Time Course of Morphological Alterations of Fungiform Papillae and Taste Buds Following Chorda Tympani Transection in Neonatal Rats

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ABSTRACT: The time course of structural changes in fungiform papillae was analyzed in rats that received unilateral chorda tympani nerve transection at 10 days of age. Morphological differences between intact and denervated sides of the tongue were first observed at 8 days postsection, with an increase in the number of fungiform papillae that did not have a pore. In addition, the first papilla with a filiform-like appearance was noted on the denervated side at 8 days postsectioning. By 11 days after surgery, the total number of papillae and the number of papillae with a pore were significantly lower on the transected side of the tongue as compared to the intact side. At 50 days postsection, there was an average of 70.5 fungiform papillae on the intact side and a mean of only 20.8 fungiform papillae the denervated side. Of those few remaining papillae on the cut side, an average of 13.5 papillae were categorized as filiform-like, while no filiform-like papillae occurred on the intact side. Significant reduction in taste bud volume was noted at 4 days posttransection and further decrements in taste bud volume were noted at 8 and 30 days postsection. Electron microscopy of the lingual branch of the trigeminal nerve from adult rats that received neonatal chorda tympani transection showed normal numbers of both myelinated and unmyelinated fibers. Thus, in addition to the well-characterized dependence of taste bud maintenance on the chorda tympani nerve, the present study shows an additional role of the chorda tympani nerve in papilla maintenance during early postnatal development. © 2002 Wiley Periodicals, Inc. J Neurobiol 51: 223–236, 2002

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Transection of the chorda tympani nerve (CTX) in the adult rat results in an initial degeneration or loss of taste buds that are contained within fungiform papillae (Hård af Segerstad et al., 1989). Upon regeneration of the chorda tympani nerve (CT), taste buds reform structurally and functionally (St. John et al., 1995). While taste buds degenerate within fungiform papillae after CTX in adult rats, there are few, if any, changes noted in the general morphology of the papillary structure itself. The exception is that, in some instances after transection, fungiform papillae form a conical structure on their apical surface. This feature is described as "filiform-like" because it resembles the conical epithelial surface of filiform papillae. However, filiform-like papillae are morphologically distinct from true filiform papillae because of their broad circumference and spatial orientation (Oakley et al., 1990; Nagato et al., 1995). Even after chronic CT denervation in the adult rat wherein the CT is not

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allowed to regenerate, only approximately 28% of fungiform papillae become filiform-like in appearance (Hård af Segerstad et al., 1989).

While these relatively minor alterations in fungiform papilla morphology occur following sectioning of the CT in adults, other manipulations produce more widespread and profound effects. The CT joins with the lingual branch of the trigeminal nerve as it travels into the tongue epithelium. Both nerves enter fungiform papillae; the CT transverses the core area and contacts the taste bud, while the lingual nerve bifurcates throughout the perigemmal area of the papillae but does not contact the taste bud (Miller, 1974). After transection of both the CT and lingual nerves, the number of filiform-like papillae increases to an average of 60% (Hård af Segerstad et al., 1989).

The severity of this effect on papillae morphology becomes more apparent when the combined CT/lingual nerves are transected during neonatal ages in the rat (Nagato et al., 1995). Interestingly, when the CT is transected alone (i.e., the lingual nerve remains intact) at 10 days postnatal, the effects on tongue morphology are similar to those observed after combined CT/lingual nerve transection; 65% of fungiform papillae either degenerate to where they can no longer be detected or they become morphologically indistinguishable from filiform papillae (Sollars and Bernstein, 2000). Of the fungiform papillae that remain, nearly 80% are filiform-like in appearance. These results are in sharp contrast to the 70% or more fungiform papillae that retain their distinctive flattened appearance even after long-term CT denervation sustained at adulthood (Hård af Segerstad et al., 1989). Therefore, during early postnatal development, the lingual nerve alone is not sufficient to maintain papillae morphology. In young rats, the presence of the CT appears necessary to maintain both taste bud and papilla structure. In other words, it appears that during development, the presence of a gustatory nerve (the CT) is a requirement for the normal maintenance of extra-gustatory epithelia tissue (the fungiform papillae). Adult fungiform papillae are less dependent upon the CT.

In a previous study, the morphological changes in fungiform papillae were examined only at 110 to 130 days after bilateral CTX at 10 days of age (Sollars and Bernstein, 2000). Thus, the time course of the degenerative process occurring in tongue morphology was not examined. Consequently, it is not clear whether the major morphological effects occur soon after CT denervation or if there is a prolonged stage-related change in morphology. Knowing the time course of papilla degeneration may provide insights into nerve/target relationships and underlying molecular mechanisms. Accordingly, the present study was designed to characterize the time course of morphological changes. It is important to note that a detailed structural analysis of the surface of the tongue was used to characterize papillae morphology. Because change in papillae structure is likely to be highly indicative of concurrent changes in taste bud morphology (Ganchrow and Ganchrow, 1989; Nagato et al., 1995), we also analyzed taste bud volume to determine the extent and time course of taste bud degeneration following neonatal CTX. In addition, electron microscopy was used to obtain lingual nerve fiber counts in intact rats and adult rats that received CTX at 10 days of age to verify that the lingual nerve was not damaged as a result of CTX.

MATERIALS AND METHODS

Animals

Litters were obtained from untimed pregnant Sprague-Dawley rats ordered from Harlan Sprague-Dawley (Dublin, VA). Dams were received into the University of Virginia vivarium during midgestation. The date that pups were born was designated as Day 0. Litters were culled on Day 1 to a maximum of eight pups per litter. Pups were weaned around Day 25 and maintained on standard rat chow and water. A total of 70 rats from 19 litters were used for these studies. Other rats from these litters were used for other studies. All procedures were carried out under the approval of the University of Virginia Animal Care and Use Committee and in full accordance with NIH guidelines.

CT Transection

At 10 days of age, unilateral CT transection (NeoCTX) was performed. Each pup was anesthetized with methohexital sodium (Brevital® Sodium; 50 mg/kg, i.p.) and given atropine (0.01 cc, i.m.). An incision was made on the ventromedial portion of the neck. The digastricus and masseter muscles on either the left or right side were bluntly dissected using microfine jeweler's forceps until the lingual nerve was visualized. The CT/lingual nerve was traced to its point of bifurcation. The CT was crushed immediately proximal to the lingual nerve and evulsed, resulting in removal of the CT to its point of entrance at the tympanic bulla. Crushing the CT prior to its removal helped eliminate potential injury to the lingual nerve. Surgery was not performed on the side contralateral (intact) to the transection. Previous studies (Sollars and Bernstein, 2000) showed that sham operations resulted in no noticeable alteration to papillae morphology; counts were similar to that noted for the intact side of the tongue in the present study. Following surgery, the incision was sutured and the rat allowed to recover from the anesthetic on a warm heating pad. The

total time rats were away from their dams was an average of 30–60 min and pups were often observed suckling within an hour of their return to the dam.

Fungiform Papillae Histology

In order to examine the time course of morphological changes to fungiform papillae, histology was performed on tongue tissue of NeoCTX rats at 0, 2, 8, 11, 14, 17, 20, 25, 30, or 50 days after NeoCTX. Five rats were used for each time point except the 50 day postsurgical group in which four rats were used. Following an overdose of sodium pentobarbital, rats were perfused with modified KREBS solution and the tongues were removed and postfixed in 8% paraformaldehyde for at least 2 days. After adequate fixation, tongues were cut immediately posterior to the intermolar eminence and the anterior portion prepared for histology. Ventral muscle layers were removed, leaving a thin strip of dorsal epithelium. In order to achieve consistency across samples, none of the tongue surface on the ventral side was included. Because approximately 80% of fungiform taste buds are on the dorsal epithelium of the anterior tongue (Miller and Preslar, 1975) examination of the remaining tongue tissue provided a large and consistent sample area. The epithelium was dipped in a 5 or 15% solution (w/v) of methylene blue, dried, flattened, and sealed between two microscope slides. Using darkfield or phase-contrast microscopy, counts were made of fungiform papillae with a pore (pore), fungiform papillae without a pore (no pore), and filiform-like papillae. Topography and counts of fungiform papillae and taste pores were made with a computer (Neurolucida; MicroBrightField, Inc., Colchester, VT) by an observer who was not informed of the surgical condition of the rat. Methylene blue stained fungiform papillae and taste pores were easily differentiated from filiform papillae under darkfield and phase-contrast microscopy.

Fungiform Papillae with Pores. Fungiform papillae appeared as circular eminences on the surface of the epithelium (Miller and Reedy, 1990). Taste pores appeared as small, distinct circular concavities on the surface of the papillae. Previous reports using the dorsal structure of fungiform papillae to quantify pores have indicated that taste pores stained with methylene blue are darker than the surrounding epithelium (Miller and Reedy, 1990; Parks and Whitehead, 1998). Those articles used a different methodology for preparing the tongue and used standard transmitted light microscopy to visualize the tissue. In the present study, ventral muscle layers were removed as completely as possible, leaving only a thin layer of the dorsal epithelium. Compared with previous methodologies, this procedure allowed for better visualization of the detailed structure of the papillae. By the use of darkfield microscopy, the pore appears translucent and is usually lighter in color than the tissue that surrounds it [see Figs. 1(A) and 2(A)]. However, our criteria for defining the pore are based on morphological analysis instead of color variation. Using the terminology established in a recent article that examined tongue morphology of hamsters (Parks and Whitehead, 1998), a fungiform papilla with a pore was defined as containing three distinct morphological characteristics: a rim that separated the large border of the papilla from the taste pore region, an indentation just inside the rim, and a hillock that protruded slightly from the indentation and contained the pore [see Figs. 1(A,B) and 2(A)]. In younger rats, the pore structure is usually less evident, but the other features of the papillae are similar to that noted in adult rats. Figure 2(B) shows an example of the surface structure of a fungiform papilla from a 10-day-old rat. Note that although the rim, indentation, and hillock are less evident than in the adult papilla shown in Figures 1(A) and 2(A), all features are easily identifiable. Thus, these papillae were counted as pore papillae.

No Pore Fungiform Papillae. As defined in the present study, no pore papillae have an epithelial surface with a relatively smooth appearance [Figs. 1(C,D)]. In these papillae, the rim and the indentation between the outer border and the rim are generally absent or very shallow, and thus they are easily differentiated from pore papillae. Rarely, papillae were noted that had a small concavity reminiscent of a pore, however, they were distinctly smaller and lacked definition as compared to the pores in pore papillae. In the absence of the other features that defined a pore papilla, these papillae were categorized as no pore papillae.

Fungiform Papillae with a Filiform-like Appearance. Denervated fungiform papillae can develop a cornified epithelial surface that has been described as filiform-like because of the conical protrusion that rises from the normally flat surface epithelium of the papilla. However, filiform-like papillae are easily differentiated from actual filiform papillae because the base of the filiform-like papillae is much larger than true filiform papillae, and the "cone" is typically oriented in a direction disparate from filiform papillae [Fig. 1(E,F); Ganchrow and Ganchrow, 1989; Hård af Segerstad et al., 1989; Oakley et al., 1990, 1993; Nagato et al., 1995; Iwasaki et al., 1997]. In the present study, counts were made of fungiform papillae with and without pores and filiform-like papillae on both the NeoCTX and intact sides of the tongue.

Taste Bud Morphology

In order to further reveal changes in taste organs following NeoCTX, taste bud morphology was examined 2, 4, 8, and 30 days after CTX at 10 days of age ($n = 2$ at each age). Surgical procedures were identical to those described above. At the specified intervals following surgery, rats were overdosed with sodium pentobarbital and perfused with modified KREBS solution followed by 8% paraformaldehyde. The tongues were removed and postfixed for 1 week in 8% paraformaldehyde and then cryoprotected in sucrose prior to sectioning. Serial sections (10 μm thick) were obtained starting 2 mm posterior from the tongue tip and extending caudally for the next 2 mm. Sections were stained with hematoxylin and eosin and taste bud volumes were mea-

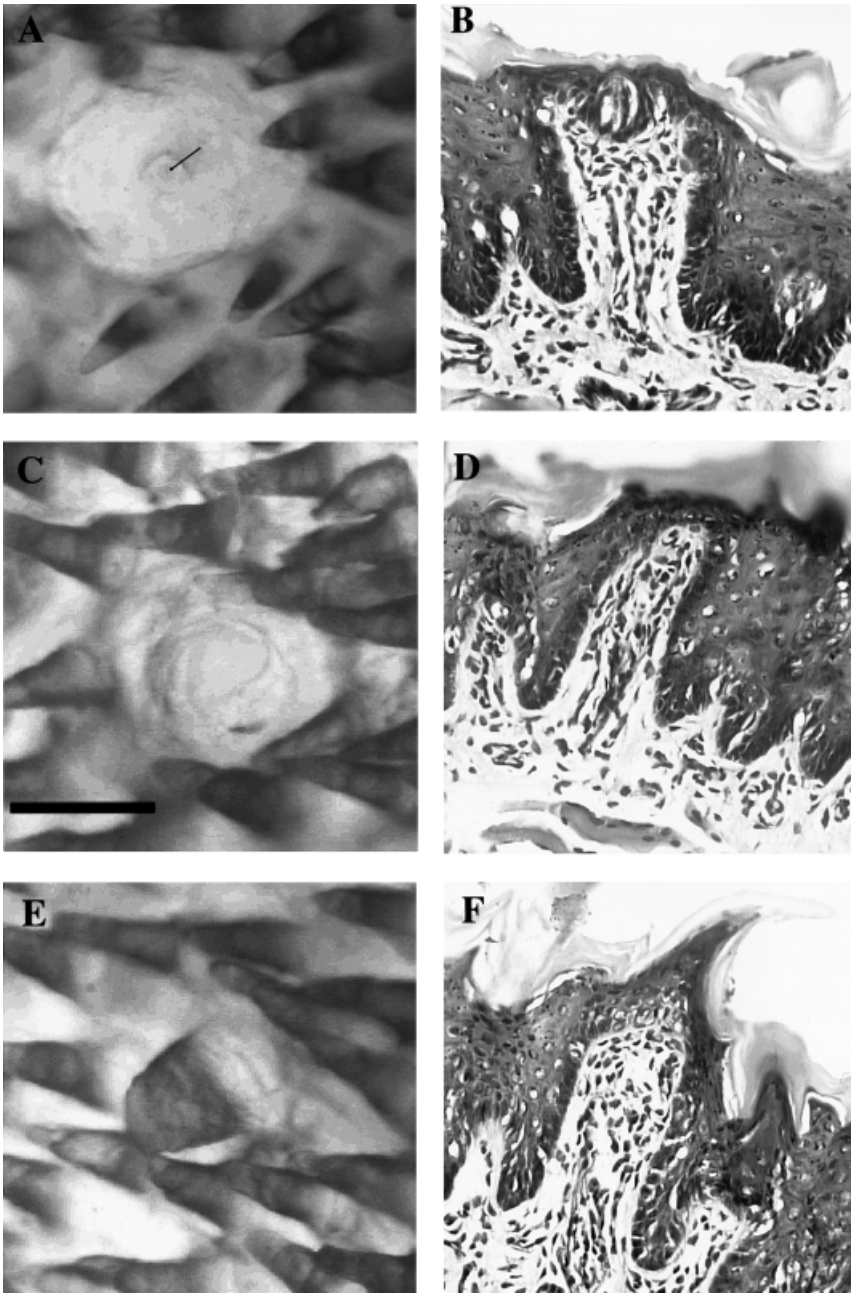

Figure 1 Examples of fungiform papillae from NeoCTX and Sham rats [(A, C, E) The dorsal surface of the tongue stained with methylene blue. (B, D, F) 10 μm sections of tongue stained with hematoxylin]. Each figure shows a single fungiform papilla surrounded by filiform papillae. (A) and (B) are examples of fungiform papillae with a pore. The arrow in (A) indicates the pore in the center of the hillock. (C) and (D) are examples of fungiform papillae without a pore. (E) and (F) are fungiform papillae that are filiform-like in appearance. The scale bar (100 μm) in (C) applies to all sections.

sured within all papillae that contained presumptive taste receptor cells. Taste bud measurements were obtained on both the intact and denervated sides of the tongue by an observer who did not have direct knowledge of the surgical condition. Computer reconstructions of taste buds were done using Neurolucida software attached to an Olympus microscope. Briefly, the border around the taste bud was outlined and digitized on the computer monitor using X, Y, and Z coordinates. Measurements were obtained across serial sections, so that the entire extent of the taste bud was

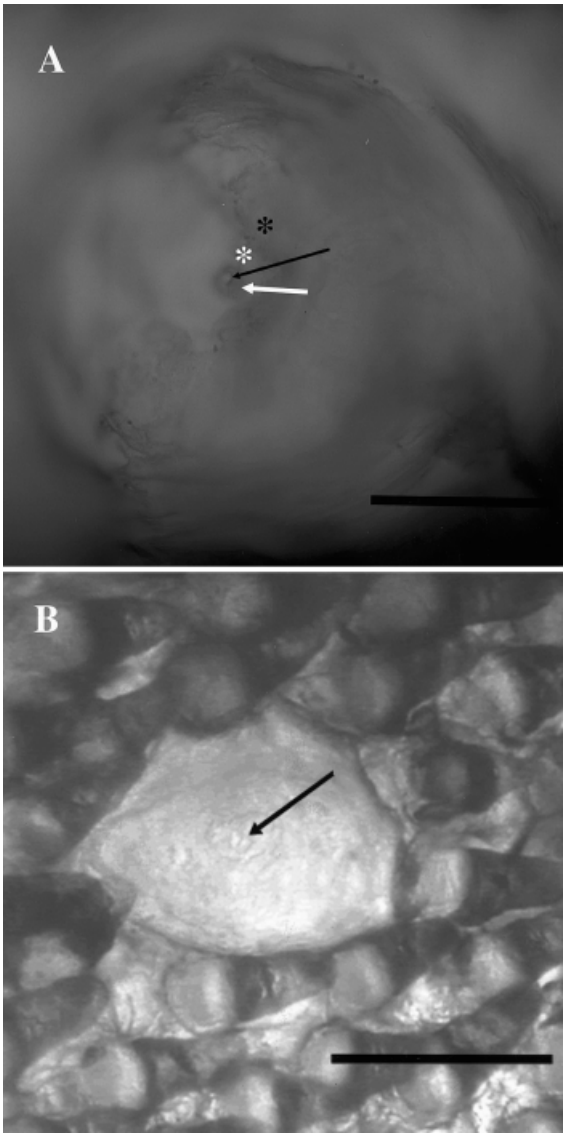

Figure 2 "Pore" fungiform papillae. (A) High magnification image of a fungiform papilla on the intact side of the tongue. Black arrow indicates pore; white arrow points to hillock; white asterisk is on portion of indentation; black asterisk is on portion of rim. (B) A fungiform papilla with a pore from a 10-day-old rat. The arrow indicates the region of the pore. Scale bar in each image represents 100 μm.

included in the analysis. Area measurements were calculated as volumes by multiplying the total area obtained for each taste bud by the section thickness. Because denervated taste receptor cells often lose their characteristic elongated orientation within the taste bud (Oakley et al., 1993), remnant taste buds were operationally defined as the region immediately below the apical surface of a fungiform papillae that had a distinct, darkly stained border [see Fig. 1(B)]. Inclusion of border cells in taste bud measurements is consistent with previous studies (Krimm and Hill, 1998). If the papilla was "empty," the surface was often heavily keratinized and did not have the darkly stained invaginated region [Fig. 1(D)].

The terminology used to describe classifications of papillae was changed slightly from that used to describe the surface structure of papillae. Sectioned papillae were classified as: with a taste bud; empty, with no apparent taste receptor cells; or filiform-like. Presumably, "taste bud" papillae encompass a combination of pore and no pore papillae, "empty" papillae would be classified strictly as no pore papillae, and filiform-like papillae are in the same category in both types of analyses. Given that the pore is small and it is occasionally difficult to visualize the taste pore in thick-sectioned tissue, no analyses were attempted based on the existence of a pore. Additionally, no direct comparisons were made of the appearance of a fungiform papilla from the surface analysis and in histological sections.

To show whether remnant taste buds contained mature taste receptor cells, cytokeratin-19 (CK-19) staining (Wong et al., 1994; Zhang and Oakley, 1996) was used on tongue tissue from two rats at 4 days post-CTX and one rat at 8 days post-CTX. Briefly, 10 μm sections of tissue from the tip region of the tongues were processed overnight with mouse anti-CK-19 (1:400; Sigma Aldrich) followed by a 2 h incubation in rhodamine-conjugated antimouse IgG (1:250; Jackson ImmunoResearch Laboratories, Inc.). The uncut side of the tongue served as a positive control for immunoreactivity.

Lingual Nerve Fiber Counts

The following study was conducted to demonstrate that no damage is done to the lingual nerve as a result of NeoCTX. The lingual nerves of three adult (approximately 50 days after transection) NeoCTX and three control rats (intact) were removed and processed for electron microscopy. The control rats received no surgical procedures prior to the lingual nerve removal. Rats were overdosed with sodium pentobarbital and perfused with modified KREBS solution followed by a solution of 4% paraformaldehyde and 2.5% glutaraldehyde. The lingual nerves were transected medial to the mandible and sections were taken of the lingual nerve immediately proximal to the point where the CT and the lingual nerve normally join. The nerves were postfixed in a solution of 4% paraformaldehyde and 2.5% glutaraldehyde for approximately 1 week. They were then postfixed in 2% osmium tetroxide, dehydrated in graded acetones and embedded in Epon 812. Semithin (0.5 μm) cross sections were cut from the hardened tissue blocks and stained with 0.5% toluidine blue to ensure that the tissue blocks yielded intact cross sections of the nerves. The blocks were trimmed and thin-sectioned for electron microscopy. One section was used for analysis from each rat. Each section analyzed was approximately 1 mm from the lingual nerve/CT juncture. Sections were stained with 0.25% lead citrate and 5% uranyl acetate in 50% acetone and observed using a JEOL 100S electron microscope. Photomicrograph images (1000X) were scanned onto a computer and imaged on PhotoShop (Adobe Systems, Inc.). Counts of myelinated and unmyeli-

nated fiber profiles were made from reconstructed montages of the entire cross sectional area of the nerve.

Control CT Transection

In all NeoCTX rats, the CT was pulled out and freed of its proximal connection. This evulsion procedure results in the removal of approximately 5 mm of the CT by removing it at the tympanic bulla, leaving intact its proximal juncture with the facial nerve. Thus, the geniculate ganglion remains intact, allowing for the possibility that the nerve could regenerate. However, there is little evidence that the nerve regenerates. Months after NeoCTX, the CT is discernable within the tympanic bulla, but we have been unable to detect it distal to the tympanic bulla (unpublished observations).

"Control" transections were carried out in four rats to determine if the extreme changes in the morphology of fungiform papillae after NeoCTX are the result of the large portion of nerve that is removed. At 10 days of age, four rats were prepared for surgery as described above. Instead of evulsing the CT, microdissection scissors were used to place a single cut in the CT just proximal to the lingual nerve/CT juncture. All other procedures were identical to those described above for NeoCTX. Tongues were removed at approximately 50 days after transection and processed according to the histological procedures described above.

Data Analysis

Analysis of variance was used to analyze differences in papillae counts for each of the categories across all time points. Posthoc analysis using Bonferroni tests was used to determine statistical differences between papillae types and differences in taste bud volumes at individual time points. Student's t tests were performed on counts of myelinated and unmyelinated lingual nerve fibers and to compare numbers of papillae on intact and CT transected sides of the tongue.

RESULTS

Time Course of Changes in Fungiform Papillae

There were striking morphological changes in fungiform papillae that occurred on the denervated side of the tongue after NeoCTX. See Figure 3 for the changes in numbers of each type of fungiform papillae for each of the periods after surgery.

Total Number of Papillae. Overall, the total number of fungiform papillae decreased significantly after sectioning [$F(9, 48) = 31.03$; $p < .00001$]. The first significant decrease in total number of papillae on the transected side as compared to the intact side occurred at Day 11 [intact side, 68.2 ± 1.28; NeoCTX side, 52.6 ± 2.56; $t(8) = 4.71$, $p < .002$]. Thus, by 11 days after NeoCTX, the morphology of fungiform papillae was changing to such a degree that many were no longer identifiable as having been fungiform papillae. That is, denervated fungiform papillae either degenerated such that they could no longer be detected, or they completely reverted to a filiform appearance that could not be differentiated from the morphology or spatial orientation of regular filiform papillae. The total number of fungiform papillae continued to decrease such that there were only 20.8 ± 3.6 papillae at 50 days postsectioning as compared to 70.5 ± 4.0 papillae on the intact side.

Pore Papillae. On the transected side, the number of pore papillae decreased as the number of days after NeoCTX increased [$F(9, 48) = 98.84$; $p < .00001$]. As compared to the intact side, the first significant drop in number of pore papillae occurred on Day 11 after surgery [intact side, 66.4 ± 1.03; NeoCTX side, 29.2 ± 2.76; $t(8) = 12.61$, $p < .0001$]. The tendency for a continuing reduction in numbers of papillae with a pore continued until the lowest occurrence at Day 30 (2.25 ± 0.77), although by Day 17 there was already an average of only 4 ± 0.71 pore papillae.

No Pore Papillae. The initial effect of CTX was noted by Day 8 after surgery when the number of no pore papillae increased significantly above that noted on the intact side [intact side, 2.8 ± 0.58; NeoCTX side, 13.4 ± 2.62; $t(8) = 3.89$, $p < .005$]. This tendency toward increasing numbers of no pore papillae continued through Day 14 to a maximum of 28.2 ± 0.97. Beyond that time, the number of no pore papillae decreased concurrent with an increase in the number of filiform-like papillae.

Filiform-Like Papillae. The first filiform-like papilla was noted in one rat at Day 8. Two other rats each had a single filiform-like papilla on Day 11. However, by Day 14 after surgery, all rats had at least two filiform-like papillae, for an average of 4.8 ± 1.02, and there was a significant difference between the intact and NeoCTX side of the tongue [$t(8) = 4.71$, $p < .002$]. The number of filiform-like papillae increased to a maximum of 26 ± 2.15 by Day 30.

Intact Side of Tongue

In contrast to the profound effects noted on tongue morphology after CT transection, there were no apparent effects to the side of the tongue contralateral to the transection. The total number of papillae [$F(9,$

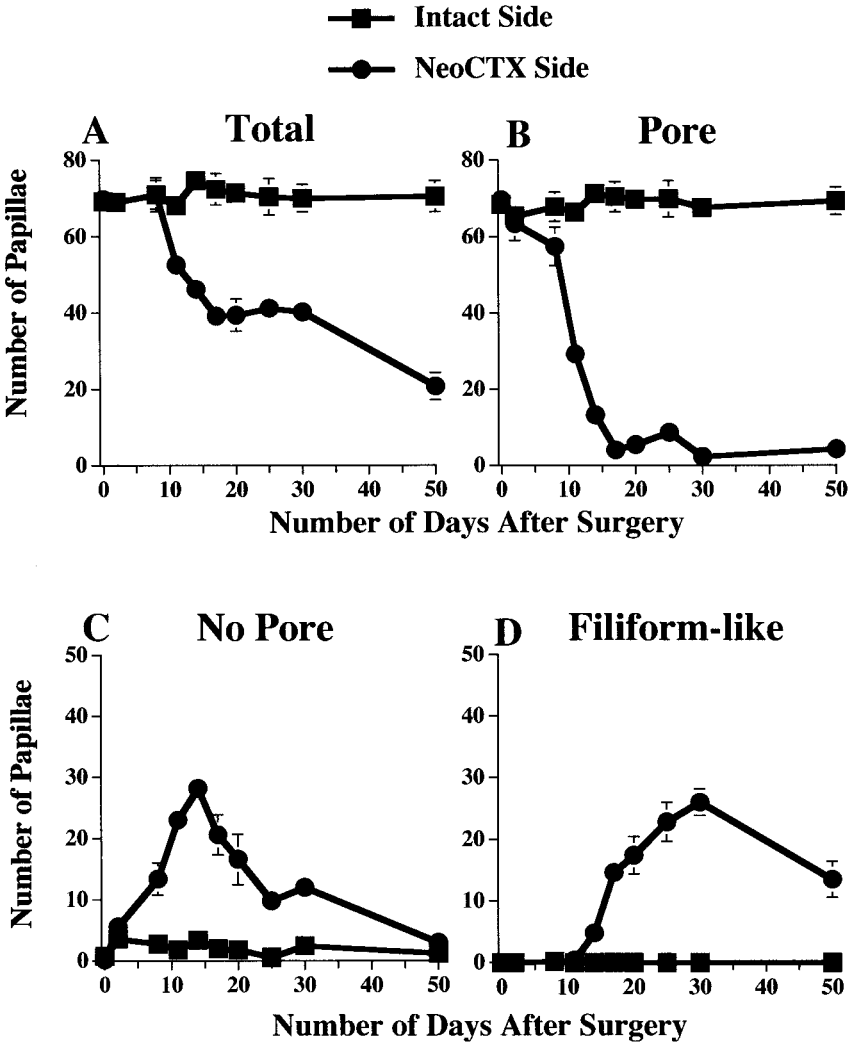

Figure 3 Time course of morphological changes of fungiform papillae of rats that received unilateral chorda tympani nerve transections at 10 days of age. Counts were made of (A) total number of fungiform papillae; (B) fungiform papillae with a taste pore; (C) papillae with a smooth, flattened surface and no pore; and (D) fungiform papillae with a filiform-like spine. Transected side = NeoCTX; uncut side = intact. Numbers are means ± S.E.M. In some instances S.E.M. is too small to be evident in the graph.

48) = 0.44; $p > .1$] and the number of fungiform papillae with a pore [$F(9, 48) = 0.46$; $p > .1$] were consistent, with a range from 65.4 ± 2.89 to 71.2 ± 2.97. There was a significant overall variation in the number of no pore papillae [$F(9, 48) = 2.25$; $p < .04$], but Bonferroni tests indicated no significant degree of difference at any two time points ($p > .05$; range: 0.6 ± 0.24 to 3.6 ± 1.21). On the intact side, only one filiform-like papilla was noted in one rat on Day 8 after surgery. No other filiform-like papillae were noted at any other time on the intact side. These results indicate that, despite the profound morphological changes that occur on the side ipsilateral to neonatal CTX, there is an extremely limited effect on papillae morphology on the side contralateral to the transection.

Taste Bud Morphology

Taste buds degenerated with a time course similar to that noted for fungiform papillae degeneration (see Fig. 4). As compared to the intact side, there was a noticeable, though insignificant, decrease in the taste bud volume on the denervated side of the tongue by 2 days post-NeoCTX. No filiform-like papillae were noted at this age, although a total of three empty papillae were counted within the sectioned regions. At 4 days post-NeoCTX, there was a significant differ-

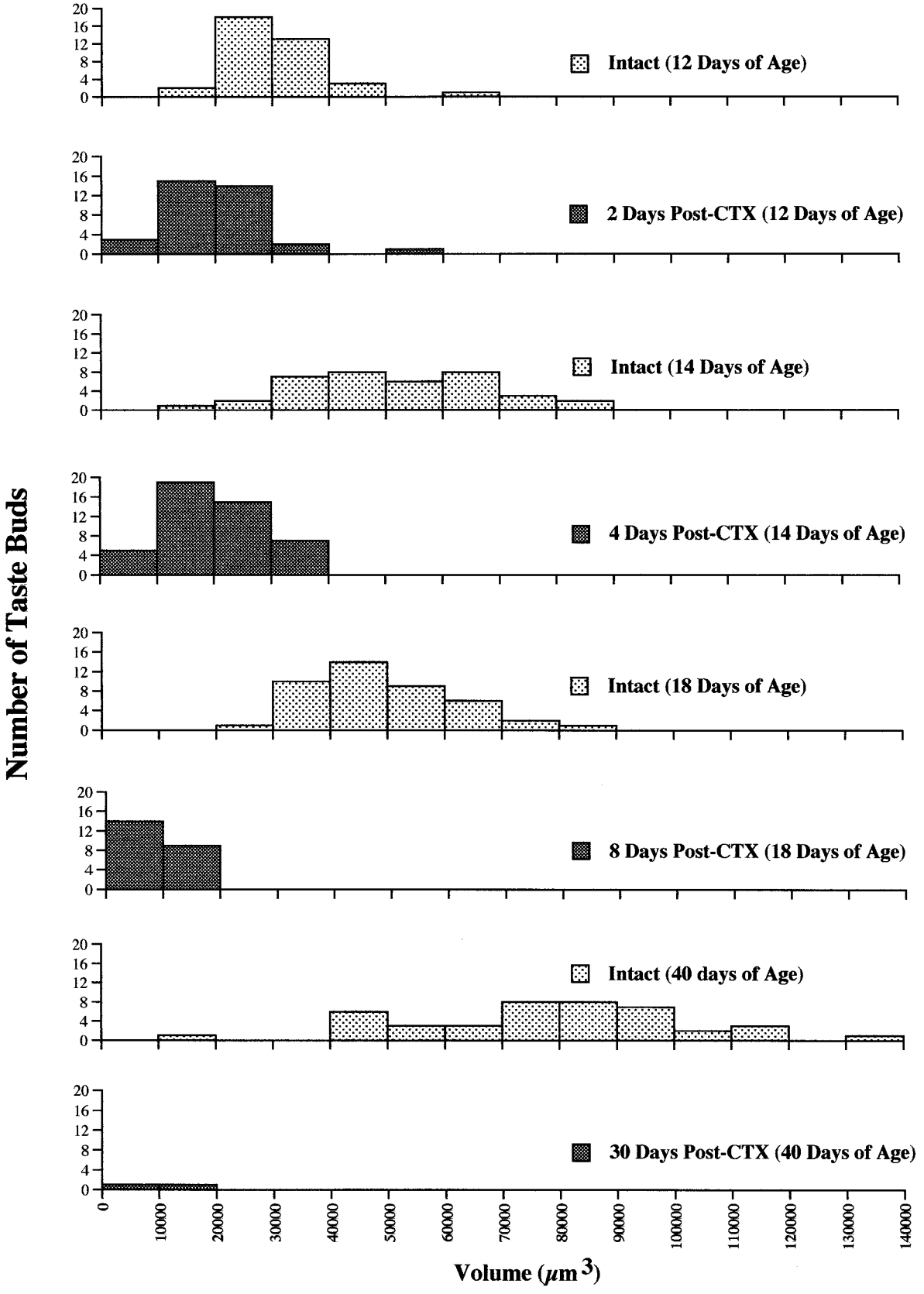

Figure 4 Histograms of the number of taste buds per volume range. Volume measurements were made on the control side of the tongues (intact) and on the side of the tongues that were CT-denervated at 10 days of age (post-CTX). Tongues were examined 2, 4, 8, or 30 days after CT surgery.

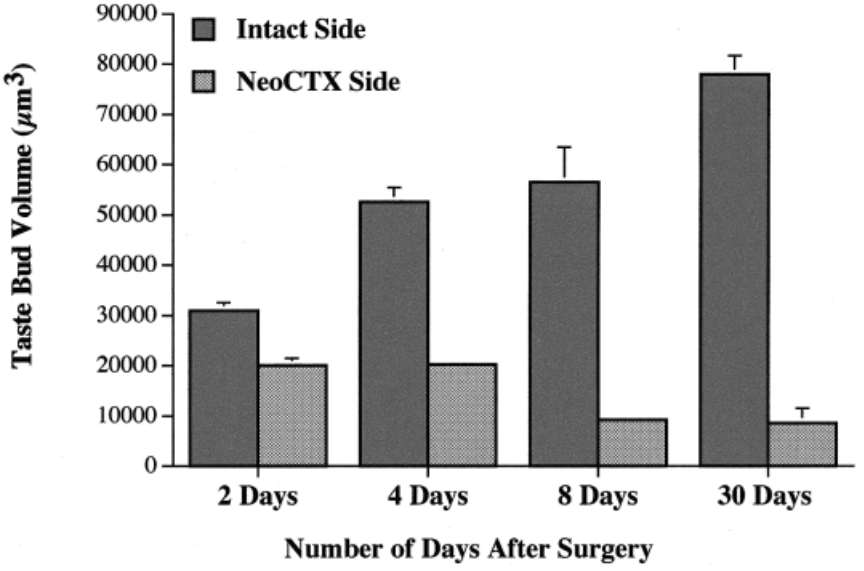

Figure 5 Average taste bud volumes (±S.E.M.) on the intact and denervated sides of the tongues at 2, 4, 8, and 30 days after CTX at 10 days of age.

ence in taste bud volume between the intact and denervated sides of the tongue (Fig. 5; $p < .05$). However, the difference between the 2-day post-NeoCTX taste buds and the 4-day post-NeoCTX taste buds was due to a developmentally related increase in the volume on the intact side and not to a further decrease in the average volume on the denervated side. On the cut side at 4 days post-CTX, CK-19 staining revealed few normal-appearing taste receptor cell populations, with many papillae containing only one or two labeled cells [see Fig. 6(C–F)]. In contrast, on the uncut side of the tongues, all available fungiform papillae contained normal-appearing aggregates of immunopositive taste receptor cells [Fig. 6(A,B)]. On the denervated side, there were a few additional empty papillae at this stage (total of six) than were noted at the 2 day post interval. By 8 days after transection, a greater degree of degeneration was noted on the denervated side of the tongue. Denervated taste bud volumes were significantly smaller than volumes noted at the earlier stages and significantly smaller than the intact side ($p < .05$). Taste buds on the denervated side were fewer in number (23 total as compared to 47 at 2 days postsection and 42 at 4 days postsection) and there were more empty papillae (a total of 16). CK-19 staining showed no labeled cells (i.e., only empty papillae) on the CT-denervated side, but robust label on the intact side. These results suggest that the taste bud volume measurements indicated at 8 days post-CTX are most likely composed of support cells with few, if any, functional taste receptor cells. In addition, similar to that noted for the surface structure papillae counts, the first filiform-like papillae were noted at 8 days post-transection. A total of nine filiform-like papillae were counted; however, all but one were more rounded on the surface than filiform-like papillae noted at later stages after denervation. It appears likely that these papillae were in a state of transition to the conical surface structure noted in later-stage, filiform-like papillae. Taste bud volume on the intact side of the tongues did not increase significantly between 4 and 8 days postsurgery. At 30 days after NeoCTX, a total of only two papillae with taste buds were found on the denervated side of the tongue. The taste buds were significantly smaller than the volumes on the intact side, but not significantly smaller than the taste buds noted at 8 days post-transection. A total of four empty papillae and 22 filiform-like papillae were noted, corroborating the surface structure analysis that there is a loss in the number of identifiable fungiform papillae after NeoCTX. On the intact side, taste buds were larger at 30 days postsurgery than taste buds at 8 days postsurgery due to the age-related increase in taste bud size (Krimm and Hill, 1998).

Lingual Nerve Fiber Profile Counts

There were no differences in the lingual nerve fiber counts between intact and NeoCTX rats. The number of myelinated fiber profiles varied by less than 5% overall, and the number of unmyelinated fiber profiles varied by 7.4% (see Table 1 for detailed averages of myelinated and unmyelinated fiber profile counts.)

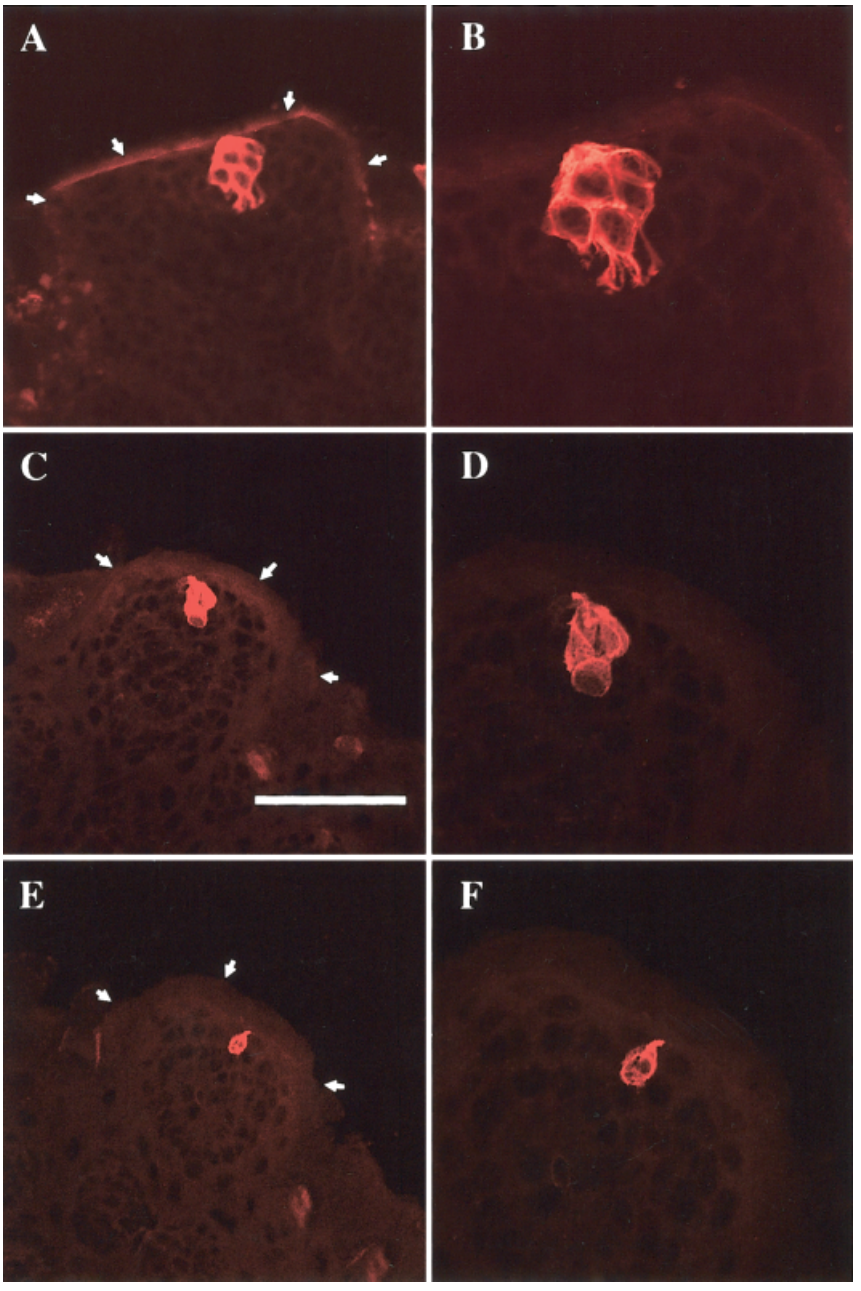

Figure 6 Cytokeratin-19 positive cells in fungiform papillae. (A) and (B) Intact side of the tongue at 14 days of age (4 days after surgery on the contralateral side). (C) and (D) An aggregate of taste receptor cells 4 days after CTX at 10 days of age. (E) and (F) A single taste receptor cell in a fungiform papillae 4 days after CTX at 10 days of age. (B), (D), and (F) are higher magnification images of (A), (C), and (E), respectively. Scale bar in (C) represents 100 μm for (A), (C), and (E) and 50 μm for (B), (D), and (F). Arrows in (A), (C), and (E) indicate the borders of the fungiform papillae.

Both myelinated [$t(4) = 0.60$, $p > .1$] and unmyelinated [$t(4) = 0.62$, $p > .1$] fibers were similar in number between groups. Additionally, there were no statistical differences in total number of fiber profiles [$t(4) = 0.61$, $p > .1$]. These results strongly suggest that there was no secondary damage to lingual nerve fibers as a result of the neonatal CTXs. Thus, it is unlikely that the morphological changes noted in fungiform papillae are the result of compound damage to lingual and CT fibers.

Table 1 Lingual Nerve Fiber Counts from NeoCTX and Intact Control Rats

Surgical Condition	Number of Myelinated Fibers	Number of Unmyelinated Fibers
Intact CT	3872.3 ± 224.2	1951.3 ± 185.0
NeoCTX	3689.7 ± 205.1	1806.7 ± 141.3

Numbers are means ± S.E.M.

Control CTXs

The tongues of rats that received CT section at 10 days of age showed changes in fungiform papillae morphology that were similar to those observed after CT evulsion (see Fig. 7). At approximately 50 days postsection, there were few pore papillae (6.2 ± 1.2) and no pore papillae (3.2 ± 1.2) and the majority of fungiform papillae that remained were of the filiform-like type (19.5 ± 2.3). Overall, there was a 61.8% loss in the total number of papillae that could be categorized into any of the three groups. In each category, there was a significant difference between the sectioned side and the intact side ($p < .05$). The tongues of rats from this group were compared to those that were examined 50 days after CT evulsion in the time course study. No significant differences occurred as a result of the type of transection ($p > .05$). There were similar numbers of pore, no pore, and filiform-like papillae on the sectioned side as compared to the evulsed side of the tongues. In addition, the total loss in fungiform papillae was similar between sectioned and evulsed tongues ($p > .05$ for each category). Therefore, simply placing a single cut in the CT at 10 days of age is sufficient to cause radical changes in fungiform papillae morphology.

DISCUSSION

The present findings indicate that the structural integrity of rat fungiform papillae is highly dependent on innervation by the CT in early postnatal ages. After only 30 days following CTX at 10 days of age, there were 42% fewer fungiform papillae on the NeoCTX side as compared to the intact side. In addition, 64% of the remaining papillae were of the filiform-like type. The effects appear to be permanent, continuing to 50 days post-transection in the present study and as much as 130 days after transection in a previous study (Sollars and Bernstein, 2000). These results are in contrast to reports of CTX in adult rats wherein over 70% of fungiform papillae retain their general characteristic morphology even when the CT is not allowed to regenerate (Hård af Segerstad et al., 1989). Although a relatively minor degree of CT-dependence appears in the adult rat, gustatory nerve regulation of papillae morphology is largely a developmental phenomenon. No studies of adult CTX have been done that are directly comparable in method of analysis to the present study. However, a decrement in number of papillae such as we observed would be noticeable even in studies where small sections of tongue tissue were analyzed. In a study of CT/facial nerve transection in juvenile rats aged 28 to 35 days, the total loss in papillae was estimated at "about 30%" (Ganchrow and Ganchrow, 1989). In adult rats, Hård af Segerstad and colleagues (1989) found no differences in total numbers of identifiable fungiform papillae across the time points (5 to 100 days) examined following CT ablation. Furthermore, despite the extreme effect on papillae morphology observed after CT/lingual nerve transection in adult rats, Oakley and colleagues (1990) observed only a 20% reduction in discernable fungiform papillae.

Neonatal CTX produced rapid changes in the morphology of fungiform papillae. As early as 2 days after transection, there was a slight increase in the number of no pore papillae. This tendency toward increased no pore papillae continued throughout the 14 days after NeoCTX. At the same time that no pore papillae reached their maximum number, the number of filiform-like papillae started to increase. Along with the increase in numbers of filiform-like papillae, there was a decrease in the total number of fungiform papillae that could be classified into one of the papillae categories. In combination, these results suggest that there is a transition in fungiform morphology such that pore papillae become no pore papillae that

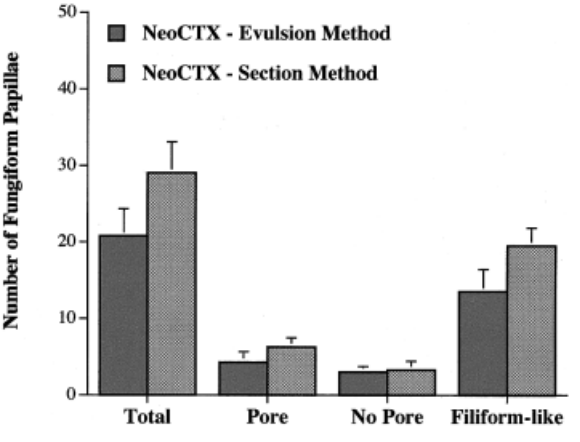

Figure 7 Number of fungiform papillae (means ±S.E.M.) at 50 days postsection. At 10 days of age, approximately 5 mm of the CT was removed (evulsion method) or a single cut was placed in the nerve (section method).

subsequently become filiform-like papillae. At the present time, there is no clear evidence that filiform-like papillae further transform such that they become indistinguishable from true filiform papillae. It is also possible that instead of taking on a filiform-like appearance, no pore papillae degenerate such that they are no longer detectable on the tongue surface.

The evidence for neural influence on taste bud maintenance is largely known, and it is no surprise that taste buds degenerate quickly after neonatal CTX in a manner similar to that noted after adult CTX (Oakley et al., 1993). However, the present study demonstrates a novel finding; during development, the extra-gustatory epithelial tissue of fungiform papillae is profoundly dependent on the presence of a gustatory nerve (the CT). Furthermore, because the CT does not directly innervate the papilla, but contacts the taste bud only (Miller, 1974), this suggests that structural or molecular factors specific to the CT and/or the taste buds are providing support for the maintenance of normal papillary morphology. Complementary results have been observed in studies of brain derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT4/5) null mutant mice. BDNF and NT4/5 null mutants have a smaller quantity of fungiform papillae (Liebl et al., 1999; Mistretta et al., 1999) and reduced geniculate ganglion volume, but there is only a small disruption in volume of the trigeminal ganglion (Jones et al., 1994; Liu et al., 1995). Given the deficits in numbers of fungiform papillae in BDNF and NT4/5 null mutant mice, the results suggest that innervation via the lingual branch of the trigeminal nerve is not sufficient to maintain fungiform papillae. Similarly, in NeoCTX rats, the lingual nerve remains intact and is not able to function alone to support fungiform papillae maintenance.

The morphology of fungiform papillae is immature at 10 days of age. In previous studies, approximately 20% fewer taste pores were observed in papillae of neonatal rats (Mistretta, 1972; Harada et al., 2000). Fungiform papillae continue to mature morphologically for at least 30 days postnatal (Iwasaki et al., 1997; Harada et al., 2000). We observed taste pores within most fungiform papillae at 10 days of age, but it was visually clear that the epithelium was immature (see Fig. 2). Although the mechanisms that underlie papillae maintenance are not known, it is possible that this state of immature development makes the epithelium particularly susceptible to the effects of denervation. Initial induction of fungiform papillae in embryonic rats and mice is regulated by signaling proteins that are also morphogens in other sensory epithelia (Mistretta, 1998). For example, the sonic hedgehog signal transduction pathway has emerged as a major contributor to early papillae formation and patterning (Hall et al., 1999a, b; Mistretta et al., 2000). At 10 days of age in the rat, it is likely that these and/or other morphogens continue to contribute to the regulation of papillae maturation and maintenance.

As noted, there appears to be little, if any, regeneration of the CT after NeoCTX (unpublished observations). In most of the rats, a large portion of CT was removed, leaving open the possibility that the severity of effect noted on fungiform papillae was because of the extensive degree of neural tissue removed. However, a single cut in the CT without the removal of any tissue in 10-day-old rats resulted in long-term changes in tongue morphology that were nearly identical to the rats that had a large portion of nerve removed. Although the degree of regeneration has yet to be quantified, these results suggest the possibility that there is a failure of regeneration because of the death of CT cell soma in the geniculate ganglion. In other systems, neural transection early in development results in a greater amount of ganglion cell death and less neural regeneration than transections in adulthood (Waite, 1984; Himes and Tessler, 1989). However, it is not clear if the changes in fungiform papillae morphology are the result of a failure of CT regeneration or if they are due to other factors. The rapid morphological changes in fungiform papillae could preclude CT regeneration. That is, it could be that the lack of a target site (i.e., fungiform papillae) inhibits the reinnervation of the CT into tongue tissue.

In all tongues, some papillae with a pore remained on the transected side. Because the transections were unilateral, there could be a small degree of contralateral innervation to maintain those taste buds. However, even after bilateral NeoCTX, a few pore papillae were observed (Sollars and Bernstein, 2000). Thus, it appears unlikely that there was any sprouting of the contralateral, intact nerves into the transected side of the tongues in the current study. Other researchers have suggested that taste buds may receive a small degree of support from nerves other than the CT, accounting for the observance of taste buds even after chronic CT denervation (Hård af Segerstad et al., 1989). For example, lingual nerve fibers have been noted in the taste buds of rats 8–12 weeks following CTX wherein the CT was not allowed to regenerate (Kinnman and Aldskogius, 1988). The limited number of taste pores seen in the present study could be the result of lingual innervation into taste buds or the regeneration of a few CT fibers.

It is evident that in neonatal rats, the epithelial structure of fungiform papillae is dependent on the presence of the CT. This is a highly intriguing finding

especially considering that the CT is a sensory nerve and does not directly innervate the papillae, but rather synapses with the taste receptor cells of the taste bud. It is possible that the CT acts synergistically with the lingual nerve to maintain papillary structure. Preliminary evidence suggests that this is a strong possibility, because lingual nerve transection alone in 10-day-old rats has a more severe effect on fungiform papillae structure than the same surgery in adult rats (Guagliardo et al., 1999). Because the lingual nerves examined in the present study appear to be undamaged after neonatal CTX, it is unlikely that NeoCTX produces a degenerative effect on the lingual nerve. However, the lingual nerve may retract from fungiform papillae secondary to the degeneration of the transected CT. For example, an increase in phagocytotic activity at the taste bud may occur as a result of CT transection. Although there is no current evidence for macrophage or fibroblast activity in fungiform papillae as a result of denervation, an increase in the number of fibroblasts was observed after denervation of circumvallate papillae in the rat (Suzuki et al., 1996). Given the much more severe effect of neonatal versus adult CTX, it seems unlikely that such an explanation could fully account for the extreme degree of change in papillae morphology that results after NeoCTX. If an increase in phagocytotic activity caused degeneration of lingual fibers within papillae, it seems likely that such changes would also be observed after adult CTX.

Development of taste buds occurs subsequent to the appearance of fungiform papillae around embryonic day 14 in the rat (Mbiene et al., 1997; Mbiene and Mistretta, 1997). While fungiform papillae appear to develop independent of innervation (Farbman and Mbiene, 1991; Mbiene et al., 1997) and more than 80% are maintained in adulthood even after transection of both the lingual and CT nerves (Oakley et al., 1993), the present study indicates that a sensitive period exists wherein neural support is needed to maintain papillae structure. That is, papillae morphology is initially independent of the CT, shifts to being dependent on the CT in early postnatal ages, and then becomes relatively independent again at adulthood. The mechanism behind the transition from total neural independence in the embryonic rat to an almost complete dependence on the CT in the early postnatal rat is unknown. However, it points to a complex interplay of molecular and cellular signals among innervating neurons and their targets.

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