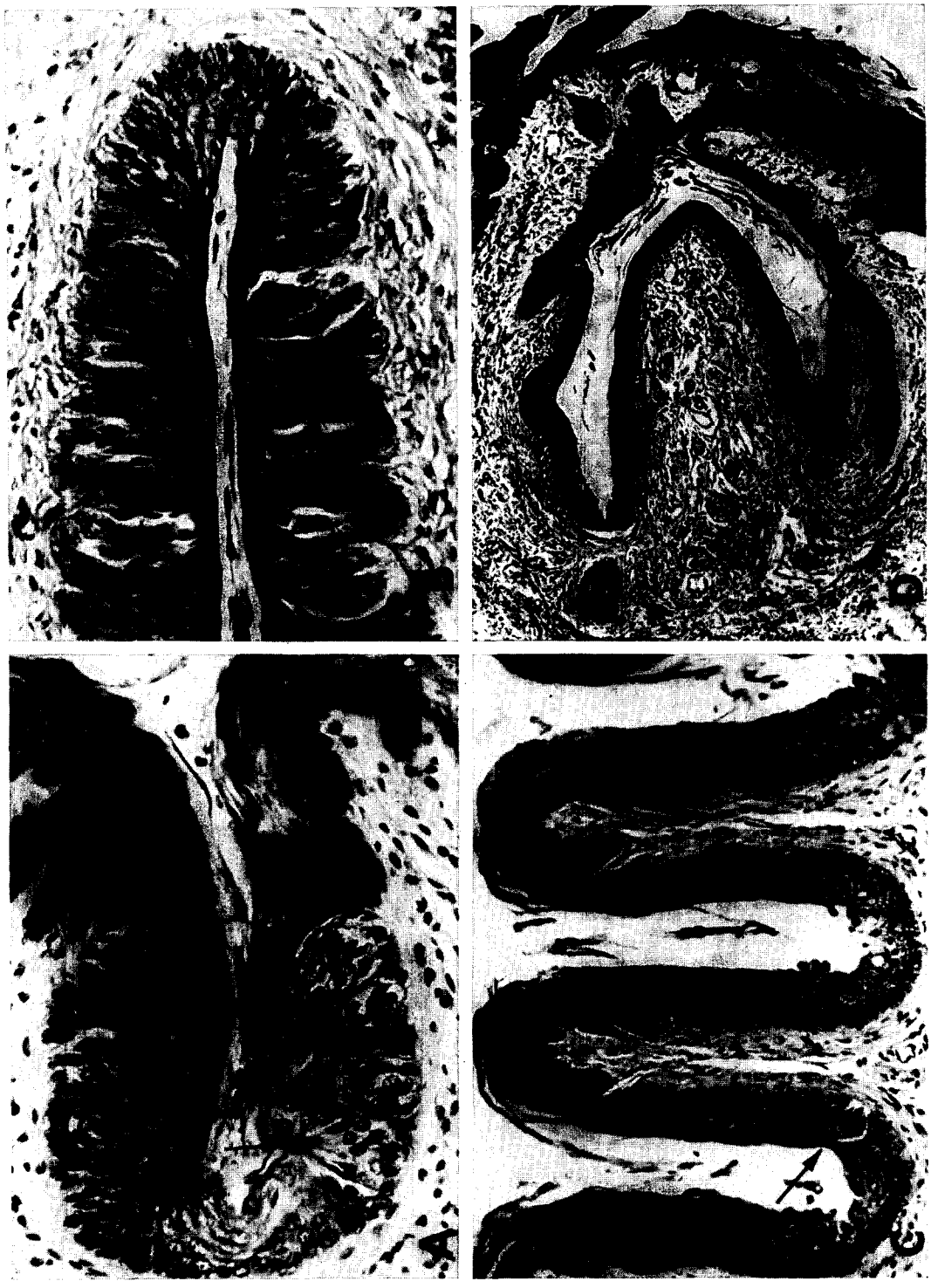

Fig. 5. Photomicrographs of foliate and circumvallate taste papillae. Paraffin, horizontal sections (10 μm), Heidenhain's iron hematoxylin. A: posterior fold of right foliate papillae with taste buds reformed by control regenerated IXth nerve. The arrow indicates the dark staining at a taste pore seen with Heidenhain's iron hematoxylin; $\times$ 330. B: portion of circumvallate papillae innervated by M–H and left IXth nerves; $\times$ 350. C: right foliate papillae, A–T nerve with left IXth cut. Arrow points to a single taste bud probably innervated by the chorda tympani nerve (see text); $\times$ 215. D: circumvallate papillae innervated by cross-regenerated XIIth nerve, left IXth nerve cut; $\times$ 105.

fibers to act as taste fibers was not a failure in regeneration of functional endings into the taste papilla but more likely was a reflection of an intimate incompatibility between non-taste fiber terminal and receptor cell precursor. Recent research on the development of function in reinnervated Pacinian corpuscles indicates that important interactions also occur between a touch fiber ending and its accessory receptor cells. Schiff and Loewenstein[12] have shown that denervated Pacinian corpuscles can be reinnervated by inferior mesenteric nerve fibers which are not normally associated with Pacinian corpuscles. In these experiments with cats the accessory receptor cells that form the lamella of the corpuscle did not degenerate after denervation, but rather remained until reinnervation by a new axon. Schiff and Loewenstein suggest that during reinnervation an interaction between the regenerating nerve fiber and the lamella cells occurred which caused transducer differentiation in the nerve ending. Apparently, not every fiber that enters a corpuscle can interact appropriately with the lamella cells since fewer than 40% of the successfully regenerated fibers responded to adequate mechanical stimulation of the corpuscle, even though every regenerated fiber was electrically excitable and conducted action potentials. Thus, both in the case of Pacinian corpuscles and taste receptors, some nerve fibers are unable to interact with the secondary sensory cells and fail to establish normal transducer function.

Nerves which failed to reform taste buds also failed to maintain them. This is reasonable if one views maintenance as itself a process of continual reformation of short-lived receptor cells[2,4] within the taste bud. Thus, it may be that the trophic processes which continually reform individual receptor cells in maintenance represent a part of the trophic processes which reform the entire taste bud.

It might be argued that the failure to reform or maintain taste buds (trophic properties) lay in a quantitative deficiency of axoplasm supplied to the epithelium. Singer *et al.*[14], on the basis of work with limb regeneration in *Xenopus*, found that the trophic effectiveness of a nerve was correlated with the total cross-sectional area of axoplasm. Thus, a possible explanation for the absence of reformation or maintenance of taste buds is that the various non-taste nerves had insufficient axoplasm. However, the chorda tympani nerve, which has only one-ninth the cross-sectional area of the auriculo-temporal nerve, reforms approximately the normal number of taste buds in the foliate papillae when crossed to the IXth nerve[10]. Even innervation of the posterior portion of the tongue by two auriculo-temporal nerves fails to reform taste buds[16]. Therefore, if the axoplasm of the non-taste sensory and motor nerves contained the postulated trophic chemical(s), this chemical was present in low concentrations or was extruded at low rates in comparison with the taste nerves. The electrophysiological responses to mechanical stimulation localized to the foliate and circumvallate papillae also suggest that the failure of taste bud reformation did not result from excessive distances for diffusion of axoplasmic chemicals.

Given that the non-taste nerves were unable to form their own taste receptor cells, the electrophysiological test of functional innervation by non-taste fibers was critically dependent upon the capacity of existing taste receptor cells to accept additional (plural) innervation from non-taste fibers. In a normal rat when one IXth nerve is cut 12% of the taste buds are lost[7] and the remaining 88% suffer a 33% cell

loss. (33% cell loss is arrived at by converting the 23% cell loss per planar section through the middle of the taste bud to a cubic function for the total taste bud volume. Bud shape is not critical.) From this it can be calculated that a maximum of one third of the circumvallate taste cells normally have bilateral IXth nerve innervation. Inasmuch as such plural innervation normally exists, it can be concluded that the procedures of tongue cross-innervation which were utilized were appropriate tests of the capacity to innervate or maintain existing taste buds. The significance of the observations that non-taste sensory and motor fibers could regenerate viable terminations in the tongue but could not form, maintain or innervate taste buds, will now be considered in relation to the formation of sensory connections.

There are 3 fundamental ways in which primary sensory neurons and epithelial cells could come to be specified as taste fibers and taste receptor precursors in embryogenesis (which has not been studied) or regeneration. (1) The receptor cell precursors, already destined to be taste cells, could impose their character upon any sensory fiber which subsequently happened to innervate them. (2) Alternatively, a fiber specified as a taste fiber could impose its sensory modality (*i.e.*, taste) upon any arbitrary nearby epithelial cell. (3) Finally, it is possible that both fiber and epithelial cell are pre-labeled as taste prior to the act of innervation, an act that occurs by a process of cell recognition between matched cells that are already designated as part of the taste system.

The present experiment on regeneration in the adult has directed its attention to the first alternative and has shown that many sensory and motor fibers of Vth and XIIth nerve origin cannot act to reform taste receptor cells and, granting plural innervation of circumvallate cells, cannot maintain or innervate existing taste receptor cells. It will be necessary to test other cranial and spinal nerves to catalogue the distribution of neurons which can serve as taste neurons. Nevertheless, an important conclusion can be drawn from existing data, namely, that many sensory and motor neurons cannot serve as taste neurons. Consequently, there must be a set or class of neurons which have been specified as taste neurons prior to reinnervation of the tongue.

Earlier results argue against the second alternative that taste fibers can innervate any arbitrary epithelial cell. Taste buds are restricted to specialized papillae in the tongue[3]. Only tongue epithelial cells in taste papillae respond to the influences of the chorda tympani or IXth nerves in reinnervation[10]. Furthermore, it has been shown with cross-reinnervation of the tongue that both the number and distribution of taste buds on the tongue are characteristic of the tissue, not the type of taste nerve innervating them. For example, although the IXth nerve is normally associated with hundreds of taste buds in two large papillae (foliate and circumvallate), when it innervates the front of the tongue it always duplicates the pattern of this new tissue—single taste buds, each located in a fungiform papillae[10]. It has been possible to form a few new taste buds with testosterone treatment, but this is actually a reestablishment in the adult of taste buds at locations where buds are found in the fetus[1,17]. Other experimental manipulation of epithelium such as transplantation or excision of the circumvallate papilla can also result in the formation of a few taste buds in abnormal locations[18,19]. Nevertheless, when the rat tongue epithelium is left undisturbed, only

a limited population of discretely localized cells in the dorsal epithelium is capable of differentiating into taste receptor cells. These particular cells are therefore labeled as taste receptor cell precursors and are not an arbitrary sample of epithelial cells. Thus, the evidence favors the remaining alternative that in regeneration in the adult both fiber and receptor cell precursor are pre-labeled as taste fiber and taste receptor and join together through a process of cell-recognition.

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