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The gustatory competence of the lingual epithelium requires neonatal innervation

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The rat vallate papilla is bilaterally innervated by the IXth nerve whose axons are required for the normal development of its several hundred taste buds. Temporary denervation during the developmental sensitive period for taste buds prevented most vallate taste buds from forming. Specifically, removing one IXth nerve and crushing the other in 3 day old neonates eliminated axons from the vallate papilla for about 10 days and by adulthood resulted in a mean ± 1 S.E.M. of 48 ± 12 vallate taste buds. Two explanations for the shortfall of adult vallate taste buds were evaluated: either 10 days of neonatal denervation impaired the gustatory competence of the vallate papilla, or the IXth nerve's trophic support of taste buds failed to recover after nerve crush on day 3. In adults, it was found that a IXth nerve previously crushed on day 3 would support numerous vallate taste buds (183 ± 27), provided that the vallate papilla had been continuously innervated by the contralateral IXth nerve during neonatal development. Consequently, taste neurons, whose axons had been crushed on day 3, seemed to survive and retain their trophic capacity to support taste buds in adults. To test for diminished competence of the gustatory epithelium, one IXth nerve was crushed on day 3 while the contralateral IXth nerve was removed. Beginning on day 75, the chorda tympani nerve was substituted for the re-innervating axons of the crushed IXth nerve. The cross-innervating chorda tympani ultimately supported only 51 ± 10 vallate taste buds. In contrast, in vallate papillae that developed without interruption of the contralateral IXth nerve during the sensitive period, the cross-innervating chorda tympani by itself supported more than four times as many vallate taste buds (214 ± 22). Evidently, a neonatal period of denervation permanently restricts the gustatory competence of the vallate epithelium; nerve-dependent precursors of taste receptor cells probably died or gpermanently changed their fate.

INTRODUCTION

It has been recognized for more than a century that adult mammalian taste buds are nerve-dependent – denervated taste buds degenerate, yet readily re-form if fungiform, foliate or vallate papillae are subsequently re-innervated[11]. In contrast, the development[3] and maintenance of the tongue's filiform papillae does not require innervation. However, it has been shown recently that gustatory innervation can negatively regulate filiform spine outgrowth[15]. An ectopic filiform spine will grow and extend from the apical surface of a denervated adult fungiform papilla[15]. Hence, in adults, re-innervation by gustatory axons not only re-forms the fungiform taste bud, but also suppresses the morphogenetic program for a filiform spine. Neuronal–epithelial interactions affecting the development of taste buds need to be further examined. The present study focuses on the gustatory nerve dependency of the developing vallate papilla of rat.

On the midline of the tongue of albino rats lies a single vallate papilla consisting of a 1-mm diameter island of tissue nearly surrounded by an elliptical trench whose walls are innervated by both the right and left IXth nerves. Vallate taste buds mature postnatally, as there are no mature vallate taste buds at birth in rat or hamster[4,9,17]. The full complement of about 600 vallate taste buds is not attained until rats are about 3 months old. Within the day 10–45 post-partum period, a given taste bud requires about 10 days to mature, i.e. form a taste pore[16]. In development vallate taste buds are neurally induced[5] during a sensitive period that is maximal from day 0–10 post-partum[6]. After avulsion of one IXth nerve on day 3, a mean ± 1 S.E.M. of

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228 ± 10 vallate taste developed, rather than the 496 ± 22 taste buds that one IXth nerve will support in adults. Removing one IXth nerve while crushing the other on day 3 led to an even more profound shortfall of vallate taste buds. Even though axons from the crushed IXth nerve began to re-invade the gustatory epithelium by day 13, the period of denervation from day 3–13 caused a conspicuous shortfall of taste buds – fewer than 50 taste buds developed by day 90[5,6].

The primary experimental objective of the present investigation was to determine whether the shortfall of vallate taste buds after temporary neonatal denervation could be reversed by other taste axons or whether it reflected a permanent developmental impairment of the vallate papilla. Since neonatal nerve crush can eliminate interactions between gustatory axons and the epithelium for about 10 days, this treatment might not only diminish the *competence* of vallate cells to form taste buds, but might also diminish the regenerated IXth nerve's capacity to *induce* taste buds in development or diminish the IXth nerve's capacity to *support* taste buds trophically in adults. Cross-innervation in adults was used to evaluate both the gustatory competence of the neonatally denervated vallate papilla and the inductive and trophic capacities of a neonatally crushed IXth nerve. It was hypothesized that temporary bilateral denervation during the sensitive period would permanently diminish the gustatory competence of the vallate epithelium.

MATERIALS AND METHODS

Surgical procedures

Sprague–Dawley albino rats *Rattus norvegicus* (obtained from Harlan Co., Indianapolis, IN, USA) were used to study the effects of neonatal interruption of the IXth (glossopharyngeal) nerve supply to the circumvallate papilla. Anesthesia was induced with ether masks for 3-day-old neonates or with sodium pentobarbital i.p. (5 mg/kg), combined with ketamine-HCl, 125 mg/kg body weight i.m. for adults. Adults also received a 50,000 U/kg i.m. injection of Bicillin antibiotic 12–24 h prior to surgery.

The IXth nerve was crushed immediately proximal to its pharyngeal branch by pinching it 10 times with the tips of fine forceps, followed by visual inspection to verify that this procedure had crushed but not transected the IXth nerve. Nerve avulsion consisted of using forceps to remove much of the IXth nerve between the petrosal ganglion and tongue. These operations did not seriously impair feeding, as neonates began to suckle within 4–8 h and adult animals were eating and drinking normally by the second postoperative day.

Cross-innervation of the adult tongue[10] was aided by using a nerve splice, removed from the ipsilateral mylohyoid (MH) nerve, to join the severed ends of the chorda tympani (CT) and IXth nerves. (The CT nerve supplies taste fibers to the fungiform papillae located in the anterior two-thirds of the rat tongue. It does not innervate the vallate papilla, which is exclusively and bilaterally innervated by the IXth nerve in normal rats.) A single 11–0 nylon suture (Ethicon Co.) was used to join the opposed nerve ends at each junction between the MH splice and the taste nerves (Fig. 1).

Nine groups of operated animals were used to evaluate whether neonatal denervation diminished the gustatory competence of the vallate epithelium or eliminated the capacity of the IXth nerve to induce or trophically support taste buds. Seven of these groups underwent two-stage operations (days 3 and 75). Table I indicates the various operative procedures, their timing, and the number of animals in each group. In groups 1–6, the left IXth nerve was avulsed (AV) on day 3 or day 75 and the right IXth nerve crushed (CR) on day 3. In group 1, the vallate papilla must have lacked IXth nerve axons for about 10 days, since it has been shown[6] that axons crushed on day 3 reappear in the epithelium beginning about day 13. On day 75 control self-reconnection was made (group 3) or experimental nerve crosses were made between the normal chorda tympani and the neonatally crushed IXth nerve (groups 4–7). The left IXth nerve was sham crushed (exposed but not manipulated) in groups 7 and 8. Animals were euthanized between 170 and 270 days of age, except those in group 8 which were euthanized between 290 and 385 days of age.

Histological procedures

Animals were deeply anesthetized with an i.p. injection of sodium pentobarbital. An infusion pump was used to perfuse the anes-

TABLE I

Methods: nerve operations

AV, avulsion of a IXth nerve on day 3 or 75; CR, crush of a IXth nerve on day 3 or 75; CT, chorda tympani nerve; MH, mylohyoid nerve.

(A) Operations to evaluate the vallate papilla

Group	*Day of nerve operations*		*Number of rats*
	Day 3	*Day 75*	
1	AV3/CR3	–	10
2	SHAM/CR3	AV75/CR3	7
3	SHAM/CR3	AV75/CR3-MH-CR3	5
4	AV3/CR3	AV3/CT-MH-CR3	8
5	SHAM/CR3	AV75/CT-MH-CR3	7

(B) Operations to evaluate the IXth nerve

Group	*Day of nerve operations*			*Number of rats*
	Day 3	*Day 75*	*Day 180*	
6	AV3/CR3	AV3/CR3-MH-CT	–	10
7	SHAM/CR3	SHAM/CR3-MH-CT	–	4
8	–	SHAM/CR75	SHAM/CR75-MH-CT	7
9	–	CT AVULSION	–	5

thetized animals intracardially with 250 ml of buffered 0.9% NaCl (pH 7.4) containing 0.02% heparin and 0.5% procaine-HCl, followed by a 250-ml mixture of 4% formaldehyde, 1% NH_4OH and 15% sucrose, final concentrations.

Serial, 10-μm transverse sections of paraffin embedded tongues of 64 rats were stained with Heidenhain's iron hematoxylin. Vallate taste buds were counted in normal animals at 90 and 180–225 days of age. The counts of fungiform taste buds were derived from examination of serial sections of the entire anterior portion of each animal's tongue from the tip to the dorsal median eminence.

RESULTS

Normal Sprague–Dawley rats have 573 ± 40 (mean ±1 S.E.M.) vallate taste buds at 180 days of age[4]. As shown previously, avulsing one IXth nerve and crushing the other on day 3 (AV3/CR3) temporarily denervates the vallate papilla during much of the developmental sensitive period and markedly reduces the number of taste buds that develop[5,6]. These results were replicated in the present experiment; CR3 IXth nerves supported a mean of 48 ± 12 taste buds in adults. Can this shortage of taste buds be attributed to the death of taste neurons having crushed axons or to barriers that regenerating axons encounter in navigating through the distal stump of a crushed nerve? Provided that the vallate papilla had been continuously innervated by one IXth nerve until day 75, the CR3 nerve alone sustained numerous vallate taste buds in adults (183 ± 27). Even after the CR3 nerve was required to regenerate a second time, it supported numerous vallate taste buds in adults (173 ± 23; compare groups 1–3 in Table II). Hence, factors other than the death of damaged taste neurons or an obstructed nerve conduit must have been responsible for the limited number of taste buds. To test for diminished vallate gustatory competence in adults, nerve anastomosis was used to direct chorda tympani taste fibers into a vallate papilla that had previously been temporarily denervated in neonates. Axons of the cross-innervating CT supported no more vallate taste buds (51 ± 10; Fig. 2B and group 4 in Table II) than a CR3 nerve after temporary neonatal denervation (group 1 in Table II). In contrast, in a continuously innervated control vallate papilla (only one IXth nerve had been interrupted on day 3), the cross-innervating CT nerve alone supported more than four times as many adult vallate taste buds (214 vs. 51 buds, $P < 0.001$, t test; group 5 in Table II; Fig. 2A).

To evaluate whether the trophic capacity of adult IXth nerves required interaction between its axons and the gustatory epithelium during the sensitive period, CR3 nerves were sutured to the distal stump of the normal CT on day 75. CR3 nerves associated with neonatally denervated vallate papillae re-formed 57 ± 4 fungiform taste buds when directed to the front of the adult tongue (Table II). CR3 nerves associated with vallate papillae that had been continuously innervated by the contralateral IXth nerve reformed 66 ± 6 fungiform taste buds when directed to the front of the adult

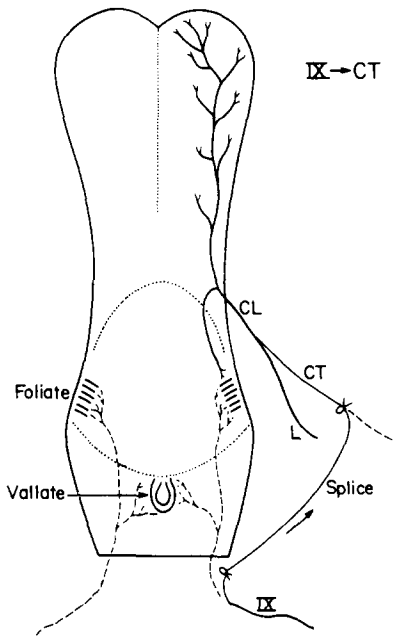

Fig. 1. A schematic drawing of the sensory innervation of the rat tongue depicts how the proximal portion of the transected IXth nerve was connected via a splice of the MH nerve to the distal stump of the CT nerve. The distal portions of right IXth nerve and the proximal portion of the right CT nerve were avulsed (dashed lines on the right side). In the animals whose proximal CT was connected via a MH splice to the distal stump of the IXth nerve (not shown), the left IXth nerve was also removed (dashed line on the left side). L, intact lingual nerve; CL, combined chorda–lingual nerve. The tongue is about 2 cm long.

TABLE II

Early denervation and taste bud induction

For simplicity only, the final status of the gustatory nerves is given under operative status. Abbreviations as in Table I.

Group	*Operative status*	*Vallate (mean ± 1 S.E.M.)*	*Fungiform*
–	Normal vallate	573 ± 40 (n = 11)	–
1	AV3/CR3	48 ± 12 (n = 10)	–
2	AV75/CR3	183 ± 27 (n = 7)	–
3	AV75/CR3–CR3	173 ± 23 (n = 5)	–
4	AV3/CT–CR3	51 ± 10 (n = 8)	–
5	AV75/CT–CR3	* 214 ± 22 (n = 7) * $p < 0.001$ (214 vs. 51)	–
–	Normal fungiform	–	82 ± 2 (n = 22)
6	AV3/CR3–CT	–	57 ± 4 (n = 10)
7	SHAM/CR3–CT	–	66 ± 6 (n = 4)
8	SHAM/CR75–CT	–	63 ± 6 (n = 7)
9	Control CT avulsion	–	17 ± 4 (n = 5)

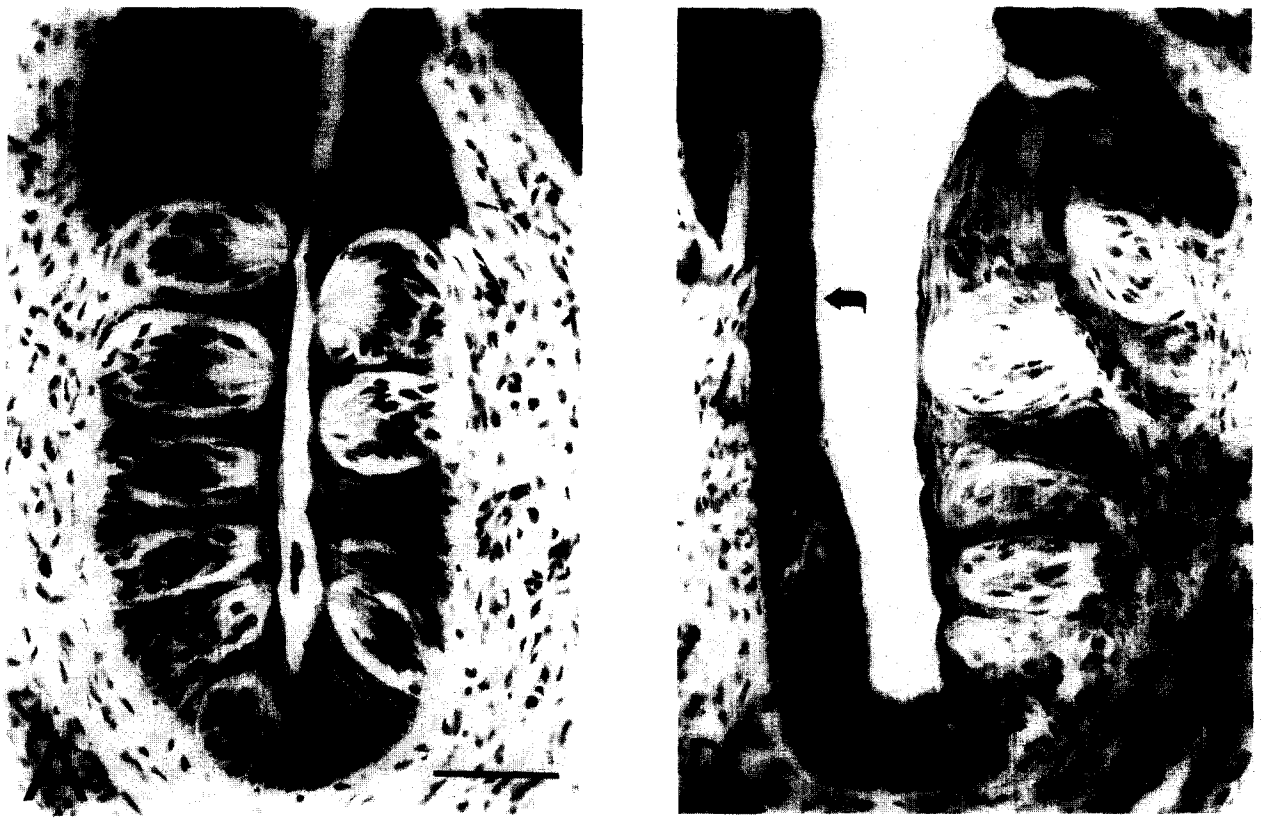

Fig. 2. A: about 10 of 207 taste buds are evident in this section of the left trench of a control vallate papilla examined on day 185. Innervation was supplied by the CT nerve after crush of one IXth nerve on day 3 and avulsion of the other on day 75 (AV75/CT–CR3). During the sensitive developmental period, the vallate papilla had been continuously innervated by an intact IXth nerve. B: in contrast, after temporary neonatal denervation in experimental animals (AV3/CT–CR3), the vallate papilla was characterized by fewer taste buds and stretches of thinner epithelium where there were no taste buds, e.g. arrow. 10-μm transverse paraffin sections stained by Heidenhain's iron hematoxylin method. Bar, 50 μm for A and B.

tongue. Neither 57 nor 66 differs significantly from the 63 ± 6 fungiform taste buds supported by IXth nerves crushed on day 75 (groups 6–8 in Table II; $P > 0.05$).

DISCUSSION

The competence of the gustatory epithelium

At every age a prompt outcome of denervation of the vallate papilla is the loss of all vallate taste buds[5,6,11]. In adults such denervation-triggered taste bud losses can be readily reversed, e.g. regeneration of a IXth nerve crushed on day 75 led to the re-formation of 405 vallate taste buds (AV75/CR75) [5]. However, after the same operation during the neonatal sensitive period (AV3/CR3), the adult IXth nerve came to support just 48 taste buds. The CT was more effective than AV3/CR3 nerves in reducing the shortage of vallate taste buds only if the CT invaded a vallate papilla that had developed with an intact contralateral IXth nerve. Thus, whether gustatory axons will support taste buds depends upon the developmental competence of the gustatory epithelium which in turn depends upon continual neonatal innervation of the vallate epithelium.

The inductive capacities of CR3 IXth nerves

In development it is known that by day 3 17 vallate taste buds are normally mature[4], that these taste buds rapidly disappear after AV3/CR3, and that mature taste buds reappear only after day 30 in AV3/CR3 rats[5]. In the present experiment such taste buds accumulated to a mean of 48 taste buds after 6–9 months. Although these observations are consistent with CR3 induction of new taste buds in late post-natal development[4,5], the progressive trophic activation of previously induced taste cells cannot be ruled out.

The neurotrophic capacities of CR3 IXth nerves

In adults, 183 of the vallate taste buds that had developed in conjunction with a normal nerve could be supported by the CR3 nerve (AV75/CR3). Nearly as many taste buds were supported by a CR3 nerve that regenerated twice (AV75/CR3–CR3; 173 taste buds). These numbers are only slightly less than the number of taste buds supported by one normal IXth nerve (AV3/NORMAL; 228 taste buds)[6]. It would seem that CR3 nerves have significant neurotrophic capacity. Of course, it is conceivable that the more abnormal vallate

environment produced by the experimental combination of avulsion of one IXth nerve and crush of the other (AV3/CR3) might lessen the CR3 axons' trophic capacity. However, as assessed by support of fungiform taste buds, the neurotrophic capacity of experimental CR3 nerves was not significantly different from that of control CR3 nerves (group 6 vs. 7 and 8; $P > 0.05$).

The present experiments demonstrate that neither the survival nor the trophic potency of taste axons depends absolutely upon *post*natal tissue interactions. Nonetheless, given that early epithelial interactions are required to prevent the death of sensory neurons in cranial ganglia in chick embryos[18], it is certainly possible that taste neuron survival may depend critically upon *pre*natal neuronal–epithelial interactions.

Cell dynamics associated with taste buds

In the adult vallate papilla, taste receptor cells are neurotrophically dependent upon gustatory innervation[6,12]. Even with trophic support most taste receptor cells live for < 2 wk, owing to the normal turnover of epithelial cells, including vallate taste cells[2,7]. It is believed that the ongoing division of specialized basal cells – taste bud stem cells – generates the young cells that replace aged taste cells[13,14]. After an operation to encourage CT axons to invade the vallate papilla, the pace of axon outgrowth (about 2 mm/day) ensured at least a 2–3-wk period of vallate denervation[1,6]. Since all vallate taste buds degenerate within 1 wk after denervation[5,8], few denervated mature taste receptor cells would survive for 2–3 wk. Instead, the cross-innervated vallate papilla must be re-populated by new taste cells derived from emerging or surviving stem cells. The present results have shown that the neonatally denervated vallate epithelium formed few taste buds, owing to the permanent loss of vallate gustatory competence. Presumably, after limited innervation during the sensitive period, only a few stem cells were committed to a gustatory fate[6,13,14]. During normal development, taste axons probably induce a large permanent population of gustatory stem cells from precursors[5,13,14].

Summary and conclusions

This study examined some of the developmental neuronal–epithelial interactions required to establish mammalian taste buds. It was already known that few vallate taste buds will develop after temporary neonatal denervation of the vallate papilla. The present experiments tested whether this taste bud shortage could be attributed to deficiencies in the taste axons or deficiencies in the taste epithelium. Crushing one IXth nerve in neonates failed to prevent that nerve from supporting numerous vallate and fungiform taste buds in adults. This indicates that taste neurons survived nerve crushing and retained much of their neurotrophic capacity. In contrast, after the neonatal vallate was temporarily denervated by both crushing one IXth nerve and avulsing the other, neither the adult CT nor the adult IXth nerve supported many vallate taste buds. It was concluded that the vallate papilla's gustatory competence had been permanently reduced by temporary neonatal denervation. Evidently gustatory innervation is a critical environmental influence whose actions on neonatal gustatory tissue are required to establish the cell lineage leading to mature taste receptor cells[19]. In the absence of timely innervation during development the precursors of taste cells probably die or irreversibly adopt a non-gustatory fate[14,15].

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